DEVELOPMENT OF DETECTION ASSAYS FOR SINDBIS VIRUS AND INVESTIGATING IN VITRO INFECTION OF MAMMALIAN CELLS

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MMedSc (Virology)

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DECLARATION

Ek verklaar dat die verhandeling wat hierby vir die kwalifikasie M.Med.Sc (Virologie) aan die Universiteit van die Vrystaat deur my ingedien word, my selfstandige werk is en nie voorheen deur my vir ’n kwalifikasie aan n ander hoer onderwys inrigting ingedien is nie.

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Hermanus Albertus Hanekom

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ABSTRACT

Sindbis virus (SINV) is a member of the *Alphavirus* genus and belongs to the family *Togaviridae*. The virus has a positive sense RNA genome of 11700 bases which encodes for both structural and non structural proteins. Infections are frequently diagnosed based on clinical, epidemiological and laboratory criteria. Laboratory confirmation is essential as SINV infections must be distinguished from various conditions that share similar clinical manifestations. The most frequently used methods for identification are haemagglutination inhibition, enzyme-linked immunosorbent assay, plaque reduction neutralization tests as well as conventional *in-vitro* neutralization assays. Serological assays for the detection of SINV are not readily available commercially and due to the non-specific symptoms caused by SINV infection the number of infections per annum may be under diagnosed. The purpose of this study was to develop serological assays such as ELISA and a novel neutralization assay that could be used in serological surveys for the detection of IgG antibodies against SINV. Furthermore to develop assays that could be used to determine the level of viral replication in mammalian cells for characterizing infection in mammalian cells as well as investigate the influence of interferon on viral replication and look for evidence of apoptosis caused by SINV infection.

An in house ELISA was developed and used to screen 146 sera for IgG antibodies against SINV. The *in-vitro* neutralization assay is the gold standard for serology and 43 samples in total were tested in both the ELISA and the *in-vitro* neutralization assay. Analysis and comparison of the results obtained using the in-house ELISA and the neutralization assay indicated that the sensitivity of the ELISA was 68.9% and the specificity of the in house ELISA was 78.57 - 85.71% depending on the use of the percentage positive or optical density values to differentiate positive and negative samples. A forward and reverse primer for the amplification of a conserved 181bp region of the nsp2 gene encoding the nsp2 protein of SINV were designed along with a TaqMan hydrolysis probe to be used in a real time quantitative TaqMan PCR. The infection of mammalian cells, human macrophages and HeLa cells, was determined by measuring viral loads with a real time quantitative TaqMan RT-PCR. Two strains of SINV were used in attempts to infect macrophages, a strain from Egypt and a strain from South Africa. Small increases in viral load suggested possible low levels of viral replication but were considered insufficient to warrant further investigation and insufficient to investigate occurrence of antibody dependent enhancement of disease in macrophages. The mechanism possibly interfering with replication of virus in the human macrophages was investigated.

Supernatant fluid samples from macrophage infections were tested for the release of interferon gamma which could inhibit viral replication. There were nine to fifteen fold differences in the concentration of

interferon gamma detected in the supernatant fluid at baseline and 24h after infection. HeLa cells were treated with similar concentrations of human interferon gamma at different time intervals. Pretreatment and concurrent treatment with infection showed reduced levels of viral load compared with no treatment or delay in treatment. Hence the suggestion that interferon could have played a role in inhibiting viral replication in the human macrophages. DNA was extracted from HeLa cells infected with SINV and the DNA fragments separated through agarose gel electrophoreses. There were multiple bands visible in the infected samples whereas the negative control did not show multiple bands, only one large band of genomic DNA. The presence of multiple DNA fragments in infected cells and absence of those fragments from uninfected cells were suggestive of virus induced apoptosis.

CHAPTER 1 LITERATURE REVIEW

1.1 Introduction

Insects play an important role in the distribution and spread of infectious pathogens. These insects include ticks and mosquitoes that are responsible for the spread of viruses, bacteria as well as parasites. The viruses that are spread by mosquitoes and ticks are collectively referred to as arthropod borne viruses or arboviruses. There are more than 500 viruses that fall into this group. These arboviruses are responsible for causing infections ranging from arthritis to more serious conditions such as encephalitis and haemorrhagic fever (Laine et al., 2004).

Alphaviruses, particularly the Old World alphaviruses transmitted by mosquitoes, are frequently overlooked as causes of disease because the infections are often not fatal and therefore do not represent a major health priority. Alphaviruses can however present a major health risk to humans. Japanese encephalitis virus (JEV), Eastern equine encephalitis (EEE) virus and Venezuelan equine encephalitis (VEE) virus have been known to cause fatal infections in humans. Other alphaviruses capable of causing severe or debilitating disease include chikungunya virus (CHIKV), Sindbis virus (SINV) and Ross River virus (RRV) which causes symptoms such as fever, rash and painful arthralgia (Hoarau et al., 2010; Ryman & Klimstra, 2008; Strauss & Strauss, 1994). SINV is considered as the prototype of the alphaviruses (Lloyd, 2009).

1.2 History

SINV is a member of the *Alphavirus* genus and belongs to the family *Togaviridae* (Calisher et al., 1980). The alphaviruses were some of the first arboviruses to be isolated and characterized. Arboviruses come from the term arthropod-borne virus which describes a transmission cycle between susceptible vertebrate hosts as well as arthropod insects and cause mild to severe disease in humans (Calisher et al., 1988a; Clarke & Casals, 1958; Karabatsos, 1975; Navaratnarajah, 2007; Porterfield, 1961). There are more than 530 viruses listed in the International Catalogue of Arboviruses which have been categorized according to antigenic, morphological, biochemical and genetic characteristics (Calisher & Karabatsos, 1988). Both humans and animals are susceptible to infection by these viruses (Gould *et al.*, 2010). Arboviruses have been found to be present on all the continents of the world with the geographic distribution dependent on the presence of competent vectors and conditions favoring vector populations (Navaratnarajah & Kuhn, 2007).

SINV was first isolated in 1952 from *Culex pipiense* and *C. univittatus* mosquitoes collected 30 km north of Cairo in the village of Sindbis. The virus was subsequently named from the area in which it was first

identified (Taylor et al., 1955). SINV was later detected in Europe, Australia, Asia and the rest of Africa through serological surveys. It was only in 1961 that SINV was confirmed as an etiological agent of disease when patients in Uganda presented with fever and attempts to isolate the virus yielded SINV (McIntosh et al., 1964; Tesh, 1982; Woodall & Williams, 1962). In 1963 the virus was isolated from skin lesions from a patient in South Africa presenting with malaise, fever and maculopapular rash (McIntosh et al., 1964). Antibody surveys have shown evidence that SINV is widely distributed throughout southern Africa (Storm et al., 2012). Further surveys conducted by testing host mosquitoes identified SINV circulating in the mosquito populations in several South African provinces including Gauteng, Free State and KwaZulu Natal (McIntosh et al., 1964).

SINV was first identified in Europe in 1965 after two Finnish patients tested positive for antibodies against SINV (Brummer-Korvenkontio & Saikku, 1975). SINV infection in Europe was first associated with illness in 1980 when a rise in antibody titers was detected in Swedish patients that suffered from Pogosta disease (Brummer-Korvenkontio et al., 2002). SINV was identified as the etiological cause of Ockelbo disease in Sweden and was isolated from *Culex sp*. mosquitoes and resident birds (Niklasson et al., 1984). In the 1980's SINV was implicated as the cause of a disease in Russia known locally as Karelian fever (L'vov et al., 1982). Epidemics in Finland occur every seven years and thousands of people are infected in each seven year cycle (Kurkela *et al*., 2004). SINV infections occur seasonally in South Africa, Australia and Scandinavia particularly after periods of heavy rainfall that favors mosquito breeding (Uejio et al., 2012).

1.3 Taxonomy and phylogeny

The *Alphavirus* genus currently includes 29 recognized species of viruses that are classified into eight antigenically related serocomplexes: Eastern, Western, Venezuelen equine encephalitis, Middleburg, Ndumu, Semliki Forest and Barmah Forest (Calisher & Karabatsos, 1988; Karabatsos, 1985; Strauss & Strauss, 1994). The alphaviruses are described as either Old World or New World alphaviruses depending on where they were first isolated (Grywna et al., 2010; Leung et al., 2011). The antigenic groups also reflect the clinical presentation of the disease associated with the viruses as shown in Table 1, with the exception that SINV, although grouping with WEE group, has not previously been associated with encephalitis. It has been proposed that recombination events between EEE and Sindbis-like virus have resulted in Western equine encephalitis (WEE) (Hahn et al., 1988; Levinson et al., 1990; Weaver et al., 1993; Weaver et al., 1997).

The grouping of the viruses within this complex, initially based on serological cross reactivity, have been substantiated with phylogenetic analysis using partial and complete genome sequences (Calisher et al., 1988a; Luers et al., 2005). Within the complex there are a group of recombinant viruses referred to as "WEE complex recombinants". Neutralization assays do however distinguish the WEE complex recombinants from SINV and other members of the complex (Powers et al., 2001). Based on analysis of complete and partial sequence data reclassification of the alphaviral compex has been proposed in which Barmah Forest virus and Middleburg virus are grouped in the Semliki Forest complex and reduces the cladogram to three clades designated Semliki Forest clade, Sindbis-Equine Encephalitis clade which includes a recombinant subclade and Aquatic virus clade which include the alphaviruses found in aquatic organism (Luers et al., 2005).

The Old World refers to viruses that occur in Africa, Europe, Asia and Australia whereas New World refers to the Americas. Old World viruses, such as SINV and RRV, are known to be less virulent than New World viruses such as VEE. Infections by Old World viruses are usually characterized by arthritis and rash whereas New World viruses cause significant disease as shown in Table 1.1. (Paredes et al., 2005). Genetic relationship based on the nucleotide sequences of the nonstructural proteins supports the grouping of New World and Old world alphaviruses (Weaver et al., 1993).

Although RNA viruses have the potential for rapid evolution due to the lack of proof-reading after replication the actual rate of divergence in alphaviruses has been found to be much lower than expected. One of the reasons postulated for the low rate of mutation is that the viruses are spread by mosquito vectors and genetic alterations to the virus may not be beneficial, inhibiting viral amplification inside the vector and therefore restricting its ability to spread within the mosquito population (Strauss & Strauss, 1994).

SINV is regarded as the most globally distributed *Alphavirus*. SINV has been identified on the basis of mosquito isolations, human serology and/or human infections in Africa, including North Africa, Cameroon, Uganda and South Africa, European countries including Sweden, Finland, Germany, United Kingdom and Italy, and Australasian countries including Australia, New Zealand, Malaysia, the Phillipines (Jöst et al., 2010; Lundstrom & Pfeffer 2010; Marchette et al., 1978; Sammels et al., 1999; Strauss & Strauss, 1997; Tesh et al., 1975; Weaver et al., 1997; Lloyd, 2009).

Table 1.1. Antigenic complex, distribution and where applicable the disease syndromes associated with the medically significant alphaviruses (Burt et al., 2012; Calisher et al., 1988a; Laine et al., 2004; Powers et al., 2001; Lloyd, 2009)

Antigenic complex	Virus species	Geographic distribution	Disease syndrome associated with complex
Barmah Forest	Barmah Forest virus	Australia	Rash, polyarthritis, myalgia
Eastern equine encephalitis	EEEV	North and South America	Encephalitis
Middelburg	Middleburg virus	Africa	Unknown
Ndumu	Ndumu virus	Africa	Unknown
Semliki Forest	Semliki Forest virus CHIKV O'Nyong-nyong virus Getah virus Mayaro virus Bebaru virus RRV Una virus Sagiyama	Africa, Asia Africa, Asia, Indian Ocean Africa Asia, Australasia South America Asia Australia, Papua New Guinea South America Japan	Rash, arthritis
Western equine encephalitis (*including recombinants)	WEEV SINV Aura virus *Fort Morgan virus *Highlands J virus Whataroa virus	North and South America Europe, Africa, Asia, Philippines, Australia North America Western North America Eastern North America New Zealand	Encephalitis associated with WEEV Rash, polyarthritis, fever associated with SINV
Venezuelen equine encephalitis	Venezuelen equine encephalitis virus	North and South America	Encephalitis
Trocara	Trocara virus	Atlantic Ocean	Not applicable

As mentioned above, SINV appears to comprise of various subtypes based on geographic location, Ockelbo virus in Sweden, Karelian virus in Russia and Whataroa virus in Australia and New Zealand. Genetic analysis of nucleotide sequence data for geographically distinct isolates of SINV have identified two distinct genetic lineages that comprise of viruses originating in Palaearctic/Ethiopian region and Oriental/Australian region (Liang et al., 2000; Sammels et al., 1999). Five genotypes have been proposed for SINV isolates worldwide based on analysis of the highly variable E2 glycoprotein gene of 59 isolates (Lundstrom & Pfeffer, 2010). Genotype 1 strains are found in Europe and Africa, Genotype II and III strains are found in Australia and East Asia, Genotype IV is found in Azerbaijan and China and Genotype V in New Zealand. Divergence at the

amino acid level has been shown to range from 12 to 22%. Birds play an important role in the natural cycle of SINV and the genetic relationship of the isolates from geographically distinct regions supports the role of migratory birds in the worldwide distribution of these strains (Jost et al., 2010; Kurkela et al., 2004; Norder et al., 1996; Sane et al., 2012).

1.4 Alphavirus structure

Alphaviruses have a spherical shaped nucleocapsid of approximately 70nm in diameter. The virus has a host cell acquired lipid membrane that surrounds the capsid making it an enveloped virus as illustrated in Figure 1.1 (Strauss & Strauss, 1994).

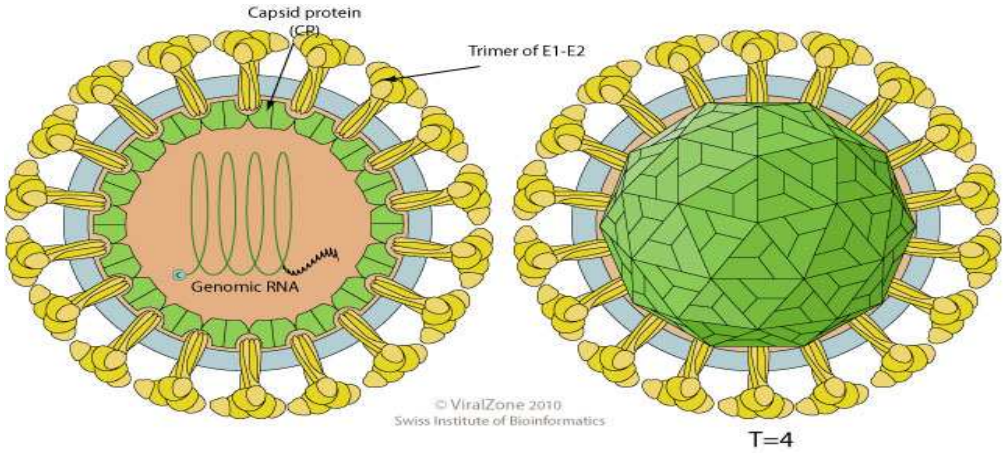

Figure 1.1. An illustration of the *Togaviridae* virus structure. (www.expasy.ch/viralzone/all_by_species/625.html).

The composition of the virus phospholipid membrane is dependent on the composition of the host cell membranes from where the membrane is acquired (Waarts, 2004). Numerous proteins are inserted in the membrane. The E1 and E2 glycoproteins of the virus which are embedded in the membrane have been shown to have antigenic properties. The E1 protein facilitates virus fusion to cells and the E2 protein is responsible for receptor binding between the virus and the host cells (Navaratnarajah & Kuhn, 2007). The mechanism that alphaviruses use to enter cells has been extensively studied. The E2 protein binds to receptors on the cell surface which causes a lowering of the pH and that advances the formation of the endosome through which the viral and cell membrane fuse and the virus is released inside the host cells (Paredes et al., 2005).

The presence of antigenic epitopes on the surface of viral proteins is important as they are recognized by the immune system and induce a protective antibody response. Numerous investigations have shown that antibodies directed against E2 are usually more reactive than those directed against the E1 glycoprotein

(Das et al, 2007). Most of the monoclonal antibodies that are capable of neutralizing viruses have been mapped to the E2 protein (Navaratnarajah & Kuhn, 2007).

1.5 Alphavirus Genome

Alphaviruses such as SINV have a single stranded positive sense RNA genome of approximately 11.7 kb in size which is capped at the 5' end and polyadenylated at the 3' (Levis et al., 1990; Strauss et al., 1984). There are two rounds of translation namely the positive sense and negative sense as illustrated in Figure 1.2. The positive sense refers to where the genomic RNA acts directly as mRNA and is partially translated to produce non-structural proteins which act within the cytoplasm of the infected cells as replicating enzymes of the virus that are responsible for viral replication. The negative sense refers to the positive sense RNA being transcribed into subgenomic intermediary 26S RNA which encodes for the structural proteins 6k, E1, E2, E3 and the capsid protein. The structural proteins are responsible for the physical structures and characteristics of the virus (Laine et al., 2004; Levis et al., 1990; Manni et al., 2008; Thiboutot et al., 2010).

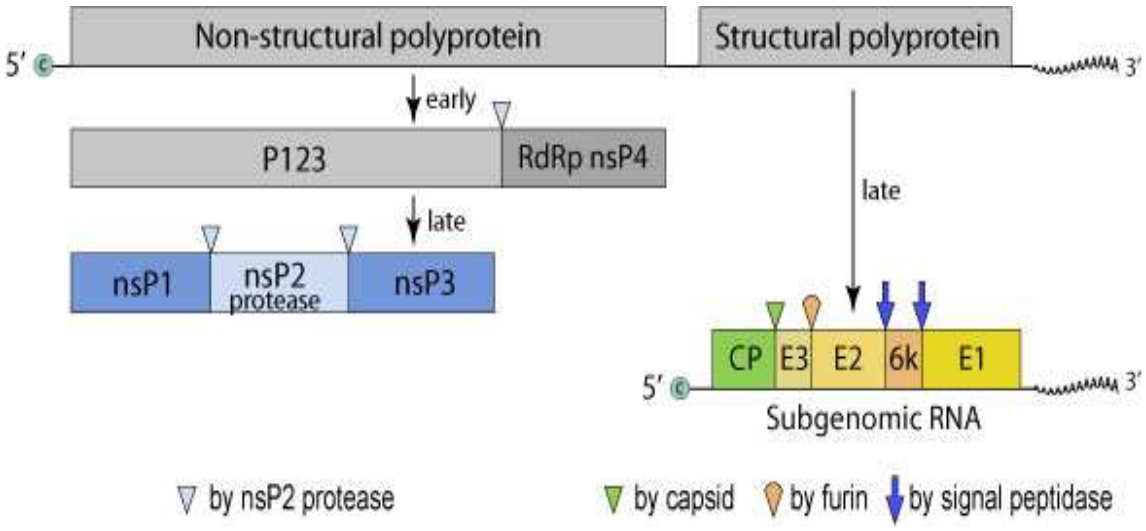

Figure 1.2. Genome of the SINV encoding the structural and non-structural proteins. The Figure illustrates how both the structural and non structural proteins are translated from their respective RNA. (www.expasy.ch/viralzone/all_by_species/625.html)**.**

Figure 1.3 explains the basic replication cycles of alphaviruses. The virus enters the cell through receptor medicated endocytosis and the positive sense RNA genome of the virus is translated directly as mRNA from the 5' end into a polyprotein that contains all four non-structural proteins for viral replication (Strauss & Strauss, 1994).

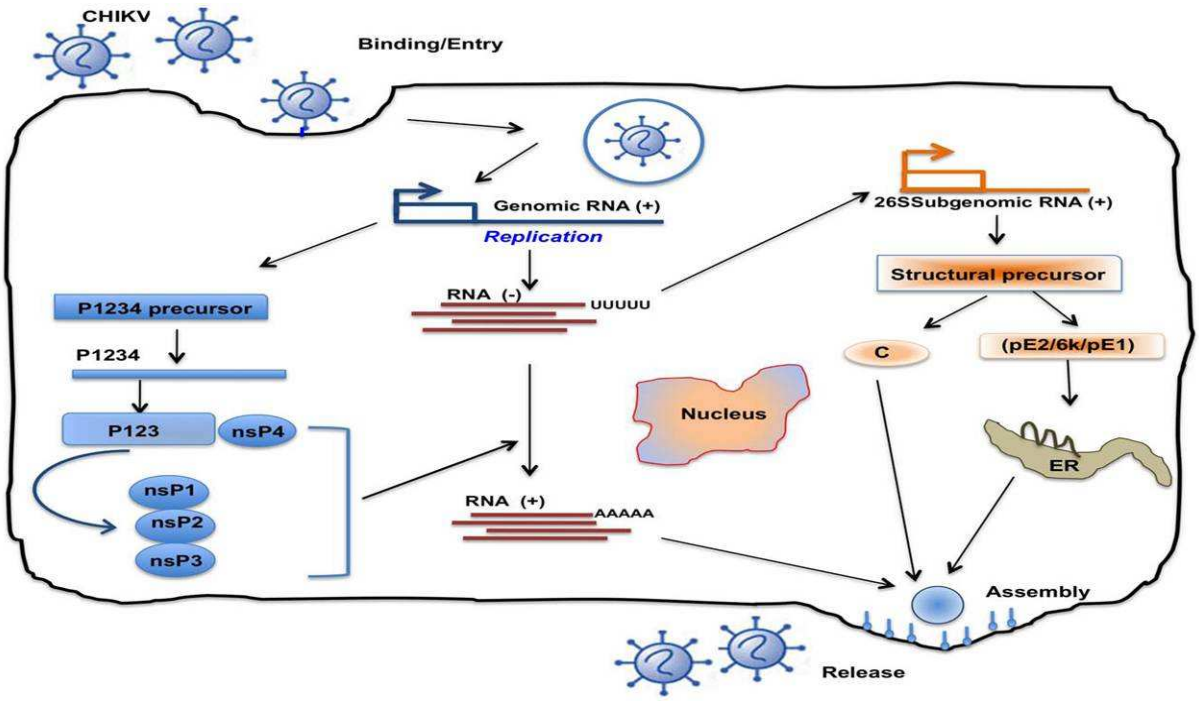

Figure 1.3. The life cycle of alphaviruses inside the cell (Thiboutot et al., 2010)**.**

The polyprotein first undergoes cis-cleavage between the nsP3 and nsP4 proteins to produce a complex containing the polyprotein nsP1-nsP3 and nsP4. In the later stages of infection the non-structural proteins are completely cleaved into the four separate non structural proteins nsP1, nsP2, nsP3 and nsP4 by means of proteolitic cleavage (Strauss & Strauss, 1994). The 3' end of the viral genome encodes for the structural proteins but has to undergo replication and transcription first to be transformed into the negative strand sub-genomic RNA. The subgenomic RNA is translated to produce a polyprotein containing all of the structural proteins. The polyprotein is cleaved into the different structural proteins by host cell proteases, processed by the endoplasmic reticulum (ER) and assembled at the host cell membrane where it exits the infected cell with a host-acquired cell membrane as illustrated in Figure 1.3 (Levis et al., 1990; Strauss & Strauss, 1994).

1.6 Epidemiology

SINV is the most globally distributed of all the alphaviruses (Laine *et al.,* 2004). Since the virus was discovered in the 1950's it has been isolated from birds like the juvenile hooded crow (*Corvus corone sardonius)*, *Culex sp.* mosquitoes and various vertebrates. SINV is transmitted to mosquitoes in nature when they feed on viraemic vertebrates. After the mosquito ingests the virus it is able to replicate inside the mosquito epithelial cells after which the virus moves to the salivary glands where it allows the mosquito to

infect new hosts when feeding (Theilmann et al., 1984a). There are various species of mosquitoes around the world that are able to act as competent vectors for spreading SINV infection.

The occurrence and abundance of mosquito vector species, or potential vector species, is a prerequisite for enzootic transmission of mosquito-borne viruses. SINV was detected in Anopheles mosquitoes in China. Mosquitoes were captured, pooled and ground up for infecting cells as well as serological testing. The testing lead to the discovery of a new strain of SINV China XJ-160 (Liang et al., 2000). During 1983-1985 field studies were conducted in Sweden to determine the etiological agent of disease that caused Ockelbo disease. A total of 63 644 mosquitoes were collected during the three year period and testing led to SINV being isolated from *C. pipiens*, *C. torrentium*, *Culiseta morsitans* and *Aedes cinereus* mosquitoes (Francy et al., 1989).

Figure 1.4. A photo showing a *Culex pipiens* mosquito vector responsible for the spread of SINV infection. (http://bugguide.net/node/view/35533)

In Russia and Norway SINV has been isolated from mosquitoes of the genus *Aedes* and *Ochlerotatus* (Lvov et al., 1984; Norder et al, 1996). During the period of July to September in 2009 a total of 16 057 mosquitoes were collected and molecular analysis demonstrated SINV present in three different mosquito species namely *C. torrentium*, *C. pipiens* and *Anopheles maculipennis sensu lato.* SINV was first isolated from the arthropod *Hyalomma marginatum* tick during 1975 in Italy (Gresikova et al., 1978). The role ticks play in the epidemiology of SINV infection in terms of spread of disease has yet to be determined.

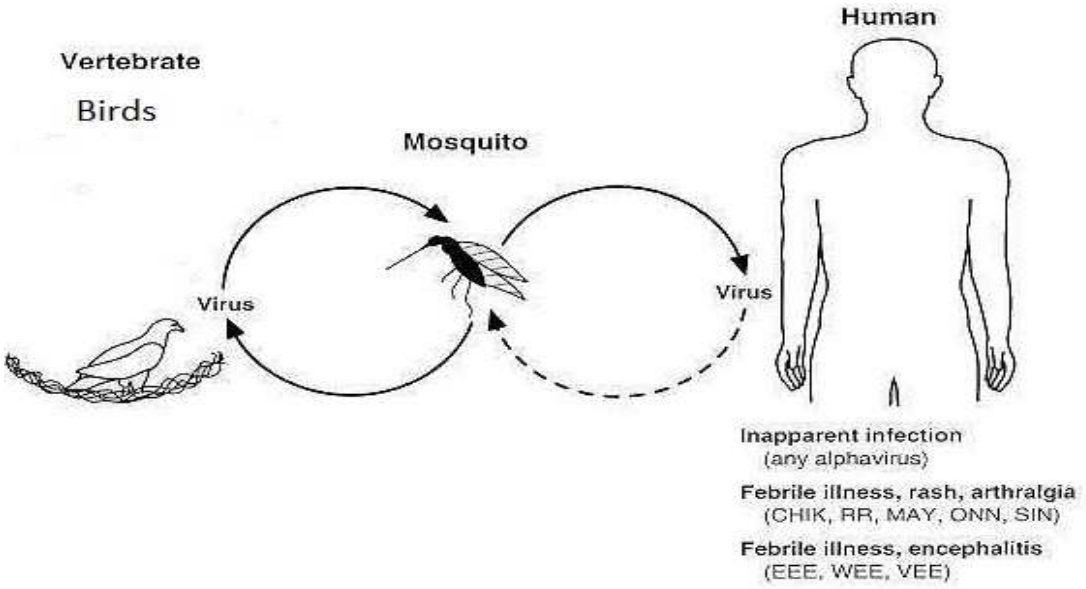

Figure 1.5. Life cycle of alphaviruses; An illustration of the basic *Alphavirus* life cycle starting from the vertebrate host to their mosquito vector. (http://www.ncbi.nlm.nih.gov/bookshelf/br.fcgi?book=mmed&part=A2918).

Figure 1.5 shows the life cycle of alphaviruses in nature. South Africa has had a long history with both SINV and West Nile virus infections. Both are transmitted between birds by the *C. univittatus* with accidental human transmission seen for both. The vector has been known to be sensitive to seasonal changes such as temperature and moisture. Tropical events such as El Nino lead to an increase in dryness in southern Africa which cause a decline in mosquito populations. El Nina has the opposite effect with increased rain which cause more wetlands to form as more mosquitoes reproduce in those conditions (Uejio et al., 2012).
Alphaviruses can infect various vertebrates ranging from birds to humans and the spread of the virus is dependent on mosquito vectors. Infection of each host is dependent on the ability of the virus to recognize receptors on the host cells and to bind to them. Mammals have a variety of cells such as neurons, muscles, lymphoid cells and synovial cells. The more receptors the virus can recognize the more difficult it is for the host organism's natural defenses to eradicate the virus. When mammalian cells are infected it leads to host macromolecular synthesis being used for viral transcription and translation of proteins (Sokoloski et al., 2012).

The receptors used by SINV for entry into host cells have been extensively studied by electron microscopy and the quantification of labeled viral particles that attach to cells (Strauss & Strauss, 1994). Experiments were performed *in-vitro* to determine whether neuronal cells have the same number of receptors for a neurovirulent (SINV S.A.AR86) strain and avirulent (SINV EgAR339) strain of the same virus (Heise et al,

2000; Smith & Tignor., 1980). Saturation studies were used to determine the number of cellular receptors for a neuronal cell line and it was shown that the cells had 1.3×10^6 receptors for the SINV S.A.AR86 strain and only 5×10^4 for the SINV EgAR339 strain. The reason for different amount of receptors between the two strains was likely that the SINV S.A.AR86 strain were less sensitive to enzymatic inactivation than those for the SINV EgAR339 strain. The nature of the receptors were examined by studying their enzyme sensitivity. When cells were treated with phospholipases or neuraminidases there were no differences in the binding efficiency. Only treatment of cells with proteases resulted in greatly reduced levels of binding and therefore it was concluded that the receptors for *Alphavirus* binding were proteinaceous (Smith & Tignor., 1980).

There are several other alphaviruses, such as CHIKV and RRV that have been associated with large outbreaks of disease (Das *et al.*, 2007). Chikungunya fever which presents with similar symptoms as seen with SINV infection was first described in 1955 after an outbreak in Tanzania. A very large outbreak of chikungunya fever occurred between 2005 and 2006 on the island of La Reunion during which at least 40% of the island population was affected. The virus subsequently spread though the neighboring Indian Ocean Islands. Outbreaks have subsequently since occurred in India, Sri Lanka and Singapore with the virus adapting to other mosquito vectors such as the *Aedes albopictus* from the usual *Aedes aegypti* vector in the Asian regions (Wikan et al., 2012).

RRV is responsible for approximately 4800 annual cases of infection in Australia (Lidbury & Mahalingam, 2000). Between 1979 and 1980 more than 60 000 patients in the southern Pacific were involved in an outbreak of RRV (Morrison et al., 2006).

1.7 Alphaviruses in South Africa

Evidence of SINV infection was first detected in human sera in South Africa in 1963 through serological surveys. Haemagglutination-inhibition tests were used to analyze 16 patient samples for antibodies against SINV of which 14 tested showed a rise in antibody titer. There were no successful virus isolations which could be attributed to low viral loads, time sample was taken or a combination of both (McIntosh et al., 1964). In 1974 an extended period of heavy rains caused an increase in the mosquito population which lead to an epidemic in the Northern Cape Province in southern Africa (Laine et al., 2004). SINV and other arboviruses are in circulation every year and epidemic severity is dependent on rainfall and mosquito populations (Uejio et al., 2012).

There is little known about the risk factors and epidemiology of SINV infection in southern Africa. Cases of SINV infection are laboratory confirmed each year, but many positive cases may be missed due to SINV not always being considered as part of the diagnosis and hence samples not being submitted for testing laboratory diagnosis (Uejio et al., 2012). The Center for Emerging and Zoonotic Diseases, National Institute for Communicable Diseases (NICD), tested 3631 human samples submitted from patients with suspected arbovirus infections between 2006-2010. Between 2006-2009 there was a positive detection rate of 1.3%, with 21/1606 sera testing positive for IgM antibodies. In 2010 a higher percentage of positive samples was recorded, 10% with 208/2025 samples tested positive. The higher incidence was related to heavy rainfall and increased mosquito populations with recurrent outbreaks of Rift Valley fever virus and West Nile virus. Symptoms of SINV infection are frequently non-specific which may explain why in the absence of large outbreaks some infections are frequently missed (Storm et al., 2012).

1.8 Disease

Virus infections that lead to serious conditions such encephalitis or hemorrhagic fever are given serious attention. Viruses that cause arthritides and musculoskeletal symptoms on the other hand are not generally well known even though infections can lead to acute diseases that may progress into chronic forms (Laine et al., 2004). Although the latter is not as lethal as encephalitis, there can be problems when identifying the causative agent as the clinical signs and symptoms are non-specific. SINV is known as one of the least virulent alphaviruses with clinical signs and symptoms usually unapparent (Lloyd, 2009). SINV can cause mild to severe disease and frequently presents with fever, rash and arthritis (Manni *et al*., 2008). The virus can also cause debilitating and chronic arthralgia or polyarthralgia which presents as chronic joint inflammation usually in small joints (Heise et al., 2000). Arthritis is defined as inflammation of joints whereas arthralgia is joint pain. The association between chronic arthritis and SINV infections in Europe has been well documented and although it is assumed to be similar in southern Africa, to date there is little documented evidence of patients with chronic arthritis being caused by SINV (Malherbe *et al*., 1963).

1.9 Pathogenesis

Acute and persistent polyarthritis is a common symptom of disease caused by members of the *Alphavirus* genus including SINV, RRV and CHIKV (Harley et al., 2001) . Typically the disease presents as a febrile illness with maculopapular rash, fatigue, muscle pain and arthritis. The mechanism of *Alphavirus* induced arthritis is not clear. Recent studies using a mouse model have shown that RRV has a specific tropism for bone, joint and skeletal muscle accompanied by severe inflammation. Characterization of inflammatory infiltrates

identified inflammatory macrophages and natural killer cells, and CD4+ and CD8+ lymphocytes. The kinetics of recovery for experimentally infected mice lacking functional T and B lymphocytes suggested that the adaptive immune response does not play a critical role in development or recovery from disease (Morrison et al., 2006).

The antibody response against SINV is detectable after viremia has resolved suggesting that the humoral response is not the most significant mechanism for clearing the virus. Further studies are required to determine if the host innate response is adequate to control the infection. The innate response certainly appears significant in development of disease with RRV as there was an absence of inflammation in experimentally infected mice treated with macrophage-toxic agents (Lidbury & Mahalingam, 2000). Very few vaccines are currently available for arboviral infections and the potential for antibody dependent enhancement (ADE) of infection poses a significant problem for the development of vaccines. This underlines the importance of surveillance studies, increased awareness and diagnostic capacities and improved knowledge on the mechanisms of pathogenesis is important for development of novel drugs or safe vaccines for arboviral diseases.

RRV is capable of infecting macrophages via a natural receptor. It has also been shown to infect macrophages *in-vitro* by Fc receptor mediated ADE specifically inhibiting expression of antiviral genes and subsequently allowing increased replication of RRV (Linn *et al.*, 1996; Lidbury & Mahalingam, 2000). The role of ADE in the immunopathogenesis of disease has still to be determined. Although ADE has yet to be confirmed in patients infected with alphaviruses, the possibility that the presence of sub-neutralizing levels of antibody could have adverse effects and cause severe disease poses a significant problem in vaccine development (Tirado & Yoon, 2003). ADE has been reported for various taxonomically diverse viruses that share common features such as the ability to replicate in macrophages and establish persistent infection.
In sequential dengue virus infections, dengue hemorrhagic fever or dengue shock syndrome are frequently associated with the presence of pre-existing antibodies against a heterologous sero-type of dengue virus and this phenomenon of ADE has greatly hampered the development of a protective vaccine (Guzman & Vazquez, 2010). The mechanism of ADE is thought to be associated with an increase in viral load however in recent studies on RRV, in addition to increased virus, there was suppression of anti-viral mediators (Assunção-Miranda et al., 2010). In a recent study human macrophages were infected with SINV strain MRE16. The infection induced release of migration inhibitor factor (MIF), and induced expression of tumor necrosis factor (TNF) alpha, interleukin (Il-1) beta and Il-6.

In addition there was elevated expression of matrix metalloproteinases (MMP) 1 and 3. SINV replication in human macrophages were shown to induce release of cytokines and several MMP that have been shown to be important in joint damage. It suggests that macrophages are one of the SINV targets during infection and it helps to shed light on the mechanisms involved in the development of viral arthritis and how SINV infection can lead to painful arthralgias (Assunção-Miranda et al., 2010).

1.10 Diagnosis

SINV infections are frequently diagnosed based on clinical, epidemiological and laboratory criteria. Laboratory confirmation is essential as the SINV infections must be distinguished from various conditions that share similar clinical manifestations and are found in similar geographic locations including other alphaviruses, flaviviruses and arthritic diseases. The interpretation of laboratory results for arboviral infections is dependent on the kinetics of viremia and antibody responses. During the acute phase of illness after onset of symptoms, detection of viral nucleic acid in serum samples by reverse-transcriptase polymerase chain reaction (RT-PCR), or by isolation of the virus is important. In samples collected later, the diagnosis is confirmed by demonstration of an immune response. However depending on the incubation period, levels and duration of viremia, severity of symptoms, patients particularly with SINV infections frequently do not present for diagnosis until the virus has been cleared and antibody is detectable. Commercial assays are not readily available and most diagnostic laboratories make use of in-house reagents. Virus can be isolated from patients during the acute phase of illness using *in-vitro* techniques or mouse inoculations. However antibody testing is more useful as the period of viremia is short and patients have frequently cleared the virus when blood is submitted for testing.

The RT-PCR using conventional thermocycling or real-time PCR, provide a rapid amplification technique for detecting arboviral infections during the early stages of illness before an antibody response is demonstrable (Hoarau et al., 2010). Real-time RT-PCR assays using either SYBR green or Taqman probe-based technology targeting a specific gene sequences have been successfully developed and used for both the detection of viral RNA as well as quantitative purposes. There several different approaches for quantitative real-time PCR analysis such as using a TaqMan probe or hybridization probes. TaqMan probes make use of the 5' exonuclease activity of the *Taq* polymerase enzyme to cleave a non-extendable hybridization probe during the extension phase of the PCR reaction (Holland et al, 1991). The probe is a dual labeled molecule with a fluorofore such as 6-carboxyfluorescein (FAM) on the 5' end and 6-carboxy-tetramethyl- rhodamine

(TAMRA) or blackberry quencher on the 3' end that quenches the fluorescent emissions from the fluorofore.

During the extension phase the exonuclease degradation of the probe leads to the separation of the fluorofore from the quencher and therefore the fluorescent signal from FAM can be detected and measured (Heid et al., 1996; Moody et al., 2000). The resulting increase in fluorescent signal emission is monitored in real-time using a sequence detector. The sequence detector uses computer algorithms to compare the amount of reporter dye (FAM) emission with the quenching dye (BBQ) (Gibson et al., 1996).

Alternative to sequence specific fluorescing probes there are dsDNA dyes that can be used, but they are usually less sensitive than sequence specific probes. They are less specific as the dsDNA dyes detect all dsDNA products by intercalation and then fluorescing (Morrison et al., 1998). The SYBRGreen assays are also usually less specific than sequence-specific hybridization probes and require a melting curve analysis for detection of amplicon with Tm equivalent to a positive control. It is sometimes required for the presence of a specific amplicon product to be confirmed using agarose gel electrophoresis as well as sequencing (Drosten et al., 2002). Molecular assays can play a role during the acute stage of illness for detection of SINV viral nucleic acid. SINV can also be isolated from either blood or skin lesions of infected individuals. Kurkela et al. showed that isolating SINV from skin lesions was as effective as testing for viral RNA from the lesions. It was however shown that virus isolation from patient blood samples was five times less effective than testing for viral RNA (Kurkela et al., 2004).

Virus may be isolated using mosquito or mammalian cell cultures, or by intracerebral inoculation of day-old mice (Lennette et al., 1995). SINV will produce cytopathic effects (CPE) in various mammalian cell lines such as baby hamster kidney, HeLa and Vero cells and their derivatives, however in most laboratories Vero cells are most frequently used for routine diagnosis. The duration of viremia in a patient is relatively short and diagnosis is more frequently dependent on detection of antibody responses, but PCR can also be used for detection of viral nucleic acid during the acute phase of infection. As with other alphaviral infections like CHIKV, the presence of early antibody appears to prevent isolation of the virus. RT-PCR may have more application during acute stages as non infectious virus can be detected in the absence of infectious virus. Antigen detection assays are insensitive and unlikely to play a significant role in detecting *Alphavirus* infections except in laboratories lacking sophisticated equipment.

Hence the most frequently used method for identifying SINV infection is by means of serological assays. Most laboratories rely on haemagglutination inhibition (HI) assays for detecting antibody which does not discriminate between IgG and IgM antibody and therefore cannot distinguish between recent and previous infection. Development of ELISA and immunofluorescent assays will assist in differentiation of IgG and IgM antibody responses. Little is published on the kinetics of antibody responses in patients with SINV infections. In-house serological assays such as ELISA for the detection of SINV IgG and IgM antibodies have been developed (Brummer-Korvenkontio et al., 2002; Calisher et al., 1985; Calisher et al., 1986; Calisher et al., 1988b; Eshoo et al., 2007; Kang et al., 2012; Laine et al., 2000; Manni et al., 2008; Martin et al., 2000; Mei et al., 2012; Niklasson et al., 2008; Stanley et al., 1985).

Plaque reduction neutralization tests (PRNT) have been successfully developed and used for the detection of alphaviruses (Buckley et al., 2003; Earley et al., 1967; Johnson et al., 2011; Lloyd et al, 1983; Lopes et al, 1970; Niklasson et al., 2012). The gold standard for serology is the *in-vitro* neutralization assay and several have been developed for the detection of antibodies to alphaviruses (Brummer-Korvenkontio, 1973; Brummer-Korvenkontio et al., 2002; Calisher et al., 1985; Calisher et al., 1986; Earley et al., 1967; Hahon, 1962; Harley et al., 2001; Stanley et al., 1985; Symington et al., 1977). Although PCR is not routinely used for diagnostic purposes, it has been used extensively in surveys and for the detection of viral RNA in mosquitoes. It has its advantages for being sensitive, but is only useful during the acute stages of infection (Eshoo et al., 2007; Hernandez et al., 2004; Hörling et al., 1993; Kurkela et al., 2004; Laine et al., 2000; Pfeffer et al., 1997; Schlesinger & Weiss, 1994; Sokolova et al., 1996). Real-time PCR has the same advantages as conventional PCR, but is a more rapid technique for detection of viral nucleic acid (Buckley et al., 2003; Dash et al., 2012; Egli et al, 2001; Gibson et al, 1996; Hoarau et al., 2010; McGoldrick et al., 1999; Ramakers et al., 2003; Sane et al., 2012 Weihong & Saint, 2002).

By analogy with a related *Alphavirus*, CHIKV, a specific IgM and IgG antibody response is likely detectable within days after onset of fever. Capture ELISA have been developed for detecting IgM in patients with positive HI results. The concern is that some infections may be missed because only 40% of patients produce anti-SINV IgM antibodies during the first week of infection. It usually then results in a negative IgM ELISA result and therefore requires a second sample to establish acute SINV infection. Alternatively a second sample could be collected from the patient two weeks after the first and both could be tested for IgG antibodies to demonstrate 4 times increase in IgG antibody titer (Sane *et al.*, 2012).

An IgG antibody response likely persists for years after infection and although IgM antibody activity in many arboviral infections have a tendency to decline to undetectable levels four to six months after infection, there have been reports of persistent specific IgM antibodies over 24 months following alphaviral infections. Persistence of IgM has been associated with more severe and chronic arthritic complications in patients with RRV and CHIKV and it remains to be established if this occurs in patients with SINV in southern Africa. However persistence of IgM antibody should be considered when interpreting assay results for diagnosis of SIN infections (Malvy et al., 2009).

1.11 Effect of interferon on viral replication

Viral infection is not a single phase process, but instead includes virus entry, genome replication, transcription, translation of proteins, and the assembly and secretion of virions. The detection of one of those events may lead to the infected cell secreting interferon (IFN) alpha or beta or to activate other defense mechanisms such as apoptosis (Guidotti & Chisari, 2001).

The innate immune response is the initial host response to infection. A pathogen is recognised by effecter cells as foreign which leads to the secretion of inflammatory mediators such as cytokines and chemokines (Kawai & Akira, 2006; Malmgaard, 2004). Those inflammatory mediators lead to the attraction of more immune cells such as neutrophils, macrophages, natural killer cells and dentritic cells to the site of infection (Guidotti & Chisari, 2001). In viral infections, some of the most effective cytokines secreted are the IFN which are so named due to their ability to interfere with viral replication (Malmgaard, 2004). IFN are known for their ability to inhibit one or more of the viral replication steps as well as play an integral immunoregulatory role in both the innate and adaptive immune systems (Guidotti & Chisari, 2001; Malmgaard, 2004).

The IFN are classified as either type 1 or type 2 depending on their sequence homology and receptor complexes. The most important ones are type 1 alpha and beta IFN and type 2 gamma IFN (Malmgaard, 2004). IFN alpha has been shown to play an integral role in restricting the first steps of viral replication with viruses such as human immunodeficiency virus HIV-1 and SINV in-vitro (Shirazi & Pitha, 1992; Després et al., 1995). In a study by Hayashi 1989 the effects of IFN alpha, beta and gamma on hepatitis B virus *in-vitro* was analyzed. They found that cells treated with the three separate IFN had much lower negative strand replicative DNA present in the cells when compared with un-treated cells (Hayashi & Koike, 1989). Studies done on hepatitis C (HCV) virus show a similar tendency as seen with the DNA virus hepatitis B (HBV).

HCV has a single stranded positive sense RNA genome similar to that of SINV. It was shown that IFN gamma was able to inhibit both genomic and subgenomic HCV replicons (Frese et al., 2002).

1.12 Apoptosis

Apoptosis is a genetically controlled process that plays an important role in the regulation of homeostasis, tissue development, and the immune system by eliminating cells that are no longer useful. It also functions by eliminating cells infected by viral pathogens (Roulston et al., 1999). Apoptosis is seen to be complementary to mitosis and plays an important role in the regulation of animal cell populations. It was shown by electron microscopy that structural changes in cells due to apoptosis take part in two different stages namely the formation of apoptotic bodies and then their phagocytosis and degradation by other cells (Kerr et al., 1972). The implications of apoptosis in mammalian cells were studied by looking at nematodes. It was showed how 131 out of 1090 cells received signaling to die off at different stages of development of these nematodes demonstrating the accuracy and regulation of apoptosis. The process of apoptosis is recognized as an important mode of cellular death which can be coded for genetically or induced by infection or other agents (Elmore, 2007).

There are two common pathways for the induction of apoptosis, the extrinsic pathway, which is initiated by virus attachment to receptors, and the intrinsic pathway, which is mediated by damage to the mitochondria (Stassen et al., 2012). Once apoptosis is activated it causes various morphological changes inside the cells such as chromatin and cytoplasmic condensation and the fragmentation of the cell into membrane-bound bodies. Endonucleases are activated that cleave chromosomal DNA into fragments ranging from 180-200 base pairs. The membranes of apoptotic cells are disturbed and the cells are broken into membrane-bound apoptotic bodies that contain cytoplasma and nuclear material. That is in stark contrast to necrotic cell death in which early loss of membrane integrity is visualized which allows cytosolic spillage into surrounding tissue and random degradation of DNA (Griffin & Hardwick, 1997).

Apoptosis can be used by viruses to enhance the pathogenesis of disease by contributing to tissue damage. Apoptosis can be used by viruses to kill cells at the end of the infectious cycle, but that can limit the viral replication as the infected cells are destroyed by the immune system. Apoptosis can conversely offer various advantages that aid viruses during infections such as the packaging of cellular contents and virion progeny into membrane bound apoptotic bodies that are taken up by surrounding cells which limits the inflammatory response from the host (Roulston et al., 1999). There are several viruses that have been

shown to cause apoptosis such as herpesvirus, adenovirus, poxvirus, baculovirus, parvovirus, retrovirus, rhabdovirus, paramyxovirus, orthomyxovirus, reovirus, bunyavirus, picornavirus, and alphaviruses (Griffin & Hardwick, 1997)

1.13 Antibody dependent enhancement of disease

In 2011 it was shown that antibody dependent enhancement (ADE) of disease have the potential to influence or even modulate the severity of disease (Halstead et al., 2011). Viruses infect host cells by attaching to cell surface receptor proteins via receptor ligands on the virion surface. The humoral immune response is activated and antibodies are produced which can block the virion receptor interactions which results in reduced infectivity. ADE is the phenomena that occurs when the induced antibodies play a role in increasing the severity of infection (Takada & Kawaoka, 2003). There are numerous factors that influence the manifestation of ADE such as the configuration of the cell-antibody complexes as well as the specificity of the antibody against the pathogen epitopes. The presence of cross reactive or sub-neutralizing levels of antibodies against the pathogen is one of the requirements for ADE (Takada & Kawaoka, 2003).

The sub neutralizing antibodies interact with the virus and form immune complexes which the host is unable to destroy through complement and other pathways (Takada & Kawaoka, 2003). There are receptors on phagocytes such as monocytes and macrophages called FCR and virus-antibody complexes can bind to these receptors and are taken up by the cells (Tirado & Yoon, 2003). *In-vitro* studies have shown that ADE can be detected when sub-neutralizing levels of antibodies are present. Higher concentrations of antibodies neutralize the virus receptors. If the Fc portion of the antibody has not been blocked it is available for the enhancement of viral infection through binding of the Fc receptor on the cell surface. Viruses that rely on cellular molecules for infection of cells can infect cells via the Fc receptors. The role of Fc receptors in viral uptake explains the increase in viral uptake into the cells (Takada & Kawaoka, 2003). Enhancement of viral infection has been demonstrated with antibodies from different sources including mice, humans and other animals. IgG immunoglobulin's have been shown to be the main antibody involved in ADE, but the roles of the respective IgG subclasses are yet to be determined. There are a number of human and animal viruses depend are dependent on ADE for viral entry into macrophages such as SINV and RRV, yellow fever virus, dengue virus , human immunodeficiency virus type 1, respiratory syncytial virus, Hantavirus, Ebola virus, Getah virus, SINV, Bunyamwera virus, influenza virus, West Nile virus, Japanese encephalitis virus B, rabbitpox virus, feline infectious peritonitis virus, rabies virus, murine cytomegalovirus,

foot-and-mouth disease virus, porcine reproductive and respiratory syndrome virus, simian hemorrhagic fever virus, and Aleutian disease virus (Tirado & Yoon, 2003).

1.14 Problem identification

Cases of SINV occur annually in southern Africa. It is however unclear exactly how prevalent the virus is and how many cases go unreported. Large outbreaks of SINV occur infrequently and although there is a reference laboratory in Johannesburg offering a specific diagnostic service, most cases are likely not submitted for laboratory testing. Serological assays for detecting SINV infection are not readily available commercially and many laboratories rely on haemagglutination inhibition assays that are time consuming. Hence the development of in-house ELISA for detecting IgG antibodies in patients will be useful for both diagnosis and serological surveys.

An association with chronic joint pain and inflammation likely occurs but has not been documented in South Africa and large-scale outbreaks of SINV leading to arthritis in patients as observed in north European countries has yet to be described (Malherbe et al., 1963). Macrophages may play a significant role in the development of rheumatoid arthritis and are likely involved in the development of viral induced arthritis. SINV has been shown to infect human macrophages *in-vitro* and this could be a useful tool for investigating the role of macrophages in the pathogenesis of infection (Assunção-Miranda et al., 2010). The secretion of cytokines is the body's response to infection. Those cytokines range from inflammation inducing IFN to signal molecule IL's. The release of certain cytokines such as IFN gamma has antiviral implications as well as immunoregulatory roles. Are viruses such as SINV sensitive to IFN gamma when infecting macrophages and if so how does it affect replication? Can IFN gamma inhibit or restrict SINV replication in mammalian cells normally susceptible to SINV viral infection? The secretion of IFN and the influence of IFN on viral replication will provide knowledge regarding the mechanisms of disease. Similarly, knowledge regarding the role of programmed cell death and the pathogenesis of infection would be important for development of novel vaccines and therapies for SINV and other alphaviruses that may be more significant public health concerns.

Aims of this study

1. To develop assays that can be used for serological surveys for the detection of SINV antibodies.

- To develop ELISA and novel neutralization assays for the detection of SINV infection that could be validated for use as diagnostic and surveillance tools.

2. To develop molecular assays to determine the level of viral replication in mammalian cells for characterization of infection of mammalian cells.

- To develop methods for quantifying SINV infection *in-vitro.*

3. To characterize infection of mammalian cells with respect to IFN release and inhibition of viral infection with IFN and determine if there is evidence for occurrence of apoptosis in infected mammalian cells.

CHAPTER 2

SEROLOGICAL ASSAYS FOR THE DETECTION OF ANTIBODIES AGAINST SINDBIS VIRUS.

2.1 Introduction

SINV infections occur seasonally every year in South Africa, with an increase in the number of cases during periods of high rainfall that favor mosquito breeding. During the period from 2006-2009 the Center for Emerging and Zoonotic Diseases, NICD in Johannesburg received a total of 1606 samples submitted for tests for suspected arbovirus infections (Storm et al., 2012). A total of 21 tested positive for IgM antibody against SINV. The following year in 2010 after a particularly high rainfall the laboratory tested 2025 samples of which 208 reacted positive (Storm et al., 2012).

Although SINV does circulate in SA causing infections annually, in contrast with SINV infections in Europe, there is little information regarding its association with persistent arthritis. SA strains of SINV have been shown to be genetically similar to ones that cause outbreaks in Finland and increased awareness and greater accessibility of serological assays may help to determine a more accurate prevalence of infection in the country and its association with foreign strains (Laine et al., 2004). Serological assays for detecting antibodies against SINV are not readily available commercially and most laboratories use in-house reagents. Virus isolations and molecular tests can also be performed on specimens from patients, but only during the acute-phase of infection. The gold standard for serological assays regarding antibody detection is the *in-vitro* neutralization assay, but this is not practical for routine use. Many laboratories worldwide still use HI assays which require the use of animals for the preparation of reagents, does not distinguish between IgG and IgM and are difficult to automate for serological surveys (Sane et al., 2012).

There are various important reasons for the validation of newly developed tests, including the need for determining the diagnostic sensitivity and specificity of an assay with respect to clinical diagnosis (Greiner & Gardner, 2000). The currently recommended procedures for the validation of serological assays for diagnosis of infectious diseases are complex and subject to several limitations such as the availability of standards and representative reference sera. It is extremely important when selecting a cut-off to select sera for positive control that are strong positives and for negatives sera that have never experienced an infection form the agent in question (Paweska et al., 2003). The sensitivity of an ELISA can be determined by the serial dilution of virus or sera to ensure the lower detection limits of the assay are adequate for

detecting low levels of infections in sera. The specificity of an assay is determined by blind testing of sera which include the etiological agent in question as well as closely related samples to confirm that the assay is specific. If the newly developed assay is found to be both specific and sensitive it can be tested further to be a potential marketable assay. Additional testing includes demonstrating the repeatability of the assay by testing the same samples, stability over time after storage of reagents as well as the maximum shelf life the assay can be stored for and still be accurate (Mei et al., 2012).

ELISA is a useful alternative to HI that can provide a rapid tool for diagnostic or serological surveillance (Niklasson *et al*., 1984). ELISA are based on the principle that both antigens and antibodies can attach to surfaces through adsorption when the plates are coated. Most proteins can bind to these surfaces due to hydrophobic interactions between the plastic surface and the protein. The antigens or antibodies interact with each other to form immunocomplexes. The interactions are visualized by the addition of commercially available species specific antibodies conjugated to horseradish peroxidase, or similar, which bind to the immunocomplex and interacts with a substrate such as 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) to cause a color change that can be measured (Crowther, 1995).

For confirmation of recent infection, capture IgM ELISA, have been developed for detecting IgM antibodies that are frequently demonstrable in the first week after onset of symptoms (Sane et al., 2012). However persistent IgM over several years has been shown to occur in various *Alphavirus* infections (Niklasson et al., 2012). If this occurs for SINV infections then it may limit the usefulness of IgM in determining recent infections. Alternatively ELISA can be used for detecting IgG antibodies with confirmation of a recent infection using a second sample collected two weeks later to detect a 4-fold increase in IgG antibodies or seroconversion (Sane et al., 2012). It is likely that many cases of SINV are undiagnosed particularly with mild infections in South Africa. It is frequently not considered as a differential diagnosis for patients with arthritis.

Various methods can be used to prepare antigen for use in ELISA, including recombinant proteins, purified cell lysate antigen and mouse brain preparations (Ansari et al., 1993; Kunita et al., 2006; Roehrig, 1982). For mouse brain preparation, animals are infected intracranially, the brain tissue is harvested after the mice succumb to infection and purified using sucrose acetone extraction method. Cell lysate antigens are readily prepared using infected cell cultures (Ansari et al., 1993). *In-vitro* neutralization tests, the gold standard in serology, are performed using adherent cell lines (Konishi et al., 2010). The conventional *in-vitro*

neutralization assay is an end point assay in which CPE is monitored daily for a defined period. What happens hour to hour is not measured and monitoring is subjective. More recently real time cell analysis (RTCA) has been introduced and used for development of neutralization assays which measures cell growth in real-time (Roche, Basel, Switzerland) (Fang et al., 2011; Tian et al., 2012). The system was developed in 2004 and is based on microelectric biosensor technology to monitor cell growth and death. The system allows for the dynamic monitoring of adherent cells without the need for labeling molecules (Tian et al., 2012).

The RTCA system comprises of four components: an electronic sensor analyzer, device station (E-Plate reader), E-Plates and a control unit computer with the applied software. The device station is located inside the incubator and can be controlled to read any of the E-Plate wells individually or all 96 simultaneously. The electronic sensor analyzer on the device station takes readings for changes in electronic impedance. The control unit with software relates these changes in impedance to cell index values (CI) which represent cell growth when CI increases and cell death when the CI decreases. The results can be interpreted as raw data or converted into graphs for analysis (Tian et al., 2012). It therefore allows for the real time quantification of cell growth and death. An increase in CI due to cell growth and a decrease in CI due to cell death or CPE (Fang et al., 2011). Human sera may then be added to the plate to quantify the inhibition of CPE due to the neutralizing antibodies in the sera. The system may therefore be able to play an important in part in doing a real-time in-vitro neutralization test (Tian et al., 2012).

The aim of this chapter was to develop serological assays for detecting anti-SINV IgG antibodies for rapid screening of patient sera. In the absence of a large number of negative and positive reference sera to validate the ELISA for diagnostic purposes, we opted to compare the results using sera available with a conventional neutralization assay. The ELISA was compared with the gold standard conventional *in-vitro* neutralization assay. In addition a real-time *in-vitro* neutralization assay was investigated as a potential tool for performing neutralization assays.

2.2 Materials and methods

2.2.1 SINV strains

Two strains of SINV, EgAR339 and S.A.AR86, were used for this study. Strain EgAR339, obtained from Dr. Robert Tesh (Galveston, Texas) was originally isolated in a village called Sindbis in Egypt during 1952 (Klimstra et al., 1999). Strain S.A.AR86, obtained from Prof J Paweska (NICD, Johannesburg) was isolated from a mosquito pool in South Africa in 1952. In both the ELISA and *in-vitro* neutralization assays only the Egyptian stain of SINV was used.

2.2.2 Human serum samples

The ELISA was optimized and a cut off determined using a control panel comprising of eight serum samples collected from patients with informed consent who had no known history of infection and a sample from a patient with a confirmed infection. The immune status of patient sera used as controls was confirmed by the Centre for Emerging and Zoonotic Viruses, NICD in Johannesburg. Subsequent to optimizing the ELISA one positive and one negative sample, designated C++ and C- respectively, were included on each ELISA plate.

The C++ and C- sera were used to optimize the neutralization assays. A total of 146 samples collected in the Free State between 2006 and 2010 from patients suffering from acute febrile illness with no confirmed diagnosis were screened in the ELISA for anti-SINV IgG antibodies. Patient sera were assigned laboratory numbers (VBD) on submission to the laboratory.

2.2.3 Enzyme-linked immunoassay

2.2.3.1 Cell lysate antigen

A cell lysate antigen was prepared using SINV infected Vero 76 (Vero) cell cultures, American Type Culture Collection (ATCC) number CRL-1587. Ten T150 flasks of confluent Vero cells were infected with SINV EgAR339 strain at a multiplicity of infection (MOI) of 0.1 and adsorbed for 60 minutes. The cells were cultured in growth media, minimum essential media (MEM) (Invitrogen, Carlsbad, United States) containing 2% fetal bovine serum (FBS) (BioWhittaker™, Basel, Switzerland), 100u/ml penicillin (BioWhittaker™, Basel, Switzerland), 0.1mg/ml streptomycin (BioWhittaker™, Basel, Switzerland) and 2mM L-glutamine (BioWhittaker™, Basel, Switzerland) at 37^0C while being monitored daily for CPE.

The cells were harvested at the initial signs of occurrence of CPE and clarified for 20 minutes at 12 500 g. The cells were lysed with 1% Triton x100 in borate buffered saline (BBS) pH 8.2 and sonicated on ice at 5 minute intervals, centrifuged at 10 000 g for 20 minutes at 4^{o}C to remove the cell debris and the supernatant fluid (SNF) stored for use as antigen. Mock antigens were prepared similarly, but from un-infected cells. The SINV cell lysate antigen and mock antigen were aliquoted and stored at -80^{o}C.

2.2.3.2 Indirect ELISA for detection of IgG antibodies in patient sera

The ELISA was performed in 96 well Polysorb plates (Nunc, Koeln, Germany). The optimal concentration of reagents such as coating antigen, primary antibody and detection antibody were determined by checkerboard titrations. Throughout the assay, reagent volumes of 100 µl were used unless specified otherwise, the diluent for reagents was phosphate buffered saline (PBS) containing 2% skimmed milk powder (Elite, Johannesburg, South Africa), incubations were performed for 1 hour at 37^{0}C, wells were blocked after coating with 200µl PBS containing 10% skimmed milk powder and plates were washed thrice with PBS containing 0.1% Tween 20 (Calbiochem, Billerica, United States).

Briefly, the optimal working dilutions for mock and SINV cell lysate antigen were determined based on checkerboard titrations and protein concentrations. A 96 well microtiter Polysorb plate was coated overnight at 4^{0}C with SINV cell lysate antigen diluted 1:1000. Plates were coated similarly with mock antigen optimally diluted at 1:100. After the plates were blocked, human serum samples and controls, diluted 1:100, were added to SINV antigen coated wells and to mock antigen coated wells as illustrated in Figure 1. The plates were incubated, washed and anti-human IgG horse radish peroxidise (HRPO) (Zymed Laboratories, Cambridge, United Kingdom) diluted 1:4000 was added to each well.

After further incubation and washing, positive reactors were visualized using the substrate ABTS (Kirkegaard and Perry Laboratories, Gaithersburg, United States). The plates were incubated at room temperature (22-25^{0}C) in the dark for 20 minutes and the optical density (OD) values were read at 405nm.The net OD value for both test and control sera were determined by subtracting the OD for each sample with mock antigen from sera with SINV cell lysate antigen. Figure 2.1 shows a layout of the plates where the dark gray areas represent SINV cell lysate antigen and light gray mock antigen. The numbers 1-32 represent the patient sera samples tested on each plate, C++ the positive controls and C- the negative control.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	2	3	4	5	6	7	8	C++	C-		
B	9	10	11	12	13	14	15	16	C++	C-		
C	17	18	19	20	21	22	23	24				
D	25	26	27	28	29	30	31	32				
E	1	2	3	4	5	6	7	8	C++	C-		
F	9	10	11	12	13	14	15	16	C++	C-		
G	17	18	19	20	21	22	23	24				
H	25	26	27	28	29	30	31	32				

Sindbis cell-lysate antigen

Mock antigen

C++ positive control

C- Negative control

Figure 2.1. Plate layout for IgG ELISA using cell-lysate antigens.

2.2.3.3 Selection of cut-off values

Cut off values are calculated to separate positive results from negative results and were determined using a panel of eight negative control sera. The negative panel of samples were tested in duplicate and sample net OD values averaged for each sample to determine the cut-off value. The net OD values were determined for each sample as follows: net OD=OD in wells with SINV cell lysate antigen minus OD in wells with mock antigen. A cut off value was determined from the mean net OD of the negative panel plus 3 standard deviations (SD)(Woo et al., 2001).

2.2.3.4 Statistical analysis of data Normalization of data

OD values are absolute measurements that are influenced by variables such as temperature. To account for variability, results can be expressed as a function of the reactivity of control samples included in each run. Therefore absorbance or OD values were expressed as percentage positive (PP) relative to a high positive control serum. The following statistical calculations were used:

- Positive to negative signal ratio: Pos/Neg ratio = ODC++/ODC-
- Net OD = OD in wells with virus antigen minus OD in wells with control antigen
- Percent positivity (PP): PP = (Mean net OD of test sample / mean net OD of C++) x 100

2.2.4 End-point in-vitro neutralization assay

The endpoint is the dilution at which a certain proportion of infected cells show CPE and as there are too many interfering factors to use 100% endpoint, it is more common to compute 50% endpoints using estimates calculated using the methods devised by Reed-Muench or Kärber (Lynnette et al., 1995). Tissue culture infectious dose $_{50}$ ($TCID_{50}$) represents the dose that will cause cytopathic changes in ≥50% of the inoculated cells.

2.2.4.1 Determination of tissue culture infectious dose $_{50}$ of the virus

The $TCID_{50}$ of a stock of cell culture derived SINV (EgAR399) was determined by preparing ten-fold dilutions of virus stock using serum free growth media from 10^{-1}-10^{-7}. A 96 well tissue culture plate was seeded with Vero cells at 1 x 10^4 cells/100µl per well using growth media. A 50 µl aliquot of each virus dilution was added to wells A to G, and repeated across the plate from columns 1 to 6. A 50 µl aliquot of growth media containing virus was added to row H. A 50 µl aliquot of growth media containing 6% FBS was added to each well to a final volume of 200 µl. The plate was then incubated at 37^0C in a carbon dioxide (CO_2) incubator and CPE read daily for 3 days. The $TCID_{50}$ was calculated according to the Kärber method (Lynnette et al., 1995). The formula for the Kärber method were as seen in Figure 2.2 below.

Negative Logarithm (log) of $TCID_{50}$ endpoint titer = Negative log of highest virus dilution concentration used - sum of % cell death at each dilution / 100 - 0.5 x log of dilution

Figure 2.2. Kärber method calculation formula.

2.2.4.2 Virus neutralization protocol

Neutralization antibody titers were determined by testing two fold dilutions of sera against a standard dose of SINV. The antibody titer is expressed as the reciprocal of the highest dilution that is able to neutralize the virus, and therefore inhibit CPE in the cells. The test sera were tested in duplicate and diluted two-fold in serum free growth media from 1:4 - 1:256.

Briefly, a 50µl aliquot of each dilution of sera was added to each well in rows A-G and 50µl virus diluted to contain 100$TCID_{50}$ per 50 µl. The plate was incubated at 37^oC in a CO_2 incubator for 60 minutes with gentle shaking of the plate twice during the incubation period. A 100ul aliquot of cell suspension in growth media

containing 6% FBS (1x 10^4 cells) was added to each well in rows A-H and incubated at 37^{o}C in a humidified CO_2 incubator. CPE was read daily over a period of 3 days.

The neutralization titer was determined on the third day as the reciprocal of the highest dilution of test sera at which CPE was inhibited.

2.2.5 Neutralization assay by real time cell analysis

Neutralization assays were performed using the eXcelligence RTCA system to determine the end point. The RTCA apparatus was initially calibrated to obtain background blank readings by using 100µl aliquots of growth media/well into each well of an E-Plate (Roche, Basel, Switzerland). Changes in the electronic impedance are recorded as CI values which represent cell growth when CI increases and cell death when the CI decreases. CI values were recorded at hourly intervals for 48 to 120 hours depending on the experiment.

2.2.5.1 Real-time monitoring of cell proliferation and virus-induced cytopathogenicity

CI standard curves for cell proliferation and for virus-induced cytopathogenicity were first prepared. Briefly, a 96 well E-Plate was seeded with 48 replicates of Vero cells at a cell density of 1 x 10^4 cells per well. For cytopathogenicity, SINV was diluted ten-fold from 10^0 to 10^{-6} and each dilution added in six replicates (columns 1-6) in rows A to G. The CI values for the cell proliferation assay were measured for 45 hours whereas the virus-induced cytopathogenicity was measured over a period of 120 hours.

$TCID_{50}$ was determined as described in section 2.2.4.1 using an E-plate and monitoring CPE as a decrease in CI value. The $TCID_{50}$ was calculated using the eXcelligence software. Positive and negative controls were prepared to visualize the effect of anti-SINV antibodies in sera on virus induced cytopathogenicity.

Neutralization assays were performed as described in section 2.2.4.2 monitoring CPE as a decrease in CI. There were five human sera selected that tested positive in both the ELISA and conventional neutralization assay and were tested for neutralizing antibody using the RTCA system.

2.3 Results

2.3.1 ELISA

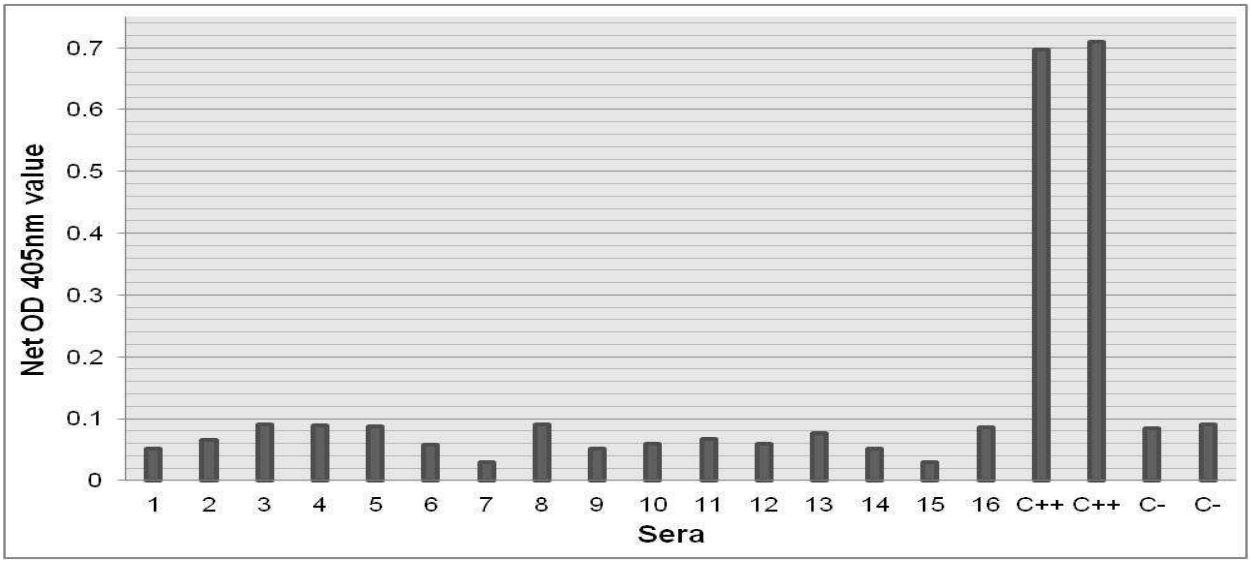

Figure 2.3. The mean net OD values for the negative control panel samples (1- 16) with two C++ positive controls and two C- Negative controls.

The negative panel was tested in a total of 18 replicates as shown in Figure 2.3 and the net OD values of the negative panel as well as the C- values tested on each plate were used to determine the cut off value as shown in Table 2.1.

Table 2.1. Cut off value determined from negative panel of sera

Serum	[a]n	[b]Mean	[c]SD	Cut off	
				[d]OD	[e]PP
Negative panel (OD)	18	0.0723	0.0275	0.1548	
Negative panel (PP)	18	9.855	3.1835		19.41%

[a]n= number of replicates

[b]Mean= mean NET OD of replicates

[c]SD= standard deviation

[d]OD= optical density

[e]PP= percent positivity of high positive control serum

The cut off value is the value that separates the positive from the negative values and was determined as the net mean OD plus 3 standard deviations (SD). The net mean OD was calculated as 0.0723 with a SD of 0.0275. Therefore the cut off value was calculated as 0.1548.

2.3.1.1 Interassay variability of cell lysate antigen ELISA

The inter assay variablilty is determined by comparing the C++ and C- ratios obtained in repeated assays performed over a period of 6 days. The net OD for C++ was divided by the net OD of C- on each given day and,the signal to noise ratio determined and compared.

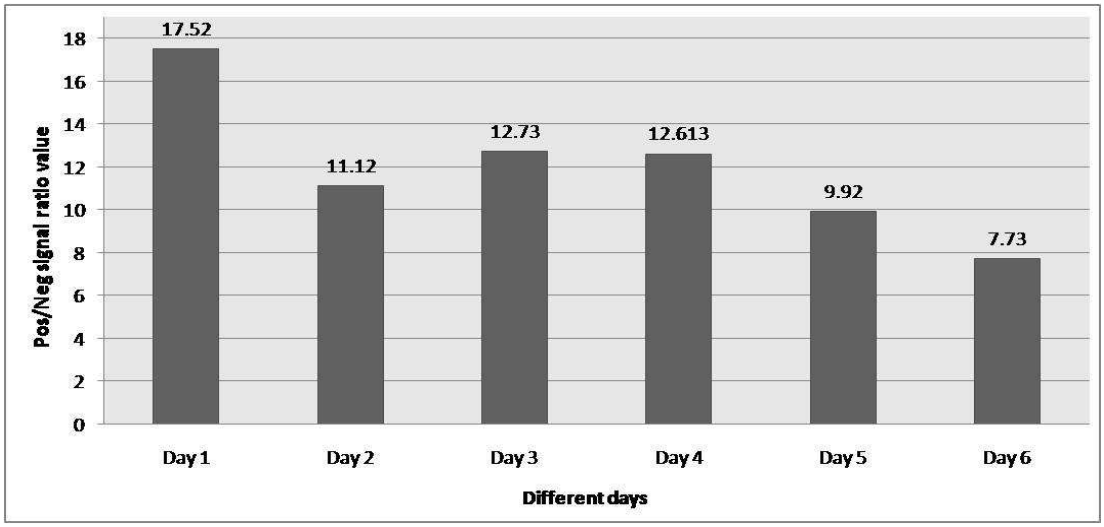

Figure 2.4. Positive to negative signal ratios of net OD values obtained for C++ and C- samples over six days.

The positive and negative signal ratios on day one to day six are shown in Figure 2.4. Comparing the signal to noise ratios indicates whether there was a uniform level of background activity each day that the test samples were tested. Variability in the signal to noise ratio was observed from day 1 to day 6. The variability shows the importance of aliquoting reagents and the use of fresh aliquots when samples are analyzed to help minimize variability due to possible reagent degredation.

2.3.1.2 Percentage positivity

Due to the variability the percentage positivity (PP) for sera was determined to correct for day to day variances. There were 146 samples tested in the ELISA, but only 43 available for testing in the *in-vitro* neutralization assay. The PP values were determined for these values and shown in Figure 2.5.

There were a total of 22 samples that tested positive using the PP cut off of 19.41 and 21 that tested negative. The PP values account for variability, but the net OD readings for each sample are absolute values and can also be used to distinguish positive and negative results. Figure 2.6 shows the net OD values for sera with a cut off at an OD of 0.155.

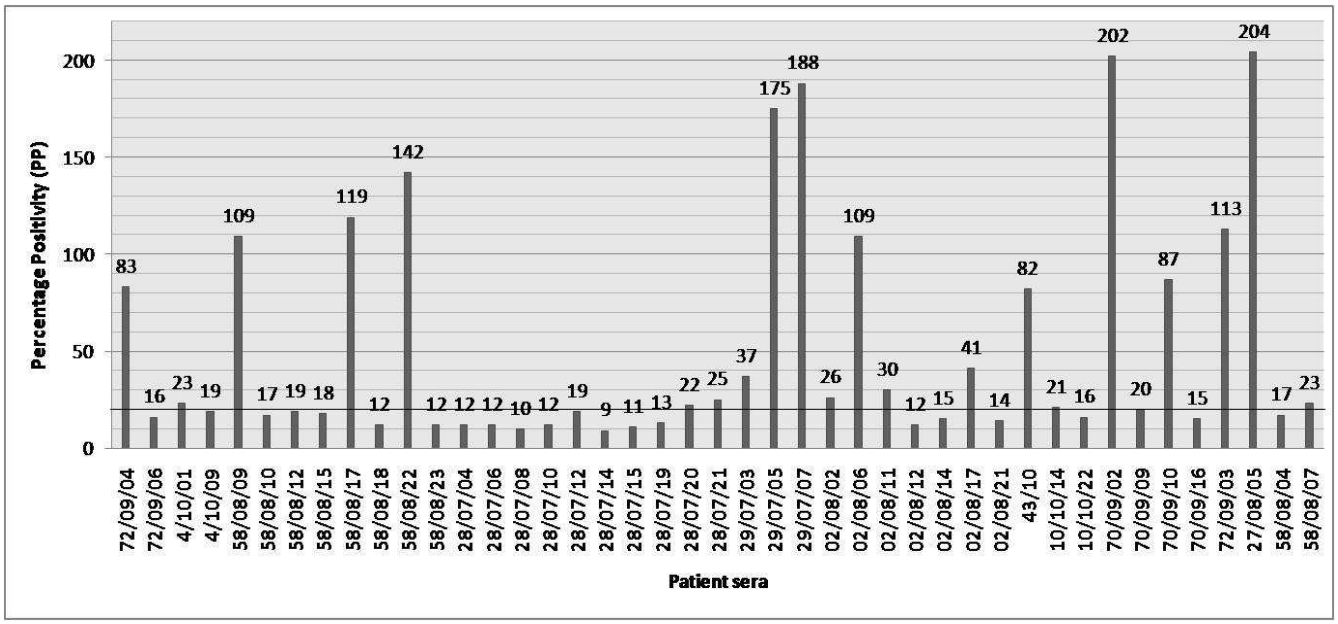

Figure 2.5. PP values for 43 samples tested in both the ELISA and neutralization assay with the cut off at 19.41.

Sera, where available, were tested in the gold standard *in-vitro* neutralization assay for comparison. Figure 2.6 shows the net OD values for sera tested in both the in house ELISA and in-vitro neutralization assays. The first 12 samples with high OD readings were grouped together to easier differentiate between strong and weak positive OD values and not the order in which they were tested. There were a total of 22 samples that tested positive for anti-SINV IgG antibodies using a cut off OD of 0.155 that are shown in Figure 2.6. In total there were 27 positive reactors from the 146 sera tested, but five of them were unavailable for testing in the gold standard and were therefore excluded from Figure 2.6.

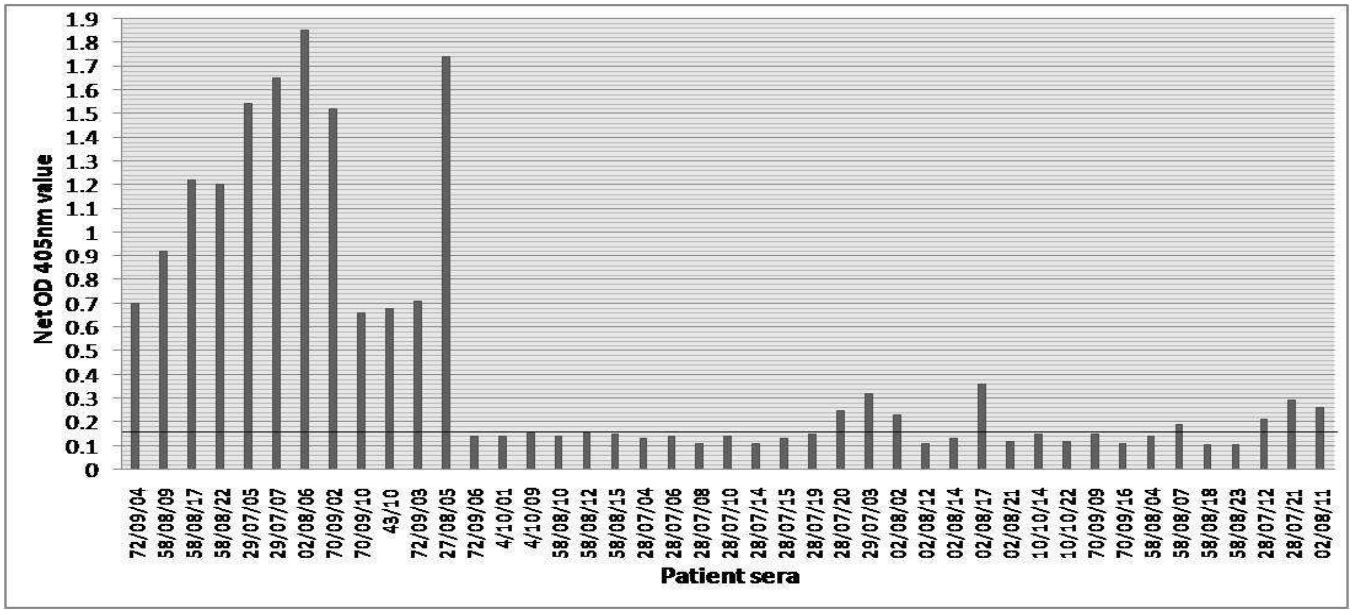

Figure 2.6. Net OD values for 43 samples tested in both ELISA and *in-vitro* neutralization test.

2.3.2 In-vitro neutralization assay

A total of 43 samples were tested for neutralizing antibodies against SINV. The $TCID_{50}$ of SINV was determined by infection of cells with 10-fold dilutions of virus performed in 6 replicates. It was determined for both EgAR339 and S.A.AR86 strains. For the EgAR339 strain the $TCID_{50}$ was calculated as 10^5. Hence in subsequent neutralization assays stock virus was diluted 10^{-3} (100 x $TCID_{50}$). For S.A.AR86 strain the $TCID_{50}$ was calculated as 10^6. Hence in subsequent neutralization assays stock virus was diluted 10^{-4} (100 x $TCID_{50}$).

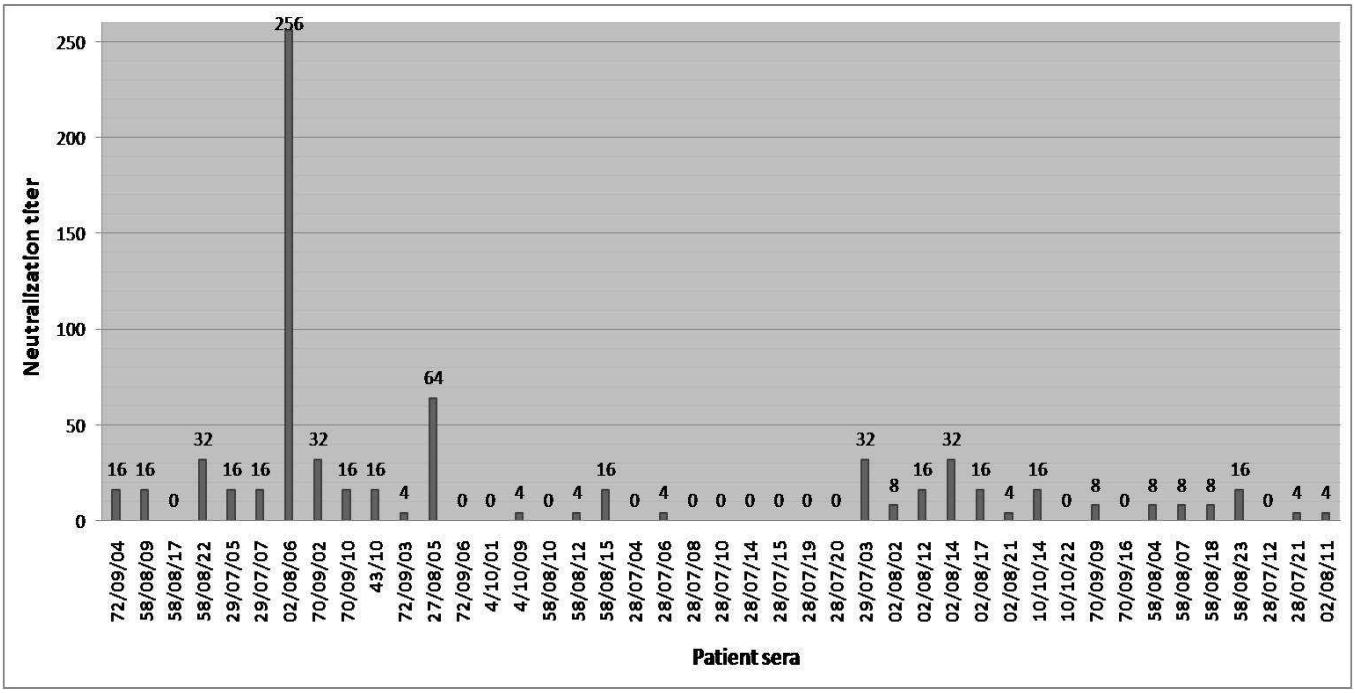

Figure 2.7. Neutralization titers for 43 samples that were tested in the in-vitro neutralization assay.

There were 43 samples tested for the presence of neutralizing antibodies against SINV. Specimens with a titer of 1≥ were considered positive. A total of 29 sera tested positive for neutralizing antibodies with titers ranging from 4-256 as seen in Figure 2.7.

Specimens with a titer of ≥ 1:4 were considered positive. A total of 14 sera tested negative for neutralizing antibodies. The golden standard is used for validation of new serological assays. The newly developed ELISA was used to screen patient sera and the sera then tested in a *in-vitro* neutralization assay. The comparison of the two assays is shown in Table 2.2. A total of 20/22 samples tested positive in both assays and 12 tested negative in both.

2.3.3 Correlation between ELISA and gold standard

The correlation between the assays were determined by looking at both methods of differentiating positive from negative results namely PP values and net OD.

Table 2.2. Summary of samples tested in the ELISA and in-vitro neutralization assays and ELISA positive determined by PP values.

	+ NEUT	- NEUT
+ ELISA	19	3
- ELISA	10	11

The sensitivity of the ELISA was determined using the data in Table 2.2 and the calculations showed below.

Sensitivity= True positives/ True positives + false negatives

= 19/ 19+10

= 65.51%

The comparison of the ELISA and the gold standard showed a specificity of 65.51%. There was a good correlation between the assays for the detection of true positive samples with 19/22 testing positive in both assays, however there were 10 sera that tested negative by ELISA.

The specificity was also calculated using the data in Table 2.2 by using the formula below.

Specificity= True negatives/ True negatives + False positives

= 11/11+3

= 78.57%

The specificity was shown to be 78.57% for the ELISA when using the PP values to discriminate between positive and negative samples.

Table 2.3. Summary of samples tested in the ELISA and in-vitro neutralization assays and ELISA positives calculated using net OD values.

	+ NEUT	- NEUT
+ ELISA	20	2
- ELISA	9	12

The sensitivity of the ELISA was determined using the data in Table 2.3 and the calculations showed below.

Sensitivity= True positives/ True positives + false negatives

= 20/ 20+9

= 68.9%

The comparison of the in house ELISA and the gold standard showed a specificity of 68.9%. There was a good correlation between the assays for the detection of true positive samples with 20/22 testing positive in both assays, however there were 9 sera that tested negative by ELISA.

The specificity was also calculated using the data in Table 2.3 by using the formula below.

Specificity= True negatives/ True negatives + False positives

= 12/12+2

= 85.71%

2.3.4 Real time *in-vitro* neutralization assay

A real-time cell analysis system was investigated to demonstrate its potential as a more accurate and rapid tool for determining virus neutralization titers then the conventional assay that relies on the laborious microscopic reading of plates.

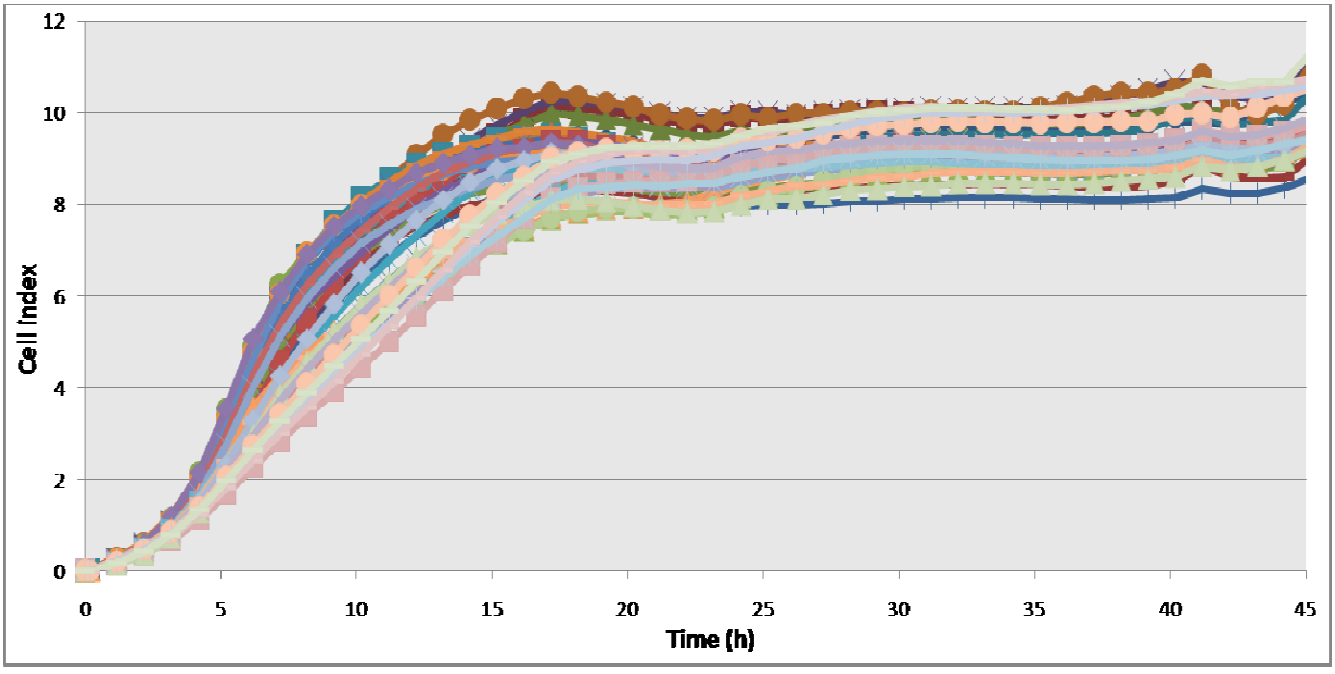

Figure 2.8. Growth curve of 48 Vero cell replicates on an E-plate monitored for 45 hours to show demonstrate a real-time increase in cell density.

The Excelligence RTCA system was used to monitor and measure the increase in cell density over 45 hours. An increase in cell density was measured as an increase of CI which was measured hourly. Figure 2.8 showed the exponential growth of Vero cells (48 replicates) from approximately 5-15 hours, the growth-rate reached a plateau and the uninfected cells reached the stationary growth phase which it maintained until 45 hours.

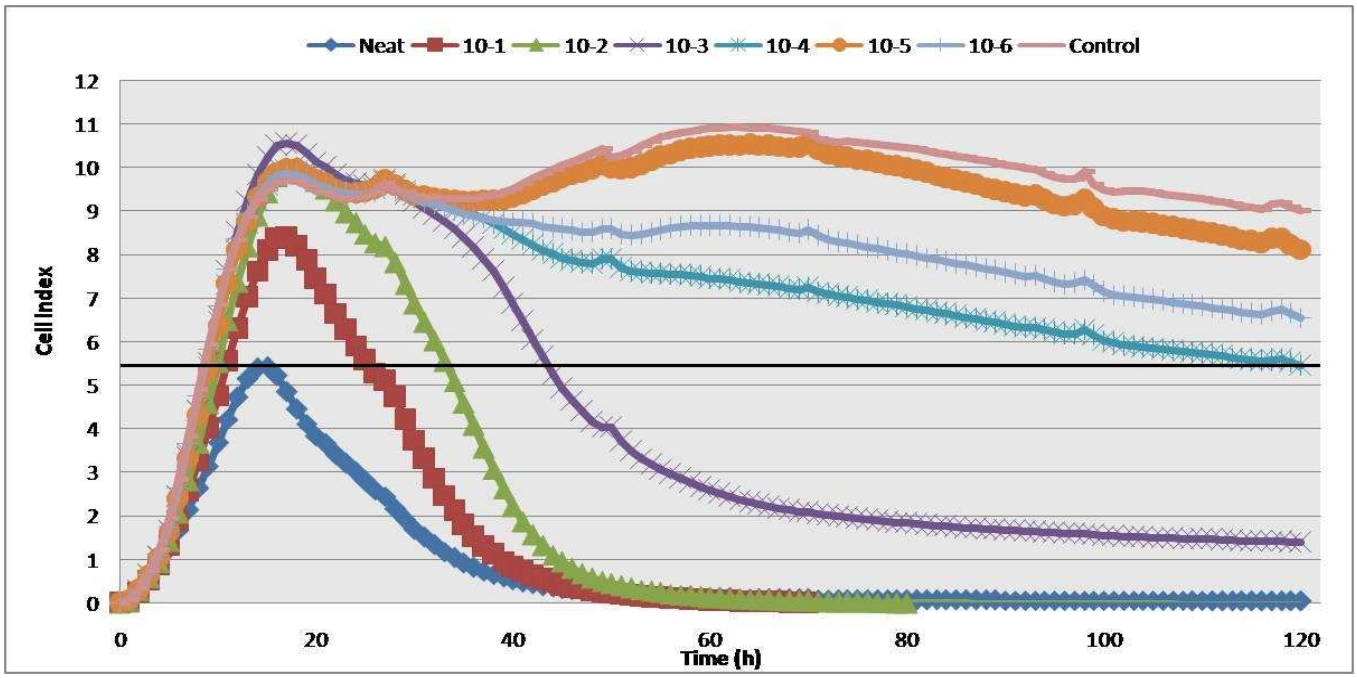

Figure 2.9. Vero cells infected with SINV diluted 10 fold dilutions to determine the virus $TCID_{50}$.

Figure 2.9 shows virus-induced cytopathic effects in Vero cells that were infected with different concentrations of SINV ranging from 10^{0} to 10^{-6}. The infections were performed in six replicates and averaged for each dilution. As observed in Figure 2.9 there an initial increase in the CI with growth of the cells occurring prior to the onset of CPE. From 20 hours the CI value declined to below the cut off within 30 hours. At 70 hours virus induced total cytopathic effects were observed in 3/7. The mean CI value in wells inoculated with virus diluted 10^{-3} decreased to 1.7 after 70 hours.

The dilutions were used to determine the virus $TCID_{50}$ using the Roche Excelligence software algorithms for calculating the $TCID_{50}$. The software allows for time-dependent physiological (effect concentrations) EC_{50} values which is equivalent to the $TCID_{50}$ calculated doing a conventional neutralization assay using the conventional Karbar method. Both the conventional neutralization assay as well as the real-time RTCA system determined the EC_{50} as a 10^{-5} dilution of the stock virus.

Quantification of neutralizing antibody titer was determined by using two-fold dilutions of sera and determining at which dilution a CI value of at least 50% of the maximum CI was detected. The maximum CI value as determined in Figure 2.9 was 10.84 and therefore the minimum CI for cells that recovered from infection to show less than 50% CPE was 5.42. CI values less than 5.42 suggest ≥ 50% CPE cell death.

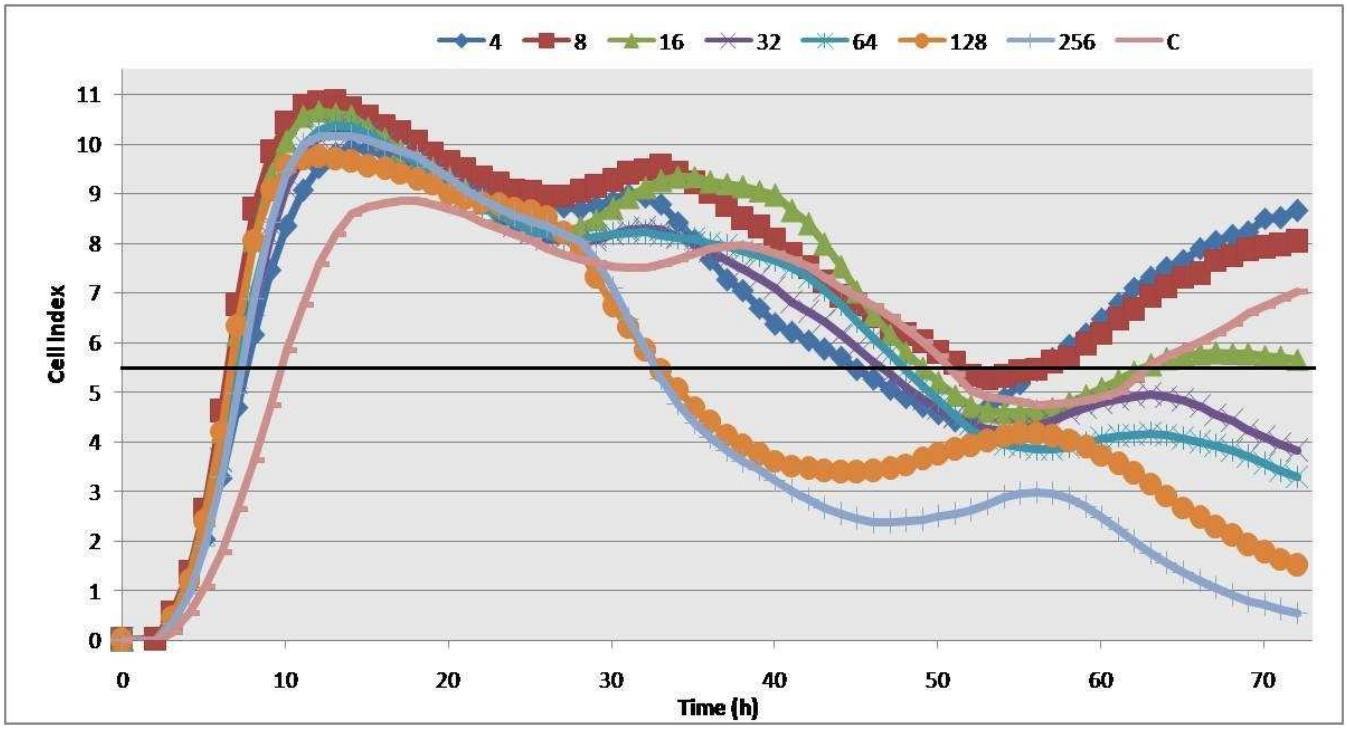

Figure 2.10. Control for RTCA system using positive serum to neutralize the virus. The numbers 4-256 in the legend refers to the dilution of positive serum diluted two fold from 1:4 to 1:256 and C was the cell control that was only Vero cells without sera or virus.

Figure 2.10 shows the outcome of a neutralization assay in which positive human control sera diluted 1:4 to 1:256 were added to Vero cells infected with 100 $TCID_{50}$ of SINV. The CI value initially increases in the presence of virus, likely representing attachment and replication of the cells prior to development of CPE caused by the virus. From 15-20 hours post infection the CI values start to decline in all wells. Using a CI value of 5.42 as the cut off to differentiate between well exhibiting ≥ 50% CPE and < 50% CPE. A neutralizing antibody titer of 16 was determined for the control sample.

At higher dilutions such as 1:32 – 1:256 the antibodies seemed insufficient in neutralizing the virus and complete virus induced cytopathogenesis was observed as could be seen by the CI that continued to decline.

The neutralization titer of the human sera was determined as the reciprocal of the highest dilution inhibiting ≥ 50% CPE. An antibody titer of 16 showed to be the highest dilution capable of inhibiting ≥ 50% CPE.

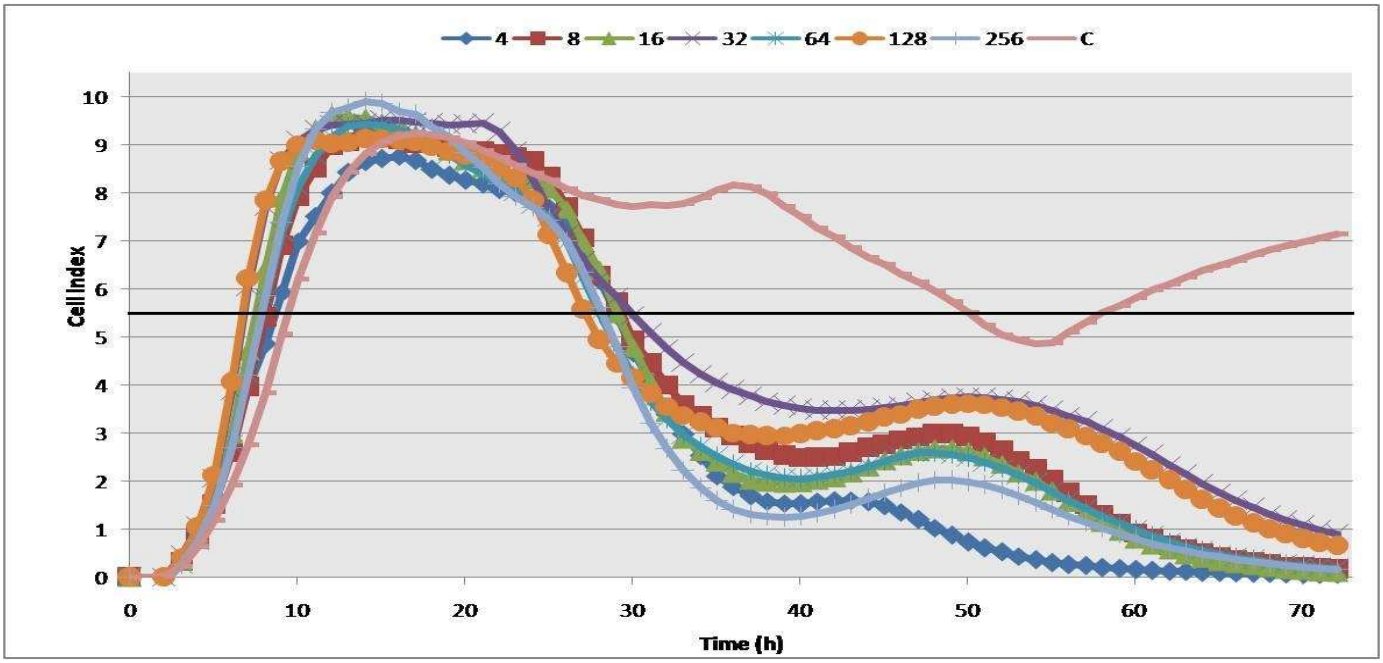

Figure 2.11. Negative control sera in the RTCA system.

Figure 2.11 shows the outcome of a neutralization assay in which negative human control sera diluted 1:4 to 1:256 were added to Vero cells infected with 100 $TCID_{50}$ of SINV. In the absence of neutralizing antibody the CI value declined to below the cut off after 30 hours to less than one between 60 and 70 hours.

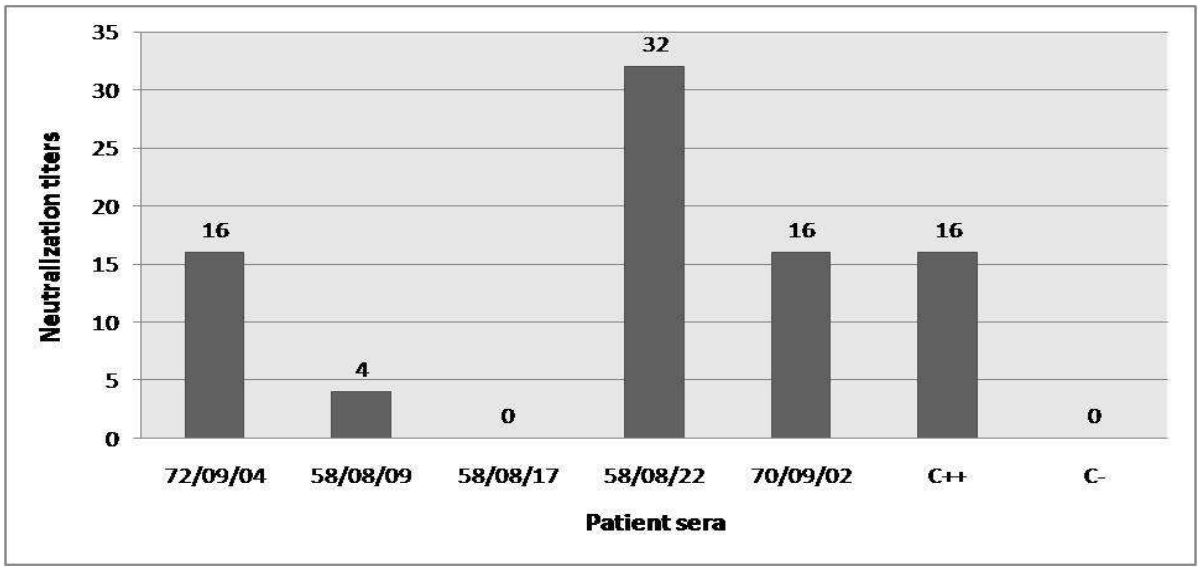

Figure 2.12. RTCA neutralization titers for patient sera samples tested with the real-time in-vitro neutralization system.

There were five serum samples tested in both the in-house ELISA and the conventional *in-vitro* neutralization test that were available for testing in the real time RTCA system. Figure 2.12 showed the neutralizing antibody titers obtained from the real time *in-vitro* neutralization assay. Positive titers ranged from 4-32 with one negative sample. Table 2.4 shows the results obtained using the ELISA and neutralization assays. Neutralizing antibodies were demonstrated in 4/5 sera using the real time *in-vitro* neutralization assay. ELISA have the ability to detect both serologically reactive and neutralizing antibodies whereas *in-vitro* neutralization assays only detects neutralizing antibodies. Sample VBD58/08/17 was serologically reactive in the ELISA, but neutralizing antibodies were not detected.

Table 2.4. Comparison of Net ELISA OD's, end point neutralization titers and real-time neutralization titers.

Sample VBD number	Net ELISA OD	Neutralization titer	RTCA neutralization assay
72/09/04	0.699	16	16
58/08/09	0.919	16	4
58/08/17	1.129	0	0
58/08/22	1.196	32	32
70/09/02	1.518	32	16
C++	0.729	16	16
C-	0.08	0	0

2.4 Summary

This chapter described the development and optimization of an ELISA using cell lysate antigen for detecting anti-SINV IgG antibodies in human sera. The assay was optimized and used to screen 146 patient samples for antibodies against SINV. The samples were submitted from patients with acute febrile disease and no confirmed diagnosis. There were 27/146 samples that tested positive for anti-SINV antibodies. Only 22/27 samples were available for testing in the gold standard so only they were included in the comparative analysis. In total 43 samples were tested in both the ELISA and the gold standard *in-vitro* neutralization assay for comparison. A total of 29 out of 43 samples tested positive for neutralizing antibodies against SINV. The *in-vitro* neutralization assay is the gold standard for serology. Analysis and comparison of the results obtained using the in-house ELISA and the neutralization assay indicated that the sensitivity of the ELISA was 68.9% suggesting that the low titered samples are likely too low for detection. Alternatively the significance of a neutralization antibody titer of 1:4 could be questionable.

Human sera at low dilutions may contain inhibitory factors preventing replication of the virus. The specificity of the in house ELISA was 78.57 - 85.71% depending on the use of the PP or OD values to determine positive and negative samples. The relatively low sensitivity and specificity of the in house ELISA limits application as a diagnostic tool. Testing of a significantly larger panel of negative samples and reference samples would help to optimize the ELISA. Comparison with a frequently used diagnostic assay such as the HI would provide information regarding sensitivity of the assay particularly for diagnostic use. Neutralizing antibody are frequently demonstratable after infection and hence not a useful assay for early diagnosis. Hence taking into consideration the limitations of the validation based on the cohort of sera, positive and negative, available for screening and lack of an additional diagnostic assay such as HI for comparison of results, the in house ELISA could have application in serological surveys with confirmation of positive results.

The samples were submitted for an unrelated study during 2006 and 2010 from patients with suspected tick bite fever, no confirmed diagnosis and presenting with symptoms similar to SINV infections. In addition, a real-time neutralization assay was investigated as a potential tool for performing neutralization assays with increased sensitivity and shorter reaction times as a result of constant real-time monitoring. Initially growth curves were established using uninfected and infected Vero cells. The $TCID_{50}$ equivalent referred to as the EC_{50} was determined as 10^{-5} for the EgAR339 strain of SINV. Five samples were tested using the real time

neutralization assay and there was a 100% correlation between the results obtained using the traditional and real time in-vitro neutralization assays. There were two samples that differed regarding the neutralization titer with both being slightly higher in the traditional neutralization assay. The difference could be attributed to the objectivity of manually reading CPE under the microscope whereas the real time assay relies on accurate impedance readings and software analysis to construct graphs that can be read and interpreted much easier.

CHAPTER 3

MOLECULAR ASSAYS FOR CHARACTERIZATION OF INFECTION OF MAMMALIAN CELLS WITH SINV

3.1 Introductions

As discussed in Chapter 1, acute and persistent polyarthritis is a common symptom of disease caused by members of the *Alphavirus* genus including SINV, RRV and CHIKV. Symptoms such as joint and muscle pain can persist for anytime from weeks to months (Morrison et al., 2006). The mechanism of SINV induced arthritis in humans is not clear although animal models have been used extensively. In mice infected with SINV it has been shown to have a high affinity for bone, joint and connective tissue which was accompanied by severe inflammation caused by the of release inflammatory cytokines that contributed to tissue destruction (Assunção-Miranda et al., 2010). RRV was used to study the kinetics of recovery in mice lacking B and T lymphocytes. It was shown that the adaptive immune response does not play a critical role in the recovery from disease (Morrison et al., 2006). In another mouse animal model the innate immune response has been shown to be significant in the development of disease with RRV as there was severe inflammation (Lidbury & Mahalingam, 2000).

RRV and SINV have previously been shown to be capable of infecting macrophages via a natural receptor. RRV has also been shown to infect macrophages *in-vitro* by Fc receptor mediated ADE specifically inhibiting expression of antiviral genes and subsequently allowing increased replication of RRV (Lidbury & Mahalingam, 2000; Linn *et al.*, 1996). The role of ADE in the immunopathogenesis of disease has still to be determined. Characterization of SINV infection of macrophages could help to determine the role of infected macrophages in the pathogenesis of arthritic disease.

Infection of mammalian cells with viruses can be determined using immunofluorescence, observing cells for CPE if the virus induces pathogenic changes, or using molecular assays to determine changes in viral load as evidence of viral replication. Real-time molecular assays have several advantages over conventional PCR techniques. TaqMan® real-time PCR is more rapid than conventional PCR and is less prone to amplified DNA contamination when tubes are opened. Conventional PCR also requires additional post and pre incubation steps than TaqMan® real time PCR. The development of TaqMan® technology also allows for not only detection but quantitative analysis. The principle of TaqMan® can be explained by the cleavage of a dual-labeled fluorogenic probe by exonuclease activity of the Taq DNA polymerase. The assay requires the

annealing of a forward and reverse primer as well as the probe which means that non-specifically amplified sequences will not lead to fluorescence (Egli et al., 2001).

The probes are fluorescently labeled on the 5' end and have a quencher on the 3' end. As the name suggests the quencher quenches the fluorescent signal from the fluorofore and no fluorescence is emitted. In the real-time reaction the probe binds to the target and 5' to 3' exonuclease activity of Taq DNA polymerase causes cleavage of the probe and allows fluorescence to be detected by the LightCycler. It allows for accurate quantification of viral nucleic acids as it correlates the required number of amplification cycles to the amount of template in the reaction. More template equals less cycles needed for detection and therefore a higher copy number is detected (Heim et al., 2003).

The replication of alphaviruses are dependent on both negative and positive strand RNA synthesis. The genome is positive strand and serves directly as mRNA and it encodes for the non-structural proteins (Strauss & Strauss, 1994). Real-time single stranded quantitative analysis can be a valuable tool for detecting and quantifying replicating virus. The single strand analysis refers to positive or negative strand cDNA (Plaskon et al., 2009). The negative strand RNA has to be synthesized early in infection and encodes for the structural proteins (Strauss & Strauss, 1994). As negative strand RNA is only synthesized during viral replication it can be used as a potential target for determining whether the virus is replicating. A quantitative real-time PCR has been successfully used to amplify strand specific viral RNA for foot-and-mouth disease virus as well as o'nyong-nyong virus (Gu et al., 2007; Plaskon et al., 2009). If the quantitative real-time PCR measures an increase of negative strand RNA over time it therefore suggests active viral replication. The accurate quantification of either positive or negative strand specific quantification assays depends on the assumption that the amount of cDNA detected by real-time quantification closely approximates the amount of specific viral RNA in the original sample (Plaskon et al., 2009).

One of the aims of this project was initially to characterize SINV infection of human derived macrophages and other mammalian cell lines. To confirm infection and replication of SINV in infected cell lines, molecular assays were developed to measure viral load.

3.2 Materials and Methods

3.2.1 Primers

A primer pair that amplified a conserved 181 base pair (bp) region of the SINV nsp2 gene encoding the nsp2 protein was identified by obtaining the genetic sequences from GenBank Accession numbers U38305, NC_001547, J02363, and aligning those sequences using ClustalX 2.0.11 as shown in Appendix 6. The oligonucleotide sequences of the primers are shown in Table 3.1. The primers were selected to contain 40%- 60% G/C content with similar melting temperature (Tm) values, which were calculated using the website www.promega.com/biomath.

Table 3.1. The nucleotide sequences of both forward and reverse primers for amplification of a 181bp region of the NSP2 gene.

Primer	Nucleotide sequence (5'-3')	Genomic Position (genomic position from NSP2 gene 1st nucleotide (5'-3')	G/C content	Tm
Sin nsp2 F	GACAGTATATCGTTGTCTCGCC	65-86	50%	50^0C
Sin nsp2 R	GAATTCTGGCCATGGTACGGC	245-225	57.14%	51^0C

The primers were designated Sin nsp2 F (forward) and Sin nsp2 R (reverse) with lengths of 22 and 21 bp respectively.

3.2.2 Reverse transcription and amplification of viral RNA

The Titan™ One Tube RT-PCR System (Roche, Basel, Switzerland) was utilized for amplifying RNA to prepare a control for quantitating viral replication. The Titan system contains Avian Myeloblastosis Virus (AMV) reverse transcriptase for complementary DNA (cDNA) synthesis and Expand High Fidelity enzymes for the amplification process. The reaction mixtures were set up as follows: 0.75µl of a 20 picomolar solution of each primer, 0.25µl (10 U/µl) of protector RNase inhibitor (Roche, Basel, Switzerland) , 2.5µl of 5mM dithiothreitol, 10µl of 5x RT-PCR buffer (7.5mM magnesium chloride ($MgCl_2$) and dimethyl sulfoxide), 1µl enzyme mix (5U/µl), 2µl template RNA. RNA extracted from SINV infected cells was used as the template. A negative control reaction was set up in the same way without the addition of template RNA.

The reactions were cycled in a Perkin Elmer thermocycler (Gene Amp PCR system 2400, Applied Biosystems, Foster City, United States) at 50^0C for 30min followed by 30 cycles of denaturation at 94^0C for 10s,

annealing at 46^{0}C for 30s, elongation at 68^{0}C for 45s, and one final elongation cycle at 68^{0}C for 7min. The samples were held at 4^{0}C.

3.2.3 Agarose gel electrophoresis

Separation of a 10µl aliquot of the PCR product by electrophoresis was done using a 1% agarose gel prepared in Tris-acetate-EDTA (TAE) buffer (pH 8.5) containing ethidium bromide (0.5µg/ml). O'GeneRuler DNA ladder mix comprising DNA fragments from 100 to 10000bp fragments (Fermentas, Burlington, Canada)) was used to determine the size of the amplicon. The samples were loaded in a 6x loading dye solution containing 60mM Tris, 10mM Ethylenediaminetetraacetic acid (EDTA), 0.02% bromophenol blue and 60% glycerol. Gel electrophoresis was performed using a BioRad PowerPac Basic system (BioRad, Hercules, United States) for 1 hour at 90V. The DNA stained with ethidium bromide was visualized with a UV transilluminator.

3.2.4 DNA purification

The Wizard®SV Gel and PCR Clean-Up System (Promega, Fitchburg, United States) was used to purify the PCR product directly from the gel according to the manufacturer's instructions. This system is based on the ability of the DNA to bind to silica membranes in the presence of chaotropic salts and removes excess nucleotides, primers and enzymes. A 40µl aliquot of the PCR product was added to an equal volume of membrane binding solution (supplied in the kit). One SV minicolumn was placed in a collection tube.

The PCR product mix was transferred to the SV minicolumn assembly and incubated for 1min at room temperature (22-25^{0}C). The column was centrifuged at 16 000 × g for 1min in a microfuge 16M Spectrafuge, (Labnet International, Edison, United States). The column was washed by adding 700µl membrane wash solution (supplied in the kit). The column was centrifuged for 1min at 16 000 × g. The wash was repeated with 500µl membrane wash solution and centrifugation of the column was at 16 000 × g for 5min. An additional spin at 16 000 × g for 5min was done to allow evaporation of any residual ethanol. The SV minicolumn was transferred to a 1.5ml microcentrifuge tube. The DNA was eluted in 50µl of nuclease free water, centrifugation at 16 000 x g for 1 min, quantified and stored at - 20^{0}C.

3.2.5 Preparation of RNA transcript

A RNA transcript was prepared to be used as a control to optimize the real-time quantitative RT-PCR and for constructing the standard curve. The DNA amplicon obtained by RT-PCR in section 3.2.2, separated by

agarose gel electrophoreses and purified as in section 3.2.4 was used to prepare the RNA transcript.

3.2.5.1 Preparation of chemically competent cells

Chemically competent cells were prepared under sterile conditions utilizing the calcium chloride method. Briefly, a 3ml overnight (O/N) culture of *Escherichia coli* (E. coli) XL-10-Gold competent cells (Stratagene, La Jolla, United States) was prepared in Luria Bertani (LB) broth without ampicillin (amp). A 100ml volume of LB broth was pre-warmed in a 250ml Erlenmeyer flask and 1ml of the O/N culture was added. The culture was incubated at 37^0C with shaking at 200rpm. The OD_{600} of the *E.coli* culture was determined at 30min intervals starting at time 0. When the OD_{600} reached between 0.45 - 0.5, the cells were transferred to ice. Cells from a 20ml culture volume were collected by centrifugation at 2000 g for 5min at 4^0C. The cells were re-suspended in half the original volume (10ml) of freshly prepared ice-cold 50mM Calcium chloride ($CaCl_2$) (0.11g Ca Cl_2/ 15ml ddH2O). The cells were then centrifuged for 5min at 2000 × g at 4^0C. The supernatant was poured off and the cells re-suspended in 1ml ice-cold $CaCl_2$. The mixture was left on ice for 1 hour, aliquoted into cryotubes in 15% glycerol and frozen at -70^0C. The transformation efficiency of the cells were determined using the formula: Colony forming units (cfu)/ ng DNA = cfu / ng DNA.

To determine the transformation efficiency, cells were transformed with 1ng pUC 19 DNA.

3.2.5.2 Ligation reactions

The SINV 181bp nsp2 gene was cloned into pGEM®T Easy (pGEM) vector (Promega, Fitchburg, United States) using T/A cloning (pGEM T-Easy vector map in APPENDIX 7). The ligation reaction was set up as shown in Table 3.2. A ligation reaction where purified DNA was replaced with deionized water was used as a negative control. The final volume of each ligation reaction was 10µl. The reactions were mixed by pipetting and incubated for 1 hour at $22\text{-}25^0$C.

Table 3.2. Ligation reaction for cloning SINV nsp2 amplicon into pGEM®T Easy vector .

Reaction component	Reaction volume
2x rapid ligation buffer (400mM Tris-HCl, 100mM $MgCl_2$, 100mM DTT, 5mM ATP, pH 7.8)	5µl
T4 DNA ligase (1U/µl)	1µl
Purified SINV nsp2 DNA (100ng/µl)	3µl
pGEM® T Easy plasmid (50ng/µl)	1µl
Total	10µl

3.2.5.3 Transformation of chemically competent XL-10-Gold cells

A 50µl aliquot of chemically competent XL-10-Gold (Stratagene, La Jolla, United States) cells were transformed with the plasmid using the heat shock method. This method uses the principle that when cells are made competent, their membranes are modified to facilitate the uptake of the DNA plasmid during the heat shock step. Briefly, 2µl of the ligation reaction was added to a 50µl aliquot of chemically competent cells, gently mixed and placed on ice for 20min. The cells were heat shocked for 45-50 seconds at 42^0C, and immediately transferred to ice for 2 min. A 950µl aliquot of room temperature SOC medium was added to the tube and incubated for 1.5 hours at 37^0C with shaking at 200rpm. Super Optimal Broth (SOC) medium was prepared as follows: 2g Bacto® tryptone, 0.5g Bacto®- yeast extract, 1ml 1M sodium chloride (NaCl) and 0.25ml 1M potassium chloride (KCl) were made up to a total volume of 97ml distilled water. The mixture was autoclaved at 121^0C and cooled to room temperature. A 2M stock solution of MgCl2.6H20 and MgSO4.7H20 and 2M stock solution of glucose were prepared and added to the tryptone mixture to give a final concentration of 20mM.

Distilled water was added to a final volume of 100ml. The final solution was filter sterilized with a pH of 7.0 and LB/amp plates were prepared as follows: 10g Bacto-tryptone, 5g-Bacto yeast, 10g sodium chloride, 15g agar and distilled water to a final volume of I liter. The broth was autoclaved at 121^0C for 30 minutes and allowed to cool to 50^0C before adding amp to a final concentration of 100µg/ml. Aliquots of 30-35ml of broth were poured into 85mm petri dishes and allowed to solidify. A 100µl aliquot of 100mM isopropyl β-D-1-thiogalactopyranoside (IPTG) (Fermentas, Waltham, United States) and 40µl of 50mg/ml X-gal (Fermentas, Waltham, United States) were spread over the surface of an LB amp plate and allowed to adsorb for 30 minutes before use. A 300µl aliquot of cells from the ligation-transformation mix was spread over the plate which was incubated O/N at 37^oC.

3.2.5.4 Plasmid purification

To identify positive transformants, three white colonies were selected from the amp/IPTG/Xgal plate and each of the three colonies were grown O/N in a 5ml volume of LB/amp at 37^0C with shaking at 200rpm. A blue colony selected and grown in the same way was used as negative control. The following day the plasmids were purified using the PureYield™ Plasmid Miniprep System (Promega, Fitchburg, United States) which includes a endotoxin removal step which removes contaminants from purified plasmid DNA allowing for improved transfection and transcription/translation reaction further upstream.

Briefly: a 1.5ml aliquot of bacterial culture was centrifuged at 16000 g for 30 seconds (s) and the SNF discarded. It was repeated once and the pellet re-suspended in 600µl ultrapure water. A 100µl aliquot of Cell Lysis Buffer (supplied in the kit) was added and the tube inverted six times. An aliquot of 350µl of cold (4–8^{0}C) Neutralization Solution was added, tube mixed by inverting and the tube centrifuged at 16000 g for 3 min. The SNF (900µl) was transferred to a PureYield™ Minicolumn and the Minicolumn/Collection tube assembly centrifuged at 16000 g for 15s. A 200µl aliquot of Endotoxin Removal Wash was added and centrifuged to the Minicolumn at 16000 g for 15s. The minicolumn was washed by adding 400µl of Column Wash Solution at centrifuging at 16000 g for 30s. The minicolumn was transferred to a clean 1.5ml microcentrifuge tube and add 30µl nuclease free water directly to the minicolumn matrix, incubate for 1 min at room temperature and centrifuged at 16000 g for 15s to elute plasmid DNA. Plasmid DNA stored at -20^{0}C.

Glycerol stocks of each recombinant plasmid O/N culture were prepared (850µl recombinant plasmid O/N culture + 150µl glycerol) and stored in cryotubes (Nunc, Koeln, Germany) at -70^{0}C.

3.2.5.5 Positive pGEM transformants

To verify insertion of the SINV amplicon DNA in the recombinant plasmid, the plasmid was tested by PCR. The GoTaq™ DNA PCR system (Promega, Fitchburg, United States) was used to test the plasmid for positive transformants. The reaction mixtures were set up as follows: 10µl of 5x green reaction buffer, 1µl deoxynucleotide mix (containing 10mM of dCTP, dATP, dCTP, dGTP), 1µl of a 20 picomolar solution of T7 (TAATACGACTCACTATAGGG) and SP6 (ATTTAGGTGACACTATAG) primers, 0.25µl GoTaq™ DNA polymerase (5u/ml), 35.75µl nuclease free water and 1µl plasmid DNA. The cycling conditions were as shown in Table 3.3.

Table 3.3. Cycling conditions for GoTaq™ DNA PCR

Step	Temperature	Time	Cycles
Initial denaturation	95^{0}C	2 min	1
Denaturation	95^{0}C	30s	30
Annealing	46^{0}C	30s	
Extension	72^{0}C	30s	
Final extension	72^{0}C	5min	1

To confirm the orientation of the insert, positive constructs obtained using SP6 and T7 primers were tested with GoTaq™ DNA PCR system using T7 primer in combination with the SINV specific reverse primer.

The reactions were set up as follows: 10µl of 5x green reaction buffer, 1µl deoxynucleotide (dNTP) mix (containing 10mM of dCTP, dATP, dCTP, dGTP), 1µl of a 20 picomolar solution of T7 and SIN nsp 2 R primers, 0.25µl GoTaq™ DNA polymerase (5u/ml), 35.75µl nuclease free water and 1µl plasmid DNA. The cycling conditions were as shown in Table 3.3.

3.2.5.6 Restriction enzyme digestion

The preparation of a RNA transcript requires that the plasmid with positive transformant to be linearized by restriction enzyme digestion. The enzyme used for restriction enzyme digestion was selected by determining which unique restriction enzyme sites were downstream (3') relative to the insert. The SINV nsp2 gene was analyzed in Nebcutter (www.nebcutter.com) showing no Pst1 restriction sites within the gene which confirmed the Pst1 enzyme would not cut the gene and was suitable for linearization. Digestion reactions were set up according to the recommendations on the Promega website (www.promega.com) as shown in Table 3.4. Reaction mixtures were incubated for 2 hours at 37^0C.

Table 3.4. Restriction enzyme analysis of SINV nsp2 by single digestion using Pst1.

Reaction component	Reaction volume
1 x restriction enzyme buffer H (90mM Tris-HCl, 10mM $MgCl_2$, 50mM NaCl, pH 7.8)	2µl
Pst1(10u/µl)	1µl
Bovine serum albumin (BSA)	1µl
100ng plasmid DNA	1µl
Nuclease free water	15µl
Total	20µl

3.2.5.7 RNA transcription

The restriction enzyme digestion reaction was terminated by adding, 1µl EDTA (0.5M), 2µl Na acetate (3M) and 40µl ethanol, the reagents were mixed and chilled at -20^0C for 15min, centrifuged at 16000 g for 1min and the SNF removed. The DNA pellet was re-suspended in 10µl nuclease free water. The MEGAscript® T7 Kit (Ambion, Carlsbad, United states) was used for the preparation of the RNA control. The reactions for RNA transcription were set up as follows: 2µl ATP, 2µl CTP, 2µl GTP, 2µl UTP, 2µl reaction buffer, 2µl enzyme mix, 5µl linear template DNA (0.5µg) and 3µl nuclease free water. The reaction mixture was incubated for 16h at room temperature. After the incubation 1µl turbo DNase was added to the reaction tube to remove any template DNA and incubated for 15min at 37^0C.

3.2.5.7.1 Purify RNA transcript

The SV Total RNA Isolation System (Promega, Fitchburg, United States) was used to purify the RNA transcript to remove any impurities as well as any residual reagent after synthesis. Briefly: the 21µl RNA transcript reaction was added to 200µl 95% ethanol, mixed, transferred to Spin Column Assembly and centrifuged for 1min at 14000 g. The flowthrough was discarded and the RNA washed by adding 600µl RNA Wash Solution to the assembly and centrifuging at 14000 g for 1min. The DNase incubation mix was prepared by combining 40µl Yellow Core Buffer, 5µl 0.09M $MnCl_2$ and 5µl of DNase I enzyme in a sterile tube. The DNase incubation mix was directly applied to the membrane inside the spin column, and incubated for 15 minutes at 20–25^{0}C.

A 200µl aliquot of DNase Stop Solution was added to the spin column and centrifuged at 14000 g for 1 min. The column was washed by adding 600µl RNA Wash Solution and centrifuging at 14000 g for 1 min. The column was washed Wash by adding 250µl RNA Wash Solution and centrifuged at 14000 g for 1 min. The spin column was transferred to an elution tube and 100µl nuclease free water added to the membrane, centrifuged at 14000 g for 1 min to elute RNA and stored at –70^{0}C.

The RNA concentration was determined using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, United States) with absorbance measured at 260nm. The concentration and RNA amplicon size were used in a calculation to determine the copy number of RNA per µl. The template size was 282 bp from the T7 binding site to the Pst1 restriction enzyme digestion site. The formula used for calculating RNA copy number was (ng RNA x 6.022x10^{23}/ length of template x (1 x 10^9) x 340.

3.2.5.7.2 Reverse transcription PCR on RNA transcript

The RNA transcript purified in 3.2.5.7.1 was reverse transcribed using Expand Reverse Transcriptase (Roche, Basel, Switzerland). The reaction volume for cDNA synthesis was 20µl and mixtures were set up as follows: 7.5µl nuclease free water, 1µl of a 20 picomolar solution of the nsp2 F (minus strand) or nsp2 R primer (positive strand), 0.5µl (40 U/µl) of protector RNase inhibitor, 2µl of 100mM dithiothreitol (DTT), 4µl of 5x Expand Reverse Transcriptase buffer pH 8.2, 1µl enzyme mix (50U/µl), 1µl dioxynucleotide nucleotide mix (10mM) and 2µl template RNA. The reactions were incubated using the Perkin Elmer Thermocycler. The following conditions were used; 30^{0}C for 10min, 42^{0}C for 50min and 4^{0}C for 5min.

3.2.6 Determining viral loads

3.2.6.1 RNA Extraction

The samples collected after infection of mammalian cells were freeze-thawed for three cycles to break the cells open and centrifuged at 5000g for 5min to remove cell debris. The supernatant fluid was used for RNA extraction.

The QIAamp® Viral RNA kit was used for viral RNA extraction (Qiagen, Venlo, Netherlands). Briefly, AVL buffer and carrier RNA mix were prepared initially by adding 5.6µl of carrier RNA to 560µl AVL buffer per sample. An aliquot of 560µl prepared AVL buffer containing carrier RNA was added into a 1.5ml microcentrifuge tube. A 140µl aliquot of supernatant fluid was added to a 1.5ml microcentrifuge tube containing the 560µl carrier RNA buffer AVL mixture.

The tube was pulse-vortexed for 15s and incubated for 10min at room temperature. The tube was briefly centrifuged, 560µl absolute ethanol added to tube and mixed by pulse-vortexing. A 630µl aliquot of the prepared solution was then added to a QIAamp Mini column in a collection tube and centrifuged at 6000g for 1min. The QIAamp Mini column was placed in a new collection tube and step repeated once. An aliquot of 500µl buffer AW1 was added to the QIAamp Mini column and centrifuged at 6000 g for 1min. The QIAamp Mini column was placed inside a new collection tube and added 500µl buffer AW2 followed by centrifugation at 20 000g for 3min. The QIAamp Mini column was put inside a new collection tube and centrifuged at 20 000g for 1min to collect residual filtrate. The RNA was eluted in 60µl elution buffer and stored at -80^{0}C.

3.2.6.2 cDNA synthesis

cDNA synthesis using RNA extracted from infectious lysates was performed using Expand Reverse Transcriptase. The RNA was reverse transcribed to cDNA using two different approaches. The one was to reverse transcribe minus strand (-) RNA using the nsp2 F primer. The second was to transcribe positive (+) strand RNA using the nsp2 R primer. Negative strand RNA (or minus strand) is detectable early after infection for approximately 4 to 6 hours, after which production of (-) strand RNA ends and is replaced with (+) strand genome and sub genomic RNA (Strauss & Strauss, 1994). An increase in (-) strand RNA is suggestive of early infection and viral replication. An accompanying increase in (+) strand RNA after infection confirms sustained replication.

3.2.6.3 Real-time quantification PCR

Real-time quantification PCR reactions were done using cDNA transcribed from (+) and (-) strand RNA prepared in section 3.2.6.2.

3.2.7 Mammalian cells

Vero 76 cells (ATCC® number CRL-1587) were infected with SINV and RNA extracted from cell cultures was used to optimize the molecular assay for detecting replicating virus. Cells were infected with SINV S.A.AR86 at MOI 1 and heat inactivated SINV. SINV inoculum was heat-inactivated at 56^0C for 30min. Briefly, Vero cells were seeded at a density of 1x 10^4 cells/100µl into a 24-well plate, SINV was absorbed onto the cells for one hour at 37^0C, virus removed and 400µl MEM containing 2% FBS, 100u/ml penicillin, 0.1mg/ml streptomycin and 2mM L-glutamine added to each well.

Samples were taken at baseline and 24h, RNA extracted as discussed in section 3.2.6.1 and cDNA synthesized using nsp2 R primer for transcription of (+) strand RNA as described in section 3.2.6.2.

3.2.8 TaqMan real-time RT-PCR

Infection and replication of SINV in mammalian cells was determined by quantifying the viral load in aliquots of cell culture media sampled at various intervals after infection. Quantification of viral load was determined using a TaqMan probe technique. The real-time RT-PCR was performed in a two step reaction. The first step was cDNA synthesis and the second was amplification and quantification of the cDNA. A TaqMan detection probe was designed to target a region of the 181bp NSP2 amplicon. The probe was 27 bp in length and fluorescently labeled on the 5' end with 6-carboxyfluorescein (6FAM) and quenched on the 3' end with BlackBerry® Quencher(BBQ) as shown in Table 3.5.

Table 3.5 Real-time PCR probe sequence

Probe	Sequence (5'-3')	Probe genomic position from NSP2 gene 1st nucleotide (5'-3')
TaqMan probe	ATCATAACGCACTCCGGAAGATCAGGA	148 - 174

The LightCycler® TaqMan® Master kit (Roche, Basel, Switzerland) was used to amplify cDNA. Each real-time reaction had a total volume of 20µl and consisted of: 12µl nuclease free water, 4µl 5x Master mix, 2µl TaqMan probe (1 pmol/µl), 0.5µl of Sin nsp2-F and Sin nsp2-R primer (20 pmol/µl) and 1µl cDNA template.

The reactions were cycled in a Roche Lightcycler® (Roche, Basel, Switzerland) pre-incubated at 95^0C for 10min followed by 50 cycles of amplification which consisted of denaturation at 95^0C for 10s, annealing at 46^0C for 30s, elongation at 72^0C for 1s, and cooling at 40^0C for 30s. The fluorescence data was acquired at the end of each extension step on the 530nm wavelength in single acquisition mode. After nucleic acid amplification the lightcycler software determined the crossing point (CP). The CP is the cycle number that corresponds to an increase of fluorescence over the threshold which was calculated with by the lightcycler software (Heim et al., 2003).

3.2.9 Standard curve

The real-time PCR was analyzed as an absolute quantitative assay. The first standard curve was constructed using the RNA transcript prepared in section 3.2.5.7.1. The RNA was diluted 10-fold from neat to 10^{-6} which correlated to 6.2 x 10^{10} - 6.2 x 10^4 copies of RNA/µl. The diluted RNA was used for cDNA synthesis as described in section 3.2.6.2. which served as template in the real-time PCR in section 3.2.8 to construct a standard curve.

The second standard curve was constructed using the DNA obtained from the RT-PCR performed in section 3.2.2. The DNA was diluted 10-fold to 10^{-7} dilution and used as template in the real-time PCR in section 3.2.8 to construct a standard curve. The standard curve was used to quantify viral nucleic acid extracted from SINV infected mammalian cells.

3.3 Results

3.3.1 Preparation of DNA control

Figure 3.1 shows a 10µl aliquot of the 181bp NSP2 gene amplicon DNA obtained through amplification using Titan Reverse Transcriptase PCR system. The PCR product was separated by gel electrophoresis on a 1% agarose gel. The amplicon in lane 2 is approximately 200bp according to the molecular weight marker, which is consistent with the expected size of 181 bp of the NSP2 gene amplicon. There is no visible band in the negative control lane 3 indicating that there was no non-specific amplification.

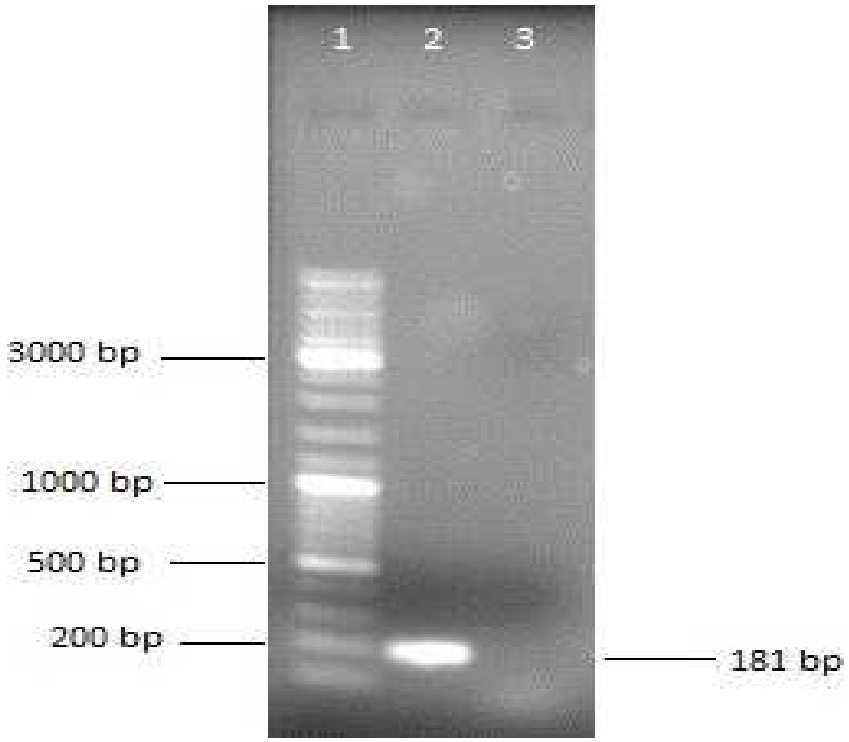

Figure 3.1. Agarose gel electrophoretic analysis of nsp2 amplicon from the one step RT-PCR, performed on SINV viral RNA. Lane 1 – O'GeneRuler DNA ladder mix comprising DNA fragments from 100 to 10 000bp fragments (Fermentas, Waltham, USA) molecular weight marker; Lane 2 - 10µl PCR product of amplified SINV RNA using nsp2 F and nsp2 R primers; Lane 3 – Negative control containing no viral RNA.

The DNA concentration was measured after purification using a NanoDrop 2000 Spectrophotometer and absorbance measured at 260nm. The DNA concentration was determined as 45.3ng/ml which was used to determine the number of DNA copies per µl. The concentration and DNA amplicon size were used in an online conversion tool (http://stanice.euweb.cz/bio/DNAtoCopy.html) and the copy number determined as 2.3×10^{11} copies of DNA/µl.

3.3.2 T/A cloning of the SINV nsp2 amplicon into pGEM® T Easy vector

Three colonies were selected from the transformation reaction described in MATERIALS AND METHODS, 3.2.5.4. Results obtained from GoTaq™ DNA PCR in section 3.2.5.5 are shown in Figure 3.2.

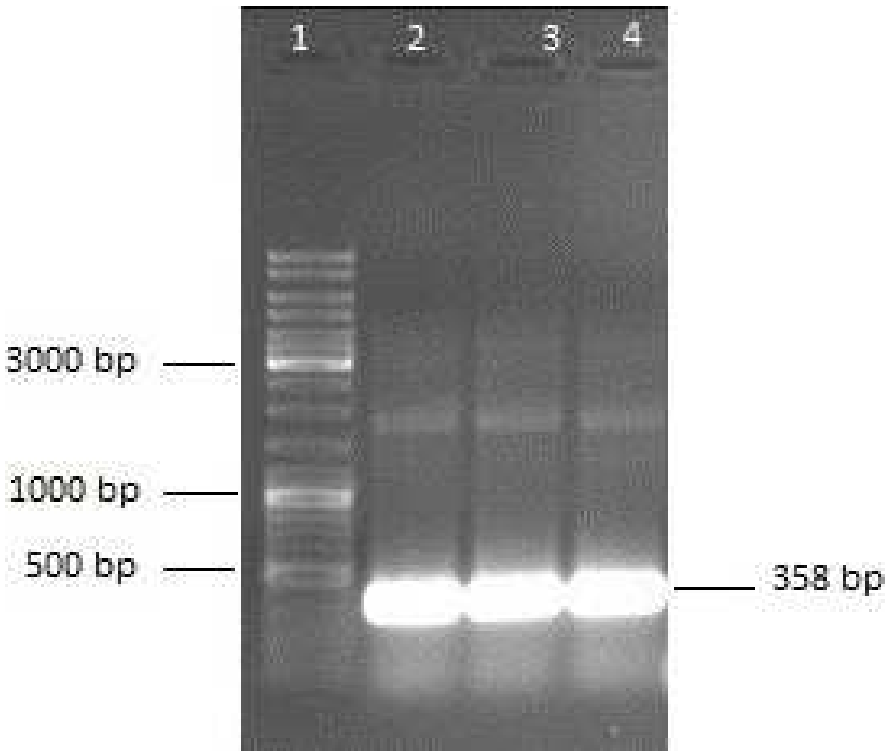

Figure 3.2. Agarose gel electrophoretic analysis of positive pGEM transformants transformed with SINV nsp2 181bp amplicon amplified by GoTaq™ DNA PCR using SP6 and T7 primers. Lane 1 – O'GeneRuler DNA ladder mix comprising DNA fragments from 100 to 10 000bp fragments molecular weight marker; Lane 2 - 10µl PCR product of amplified SINV nsp2 amplicon for colony 1; Lane 3 - 10µl PCR product of amplified SINV nsp2 amplicon for colony 2; Lane 4 - 10µl PCR product of amplified SINV nsp2 amplicon for colony 3.

Figure 3.2 showed amplification of template in lanes 2, 3 and 4 with an estimated 358bp amplicon. The 358 bp amplicon included the cloned SINV nsp2 181 bp gene with 177 bp additional nucleotides on either side of the 3' and 5' ends of the insert until the SP6 and T7 primer binding sites. When using T/A cloning the insert can be cloned in either orientation. There were non-specific bands of approximately 1600 bp which were likely non-specific amplification.

To determine the orientation of the insert a PCR was performed using the T7 and Sin nsp2 R primers.

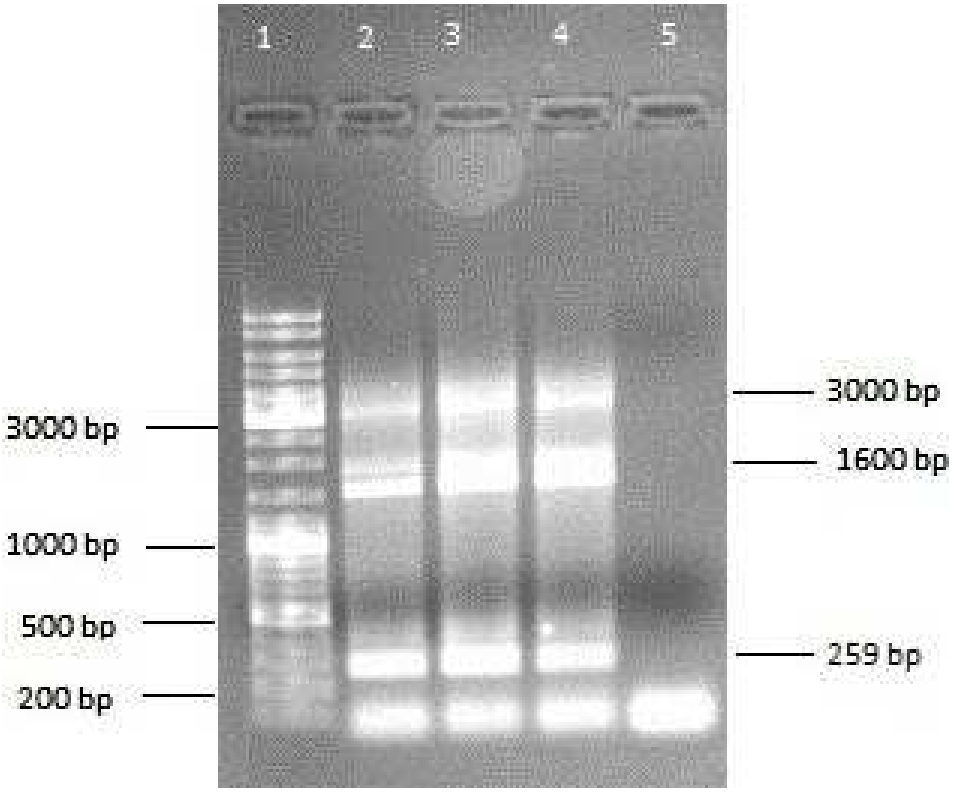

Figure 3.3. Agarose gel electrophoretic analysis of positive pGEM transformants transformed with SINV nsp2 181bp amplicon amplified by GoTaq™ DNA PCR using Sin nsp2 R and T7 primers. Lane 1 – O'GeneRuler DNA ladder mix comprising DNA fragments from 100 to 10 000bp fragments molecular weight marker; Lane 2 - 10µl PCR product of amplified SINV nsp2 amplicon colony 1; Lane 3 - 10µl PCR product of amplified SINV nsp2 amplicon for colony 2; Lane 4 - 10µl PCR product of amplified SINV nsp2 amplicon for colony 3; Lane 5 - Negative control.

The orientation of the PCR insert in pGEM was confirmed by PCR using Sin nsp2 R and T7 primers. Figure 3.3 shows positive amplification of a 259 bp amplicon for three colonies in lanes 2, 3 and 4. The 259 bp included the 181 bp nsp2 insert as well as 78 additional nucleotides on the 5' end of the insert to the T7 binding site. The positive amplification showed the gene was in the correct orientation of 5'-3' for transcription of RNA from the T7 promotor site. There were non-specific bands of approximately 3000 bp and 1600 bp which were likely non-specific amplification and pGEM plasmid DNA. One transformed plasmid was selected and designated pGEMSIN. A glycerol stock, of the culture was prepared and stored at -80^{0}C.

The pGEMSIN construct was used in subsequent reactions.

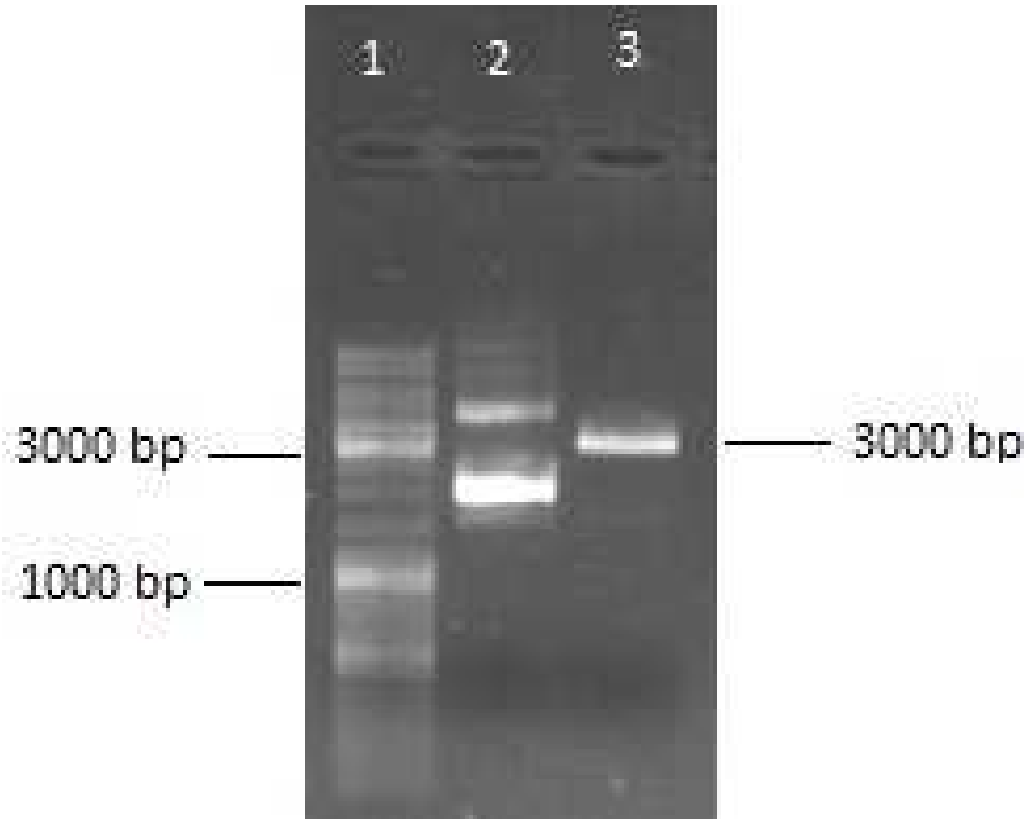

Figure 3.4. Agarose gel electrophoretic analysis of positive pGEM transformant linearized by restriction enzyme digestion using Pst1 restriction enzyme. Lane 1 – O'GeneRuler DNA ladder mix comprising DNA fragments from 100 to 10 000bp fragments molecular weight marker; Lane 2 - pGEM undigested plasmid; Lane 3- pGEM-SINV nsp2 linearized by Pst1 restriction enzyme digestion.

To transcribe RNA it was necessary to linearise the pGEMSIN construct prior to transcription. The plasmid was linearised using Pst1 restriction enzyme which cuts at a site in the multiple cloning region downstream of the 3' end of the nsp2 gene insert. Figure 3.4 shows in lane 2 uncut pGEM control with two main bands namely supercoiled and nicked circles DNA. Lane 3 showed the pGEMSIN construct being linearized by Pst1 restriction enzyme at 3000 bp.

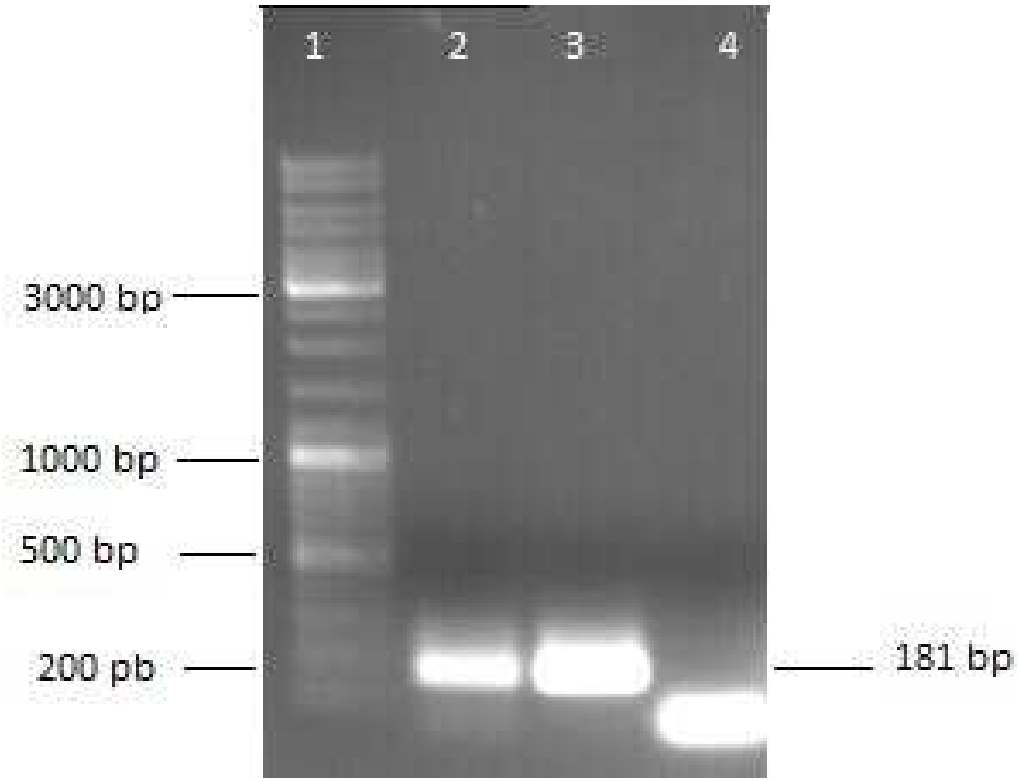

Figure 3.5. Agarose gel electrophoretic analysis of RNA transcript as detected by Titan reverse transcriptase PCR system. Lane 1 – O'GeneRuler DNA ladder mix comprising DNA fragments from 100 to 10 000bp fragments molecular weight marker; Lane 2 - pGEM undigested colony 1; Lane 3- pGEM-SINV nsp2 linearized by Pst1 restriction enzyme; Lane 4 - Negative control.

The RNA transcript was prepared using the MEGAscript® T7 Kit and purified using SV Total RNA Isolation System. To determine whether the RNA transcript was functional the Titan™ One Tube RT-PCR System was used with nsp2 F and nsp2 R primers to amplify the transcript in duplicate. Figure 3.5 shows positive amplification with a PCR product in lane 2 and 3 whereas lane 4 was the negative control and showed no amplification, only primer dimer.

3.3.3 TaqMan LightCycler real time PCR

The real-time quantitative RT-PCR was initially tested by amplifying DNA prepared from Titan reverse transcriptase amplification in 3.2.1. There was amplification as shown in Figure 3.6 where the blue line depicts the amplification of SINV nsp2 DNA with a crossing point at 21.75 whereas the green line depicts the negative control indicating no amplification.

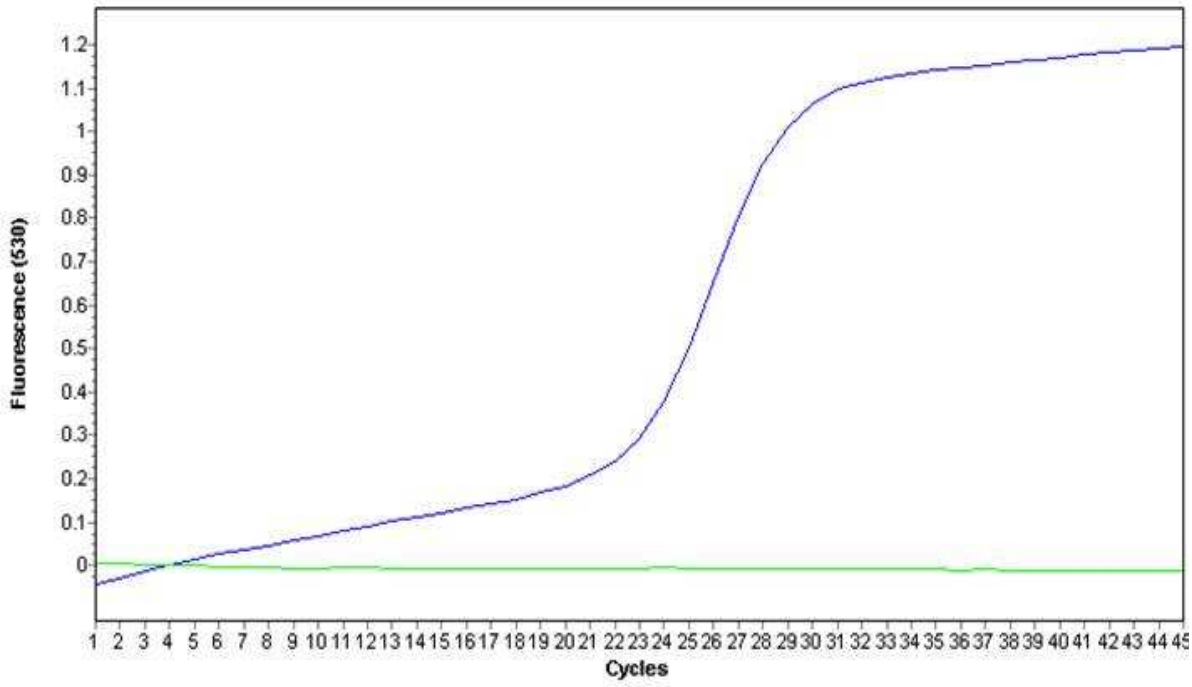

Figure 3.6. Amplification of the nsp2 DNA amplicon with TaqMan real-time PCR. The blue line shows positive amplification and the negative control is indicated by the green line.

3.3.3.1 Standard Curve using cDNA from RNA transcript

The concentration of RNA obtained in section 3.2.5.7.1 was determined using a NanoDrop 2000 Spectrophotometer with absorbance measured at 260nm. The RNA concentration was determined as 7.5ng/µl which was used to determine the number of RNA copies per µl. The concentration and RNA amplicon size were used in a calculation to determine the copy number of RNA per µl. The template size was 282 bp from the T7 binding site to the Pst1 restriction enzyme digestion site. The formula used for calculating RNA copy number was (ng RNA x $6.022x10^{23}$/ length of template x (1×10^{9}) x 340 and the copy number was determined as 4.7×10^{10} copies/RNA per µl. The RNA was diluted 10-fold from 10^{0} to 10^{-6} which correlated to 4.7×10^{10} - 4.7×10^{4} copies of RNA/µl.

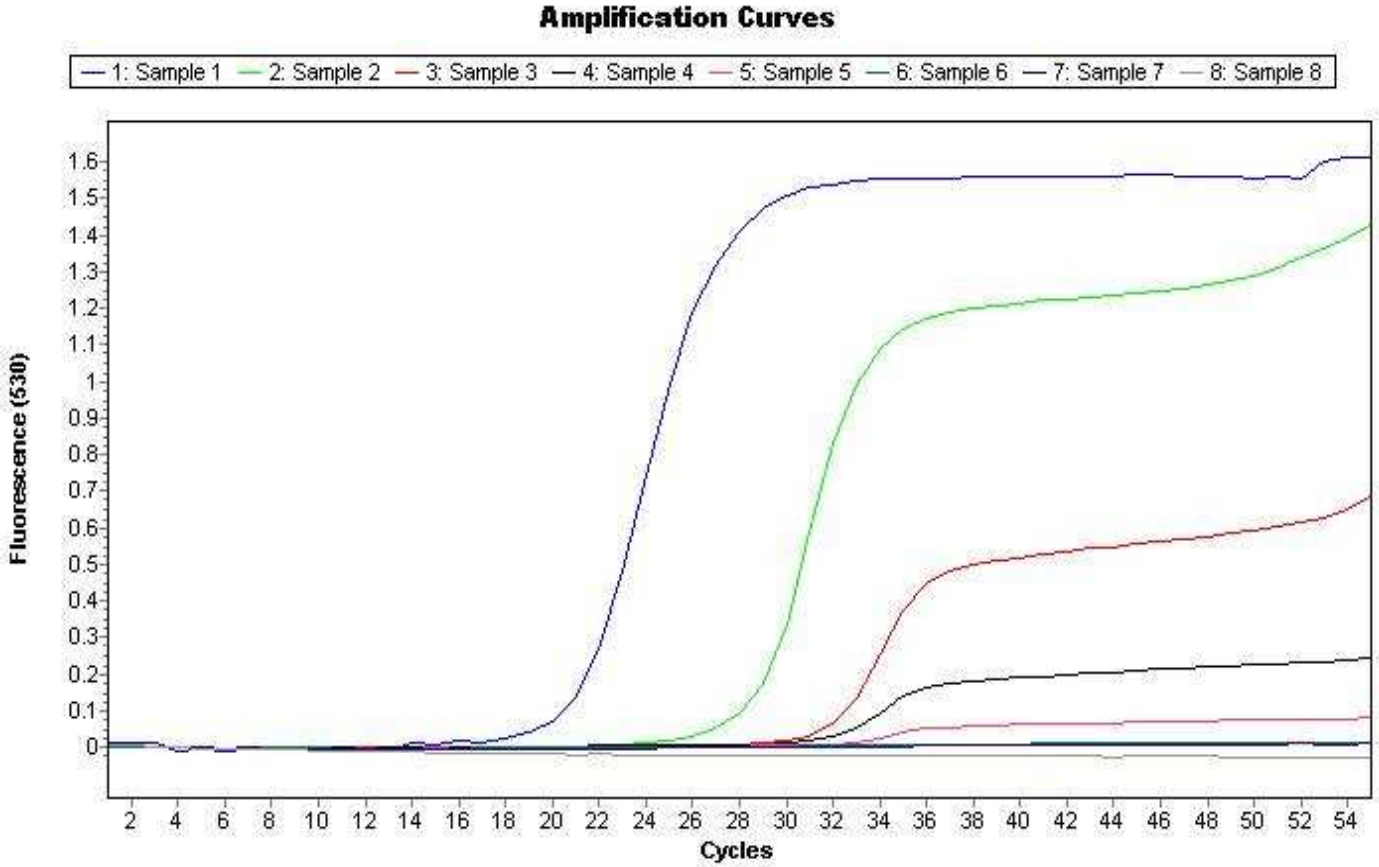

Figure 3.7. Amplification curves for the TaqMan quantitative real-time PCR with cDNA copy numbers ranging from 4.7 x 10^{10} - 4.7 x 10^{4} copies of cDNA/µl prepared from RNA transcript. Sample 1 (blue line) represented 4.7 x 10^{10} copies of cDNA/µl, Sample 2 (lime green line) represented 4.7 x 10^{9} copies of cDNA/µl, Sample 3 (red line) represented 4.7 x 10^{8} copies of cDNA/µl, Sample 4 (black line) represented 4.7 x 10^{7} copies of cDNA/µl, Sample 5 (pink line) represented 4.7 x 10^{6} copies of cDNA/µl, Sample 6 (dark green line) represented 4.7 x 10^{5} copies of cDNA/µl, Sample 7 (purple line) represented 4.7 x 10^{4} copies of cDNA/µl and Sample 8 (grey line) was the negative control.

The concentration of the RNA transcript was measured using a nanodrop and the copy number per µl determined. Dilutions were made and reverse transcribed into cDNA to use as template in the quantification PCR. The amplification of cDNA reverse transcribed from the RNA transcript at different dilutions can be seen in Figure 3.7. Amplification of cDNA at lower copy numbers did not yield satisfactory amplification curves and the assay was only able to detect and quantify 4.7 x 10^{7} copies. The curves seen for the higher cDNA concentrations with the blue (4.7 x 10^{10} copies) and green (4.7 x 10^{9} copies) lines indicate detection and amplification.

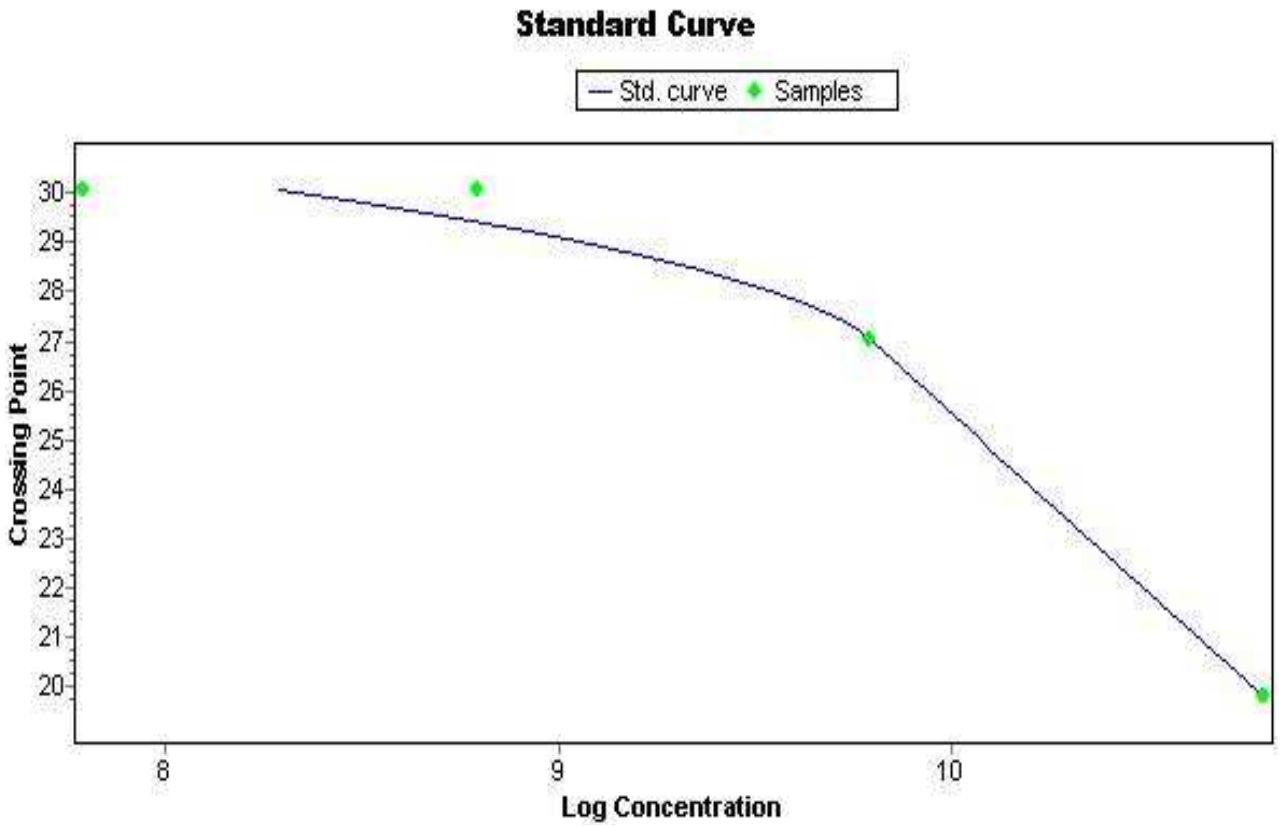

Figure 3.8. The standard curve was determined by plotting the crossing points at each dilution against the log copy numbers of cDNA/µl.

The standard curve was constructed by plotting the CP at each dilution against the corresponding copy number of cDNA. Efficient amplification should result in a linear correlation between the CP and the copy number of cDNA/µl of nucleic acid. A non-linear curve was obtained in Figure 3.8 which suggested the PCR may not be accurate for quantification of viral loads or that the copy number calculated for the RNA control used to construct the standard curve was inaccurate.

In order to identify whether there was a problem with the sensitivity of the detection probe the real-time PCR products were separated and visualized using a 1% agarose gel electrophoresis as described in section 3.2.3.

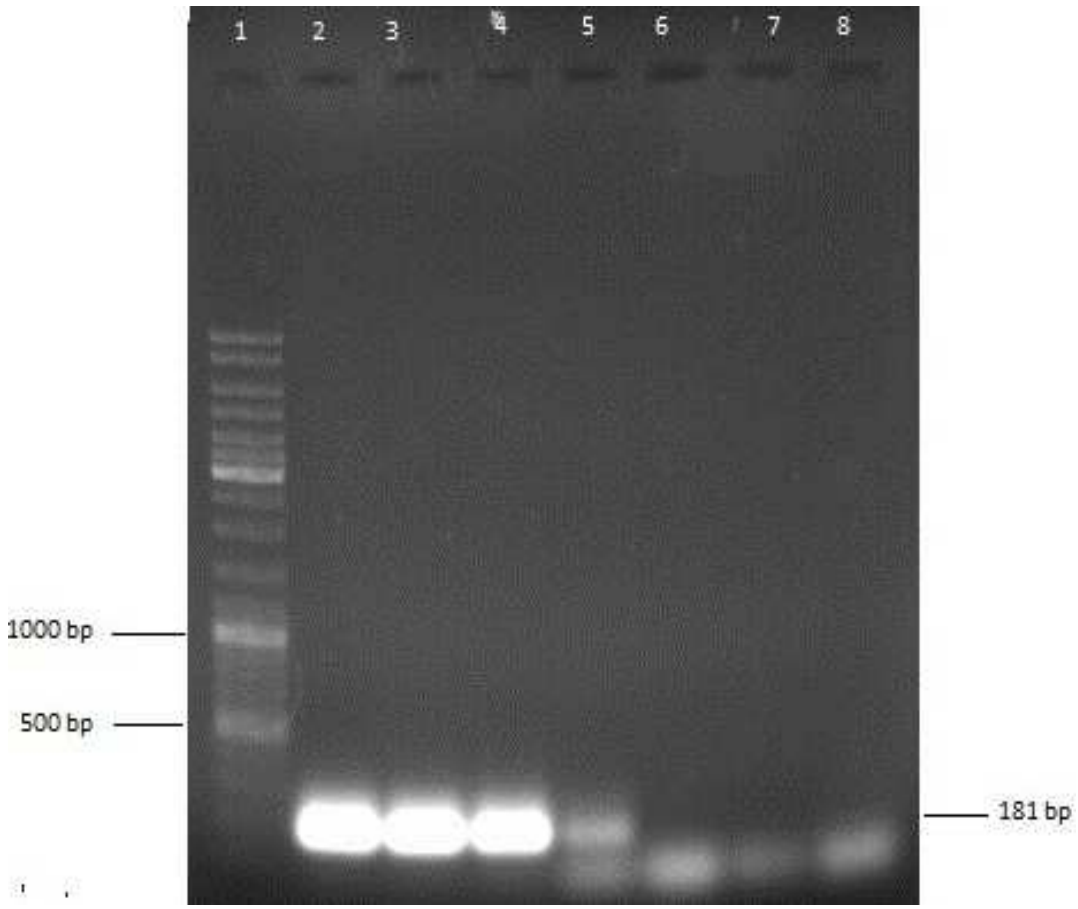

Figure 3.9. Agarose gel electrophoretic analysis of real-time PCR amplifications. Lane 1 – O'GeneRuler DNA ladder mix comprising DNA fragments from 100 to 10 000bp fragments molecular weight marker; Lane 2 - 4.7 x 10^{10} copies of cDNA/µl; Lane 3 - 4.7 x 10^{9} copies of cDNA/µl; Lane 4 - 4.7 x 10^{8} copies of cDNA/µl; Lane 5 - 4.7 x 10^{7} copies of cDNA /µl; Lane 6 - 4.7 x 10^{6} copies of cDNA/µl; Lane 7 - 4.7 x 10^{5} copies of cDNA/µl; Lane 7 - Neg control.

There were strong amplification bands present in Lanes 2, 3, and 4 with a faint band in Lane 5 of Figure 3.9 corresponding to the 181 bp SINV nsp2 amplicon. The bands in Lanes 2-4 correlated to 4.7 x 10^{10} copies of cDNA/µl - 4.7 x 10^{7} copies of cDNA/µl. The lowest concentration that resulted in a visible band was 4.7 x 10^{7} copies of cDNA/µl which correlated the lowest detectable concentration using real-time PCR detection as shown in Figure 3.7. The direct correlation indicated that the lack of sensitivity of the real-time PCR was not due to poor binding of the probe. It is possible that if linearization of the pGEMSIN construct was incomplete then the resulting RNA transcript was longer than the predicted 181bp predicted transcript. Although Figure 3.4 suggested complete linearization of the pGEM transformant it is possible that there were low concentrations of unlinearized plasmid that were not detected on the gel. Hence there was likely

run off of the transcript with sizes far greater than 181 bp which would have resulted in an inaccurate determination of RNA copy number as the calculation was based on a 181 bp transcript. Hence a standard curve was determined using DNA prepared as described in section 3.3.1.

3.3.3.2 Standard Curve using DNA from reverse transcribed viral RNA

A standard curve was prepared using a series of dilutions of DNA control and was used as a reference to determine concentrations of DNA in unknown samples. Hence subsequent quantification of viral load from experimental samples was based on determination of copies of cDNA after reverse transcription of RNA. The DNA obtained from the Titan reverse transcriptase reaction was serially diluted and dilutions ranging from 10^{-4} - 10^{-8} were used to prepare the standard curve which correlated to 2.2 x 10^{7} - 2.2 x 10^{3} copies of DNA/µl respectively.

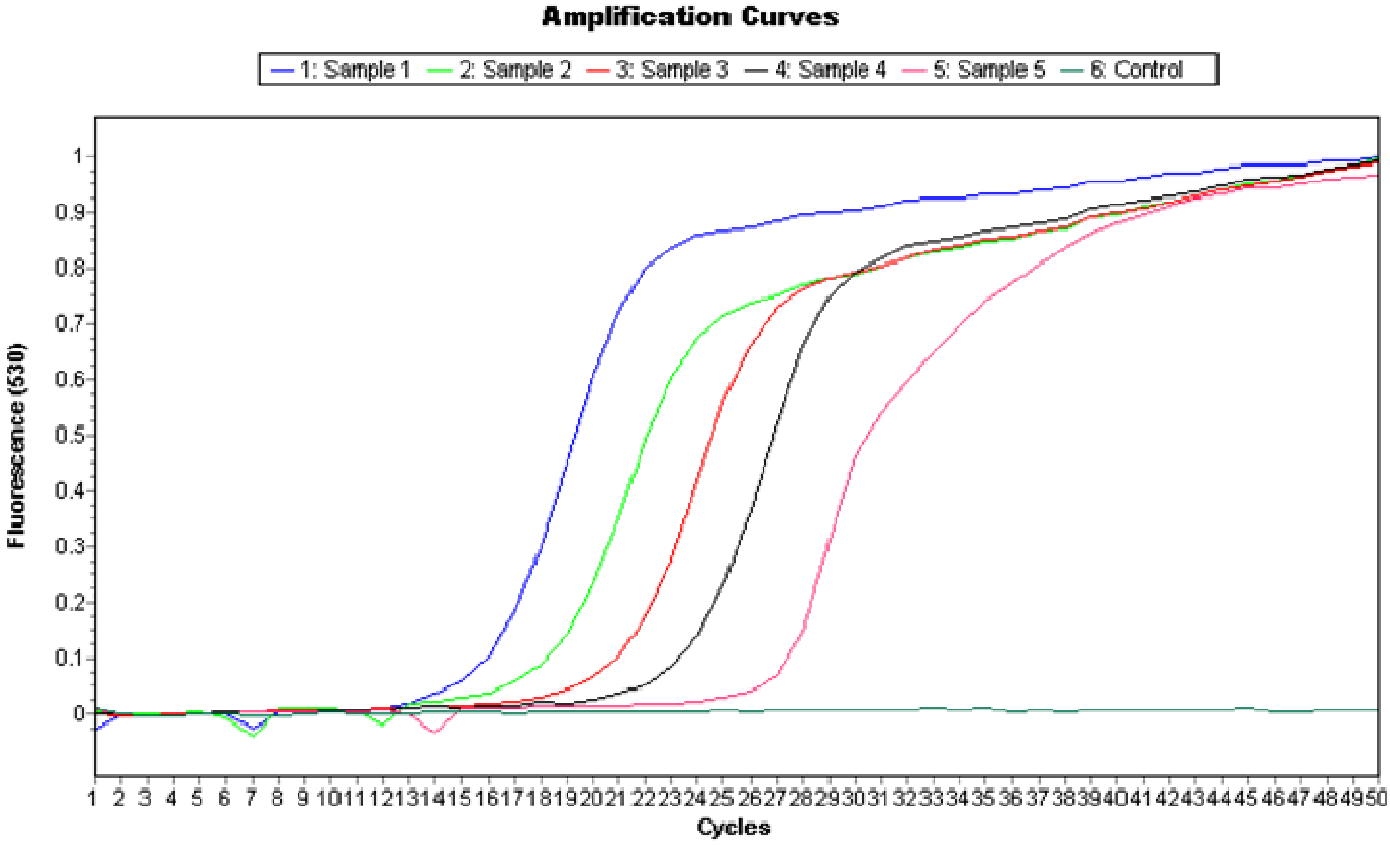

Figure 3.10. Amplification curves for the TaqMan quantitative real-time PCR using DNA. Sample 1 (blue line) represented 2.2 x 10^{7} copies of DNA/µl, Sample 2 (lime green line) represented 2.2 x 10^{6} copies of DNA/µl, Sample 3 (red line) represented 2.2 x 10^{5} copies of DNA/µl, Sample 4 (black line) represented 2.2 x 10^{4} copies of DNA/µl, Sample 5 (pink line) represented 2.2 x 10^{3} copies of DNA/µl, Sample 6 (dark green line) represented the negative control.

Figure 3.10 shows that the 10-fold diluted DNA samples were detected evenly apart and resulted in a efficiency of 2.495. The efficiency showed that the CP for each dilution was detected on average 2.5 cycles from the previous one.

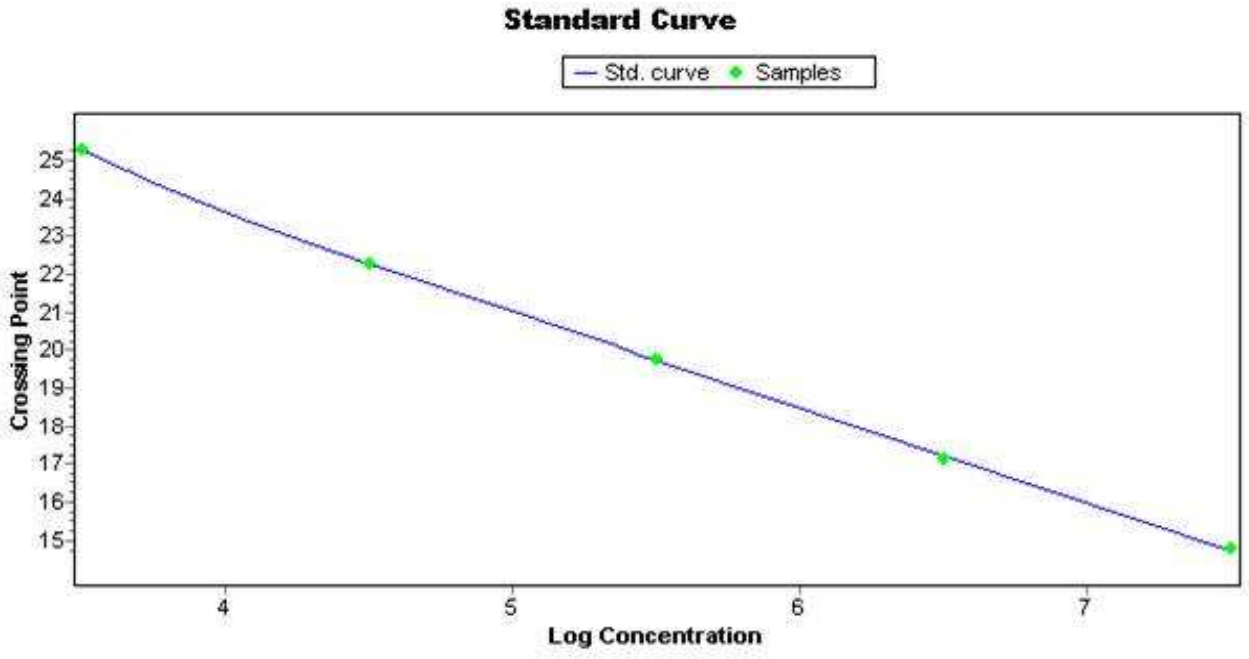

Figure 3.11. The standard curve was determined by plotting the crossing points at each dilution against the log concentration of DNA copies/µl.

When the crossing points were plotted against the DNA copy numbers it showed a linear correlation as seen in Figure 3.11. The linear correlation meant it could be used as a standard curve in the real-time quantitative PCR assay to which unknown samples could be compared to determine their DNA copy numbers.

The sensitivity of the assay was assessed by serial 10-fold dilutions of DNA to determine the lowest copy number of DNA that the assay can detect.

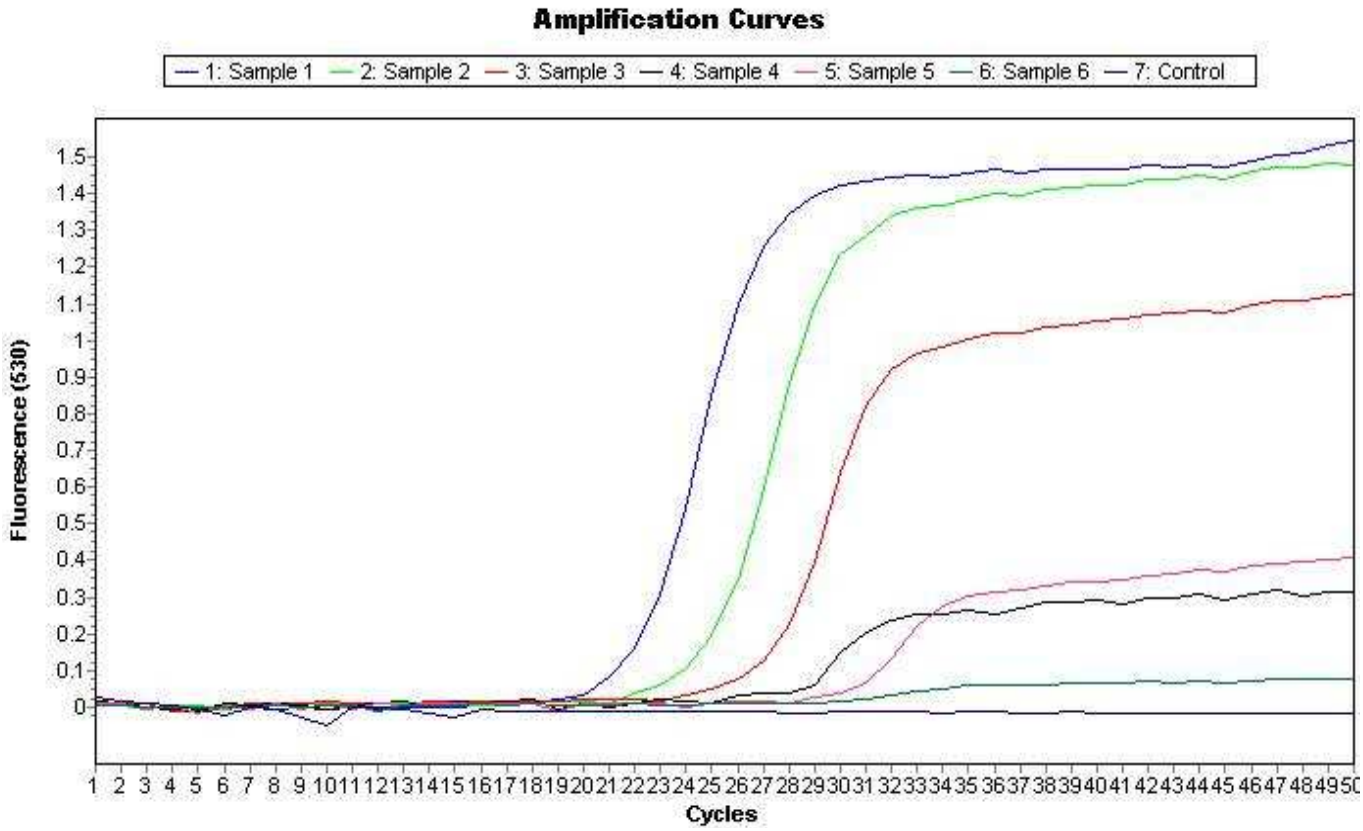

Figure 3.12. Amplification curves for the TaqMan quantitative real-time PCR with cDNA copy numbers ranging from 2.2 x 10^6 – 2.2 x 10^1 copies of DNA/µl. Sample 1 (blue line) represented 2.2 x 10^6 copies of DNA/µl, Sample 2 (lime green line) represented 2.2 x 10^5 copies of DNA/µl, Sample 3 (red line) represented 2.2 x 10^4 copies of DNA/µl, Sample 4 (black line) represented 2.2 x 10^3 copies of DNA/µl, Sample 5 (pink line) represented 2.2 x 10^2 copies of DNA/µl, Sample 6 (dark green line) represented 2.2 x 10^1 copies of DNA/µl, Sample 7 (purple line) was the negative control.

The sensitivity of the real-time quantitative PCR was determined as shown in Figure 3.12. The real-time quantitative PCR was able to successfully detect and quantify concentrations from 2.2 x 10^6 copies/DNA (blue line) to a lowest concentration of 2.2 x 10^2 copies of DNA/µl (pink line).

3.3.3.3 PCR for the detection of replicating virus

The ability of targeting (-) strand RNA in the real-time PCR for detecting replicating virus was demonstrated by infecting Vero cells with infectious SINV S.A.AR86 at MOI 1 and heat-inactivated virus with samples taken at baseline and 24h for testing.

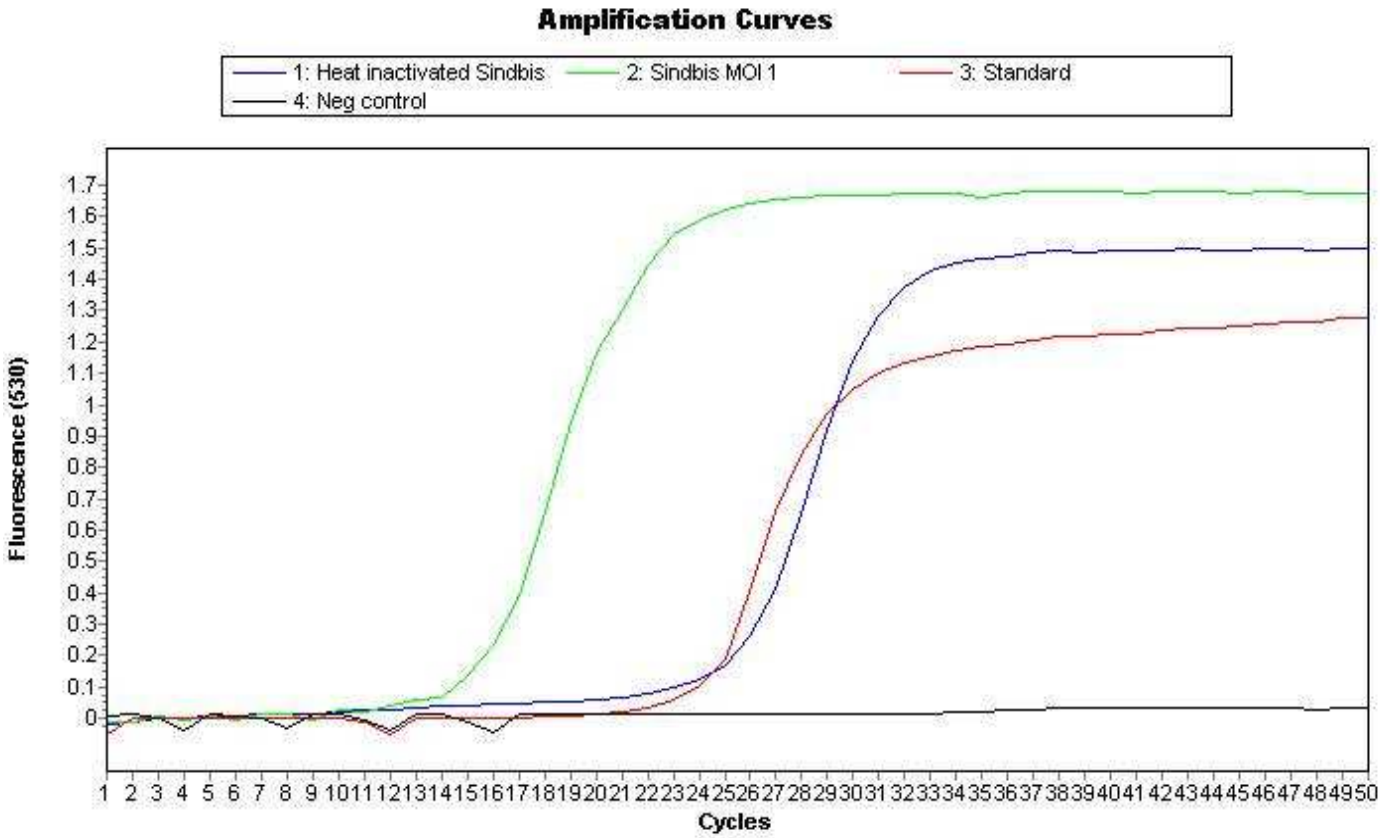

Figure 3.13. Amplification curves for Vero cells infected with SINV S.A.AR86 at MOI 1 and samples at time periods base and 24h.

Figure 3.13 shows a significant difference between the cells inoculated with infectious SINV (green line) and the heat-inactivated sample (blue line). There was a 10 000-fold difference in copy number between the heat-inactivated sample at 6.49 x 10^4 and live virus to 4.89 x 10^8 copies/µl as shown in Table 3.6. The increase in copy number likely represents replicating virus in cells infected with infectious virus compared to cells infected with heat inactivated virus. The detection of 6.49 x 10^4 copies cDNA/ µl from cells infected with heat-inactivated virus was attributed to noninfectious virus present in the inoculum.

Table 3.6. The crossing points and DNA copy numbers/µl of Vero cells infected with SINV S.A.AR86 at MOI 1 and sampled at different time intervals.

Sample	Name	CP	(Copies cDNA/µl)	Standard
1	Heat inactivated SINV	24.29	6.49 x 10^4	
2	SINV	14.31	4.89 x 10^8	
3	Standard	22.69	2.20 x 10^5	2.20 x 10^5
4	Neg control		-	-

3.4 Summary

A forward and reverse primer pair for the amplification of a 181bp region of the nsp2 gene encoding the nsp2 protein of SINV was designed. The primer pair was successfully used to amplify a 181 bp amplicon using Titan Reverse Transcriptase PCR system. The 181 bp amplicon was cloned into the pGEM® T-Easy vector using T/A cloning. The correct orientation of the insert was determined by PCR using nsp2 R and T7 primers that amplified a 259 bp amplicon. The pGEMSIN construct was linearised and transcribed to obtain a RNA control. A real time quantitative TaqMan PCR was developed. A hydrolysis probe for quantitative real time PCR analysis was designed which was fluorescently labeled with 6FAM on the 5' end and BBQ on the 3' end. The real-time quantitative PCR assay was optimized by analyzing viral nucleic acid extracted from infected Vero cells. Vero cells were infected with SINV S.A.AR86 strain at MOI 1 and samples taken at baseline and 24h. Viral RNA was extracted from the infectious SNF and reverse transcribed into cDNA. Negative strand cDNA synthesis was performed by using the Sin nsp2 F primer in order to show an increase in viral nucleic acid from that of the inoculum. The quantification showed a definite increase in the copy numbers of cDNA from baseline to 24h after infection.

The RNA control was used to construct a standard curve for the TaqMan real time quantitative PCR. Using dilutions of transcribed RNA the assay was found to be insensitive with a minimum detection limit of 4.7×10^7 copies cDNA/µl. Apart from sensitivity the 10-fold dilutions were not detected at regularly spaced crossing points as expected. The result was a non-linear correlation between the crossing points at different dilutions and the DNA concentrations. A DNA control using the Titan™ One Tube RT-PCR System was therefore prepared, diluted and used at 10-fold dilutions to construct a standard curve for the real-time quantitative PCR assay. The amplification curves were spaced evenly apart with a 2.4 cycle difference between dilutions. When the crossing points and DNA concentrations were plotted on a graph it showed a perfect linear correlation between the two. Hence a standard curve based on quantification of DNA rather than RNA was adopted for measuring viral load. The PCR was shown to detect $\geq 2.2 \times 10^2$ copies of DNA/µl

PCR amplification of cDNA transcribed from (-) and (+) strand RNA was investigated. The (-) strand was transcribed using the Sin nsp2 R primer in the cDNA synthesis and was to show early viral replication. An increase in (-) negative strand RNA should indicate early viral replication. The real time quantitative assay was optimized to be used as an analytic tool for quantifying viral nucleic acid in infected SNF. An increase in viral load based on amplifying cDNA transcribed from (+) strand RNA should indicate later stages of viral replication and transcription of genomic and sub genomic RNA. The effectiveness of the negative strand

PCR was tested by infecting Vero cells with either SINV at MOI 1 or heat-inactivated SINV at MOI 1. The quantitative PCR showed a 10 000 fold increase in viral load form baseline to 24h in cells infected with SINV at MOI 1 which was suggestive of viral replication. In comparison there was no increase in viral load in cells infected with heat-inactivated SINV at MOI 1 which suggested no replication.

CHAPTER 4

INFECTION OF MAMMALIAN CELLS WITH SINDBIS VIRUS

4.1 Introduction

SINV and other alphaviruses are known to cause arthritis in humans however the mechanisms of pathogenesis are not known (Assunção-Miranda et al., 2010). Macrophages play a crucial role in the development of arthritis, but they have yet to be identified as targets of SINV infection in viral arthritis. However monocytes and macrophages have been shown to be present in inflammatory infiltrates during RRV infection and therefore it is possible that they play a role in pathogenesis of arthritic disease (Morrison et al., 2006).

The relationship between infectious viral particles and the host cells is complicated to say the least. The virus strives to be invisible to the host cells and evade the immune system while the host tries to eliminate the virus while limiting damage to itself (Guidotti & Chisari, 2001). The innate immune response is the initial barrier of protection against infection. The host immune system uses pattern-recognition receptors on cells such as dendritic cells and macrophages to recognize foreign or viral and nucleic acid. These effector cells, after recognition, have the ability to secrete inflammatory mediators such as cytokines and IFN (Kawai & Akira, 2006).

The IFN are known to have strong antiviral activity and include alpha, beta and gamma IFN. Both IFN alpha and gamma play an important role in controlling infections. Firstly by activating and recruiting macrophages as well as natural killer cells and T cells that release cytokines. They can lead to an up regulation of antigen processing, major histocompatibility complex (MHC) expression in the infected cells as well as exert direct antiviral activity (Guidotti & Chisari, 2001). IFN gamma has been shown to be an effective treatment when given to patients with hepatitis B virus (HBV) to decrease viral loads in patient sera. IFN gamma also has the ability to lead to a decrease in viral RNA replication for RNA viruses (Costa-Pereira et al., 2002; Hayashi & Koike, 1989; Wu et al., 2011).

SINV is able to cause cell death by necrosis in most mammalian cell types and infected cells show characteristics of apoptosis (Wang et al., 2008). Apoptosis in living organisms is an important natural process for maintaining homeostasis and is a genetically regulated process.

Apoptosis has the ability to eliminate damaged or abnormal cells to maintain biological order. The cells have mechanisms to overcome pathogen-induced apoptosis, but those can be overwhelmed by certain pathogens (Roulston et al., 1999). A mouse model of SINV central nervous system infection showed that young mice exhibited very high rates of both apoptosis and mortality. The level of neurovirulence displayed by strains was a major factor in the levels of mortality and apoptosis observed (Clarke & Tyler, 2009). The exact mechanisms used by SINV to induce apoptosis are not established, but possible mechanisms have been postulated. SINV infection has been shown to be responsible for the inhibition of anti-apoptotic proteins and the stimulation of pro apoptotic proteins (Griffin & Hardwick, 1998).

The aim of this study was initially to characterize SINV infection of human derived macrophages. Macrophages isolated from human peripheral blood mononuclear cells (PBMC) were however not susceptible to infection in our study and attempts were made to determine why the cells were refractory to infection and determine if SINV can induce apoptosis in a mammalian cell line.

4.2 Materials and methods

4.2.1 Mammalian cells

Two mammalian cell lines were used in this study. Human PBMC were isolated from whole blood and differentiated into macrophages for SINV infectivity studies. HeLa cells ATCC® number CCL-2 were infected with SINV to characterize the effect of SINV infection on IFN release and to determine if SINV can cause apoptosis.

4.2.1.1 Isolation PBMC's from whole blood

An aliquot of 18ml whole blood in EDTA was obtained from a volunteer with informed consent for PBMC isolation. An aliquot of 150ml of Roswell Park Memorial Institute (RPMI) 1640 growth media containing 10% FBS, penicillin 100u/ml, streptomycin 0.1mg/ml) and L-glutamine 2mM was warmed to room temperature. Briefly, 18ml blood was aliquoted into a sterile 50ml tube and made up to 50ml with RPMI 1640 growth media. The 50ml blood/media was divided into two aliquots and layered onto Ficoll-Hypaque (AEC-Amersham, Johannesburg, South Africa) in two sterile tubes followed by centrifugation for 20min at 900g with the brake off. The buffy coat was collected and washed twice with RPMI 1640 growth media and centrifuged at 420g for 10min to obtain a pellet. The cells were re-suspended in a final volume of 10ml RPMI 1640 growth media and the cells were counted with a haemocytometer using the trypan blue exclusion method.

The PBMC were differentiated into macrophages using the optimized protocol for human macrophages (http://bio.lonza.com/fileadmin/groups/marketing/Downloads/Protocols/Generated/Optimized_Protocol_76.pdf). PBMC were seeded at a density of 2.2 x 10^5 cells per well in a 24 well Nunc tissue culture plate (Nunc, Koeln, Germany) and incubated for 24h in a CO_2 incubator at 37^0C. The growth media was removed and replaced with RPMI differentiation media which was comprised of: RPMI 1640 supplemented with 10% FBS, penicillin 100u/ml, streptomycin 0.1mg/ml, L-glutamine 2mM, sodium pyruvate solution 1mM (BioWhittaker™, Basel, Switzerland), non-essential amino acid solution 100X (BioWhittaker™, Basel, Switzerland) and recombinant human macrophage colony-stimulating factor (M-CSF) 50ng/ml (Sigma-Aldrich, St. Louis, United States). The plate was incubated in a CO_2 incubator for 7 days at 37^0C with the differentiation media being changed twice during that period. Cells were observed daily for evidence of differentiation.

4.2.1.2 Macrophage infections

Human macrophages were infected with both strains of SINV. The macrophages were initially infected with the EgAR339 strain of SINV at MOI 0.1. Briefly: The cells were seeded at 2.2 x 10^5 cells per well in a 24 well plate, differentiated and grown until confluent before infection. The RPMI differentiation media was removed from the cells and viral inoculum left on the cells for 1 hour at 37^0C. The virus was removed and 400µl RPMI 1640 growth media was added to each well. The samples were taken at baseline, 2h, 4h, 6h, 10h and 24h intervals. Baseline refers to a sample at time zero directly after virus was removed from the cells and media was added. An aliquot of media from each well was frozen at -80^0C. The samples were titrated on Vero cells to measure viral replication. Briefly: Vero cells were seeded at 1 x 10^4 cells per well in a 96 well plate and grown until confluent before infection. The infectious samples were diluted 10-fold in the wells to have final dilutions ranging from 10^{-1} - 10^{-7} to infect the confluent Vero cells and incubated at 37^0C. CPE was monitored daily for three days.

The above methodology was used in subsequent assays unless otherwise specified using S.A.AR86 strain of SINV at MOI 1 and 0.1. Initially aliquots of media without cells were collected and the viral load determined by virus titration and real time quantitative PCR. In subsequent experiments media and cells were collected as samples because virus progeny may not have been released into the media sufficiently. The samples containing cells and media were freeze thawed three times to lyse the cells. The sampled were centrifuged at 1000 g to remove cell debris and SNF diluted and titrated on Vero cells as well as in a real time quantitative PCR. For Vero cell titration the SNF samples were 10-fold diluted from 10^{-1}-10^{-7}, incubated at 37^0C and monitored daily for CPE for three days. Viral loads were determined using quantitative real time PCR analysis the RNA was extracted from SNF, cDNA synthesized and viral loads determined for evidence of viral replication.

4.2.1.3 HeLa cells

HeLa cells were selected for various reasons such as their inability to express type 2 IFN which could influence further experiments, being susceptible to virus-induced apoptosis (Del Puerto et al., 2011; Ogata & Shigeta, 1979). The cells were maintained in MEM growth media containing 2% FBS, 100u/ml penicillin, 0.1mg/ml streptomycin and 2mM L-glutamine. Confluent cells were trypsinized, counted and seeded into 24 well plates at a density of 2.2 x 10^5 cells per well and incubated at 37^0C until confluent.

The HeLa cells were infected with SINV S.A.AR86 at MOI 0.1, MOI 1 and the Egyptian strain EgAR339 at MOI 0.1 in three separate experiments. The SINV Egyptian strain EgAR339 was only used at MOI 0.1 due to the inoculum having a higher $TCID_{50}$ than the SINV S.A.AR86 strain. The higher $TCID_{50}$ of the SINV Egyptian strain EgAR339 did not allow for virus at MOI 1 in the 400µl reaction volume in one of the wells of a 24 well plate. Briefly: confluent cells were trypsinized, counted and seeded into 24 well plates at a density of 2.2 x 10^5 cells per well. The MEM growth media was removed from the cells and virus absorbed into the cells for 1 hour at 37^0C. The virus was removed and 400µl MEM growth media was added. Cells were scraped off, sampled with SNF and were taken at baseline, 2h, 4h, 6h, 10h and 24h intervals. The cells were scraped off, with media were frozen at 80^0C.

4.2.2 IFN gamma ELISA

The ELISA was performed in 96 well Corning Costar 9018 plates (Corning, New York United States). Figure 4.1 shows the 96 well plate layout for the IFN gamma ELISA.

	IFN Standards		**Macrophages MOI 0.1**	**Macrophages* MOI 1**	**HeLa MOI 0.1**	**HeLa* MOI 1**
	1	**2**	**3**	**4**	**5**	**6**
A	500 pg/ml	500 pg/ml	baseline	baseline	baseline	baseline
B	250 pg/ml	250 pg/ml	baseline	baseline	baseline	baseline
C	125 pg/ml	125 pg/ml	24h	24h	24h	24h
D	62.5 pg/ml	62.5 pg/ml	24h	24h	24h	24h
E	31.25 pg/ml	31.25 pg/ml				
F	15.63 pg/ml	15.63 pg/ml				
G	7.81 pg/ml	7.81 pg/ml				
H	C	C				

Figure 4.1. Shows the plate layout for the IFN gamma ELISA

***SINV S.A.AR86 infection only.

Throughout the assay, reagent volumes of 100 µl were used unless specified otherwise. The Mouse IFN gamma ELISA Ready-SET-Go® (eBioscience, San Diego, United States) kit was modified for testing infected cell SNF samples for human IFN gamma by substituting both the coating and detection antibodies for human IFN gamma antibodies. The human IFN coating antibody used was anti-human IFN gamma monoclonal antibody 1-D1K and the detection antibody was anti-human IFN gamma biotinylated monoclonal antibody 7-B6-1 (MABTECH, Nacka Strand, Sweden).

A standard curve was prepared by serially diluting recombinant human IFN gamma (Sigma-Aldrich, St. Louis, United States) which was expressed in *E. coli* bacteria, 2-fold from 500pg/ml to 7.81pg/ml. The diluent for reagents was the 5 x Assay diluent provided in the kit diluted in PBS (pH 7.4) 1/5 and incubations were performed for 1 hour at room temperature unless otherwise specified, wells were blocked after coating with 200µl Assay diluent/PBS containing and plates were washed five times with PBS containing 0.1% Tween 20.

Briefly, a 96 well microtiter Corning Costar 9018 plate was coated overnight at 4^0C with monoclonal anti-human IFN gamma 1-D1K (250ng) diluted 1:2000. The plates were blocked, infected macrophage and HeLa cell culture media samples at baseline and 24h were added (75µl sample and 25µl Assay diluent) with serially diluted human IFN gamma standards and incubated overnight at 4^0C. After washing, human IFN gamma detection antibody 7-B6-1 (250ng) which is biotinylated was diluted 1:2000 in assay diluent and added to each well. The plates were incubated at room temperature for one hour. After washing a 100µl of avidin- horseradish peroxidase diluted in 1x Assay diluents was added to each well and incubated for 30 min at room temperature. The wells were aspirated and washed 7 times and 100µl of substrate solution 3,3',5,5'-Tetramethylbenzidine (TMB) was added to each well. The reactions were stopped after 15 min by adding 50µl stop solution (2N H_2SO_4) to each well and readings taken at 450 nm.

4.2.3 Infecting IFN treated HeLa cells

To determine the influence of IFN gamma on the replication of SINV in mammalian cells HeLa cells were infected as shown in Figure 4.2 with SINV S.A.AR86 at MOI 0.1. The cells were treated with IFN gamma at six hours before infection, at infection and six hours after infection.

Briefly: The HeLa cells were seeded at 2.2 x 10^5 cells per well in a sterile 24 well cell culture plate and grown until confluent before infection. The maintenance media used was MEM containing 2% FBS, 100u/ml penicillin, 0.1mg/ml streptomycin and 2mM L-glutamine. The media was removed from the confluent cells and the HeLa cells were treated with human IFN gamma to have an final concentration of either 1000 pg/ml or 2000 pg/ml per well six hours before infection, at infection or six hours after infection. The cells were infected with SINV S.A.AR86 MOI 0.1. The cells were incubated for 24h at 37^0C post infection and both cells and SNF fluid sampled for analysis.

		Pre-treated IFN gamma 6h	Treated with IFN gamma at infection	Treated with IFN gamma at 6h after infection	IFN without virus	Virus No IFN
IFN gamma Concentration		**1**	**2**	**3**	**4**	**5**
2000 pg/ml	**A**	HeLa cells	HeLa cells	HeLa cells	HeLa cells	HeLa cells
2000 pg/ml	**B**	HeLa cells	HeLa cells	HeLa cells	HeLa cells	HeLa cells
1000 pg/ml	**C**	HeLa cells	HeLa cells	HeLa cells	HeLa cells	HeLa cells
1000 pg/ml	**D**	HeLa cells	HeLa cells	HeLa cells	HeLa cells	HeLa cells

Figure 4.2. The plate layout for HeLa cells pre-treated with IFN gamma.

4.2.4 Viral nucleic acid quantification

The infectious samples obtained from the IFN treated HeLa cells were freeze-thawed for three cycles to lyse the cells open and clarified by centrifugation at 5000g for 5min. The QIAamp® Viral RNA kit was used for viral RNA extraction as discussed in section 3.2.6.1. The RNA extraction was followed by the reverse transcription of (+) strand RNA as described in section 3.2.6.2. An increase in viral load shows evidence that the virus is replicating. The real-time quantification reactions were performed using the TaqMan real-time quantification assay discussed in section 3.2.8 using either cDNA transcribed from (+) or (-) strand RNA as template.

4.2.5 Apoptosis

The Apoptotic DNA ladder kit (Roche, Basel, Switzerland) was used to analyze cell culture SNF samples after infection. The kit is designed for the extraction of cellular DNA that allows the detection of typical DNA ladder which is a hallmark of apoptotic cells. In cells in which apoptosis has been induced morphological changes are associated with the appearance of nucleosome excisions from chromatin through the activation of intracellular endonuclease which results in the appearance of a nucleosomal DNA ladder (Stassen et al., 2012).

Briefly: The HeLa cells were seeded at 2 x 10^6 cells per well in a sterile 24 well cell culture plate and grown until confluent before infection. The maintenance media used was MEM containing 2% FBS, 100u/ml penicillin, 0.1mg/ml streptomycin and 2mM L-glutamine. The media was removed from the confluent cells and the HeLa cells infected with SINV S.A.AR86 MOI 0.1. The cells were incubated for 24h at 37^0C. Baseline and 24h samples containing both cells and SNF fluid were taken for analysis.

An aliquot of 200µl Lysis buffer was added to cells (2 x 10^6 HeLa cells) and incubated for 10 min at room temperature to lyse infected cells. A 100µl aliquot of isopropanol was added to each sample for precipitation of DNA, vortexed, added to the filter/collection and centrifuged for 1min at 6000g. The flowthrough was discarded and filter-bound sample washed with 500µl wash buffer for 1min at 6000g, washed and centrifuged again, the flowtrough discarded, washed and centrifuged for 10 000g for 10s. DNA was eluted in 200µl pre-warmed (70^0C) elution buffer, centrifuged at 6000g for 1min and stored at -20^0C.

The extracted DNA was analyzed by gel electrophoresis as discussed in section 3.2.3

4.3 Results

4.3.1 PBMC infection

4.3.1.1 Measuring viral replication by titration in Vero cells

The SINV strain EgAR339 was used to infect human macrophages with MOI 0.1. To determine if virus was replicating within the macrophages, samples collected at intervals after infection were diluted (10^{-1} - 10^{-7}) and the titer of virus in each sample determined by inoculating Vero cells and monitoring CPE. After three days there was ≥ 50% CPE in all wells inoculated with samples collected at baseline and after 24 hours and diluted 10^{-1} - 10^{-4}. There was no CPE observed in wells with samples diluted of 10^{-5} - 10^{-7}. Results showed that there were no significant increase in viral titer which likely meant that there was no or only low levels of replication of the virus in the macrophages. Attempts to infect macrophages were repeated on three occasions with similar results.

SINV strain S.A.AR86 was used to infect human macrophages with MOI 0.1 and MOI 1. Similarly, samples collected after infection were titrated on Vero cells to determine viral titers. After three days there was ≥ 50% CPE seen in all wells inoculated with samples collected at baseline and at intervals up to 24 hours after infection with samples diluted 10^{-1} - 10^{-5}. There was no CPE observed in wells with dilutions of 10^{-6} - 10^{-7}. Similarly for macrophages infected with SINV strain S.A.AR86 at MOI 1 an end point titer of 10^{7} was determined for samples collected from infected cells at baseline and up to 24 hours after infection. Results showed that there were no significant increase in viral titer which likely meant that there was no or only low levels of replication of the virus in the macrophages. Attempts to infect macrophages with the S.A.AR86 strain of SINV were repeated on two occasions with similar results. Infections using SINV S.A.AR86 were repeated twice and at each time point cells and media were collected and analyzed for infectious virus. Similar endpoint titers suggested little or no viral replication in the macrophages. To determine if there were low levels of replication that could not be differentiated using 10 fold dilutions, viral loads were determined using quantitative real time PCR.

4.3.1.2 Determination of viral load using (-) strand RNA template

The quantitative real time PCR was used to determine whether there was an increase in the cDNA copy number in samples collected at baseline and after 24h. SNF samples were taken initially at different intervals after infection (baseline, 2h, 4h, 6h, 10h and 24h), RNA extracted, cDNA synthesized using the forward primer to transcribe (-) strand RNA and quantitated in a real time PCR. The CP is the cycle number

that corresponds to an increase of fluorescence over the threshold calculated using the lightcycler software. In each PCR reaction the samples were tested with a sample of known copy number of 2.2 x 10^3 copies DNA/µl and a negative control.

The real time quantification reactions performed on duplicate experiments did not show an increase of cDNA copy number from baseline to 24h. The infection experiment was repeated as described in section 4.3.1.1 in which viral load was determined in cells and media. After freeze thawing the samples RNA was extracted from the SNF, cDNA synthesized and quantitated in a real time PCR.

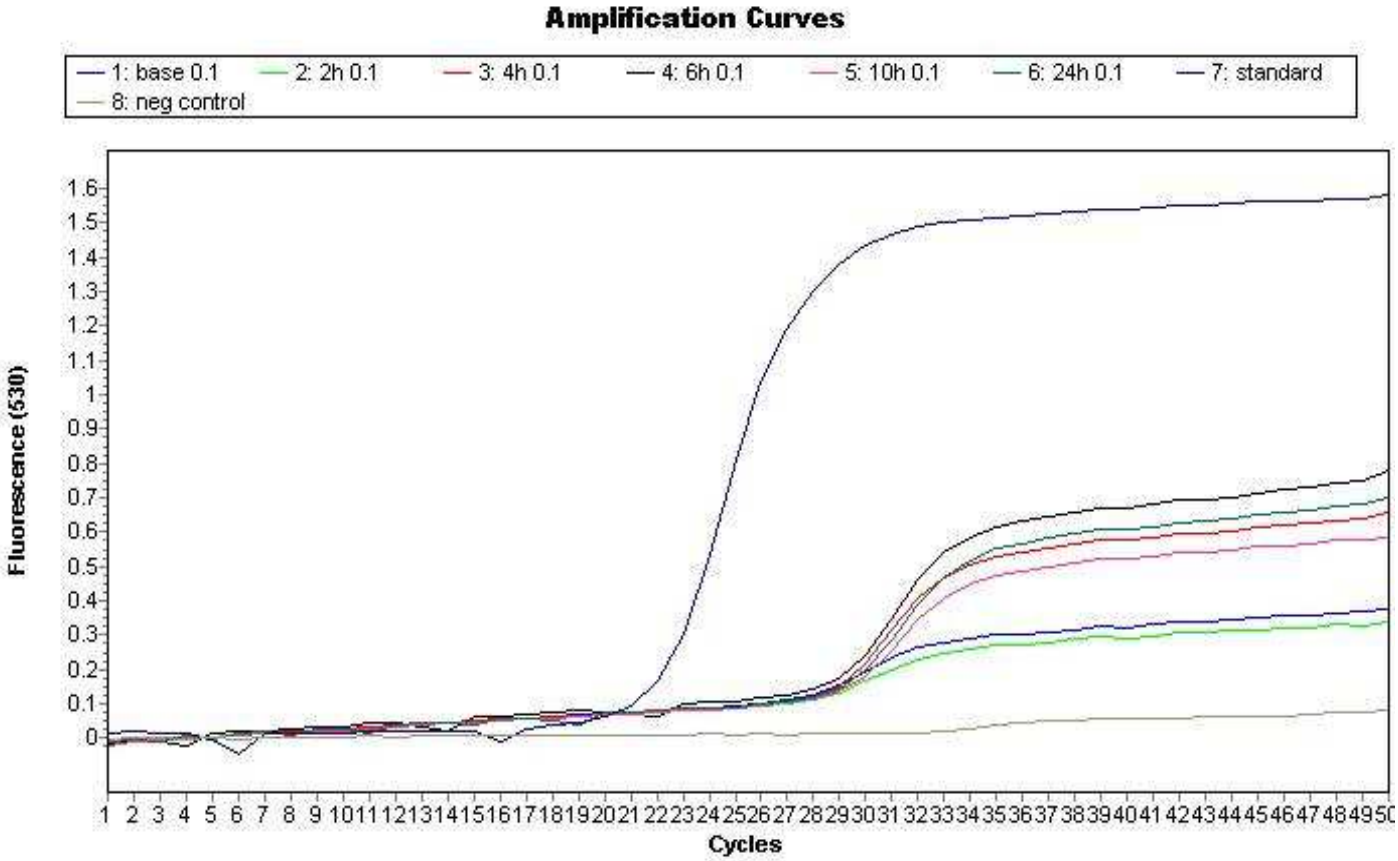

Figure 4.3. The amplification curves for macrophages infected with SINV S.A.AR86 at MOI 0.1 at time periods base, 2h, 4h, 6h, 10h and 24h.

There was no evidence of viral replication from the macrophages inoculated with SINV S.A.AR86 at MOI 0.1. The amplification curves in Figure 4.3 represent the six samples collected from baseline to 24h post inoculation. The crossing points as well as the estimated copy number of DNA in each sample are shown in Table 4.1.

Table 4.1. The crossing points and cDNA copies/µl present in macrophages infected with SINV S.A.AR86 at MOI 0.1 and sampled at different time intervals.

Sample	Name	Crossing point	(Copies cDNA/µl)	Standard
1	Base 0.1	26.11	4.41×10^3	
2	2h	25.47	3.66×10^3	
3	4h	26.96	2.88×10^3	
4	6h	27.41	2.41×10^3	
5	10h	27.50	2.33×10^3	
6	24h	27.50	2.32×10^3	
7	Standard	20.51	2.2×10^3	2.2×10^3
8	Negative control	-	-	

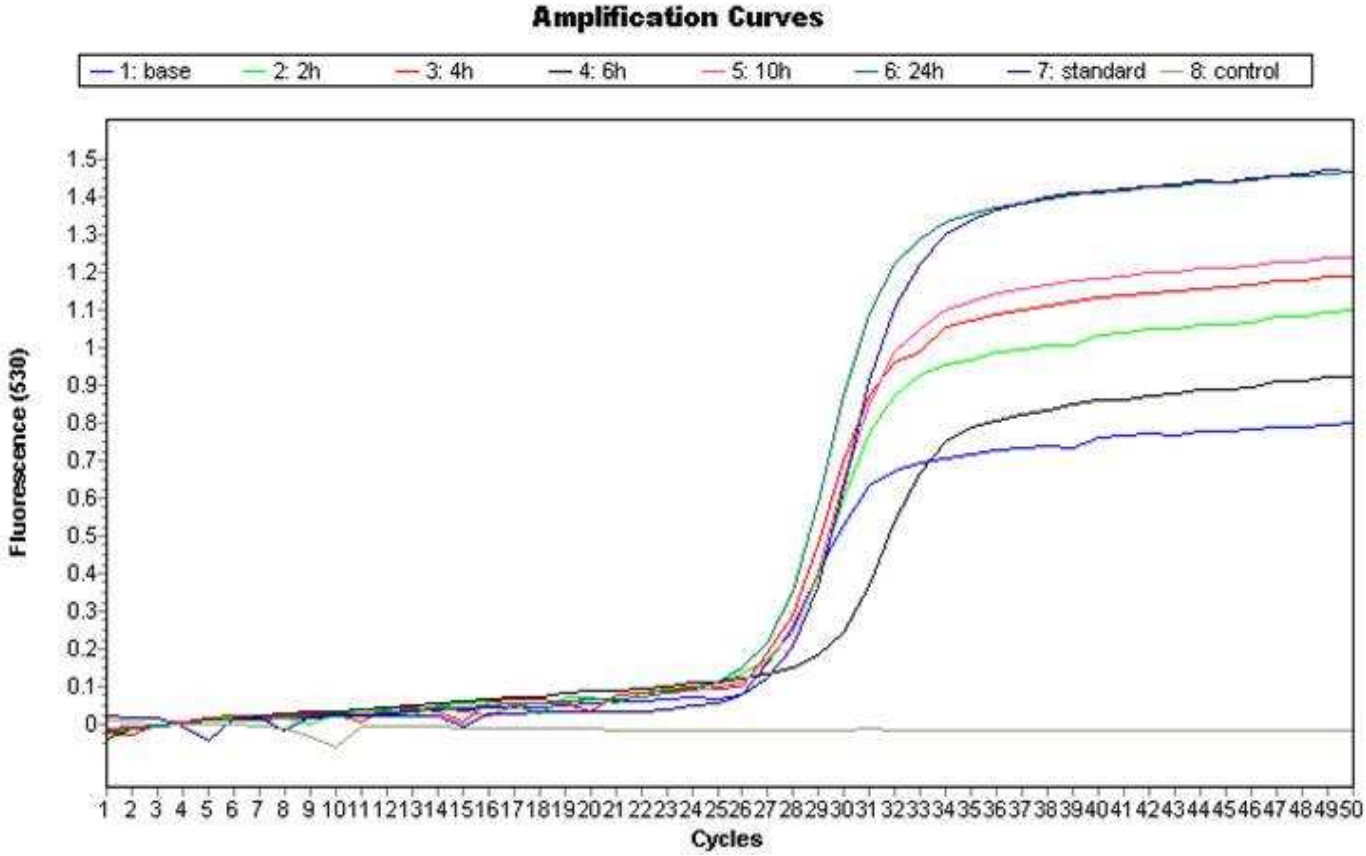

Figure 4.4. . Viral load for macrophages infected with SINV S.A.AR86 with MOI 1 at time periods base, 2h, 4h, 6h, 10h and 24h. Sample number 7 was the purple line and known standard with 2.2×10^5 copies of DNA/µl.

Similarly the macrophages infected with an MOI 1 did not show significant viral replication when looking at the amplification graphs of the different samples as can be seen in Figure 4.4. The samples from baseline (blue line) to 24h (dark green line) were detected at the same crossing point indicating similar copies of DNA template present and hence no replication. Table 4.2 allowed for more detailed analysis of amplification cycles in terms of the crossing points as well as the amount of cDNA copies/µl.

Table 4.2. The crossing points and cDNA copies/µl present in macrophages infected with SINV S.A.AR86 at MOI 1 and sampled at different time intervals.

Sample	Name	Crossing point	(Copies cDNA/µl)	Standard
1	Base 0.1	24.90	4.81×10^3	
2	2h	25.77	2.85×10^3	
3	4h	25.46	3.40×10^3	
4	6h	27.67	1.17×10^3	
5	10h	25.84	2.74×10^3	
6	24h	25.49	3.34×10^3	
7	Standard	26.16	2.2×10^5	2.2×10^5
8	Negative	-	-	

The macrophages infected with SINV at both MOI 0.1 (Table 4.1) and 1 (Table 4.2) did not show an increase in (-) strand RNA based on cDNA copy number in samples collected at baseline and at intervals up 24h. Small fluctuations were observed that were likely due to sampling. Overall there was no significant increase during the 24h period. To confirm the results an RT-PCR amplifying cDNA transcribed from (+) strand RNA was performed.

4.3.1.3 Determination of viral load using (+) strand RNA template

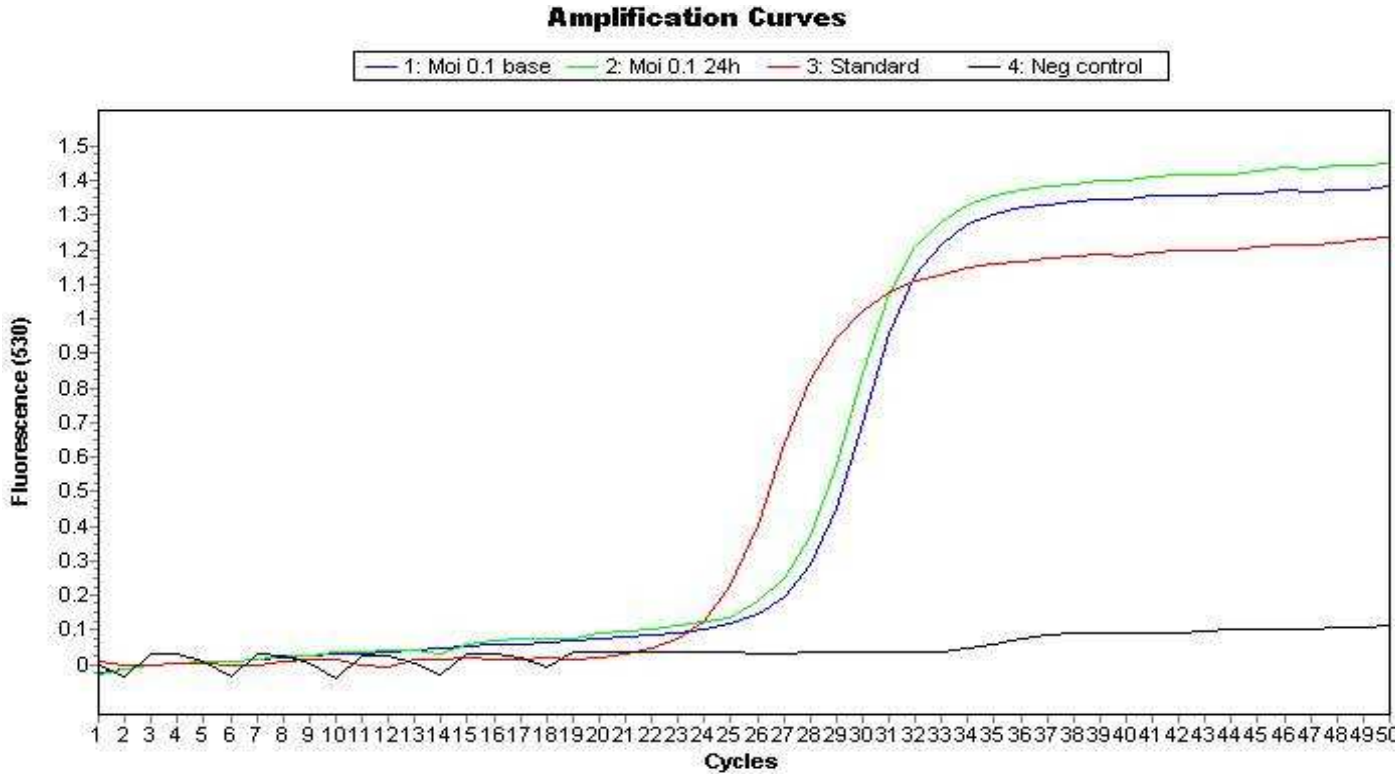

Figure 4.5. The cDNA amplification curves for macrophages infected with SINV S.A.AR86 at MOI 0.1 and samples at time periods base and 24h.

Figure 4.5 indicated a marginal increase in the viral load based on amplification of cDNA transcribed from (+) strand RNA from the baseline (blue line) to 24h (green line) indicating low levels of replication. The crossing points in Table 4.3 decreased from 25.97 at baseline to 25.56 at 24h and the viral load increased from 2.15 x 10^4 cDNA copies at baseline to 2.71 x 10^4 cDNA copies at 24h. However similar small fluctuations were observed previously and were not considered sufficient evidence that the virus was replicating in the macrophages.

Table 4.3. The crossing points and cDNA copies/µl in macrophages infected with SINV S.A.AR86 at MOI 0.1 and sampled at different time intervals.

Sample	Name	Crossing point	(Copies cDNA/µl)	Standard
1	Base	25.97	2.15 x 10^4	
2	24h	25.56	2.71 x 10^4	
3	Standard	22.54	3.20 x 10^5	3.20 x 10^5
4	Neg control		-	-

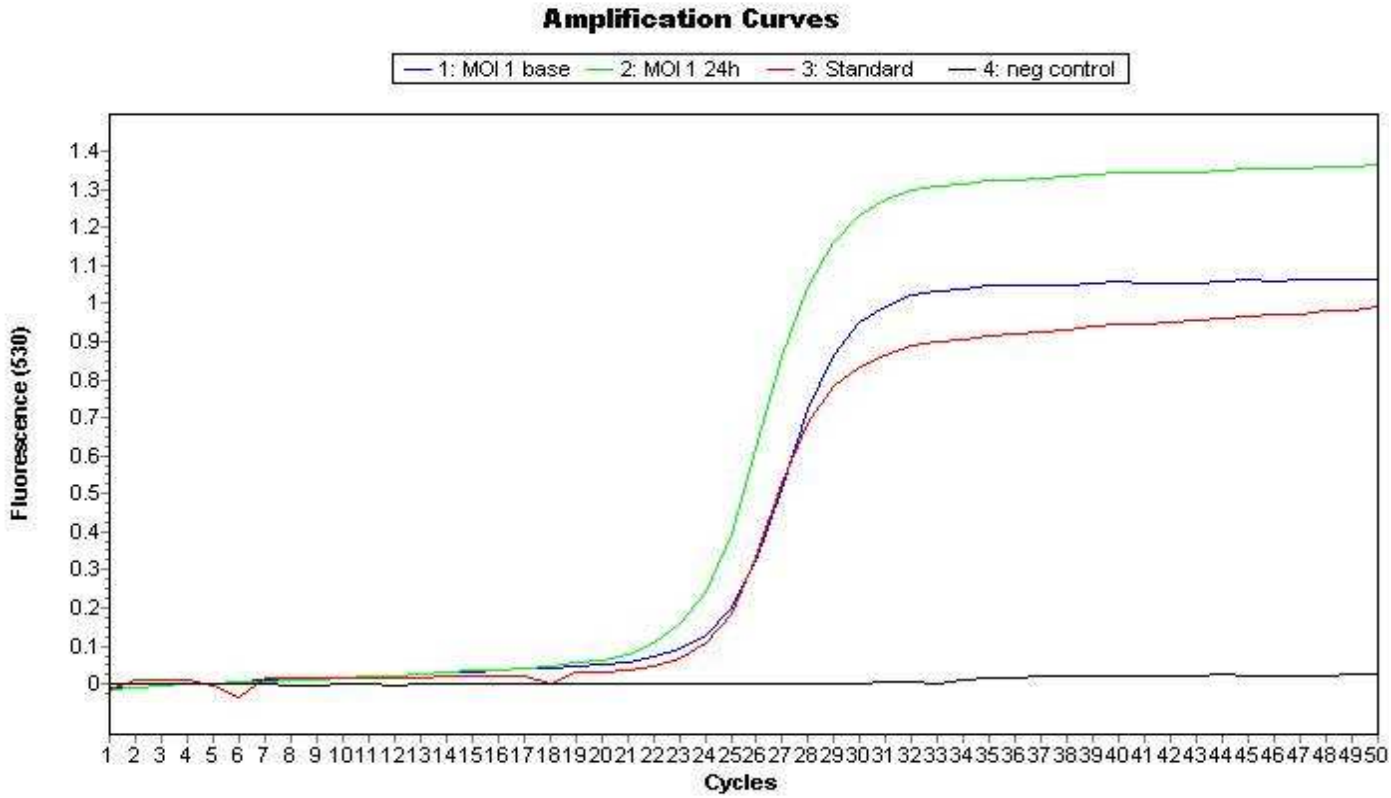

Figure 4.6. The cDNA amplification curves for macrophages infected with SINV S.A.AR86 at MOI 1 and samples at time periods base and 24h.

The amplification curves in Figure 4.6 with MOI 1 infection of macrophages showed a small decrease in the crossing point value over the 24h period indicating higher viral load in the 24h sample and therefore low levels of viral replication.

Table 4.4. The crossing points and cDNA copy numbers in macrophages infected with SINV S.A.AR86 at MOI 1 and sampled at different time intervals.

Sample	Name	Crossing point	(Copies cDNA/µl)	Standard
1	Base	23.08	1.64×10^5	
2	24h	22.11	3.88×10^5	
3	Standard	22.69	3.20×10^5	3.20×10^5
4	Neg control		-	-

There was a two-fold increase in the the viral load between the baseline sample and the 24h sample with the CP value 1 cycle higher which is indicative of low levels of viral replication. We were able to detect low levels of SINV replication in the macrophages at both MOI 0.1 (Table 4.3) and (Table 4.4). The two-fold increase in viral load was the highest level of replication that could be detected. It was not considered a high enough level of replication which could be useful in downstream experiments. In the absence of infection of the macrophages attempts were made to determine why SINV did not replicate sufficiently in the macrophages.

The infection of macrophages with SINV S.A.AR86 and measurement of viral load was repeated four times in total and all experiments showed similar results with a lack of replication over time. There was no significant increase in viral loads from baseline to 24h.

4.3.2 HeLa cell infections

4.3.2.1 Detection of viral load in HeLa cells

Prior to treatment of cells with IFN, the replication of SINV in HeLa cells was confirmed by determining viral load using the reverse primer to transcribe RNA.

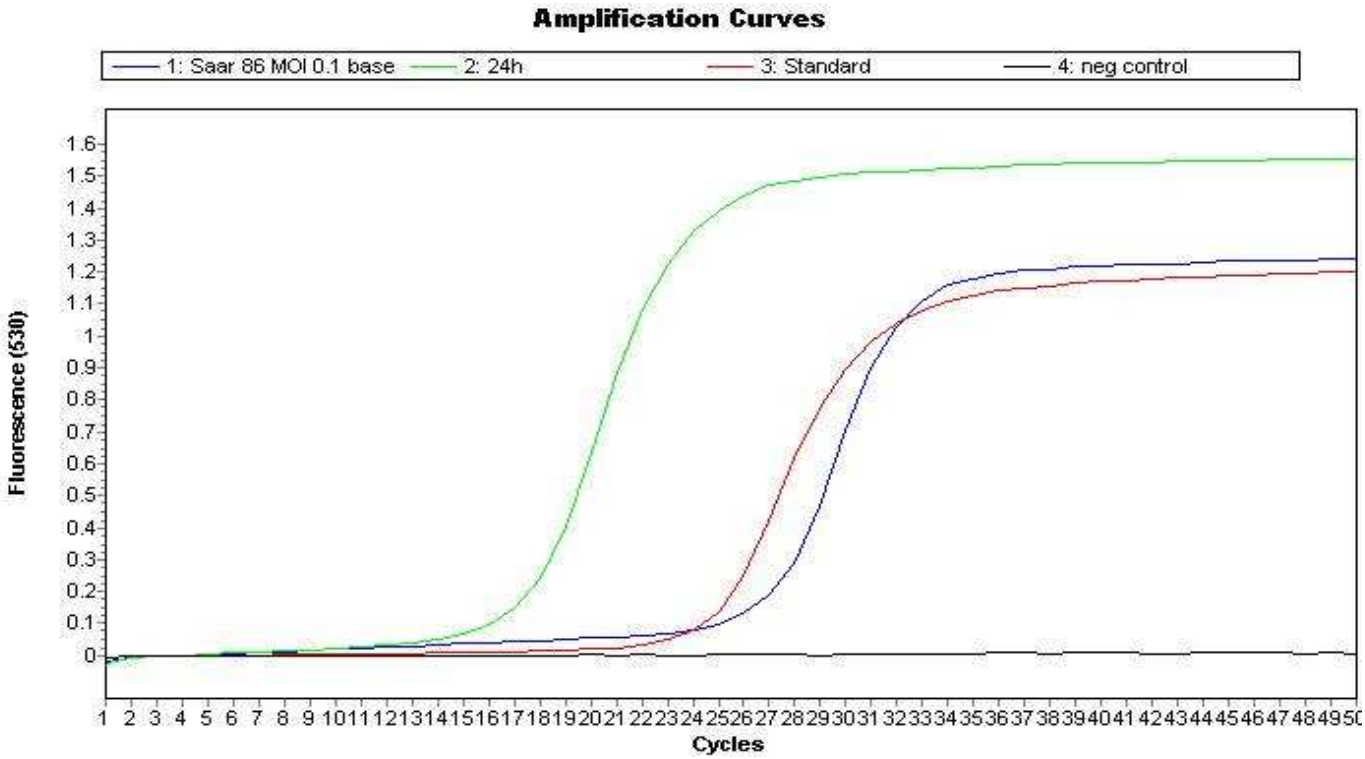

Figure 4.7. The amplification curves for HeLa cells infected with SINV S.A.AR86 at MOI 0.1 at time periods base and 24h.

Figure 4.7 indicated a substantial increase in copies of cDNA between baseline (blue line) and 24h (green line) samples. There was a difference of 9 cycles between the baseline and 24h samples as well as a 10 000-fold increase in the cDNA copy number from 5.26 x 10^4 at baseline to 1.71 x 10^8 at 24h as seen in Table 4.5.

Table 4.5. The crossing points and cDNA copies/µl present in HeLa cells infected with SINV S.A.AR86 at MOI 0.1 and sampled at different time intervals.

Sample	Name	Crossing point	(Copies cDNA/µl)	Standard
1	Base 1	25.66	5.26 x 10^4	
2	24h	16.18	1.71 x 10^8	
3	Standard	23.49	3.20 x 10^5	3.20 x 10^5
4	Neg control		-	-

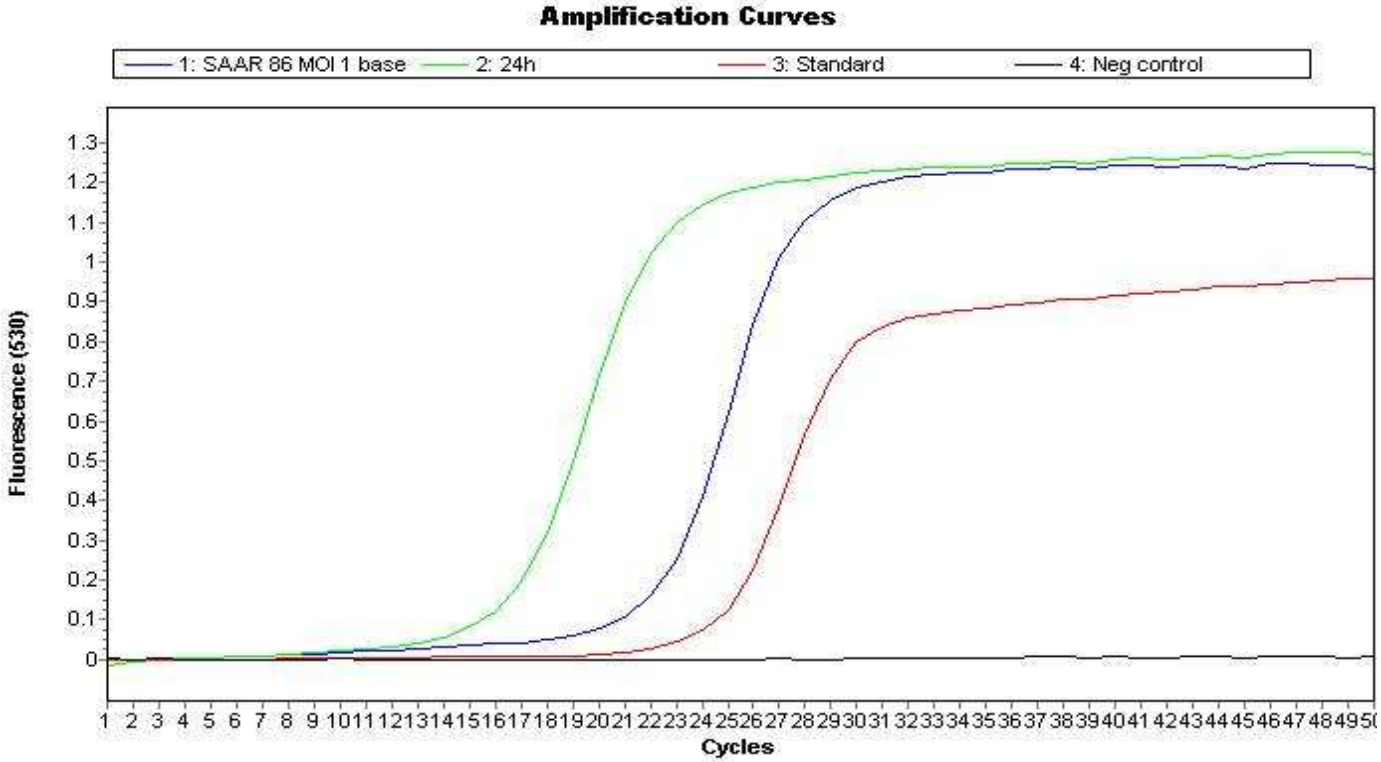

Figure 4.8. The amplification curves for HeLa cells infected with SINV S.A.AR86 at MOI 1 at time periods base and 24h.

Figure 4.8 showed substantial virus replication between the baseline (blue line) and 24h (green line) sample. There was a difference of 5 cycles as well as a 100-fold difference of in copies of cDNA from 1.93 x 10^6 at baseline to 3.14 x 10^8 for sample collected 24h after infection as shown in Table 4.6.

Table 4.6. The crossing points and cDNA copies/µl present in HeLa cells infected with SINV S.A.AR86 at MOI 1 and sampled at different time intervals.

Sample	Name	Crossing point	(Copies cDNA/µl)	Standard
1	Base 1	20.99	1.93 x 10^6	
2	24h	15.34	3.14 x 10^8	
3	Standard	23.29	3.20 x 10^5	3.20 x 10^5
4	Neg control		-	-

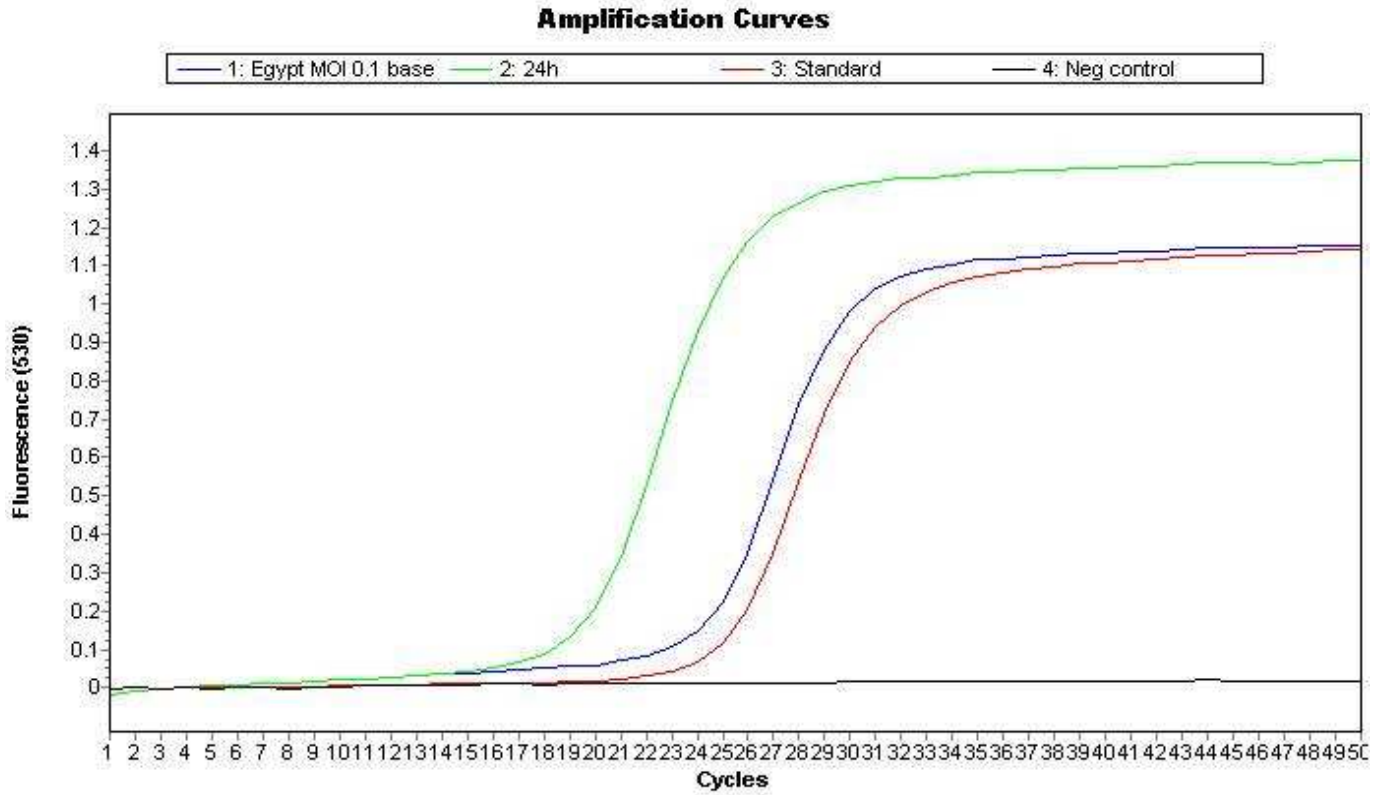

Figure 4.9. The amplification curves for HeLa cells infected with SINV EgAR339 at MOI 0.1 at time periods base and 24h.

The growth curves in Figure 4.9 showed significant virus replication between baseline (blue line) and 24h (green line) samples. The 24h sample was detected 5 cycles earlier than the baseline samples and had a 100-fold difference in copies cDNA from 4.20 x 10^5 at baseline to 3.01 x 10^7 for sample collected 24h after infection as shown in Table 4.7.

Table 4.7. The crossing points and cDNA copies/µl present in HeLa cells infected with SINV EgAR339 at MOI 0.1 and sampled at different time intervals.

Sample	Name	Crossing point	(Copies cDNA/µl)	Standard
1	Base 1	23.05	4.20 x 10^5	
2	24h	18.34	3.01 x 10^7	
3	Standard	23.80	3.20 x 10^5	3.20 x 10^5
4	Neg control		-	-

4.3.3 IFN gamma ELISA

A standard curve was prepared for the IFN gamma ELISA by preparing serial dilutions of human IFN gamma. The OD readings were plotted against the concentrations of IFN gamma and a linear correlation between the two was determined.

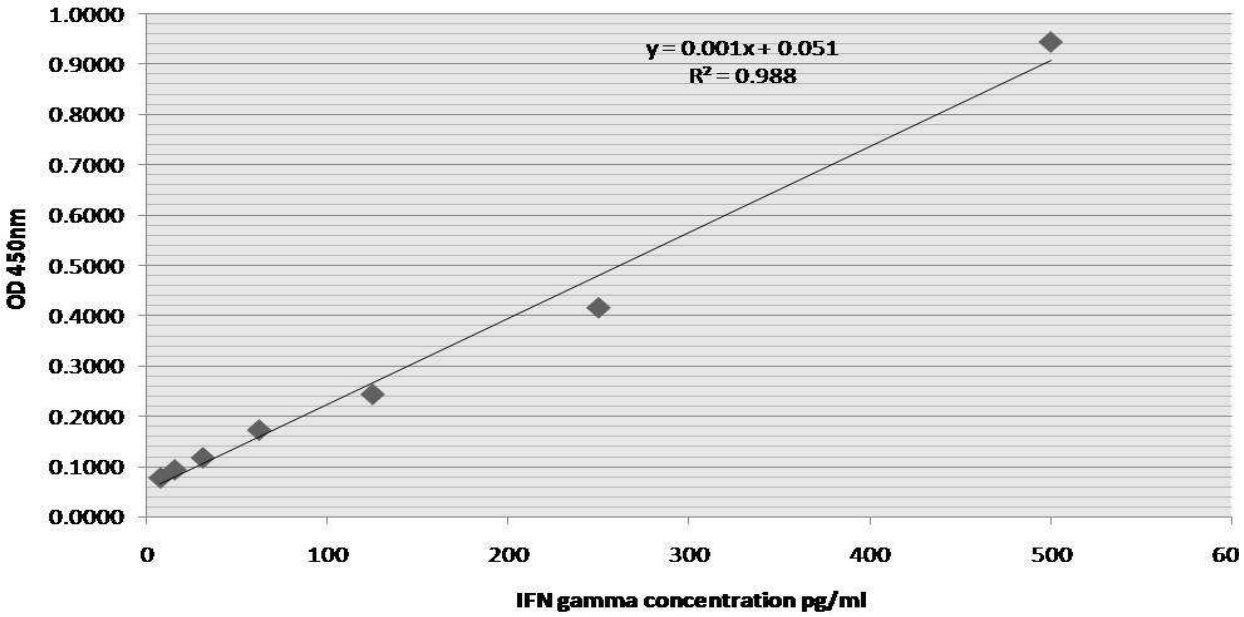

Figure 4.10. Standard curve for IFN gamma ELISA with OD at 450nm plotted against the concentration of IFN gamma in pg/ml.

The assay was able to successfully detect the complete range of IFN gamma concentrations in the standard curve from 7.81pg/ml to 500pg/ml as shown in Figure 4.10. The optimized assay was simultaneously used to test macrophage SNF samples at baseline and 24h in duplicate for the presence and concentrations of IFN gamma were detected in the samples.

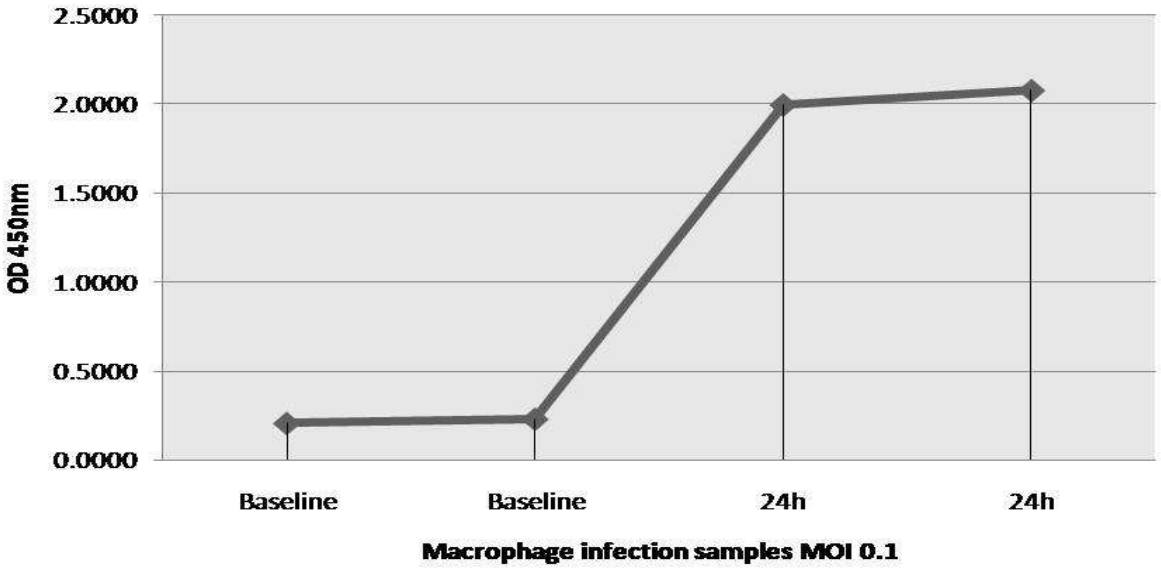

Figure 4.11. OD values measured at 450nm in macrophage cells infected with SINV S.A.AR86 at MOI 0.1 at baseline and 24h.

There was a decisive increase between the OD readings at baseline and 24h in the macrophages infected at MOI 0.1 as seen in Figure 4.11. The samples were tested in duplicate and gave similar results. The baseline OD readings, 0.2080 and 0.2310 correlated to a baseline IFN gamma concentration of 118.35pg/ml and 131.61pg/ml respectively. OD's of 1.9960 and 2.0770 which correlated to IFN gamma concentrations of 1135.71pg/ml and 1183.35pg/ml respectively were detected in samples collected 24h after infection of SINV.

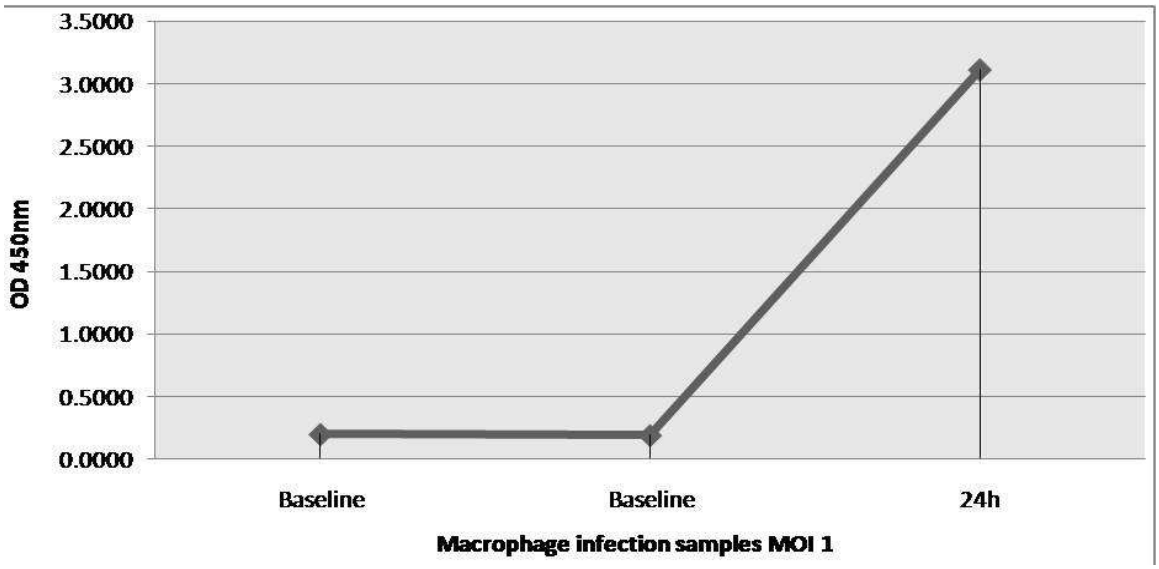

Figure 4.12. Shows the change of IFN gamma concentration in macrophages cell infected with SINV S.A.AR86 at MOI 1.

The samples collected from experiments in which macrophages were infected with SINV at MOI 1 were tested in duplicate, but the 2nd sample resulted in a reading that was too high to accurately determine. Figure 4.12 shows there was a higher increase in the OD readings in cells infected with SINV at MOI 1 than with MOI 0.1. The baseline samples tested in duplicate had OD values at 0.1990 and 0.1900 which correlated to a IFN gamma concentrations of 114.60pg/ml and 109.42pg/ml respectively whereas after 24h the OD increased to 3.11 which correlated to a IFN gamma concentration of 1792.18pg/ml. The increase in the IFN levels in SINV infected macrophages between baseline and 24h suggest that infection led to the rapid secretion of IFN gamma by the macrophages.

4.3.4 IFN treated HeLa cells infection nucleic acid amplification

To determine if IFN gamma could prevent or reduce SINV replication experiments were performed in which cells were pretreated with IFN gamma prior to infection with SINV. The effect of IFN gamma treatment at different time intervals in SINV infected HeLa cells was determined by real-time quantitative real-time PCR.

4.3.4.1 Viral load determination

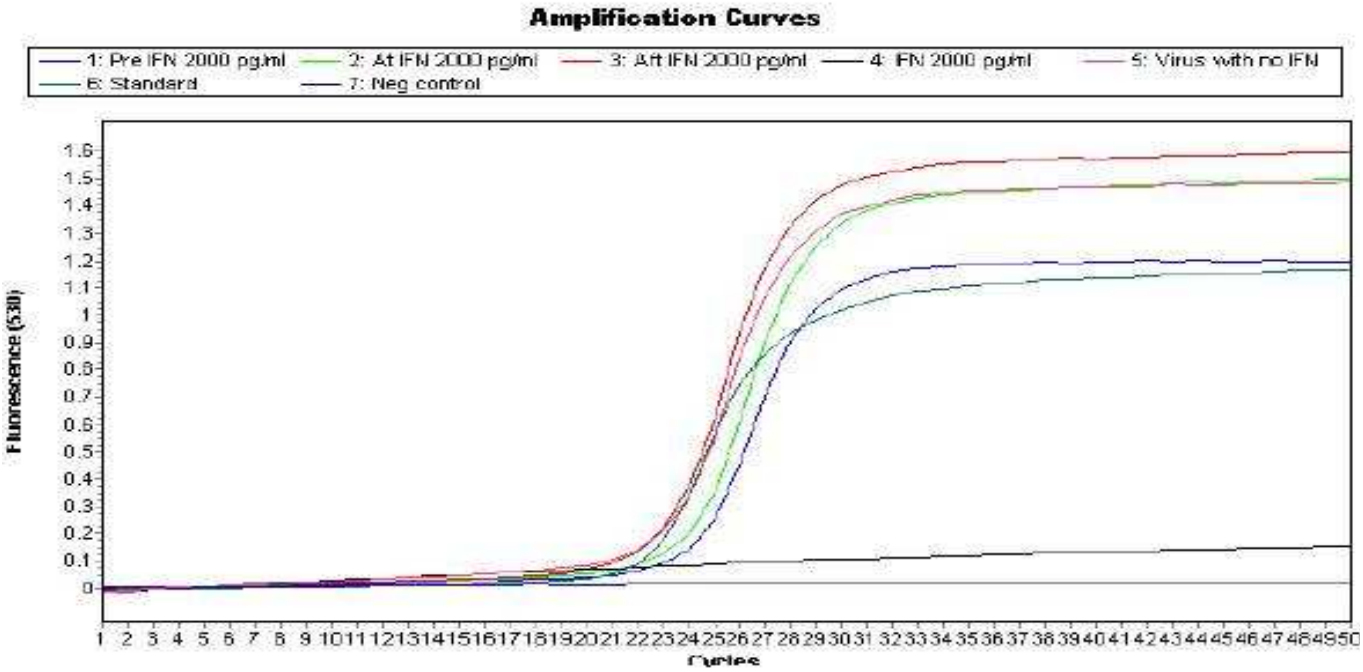

Figure 4.13. The amplification curves for HeLa cell SNF samples infected with SINV S.A.AR86 at MOI 0.1 treated with 2000pg/ml IFN gamma.

Figure 4.13 shows the amplification curves for samples collected from cells pretreated with IFN gamma. The replication as determined by the changes in copy number of cDNA was very similar with both IFN gamma pre-treatment (blue line) as well as at (green line) time of infection. In contrast there was an increase in viral nucleic acid in cells treated with IFN gamma after infection (red line) which indicated that viral replication was initialized before IFN gamma could inhibit it. It was interesting to note the similarity in viral replication between the cells treated with IFN gamma after infection and the infected cells that were not treated with IFN gamma (pink line). Both the negative control (grey line) and IFN treated cells without virus (black line) tested negative as expected

Table 4.8. The crossing points and cDNA copy numbers in HeLa cells infected with SINV S.A.AR86 at MOI 0.1 and treated with IFN gamma 2000pg/ml at different time intervals.

Sample	Name	Crossing point	(Copies cDNA/µl)	Standard
1	Pre-treated IFN	22.62	4.18×10^5	
2	At infection IFN	22.43	4.92×10^5	
3	After infection IFN	21.46	1.20×10^6	
4	IFN, no virus	-	-	
5	Virus, no IFN	21.60	1.05×10^6	
6	Standard	20.74	2.30×10^6	2.30×10^6
7	Negative control	-	-	

The similarities between the pre-treated IFN gamma infection and simultaneous infection and IFN gamma treatment is better understood when comparing the concentrations of cDNA in the SNF of each sample after infection as shown in Table 4.8. Samples from cells pre-treated as wells as treated at time of infection were detected at crossing points 22.62 and 22.43 respectively which related to 4.18 x 10^5 copies cDNA/µl and 4.92 x 10^5 copies cDNA/µl respectively. Viral loads detected in cells treated with IFN gamma after infection and cells that had no IFN treatment had similar crossing points of 21.46 and 21.60 respectively as well as cDNA copy numbers of 1.20 x 10^6 and 1.05 x 10^6. The results indicated that treating the HeLa cells with IFN gamma six hours post infection was not effective and that viral replication had already overcome the potential inhibitory effects of the IFN gamma.

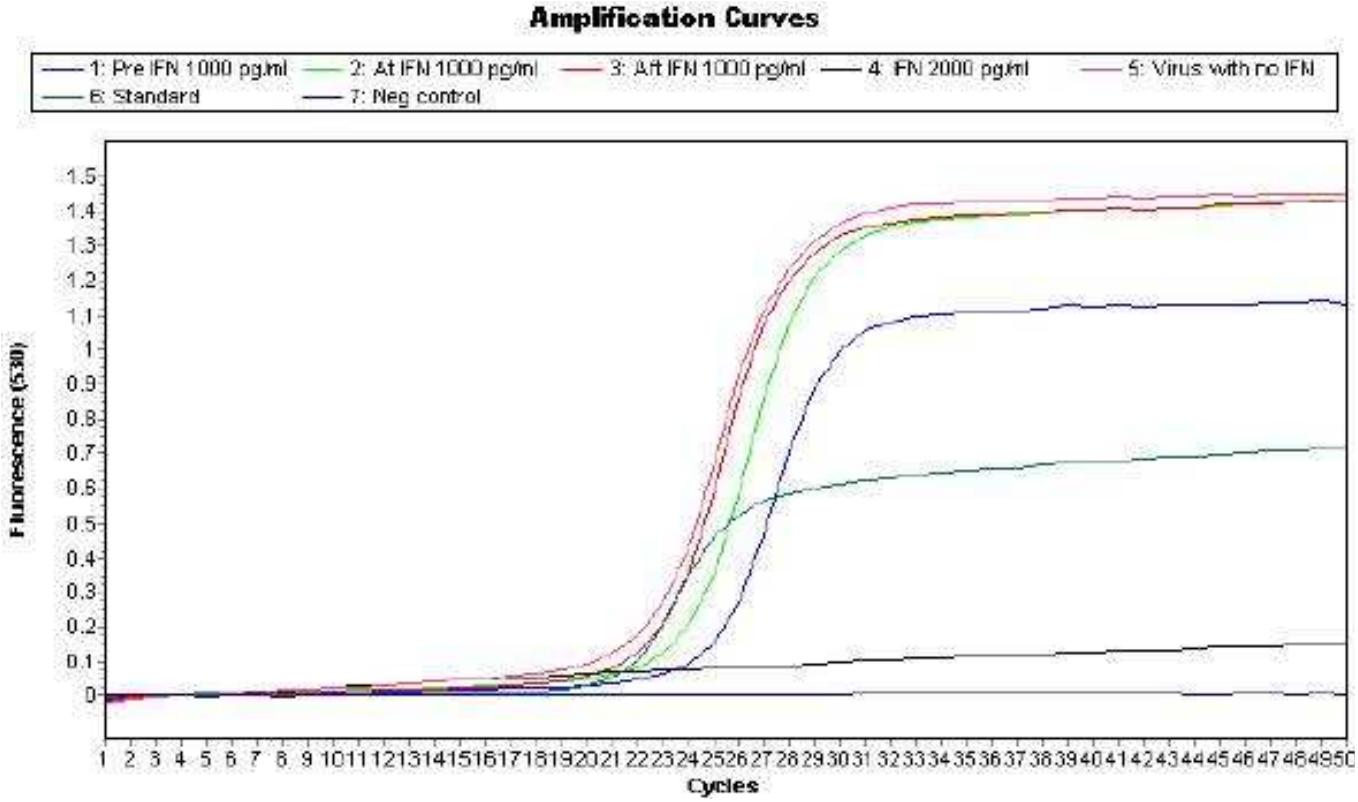

Figure 4.14. The amplification curves for HeLa cell SNF samples infected with SINV S.A.AR86 at MOI 0.1 treated with 1000pg/ml IFN gamma.

Figure 4.14 indicated more subtle similarities and differences than seen in Figure 4.16. There were subtle differences between the two which could be attributed to the lower dose of IFN gamma used, 1000pg/ml instead of 2000pg/ml. The viral load in cells pre-treated with IFN gamma (blue line) led to lower viral replication than cells treated at infection (green line). Table 4.9 showed a decrease in crossing points from 23.46 for pre-treated cells to 22.41 for cells treated at infection as well as an increase in cDNA copy numbers from 8.06 x 10^4 for pre-treated cells to 1.96 x 10^5 cells treated at infection.

Table 4.9. The crossing points and cDNA copy numbers/µl in HeLa cells infected with SINV S.A.AR86 at MOI 0.1 and treated with IFN gamma 1000pg/ml at different time intervals.

Sample	Name	Crossing point	(Copies cDNA/µl)	Standard
1	Pre-treated IFN	23.46	8.06×10^4	
2	At infection IFN	22.41	1.96×10^5	
3	After infection IFN	21.28	5.54×10^5	
4	IFN, no virus	-	-	
5	Virus, no IFN	21.06	6.74×10^5	
6	Standard	19.72	2.30×10^6	2.30×10^6
7	Negative control	-	-	

The viral load detected in cells treated with IFN gamma in cells after infection and infection without IFN gamma was similar to that observed with the 2000pg/ml IFN treatment. The crossing points were very similar at 21.28 for cells treated after infection and 21.08 for cells not treated with IFN as well as the cDNA copies/µl at 5.54×10^5 for cells treated after infection and 6.74×10^5 for cells not treated with IFN.

4.3.4.2 Viral load using (+) strand RNA template

Negative strand RNA is usually evident 4 to 6 hours after infection with (+) strand RNA in abundance later in replication (Strauss & Strauss, 1994). In addition to determining changes in viral load based on (-) strand template, viral load was determined using (+) strand RNA template.

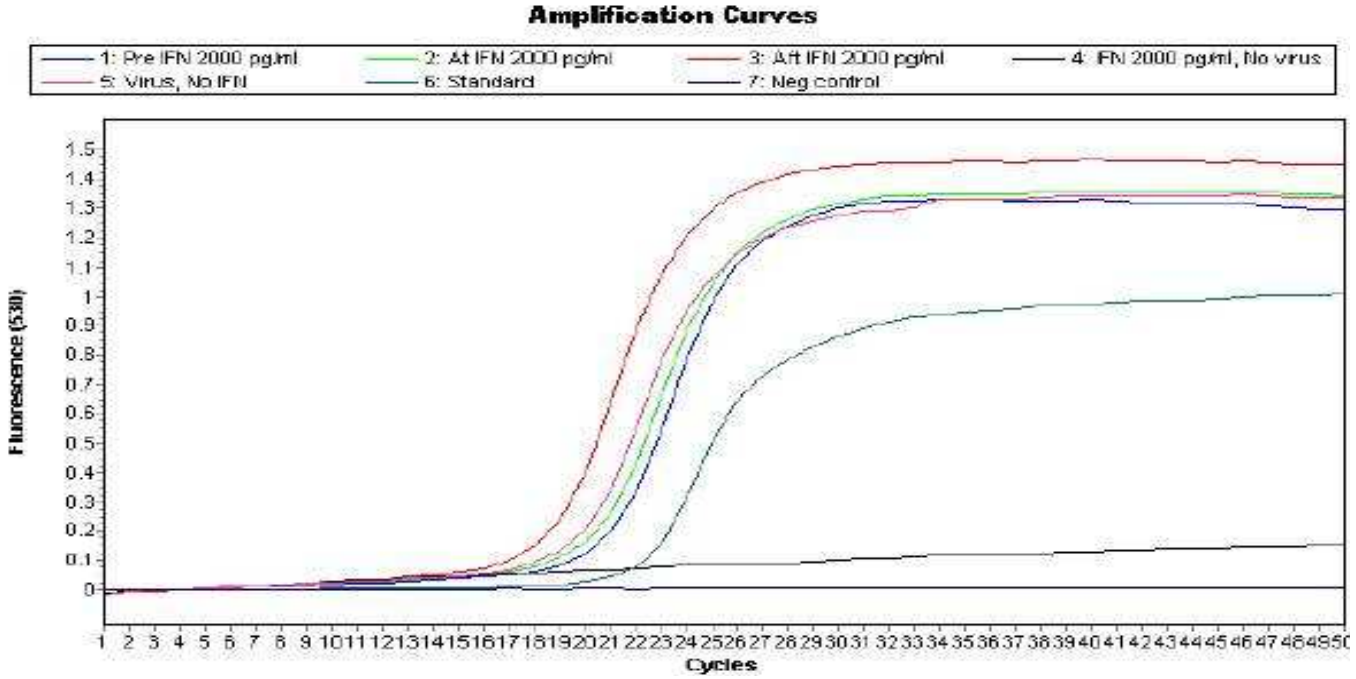

Figure 4.15. The amplification curves for HeLa cell SNF samples infected with SINV S.A.AR86 at MOI 0.1 treated with 2000pg/ml IFN gamma.

Figure 4.15 shows that pre-treating (blue line) the HeLa cells with IFN gamma was slightly more effective in reducing load and therefore viral replication than treating at time of infection (green line) or after infection (red line). Pre-treated HeLa cells showed the lowest levels of viral load and those treated after or without treatment showed the highest. Table 4.10 shows the viral load more accurately with the crossing points decreasing from 19.29 in pre-treated cells to 18.86 in cells treated at infection and then to 17.11 in cells treated after infection. That correlated to cDNA copy numbers of 7.46×10^6 in cells pre-treated to 1.10×10^7 in cells treated at infection and lastly 5.46×10^7 in the cells treated after infection.

Table 4.10. The crossing points and cDNA copy numbers/µl in HeLa cells infected with SINV S.A.AR86 at MOI 0.1 treated with IFN gamma 2000pg/ml at different time intervals.

Sample	Name	Crossing point	(Copies cDNA/µl)	Standard
1	Pre-treated IFN	19.29	7.46×10^6	
2	At infection IFN	18.86	1.10×10^7	
3	After infection IFN	17.11	5.46×10^7	
4	IFN, no virus	-	-	
5	Virus, no IFN	18.19	2.04×10^7	
6	Standard	20.58	2.30×10^6	2.30×10^6
7	Negative control	-	-	

When comparing the at infection and post treated cells with infected cells without IFN there was little or no difference indicating that treating the cells after infection had little influence on the viral replication. A comparison of the viral loads detected using (-) strand RNA with (+) strand RNA in Table 4.10 showed the copies cDNA/µl were 100-fold higher at the same MOI which suggests that early replication resulting in (-) strand RNA was likely with an abundance of (+) strand RNA during later replication.

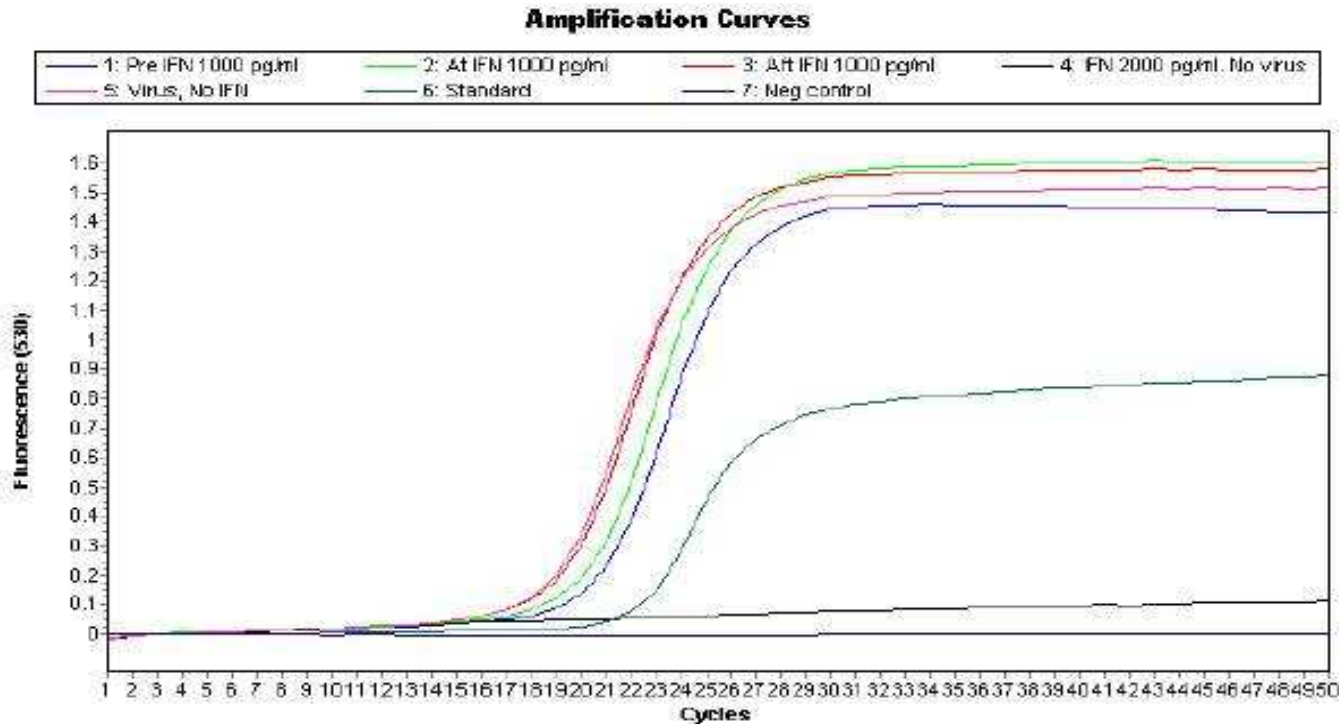

Figure 4.16. The amplification curves for HeLa cell SNF samples infected with SINV S.A.AR86 at MOI 0.1 treated with 1000pg/ml IFN gamma.

The HeLa cells treated with IFN gamma at 1000pg/ml showed the same tendencies as seen with the 2000pg/ml IFN treatment. Viral load was higher in cells pre-treated with IFN gamma compared to cells treated at infection or treated post infection as shown in Figure 4.16.

Table 4.11. The crossing points and cDNA copies/µl in HeLa cells infected with SINV S.A.AR86 at MOI 0.1 treated with IFN gamma 1000pg/ml at different time intervals.

Sample	Name	Crossing point	(Copies cDNA/µl)	Standard
1	Pre-treated IFN	19.22	7.93×10^6	
2	At infection IFN	18.80	1.16×10^7	
3	After infection IFN	17.86	2.74×10^7	
4	IFN, no virus	-	-	
5	Virus, no IFN	17.55	3.64×10^7	
6	Standard	20.57	2.30×10^6	2.30×10^6
7	Negative control	-	-	

Table 4.11 indicated how the crossing points decreased from 19.22 in pre treated cells to 18.80 in cells treated at infection to 17.86 in cells treated post infection. That correlated to viral load in the pre-treated cells being the lowest at 7.93×10^6 compared to 1.16×10^7 in cells treated with IFN at infection. The viral load in cells treated post infection and infected cells without IFN was very similar with their crossing points being 17.86 and 17.55 as well as their cDNA copies/µl 2.74×10^7 and 3.64×10^7 respectively.

4.3.5 Apoptosis DNA ladder

The extracted DNA from infected HeLa cells was separated using agarose gel electrophoreses.

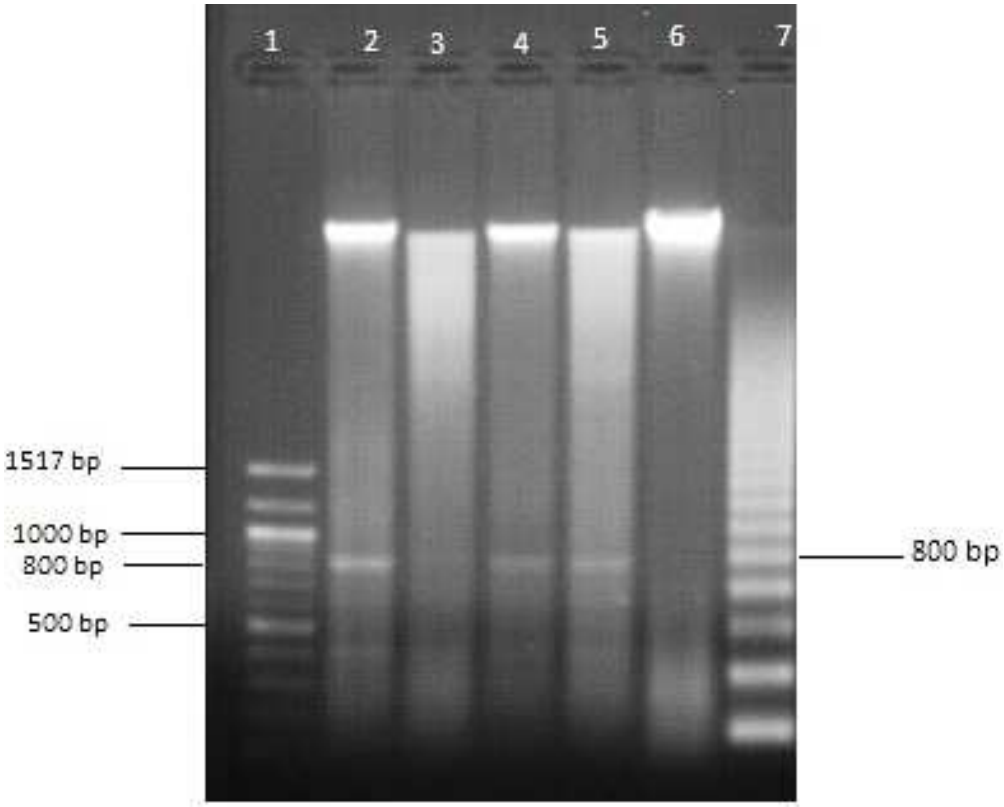

Figure 4.17. Agarose electrophoretic gel of DNA extracted from infected HeLa cells with SINV S.A.AR86 virus. Lane 1 - 100 bp DNA ladder molecular weight marker; Lane 2 - extracted HeLa DNA MOI 0.1 24h; Lane 3 - extracted HeLa DNA MOI 0.1 baseline; Lane 4 - extracted HeLa DNA MOI 1 24h; Lane 5 - extracted HeLa DNA MOI 1 baseline; Lane 6 - DNA from un-infected HeLa cells (negative control); Lane 7 - positive control.

Figure 4.17 shows DNA fragments separated with agarose gel electrophoreses. The principle of apoptosis DNA fragmentation is that host cell DNA is fragmented into smaller segments. Lane 7 shows the positive control of complete apoptosis and fragmentation of host cell DNA. At the top of lanes 2-6 there was a thick band larger than 1517 bp and representing the un-fragmented HeLa cell DNA. There was a strong DNA band present in lanes 2, 4 and 5 at 800 bp with two lesser bands seen between 500 bp and 800 bp. The presence of multiple DNA bands shows DNA fragmentation and therefore shows apoptosis. The positive control in lane 7 shows an example of what DNA fragmentation looks like. Lane 6 showed the DNA of HeLa cells that were not infected with SINV and a single un-fragmented DNA band at the top of lane 6 was clearly present which represented HeLa cell genomic DNA.

DNA fragmentation of infected HeLa cells were shown *in-vitro* using the DNA ladder apoptosis kit. There was a clear difference between the DNA separated by electrophoresis between SINV infected cells and un-infected cells. The HeLa cells that were not infected did not show any DNA fragmentation, whereas multiple bands were visible in infected cells. The implications for SINV infection *in-vitro* leading to apoptosis has yet to be determined. Whether the effects can be replicated in-vivo using animal models has to be determined and could be addressed in additional studies. The effect and implications of SINV infection leading to apoptosis on the pathogenesis of disease has not been assessed in this study, but *in-vivo* studies may help to better understand the effects.

4.4 Summary

The initial aim was to determine if SINV can infect human macrophages and determine cytokine secretion and evidence for antibody mediated enhancement of disease as this may indicate a role for macrophage involvement in the mechanism by which SINV causes arthralgia in patients. Two strains of SINV were used in attempts to infect macrophages, a strain from Egypt and a strain from South Africa. Macrophages were successfully differentiated from human PBMC and subsequent to viral infections, evidence of replication was investigated by determining viral titers in SNF from infected macrophages. In the absence of an increase in viral titer, viral loads were determined using RNA extracted from tissue culture fluid and from lysed cells. In addition viral load was determined using a PCR that targeted amplification of strand specific template that would clearly indicate replicating virus. In summary small increases in viral load suggested possible low levels of viral replication but were considered insufficient to warrant further investigation.

Hence it was decided to determine if IFN release from infected macrophages could inhibit SINV replication. Increased levels of IFN gamma were demonstrated 24h after infection of human macrophages with SINV. HeLa cells were then infected to confirm their susceptibility to SINV. Increased viral loads 24h after infection provided evidence that SINV could replicate in HeLa cells. HeLa cells were treated with human IFN gamma at concentrations similar to those detected in human macrophages. Pretreatment and concurrent treatment with infection showed reduced levels of viral load compared with no treatment or delay treatment. Hence the suggestion that IFN could have played a role in inhibiting viral replication in the human macrophages. Pretreatment reduced levels of viral load, however there was evidence that the virus did replicate. IFN could play a dose dependent role or alternatively there could be another mechanism contributing to inhibition.

The detection of apoptosis was done by extracting DNA from infected HeLa cells. The extracted DNA fragments were separated through agarose gel electrophoreses. The resulting separation compared DNA from infected samples, a negative control without virus and a positive control showing apoptotic DNA bands. There were multiple bands visible in the infected samples whereas the negative control did not show multiple bands, only one large band of genomic DNA. The presence of multiple separated DNA fragments in infected cells and absence of those bands in uninfected cells were suggestive of virus induced apoptosis.

CHAPTER 5

DISCUSSION

SINV is classified as an *Alphavirus* and belongs to the family *Togaviridae* (Theilmann et al., 1984). The alphaviruses were some of the first arboviruses to be isolated and characterized. The name arboviruses is derived from arthropod-borne virus which describes a transmission cycle between susceptible vertebrate hosts and arthropod insects and cause infections ranging from mild to severe in humans (Calisher & Karabatsos 1988; Karabatsos, 1975). Arboviruses have been found to be present on all the continents of the world with the geographic distribution dependent on the presence of competent vectors and conditions favoring vector populations (Navaratnarajah & Kuhn, 2007).

Little is known about the risk factors and epidemiology of SINV infection in southern Africa. Cases of SINV infection are confirmed in the laboratory each year, but positive cases are likely frequently missed due to lack of awareness (Uejio et al., 2012). SINV infection presents with non-specific symptoms which may explain why some infections are frequently not detected (Storm et al., 2012). It is likely that many cases of SINV are undiagnosed particularly with mild infections. It is frequently not considered as a differential diagnosis for patients with arthritis. Virus isolation from blood or skin lesions during the acute phase of infection is a viable method for identifying the infectious virus. Molecular assays such as conventional PCR can be used for the detection of viral nucleic acid.

Application of virus isolation and molecular assays is limited by only being effective during the acute stage of infection. Antibody testing is usually deemed more effective in routine diagnostics because the period of viremia is short (Kurkela et al., 2004). ELISA are a useful technique that can provide a rapid tool for diagnostic or serological surveillance however serological assays for the detection of anti-SINV antibodies are not readily available commercially and most laboratories therefore use in-house reagents (Niklasson *et al*., 1984). HI assays are commonly used, but the assay is laborious and impractical for serological surveillance and does not distinguish between IgG and IgM antibodies hence it cannot distinguish between recent and previous infections (Sane et al., 2012).

In-vitro neutralization tests are the gold standard in serology and are performed using adherent cell lines (Konishi et al., 2010). They are impractical for routine diagnosis and serological surveillance but have application in validating new assays. *In-vitro* neutralization tests currently require observation of CPE or

plaque reduction assays which again can be arduous for surveillance studies. Recently a RTCA was introduced which can be used to perform *in-vitro* neutralization assays, but which measures cell growth and death in real-time (Fang et al., 2011; Tian et al., 2012). An increase in cell density is measured as an increase in CI whereas a decrease in cell density due to CPE is represented as a decrease of CI (Fang et al., 2011).

This study aimed to develop an ELISA for application in serological surveys for the detection of SINV antibodies. In addition molecular assays were developed to assist with characterization of infection in mammalian cell lines. An in house ELISA for screening patient sera for IgG anti-SINV antibodies was developed and optimized. A SINV cell-lysate antigen was prepared by infecting Vero cells, harvesting the cells before CPE, lysing the cells and using the SNF as a coating antigen in ELISA. Mock antigen was prepared similarly using uninfected Vero cells. Polysorb microtiter plates were coated with SINV cell-lysate antigen and mock antigen. A negative panel comprising of eight serum samples were tested in a total of 18 replicates for the calculation of the cut-off value. The cut-off value is the value that separates positive samples from negative samples and was determined using the net OD values and PP values for normalization of data. The cut off was determined using mean net OD plus three SD and mean PP value plus three SD. Using the net OD values, a cut off of 0.155 was determined and using PP values a cut off of 19.41% was calculated.

The in house ELISA was used to screen 146 sera for SINV IgG antibodies. The samples were submitted for an unrelated study during 2006 and 2010 from patients with suspected tick bite fever, no confirmed diagnosis and presenting with symptoms similar to SINV infections. A total of 22 samples tested positive using the ELISA. Only 43 samples had sufficient volume for testing using the neutralization assay and 29 were positive for neutralization antibodies at titers ranging from 1:4 to 1:256. After analysis of the results the ELISA had a sensitivity of 68.9% and a specificity of 78.57 - 85.71% depending on whether PP or OD values were used to determine positive and negative samples. This suggests that samples with a low titer of antibody may not be detected using the ELISA. Testing of a significantly larger panel of negative samples and reference samples would help to optimize the ELISA. The assay was time effective and relatively simple making it a viable assay for staff that are not as technically trained. The ELISA assay was not validated for diagnostic purposes due to limitations in our cohort of samples. Viruses have shown to have the ability to be serologically cross-reactive and a diagnostic assay has to have the ability to distinguish between closely related viruses. In the case of SINV a diagnostic assay has to have the ability to distinguish between other alphaviruses that occur in southern Africa such as Middelburg virus and, sporadically, CHIKV.

An *in-vitro* neutralization assay could be more effective than ELISA in differentiating between closely related alphavirus as cross reactivity has been shown (Manni et al., 2008; Laine et al., 2004).

A new system for measuring cell-growth and virus induced cytopathogenesis in real-time was investigated. The RTCA system was compared with the traditional *in-vitro* neutralization test which relies on observation of CPE using a microscope. The real time *in-vitro* neutralization assay showed a 100% correlation relative to the traditional *in-vitro* neutralization assay for both positive and negative samples. The real-time assay is as accurate and efficient as the traditional assay without the variable of different perspectives when reading plates under the microscope.

One of the aims of this project was to characterize SINV infection of human derived macrophages and other mammalian cell lines. To confirm infection and replication of SINV in different cell lines, molecular assays were developed to quantitate viral load and to differentiate between replicating virus and inocula. A primer pair was identified to amplify conserved 181 bp region of SINV nsp2 gene encoding the nsp2 protein. They were identified by obtaining the genetic sequences from GenBank and aligning those sequences using ClustalX 2.0.11. The primers were designated as Sin nsp2 F and Sin nsp2 R and were 22 and 21 bp respectively in length.

A RNA transcript was prepared for constructing a standard curve for the real-time quantitative RT-PCR. A standard curve is prepared as a series of 10-fold dilutions with known concentrations and is used to determine the concentration of template in unknown samples. The RNA transcript was prepared by amplification of RNA template using Titan™ One Tube RT-PCR. The amplicon was purified and ligated into pGem T Easy using T/A cloning prior to transformation of chemically competent XL-10-Gold cells. The orientation of the insert in positive transformants was confirmed using PCR and primers targeting a region of the insert and a region of the pGEM T Easy plasmid. The pGEMSIN construct was subsequently linearized using the Pst1 restriction site in the multiple cloning site 3' of the inserted region and RNA transcribed using MEGAscript® T7 Kit. The standard curve was constructed using RNA in 10-fold dilutions with RNA copy numbers ranging from 6.2×10^{10} - 6.2×10^{4} copies of RNA/µl based on calculations assuming a 181 bp RNA transcript. A nonlinear curve and a minimum detection limit of 6.2×10^{7} copies of RNA/µl suggested that either the sensitivity of the PCR was inadequate or the copy number of the RNA transcript was incorrect.

On the hypothesis that the linearization of the plasmid used for RNA transcription was incomplete and that the RNA could be greater than 181 bp, a standard was prepared using DNA amplicon of known size. The DNA control was prepared using Titan™ One Tube RT-PCR to amplify RNA template. The amplicon was purified, the DNA copies calculated and tenfold dilutions from 2.2×10^7 - 2.2×10^2 copies of DNA/µl used to construct a standard curve. A linear correlation between the crossing points and the copies of DNA measured in copies DNA/µl was obtained. The PCR based on a standard curve derived from DNA of known size was able to detect 2.2×10^2 copies of DNA/µl.

The infection experiments involved inoculating cells with live virus. The inoculum was incubated on the cell cultures for a period prior to removal. Assuming that complete removal of all virus could not be guaranteed and considering that replication was not occurring at high levels it was decided to develop an RT-PCR that was strand specific to differentiate early and late replication and quantitating changes in viral load as indicative of viral replication. Alphaviruses are positive strand viruses and the synthesis of genomic and subgenomic RNA requires synthesis of (-) strand RNA. In addition it has been reported that alphaviruses synthesize (-) strand RNA for a short period early in the infection and targeting (-) strand RNA may indicate occurrence of early replication (Sawicki & Sawicki, 1980; Strauss & Strauss, 1994). The RT-PCR was performed as a two step reaction in which the cDNA was derived from (-) strand RNA using the forward primer and (+) strand using the reverse primer. To optimize the RT-PCR, HeLa cells were infected with infectious or heat inactivated SINV and viral loads determined in samples. There was a 10 000-fold difference in the viral load between cells infected with heat-inactivated and infectious virus at the same MOI. The optimized molecular assays were used to quantify viral load in infected mammalian cells, macrophages and HeLa cells, and show whether the virus was replicating.

The macrophages were differentiated from fresh PBMC that were isolated from whole blood using M-CSF. Human macrophages were inoculated with strains of SINV. Determination of the titer of infectious virus in cell culture supernatants collected at intervals after infection showed no change in viral titer using 10 fold dilutions. Previous reports of infection of human macrophages showed 3 fold increases in viral titer based on plague reduction assays (Assunção-Miranda et al., 2010). Viral loads were subsequently determined to determine changes in viral load from baseline to 24 hours post infection. The cDNA was synthesized using transcription of (-) strand RNA using the nsp2 F primer and then using the Sin nsp2 R primer for transcription of (+) strand RNA. Macrophages were infected with SINV S.A.AR 86 at two different MOI's, MOI 0.1 and MOI 1.

The quantification of viral load in macrophages did not show satisfactory viral replication between baseline and 24h samples. The volunteer who the macrophages were obtained tested negative for SINV antibodies in both the ELISA and *in-vitro* neutralization assay.

Infection of human macrophages has been shown previously using the MRE16 strain of SINV. An increase in viral titer was shown using a plaque assay (Assunção-Miranda et al., 2010). It was initially aimed to characterize SINV infection of human derived macrophages, but our attempts showed that they were not susceptible to infection even though the volunteer who the macrophages were obtained tested negative for SINV antibodies in both the ELISA and *in-vitro* neutralization assay. The MRE16 strain is a Malaysian strain of SINV and is a member of the Oriental Australian lineage whereas the South African strain of SINV S.A.AR86 and Egyptian strain AR339 belongs to the Paleoarctic Ethiopian lineage (Jöst et al., 2010; Sammels et al., 1999). Differences in strains of virus may have been responsible for insusceptibility, alternatively it may have been host derived that investigating susceptibility of macrophages from alternative humans may have been useful. Alternative to investigating other humans, it was decided to investigate possible mechanisms that influenced inhibition of infection and replication.

It was postulated that SINV infection of macrophages lead to the secretion of IFN gamma which in turn inhibited viral replication. The IFN are known to have strong antiviral activity and include alpha, beta and gamma IFN. IFN gamma has been shown to be an effective treatment when given to patients with HBV to decrease viral loads in patient sera. IFN gamma also has the ability to lead to decrease in viral RNA replication for RNA viruses (Costa-Pereira et al., 2002; Hayashi & Koike, 1989; Wu et al., 2011). SNF samples obtained from macrophages infected with SINV S.A.AR86 at MOI 0.1 and MOI 1 were tested in duplicate for the secretion of IFN gamma. There was almost a 10-fold increase in IFN gamma from baseline to 24h at MOI 0.1 infection. Infections with MOI 1 resulted in higher IFN concentrations with a 15-fold increase from baseline to 24h. HeLa cells were treated with similar levels of IFN gamma at three different time intervals to determine the effect of IFN on SINV replication. It was shown that treatment of HeLa cells before and at the time of infection was more effective in inhibiting SINV replication than treatment after infection. We were able to show that treating HeLa cells did have an inhibitory effect on viral growth and replication. It therefore gives credence to the idea that IFN gamma secreted from the macrophages may have lead to an inhibition of viral replication.

Apoptosis may also have a pathogenic role by contributing to cell death and tissue injury and occurs in a wide range of human viral infections, including central nervous system (CNS), heart and liver infections. An increase in viral titer has been associated with an increase in apoptosis. Animals models used for infections and testing of apoptosis in targeted areas of infection have revealed a positive correlation between apoptosis and disease severity that is suggestive of the idea that apoptosis is a pathogenic mechanism in virus-induced disease (Clarke & Tyler, 2009). SINV infected HeLa cells were sampled and tested to determine whether SINV infection leads to apoptosis in the cells. DNA was extracted from the infected HeLa cells and separated using agarose gel electrophoresis. The principle of apoptosis DNA fragmentation is that host cell DNA is fragmented into smaller segments (Del Puerto et al., 2011). DNA fragmentation was seen in the infected cells as there were multiple bands present between baseline and 24h samples. The control HeLa cells that were not infected showed only a large non-fragmented DNA band at the top of the gel which was probably genomic DNA. The Apoptotic DNA ladder kit has been shown to be useful in detecting virus induced apoptosis. Infection of *BHK*-21 with African horse sickness virus led to apoptosis in the BHK-21 cells that was detectable after 12 hours. Similar results to ours were shown where there were multiple bands present in infected in cells whereas mock infected cells showed only un-fragmented chromosomal DNA (Stassen et al., 2012).

Apoptosis plays an important part in the pathogenesis of infections. Virus infected cells usually either undergo cell lysis and present as CPE or can undergo apoptosis. The appearance of apoptosis correlates with disease severity in a number of different animal models of virus-induced disease. SINV infection in mice leads to central nervous system (CNS) disease which leads to apoptosis in infected neurons and therefore neurological damage (Clarke & Tyler, 2009). Studies for identifying whether virus infections lead to apoptosis, which signaling pathways are used and to which areas they are targeted can potentially provide novel targets for treatment for diseases caused by those viruses (Clarke & Tyler, 2009). As apoptosis plays an important role in the pathogenesis of disease treatment to potentially inhibit or limit apoptosis caused by viral infections may play a big role in lessening the degree of illness or decreasing the disease severity. The mechanisms and pathways involved in induction of apoptosis in SINV infection were not investigated further, but further experiments can be conducted in future studies to determine which pathways for apoptosis are followed as well as the significance in the pathogenesis of disease.

In summary, the study has indicated that infection of macrophages may be inhibited by the release of IFN. However PBMC's from only one volunteer were investigated. Additional experiments from multiple volunteers as well as other strains of SINV may help determine the clinical significance of this observation.

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APPENDIX 1

ELISA RAW DATA

Negative panel samples in duplicate

Sample	1	2	3	4	5	6	7	8	C++	C-
SINV Ag	0.106	0.11	0.14	0.127	0.123	0.106	0.095	0.125	0.735	0.16
MOCK	0.054	0.044	0.043	0.035	0.035	0.049	0.066	0.031	0.038	0.071
Net OD	**0.052**	**0.066**	**0.097**	**0.092**	**0.088**	**0.057**	**0.029**	**0.094**	**0.697**	**0.089**
PP	7.4	9.39	13.8	13.1	12.52	8.1	4.13	13.37	81.71	12.66
Sample	1	2	3	4	5	6	7	8	C++	C-
SINV Ag	0.105	0.103	0.10	0.114	0.108	0.11	0.161	0.117	0.747	0.164
MOCK	0.054	0.043	0.033	0.054	0.032	0.059	0.132	0.031	0.038	0.071
Net OD	**0.051**	**0.06**	**0.067**	**0.06**	**0.076**	**0.051**	**0.029**	**0.086**	**0.709**	**0.093**
PP	7.25	8.53	9.53	8.53	10.81	7.25	4.13	12.33	83.12	13.23

Plate 1

Sample	72/09/04	72/09/05	72/09/06	4/10/01	4/10/02	4/10/03	4/10/09		
SINV Ag	0.809	0.5210	0.290	0.0260	0.1640	0.0850	0.2680		
MOCK	0.1110	0.4260	0.1580	0.0630	0.0680	0.0650	0.1080		
Net OD	**0.699**	**0.095**	**0.132**	**0.197**	**0.096**	**0.02**	**0.16**		
PP	83.12	11.3	15.7	23.42	11.41	2.38	19.02		
Sample	58/08/09	58/08/10	58/08/11	58/08/12	58/08/13	58/08/14	58/08/15	58/08/17	
SINV Ag	1.1530	0.3010	0.1200	0.2630	0.1130	0.8880	0.2500	1.3530	
MOCK	0.2340	0.1600	0.0820	0.1050	0.0780	0.0710	0.1000	0.1340	
Net OD	**0.919**	**0.141**	**0.038**	**0.158**	**0.035**	**0.817**	**0.15**	**1.0013**	
PP	109.27	16.77	4.52	18.79	4.16	97.14	17.84	119.06	
Sample	58/08/18	58/08/19	58/08/20	58/08/21	58/08/22	58/08/23	58/08/25	58/08/26	58/08/27
SINV Ag	0.2050	0.2020	0.2450	0.1640	1.2900	0.2940	0.0430	0.4140	0.1520
MOCK	0.1010	0.1160	0.1810	0.0840	0.0940	0.1910	0.0350	0.2100	0.1420
Net OD	**0.104**	**0.086**	**0.064**	**0.08**	**1.196**	**0.103**	**0.008**	**0.204**	**0.01**
PP	12.37	10.23	7.61	9.51	142.21	12.25	0.95	24.26	1.19
Sample	58/08/28	58/08/29	58/08/30	58/08/31	C++	C++	C-	C-	
SINV Ag	0.2040	0.1830	0.3440	0.1530	0.8890	0.9430	0.1330	0.1290	
MOCK	0.1450	0.1130	0.2920	0.1000	0.0700	0.0800	0.0760	0.0900	
Net OD	**0.059**	**0.07**	**0.052**	**0.053**	**0.819**	**0.863**	**0.057**	**0.039**	
PP	7.02	8.32	6.18	6.3	97.38	102.62	6.78	4.64	

Plate 2

Sample	28/07/01	28/07/02	28/07/03	28/07/04	28/07/05	28/07/06	28/07/07	28/07/08	28/07/09	28/07/10
SINV Ag	0.3460	0.2600	0.2160	0.2580	0.1520	0.3750	0.0470	0.2520	0.2050	0.3710
MOCK	0.3900	0.1700	0.1230	0.1240	0.1130	0.2320	0.0200	0.1390	0.1410	0.2330
Net OD	**-0.044**	**0.09**	**0.093**	**0.134**	**0.039**	**0.143**	**0.027**	**0.113**	**0.064**	**0.138**
PP	-3.81	7.79	8.04	11.59	3.37	12.37	2.33	9.78	5.54	11.94
Sample	**28/07/11**	**28/07/12**	**28/07/13**	**28/07/14**	**28/07/15**	**28/07/16**	**28/07/17**	**28/07/18**	**28/07/19**	**28/07/20**
SINV Ag	0.4920	0.3940	0.2330	0.2530	0.3290	0.1650	0.4600	0.0510	0.6010	0.7290
MOCK	0.5760	0.1800	0.1550	0.1450	0.1980	0.1070	0.1100	0.0190	0.4540	0.4790
Net OD	**-0.084**	**0.214**	**0.078**	**0.108**	**0.131**	**0.058**	**0.35**	**0.032**	**0.147**	**0.25**
PP	-7.27	18.5	6.75	9.34	11.33	5.02	30.28	2.77	12.72	21.63
Sample	**28/07/21**	**28/07/22**	**28/07/23**	**28/07/24**	**28/07/25**	**28/07/26**	**28/07/27**	**28/07/28**	**28/07/29**	**28/07/30**
SINV Ag	0.4040	0.2410	0.2730	0.2730	0.3620	0.1770	0.3310	0.0490	0.2230	0.1930
MOCK	0.1130	0.1460	0.1780	0.1430	0.2610	0.1520	0.1610	0.0230	0.1470	0.1020
Net OD	**0.291**	**0.095**	**0.095**	**0.13**	**0.101**	**0.025**	**0.17**	**0.026**	**0.076**	**0.091**
PP	25.17	8.22	8.22	11.25	8.74	2.16	14.71	2.25	6.57	7.87
Sample	**29/01/01**	**29/01/02**	**C++**	**C++**	**C-**					
SINV Ag	0.9970	2.0740	1.2580	1.1810	0.02080					
MOCK	0.1080	0.1130	0.0700	0.0570	0.1040					
Net OD	**0.889**	**1.961**	**1.188**	**1.124**	**0.104**					
PP	9.34	169.64	102.77	97.23	9					

Plate 3

Sample	29/07/03	29/07/04	29/07/05	29/07/06	29/07/07	02/08/01	02/08/02	02/08/03	02/08/04	02/08/05
SINV Ag	0.3840	0.2010	1.6780	0.0710	1.7250	0.1350	0.6550	0.1840	0.1860	0.1360
MOCK	0.0620	.01360	0.1370	0.0410	0.0750	0.0820	0.4280	0.0950	0.0900	0.0830
Net OD	**0.322**	**0.065**	**1.541**	**0.03**	**1.65**	**0.053**	**0.227**	**0.089**	**0.096**	**0.053**
PP	36.65	7.4	175.41	3.41	187.82	6.03	25.84	10.13	10.93	6.03
Sample	**02/08/06**	**02/08/07**	**02/08/08**	**02/08/09**	**02/08/10**	**02/08/11**	**02/08/12**	**02/08/13**	**02/08/14**	**02/08/15**
SINV Ag	1.9450	0.1740	0.1910	0.1520	0.1130	0.3910	0.2060	0.0320	0.3310	0.1240
MOCK	0.0990	0.2250	0.1150	0.1050	0.0650	0.1280	0.1010	0.0210	0.2000	0.0830
Net OD	**0.955**	**-0.051**	**0.076**	**0.047**	**0.048**	**0.263**	**0.105**	**0.011**	**0.131**	**0.041**
PP	108.7	5.81	8.65	5.35	5.46	29.94	11.95	1.25	14.91	4.67
Sample	**02/08/25**	**02/08/16**	**02/08/17**	**02/08/18**	**02/08/19**	**02/08/20**	**02/08/21**	**02/08/22**	**30/09**	**43/10**
SINV Ag	0.0950	0.2880	0.4580	0.1890	0.0420	0.1030	0.2570	0.0520	0.1500	0.8000
MOCK	0.0660	0.2220	0.0970	0.1010	0.0490	0.0560	0.1360	0.0340	0.0680	0.0790
Net OD	**0.029**	**0.066**	**0.361**	**0.088**	**-0.007**	**0.047**	**0.121**	**0.018**	**0.082**	**0.721**
PP	3.301	7.51	41.09	10.02	0.8	5.35	13.77	2.05	9.33	82.07
Sample	**C++**	**C++**	**C-**	**C-**						
SINV Ag	0.9630	0.9330	0.1510	0.1820						
MOCK	0.0730	0.0660	0.1100	0.0850						
Net OD	**0.89**	**0.867**	**0.041**	**0.097**						
PP	101.31	98.7	4.67	11.04						

Plate 4

Sample	10/10/14	10/10/21	10/10/22	10/10/25	10/10/26	10/10/27	10/10/29			
SINV Ag	0.3150	0.1350	0.1850	0.1570	0.1160	0.1870	0.0950			
MOCK	0.1590	0.0630	0.0630	0.1090	0.0730	0.0990	0.0510			
Net OD	**0.156**	**0.072**	**0.122**	**0.048**	**0.043**	**0.088**	**0.044**			
PP	20.79	9.59	16.26	6.4	5.73	11.73	5.86			
Sample	**70/09/01**	**70/09/02**	**70/09/03**	**70/09/04**	**70/09/05**	**70/09/06**	**70/09/07**	**70/09/08**	**70/09/09**	**70/09/10**
SINV Ag	0.1300	1.5710	0.1010	0.1620	0.1210	0.0990	0.2000	0.3240	0.2840	0.8550
MOCK	0.0800	0.0530	0.0620	0.0970	0.1020	0.0630	0.2190	0.3050	0.1370	0.2000
Net OD	**0.05**	**1.518**	**0.039**	**0.065**	**0.019**	**0.036**	**-0.019**	**0.019**	**0.147**	**0.655**
PP	6.66	202.27	5.2	8.66	2.53	4.8	-2.53	2.53	19.59	87.28
Sample	**70/09/11**	**70/09/12**	**70/09/13**	**70/09/14**	**70/09/15**	**70/09/16**	**70/09/17**	**70/09/18**	**72/09/01**	**72/09/02**
SINV Ag	0.1130	0.0420	0.1970	0.1290	0.0560	0.3300	0.1110	0.0430	0.1660	0.1980
MOCK	0.0770	0.0210	0.1060	0.0920	0.0320	0.2170	0.0460	0.0250	0.0770	0.1020
Net OD	**0.0363**	**0.021**	**0.091**	**0.037**	**0.024**	**0.113**	**0.065**	**0.018**	**0.096**	**0.09**
PP	4.84	2.8	12.13	4.93	3.2	15.06	8.66	2.4	12.79	11.99
Sample	**72/09/03**	**C++**	**C++**	**C-**	**C-**					
SINV Ag	0.9310	0.8160	0.8210	0.1510	0.1170					
MOCK	0.0820	0.0690	0.0670	0.0670	0.0820					
Net OD	**0.849**	**0.747**	**0.754**	**0.084**	**0.035**					
PP	113.12	99.53	100.46	11.19	4.66					

Plate 5

Sample	02/08/23	02/08/24	02/08/25	27/08/01	27/08/02	27/08/03	27/08/04	27/08/05	27/08/06	27/08/07
SINV Ag	0.2150	0.3350	0.0360	0.1750	0.1570	0.0320	0.1360	1.8390	0.1840	0.2980
MOCK	0.1200	0.3590	0.0240	0.1480	0.1060	0.0220	0.0680	0.0970	0.0890	0.1610
Net OD	**0.095**	**-0.024**	**0.012**	**0.027**	**0.051**	**0.01**	**0.068**	**1.742**	**0.095**	**0.137**
PP	11.14	-2.81	1.41	3.17	5.98	1.17	7.97	204.22	11.14	16.06
Sample	**27/08/08**	**27/08/09**	**27/08/10**	**27/08/11**	**27/08/12**	**27/08/13**	**27/08/14**	**27/08/15**	**27/08/16**	**27/08/17**
SINV Ag	0.2450	0.1240	0.1570	0.1580	0.2990	0.0470	0.1450	0.1760	0.2830	0.1870
MOCK	0.1520	0.0670	0.1060	0.0720	0.2070	0.0350	0.0870	0.0760	0.1050	0.1370
Net OD	**0.093**	**0.057**	**0.051**	**0.086**	**0.092**	**0.012**	**0.058**	**0.1**	**0.178**	**0.05**
PP	10.9	6.68	5.98	10.08	10.87	1.41	6.8	11.72	20.87	5.86
Sample	**58/08/01**	**58/08/02**	**58/08/03**	**58/08/04**	**58/08/05**	**58/08/06**	**58/08/07**	**58/08/08**		
SINV Ag	0.1570	0.1660	0.1920	0.2630	0.1670	0.1560	0.3220	0.2320		
MOCK	0.0990	0.1810	0.1270	0.1210	0.0710	0.0780	0.1250	0.2250		
Net OD	**0.058**	**-0.015**	**0.065**	**0.142**	**0.096**	**0.078**	**0.197**	**0.0073**		
PP	6.8	-1.76	7.62	16.65	11.25	9.14	23.09	0.86		
Sample	**C++**	**C++**	**C-**	**C-**						
SINV Ag	0.8690	0.9630	0.1950	0.2000						
MOCK	0.0620	0.0640	0.0980	0.1250						
Net OD	**0.807**	**0.899**	**0.097**	**0.075**						
PP	94.61	105.39	11.37	8.79						

APPENDIX 2

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ABTS = 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)

Amp = Ampicillin

ATCC = American Type Culture Collection

ADE = Antibody dependent enhancement

AMV = Avian Myeloblastosis Virus

bp = Base pair

BBQ = Blackberry quencher

BBS = Borate buffered saline

$CaCl_2$ = Calcium chloride

CO_2 = Carbon dioxide

CI = Cell index

CHIKV = Chikungunya virus

CI = Cell index values

cfu = Colony forming units

cDNA = Complimentary deoxyribonucleic acid

CPE = Cytopathic effect

DNA = deoxyribonucleic acid

dNTP = deoxynucleotide tri-phosphate

EDTA = Ethylenediaminetetraacetic acid

EEE = Equine encephalitis virus

ER = Endoplasmic reticulum

E.coli = Escherichia coli

FAM = Carboxyfluorescein

FBS = Fetal bovine serum

HI = Haemagglutination inhibition

HRPO = Horseradish peroxidase

Ig = immunoglobulin

IFN = Interferon

IL = Interleukin

IPTG = Isopropyl β-D-1-thiogalactopyranoside

JEV = Japanese equine encephalitis virus

kb = Kilobase

KCl = Potassium chloride

LB = Luria-Bertani

MEM = Minimum essential media

MHC = Major histocompatability complex

MIF = Macrophage inhibitory factor

MMP = Matrix metalloproteinases

NaCl = Sodium chloride

NICD = National Institute for Communicable Diseases

nm = Nanometer

nsp = Non structural protein

NTR = Non translated region

OD = Optical density

O/N = Overnight

PBS = Phosphate buffered saline

PCR = Polymerase chain reaction

PRNT = Plaque reduction neutralization test

RNA = Ribonucleic acid

RPMI = Roswell Park Memorial Institute

RRV = Ross River virus

RTCA = Real-time cell analysis system

RT-PCR = Reverse transcriptase PCR

SD = Standard deviation

SINV = Sindbis virus

SNF = Supernatant fluid

TAE = Tris-acetate-EDTA

TAMRA = Carboxytetramethylrhodamine

$TCID_{50}$ = Tissue cell infectious dose

Tm = Melting point

TMB = 3,3',5,5'-Tetramethylbenzidine

TNF = Tumor necrosis factor

VEE =Venezuelan equine encephalitis

WEE = Western equine encephalitis

APPENDIX 3

Title and abstract of presentation at the Faculty of Health Sciences, Research Forum 25 to 26 August 2011, University of the Free State, Bloemfontein

OPTIMIZATION AND COMPARISON OF METHODS FOR DETECTION OF SINDBIS IGG ANTIBODIES IN HUMAN SERA

AUTHORS: Hanekom M, Burt FJ.

PRESENTER: Hanekom M

Sindbis virus is a mosquito borne virus that belongs to the Togaviridae family and Alphavirus genus. Acute and persistent polyarthritis is a common symptom of disease caused by members of the Alphavirus genus. Sindbis virus occurs annually in South Africa, however there is little information regarding its association with persistent arthritis. The aim of this study was to optimize and compare various serological assays for the detection of antibodies against Sindbis to investigate the prevalence of the virus in SA and for immune profiling studies.

MATERIALS AND METHODS: An *in-vitro* neutralization assay (the gold standard), an ELISA using whole cell lysate antigen prepared from Vero cells infected with Sindbis virus and an assay using a recombinant antigen were optimized using positive and negative serum samples. For the recombinant antigen, the E2 protein of Sindbis was targeted for expression as it is both antigenic and immunogenic. The gene was cloned into the pIEx/Bac 1 expression plasmid for direct transfection of Sf9 cells.

RESULTS: Neutralization assays confirmed the presence of anti-Sindbis IgG antibodies in sera from 3 patients and the absence of antibodies in the negative control. The positive and negative sera were used to optimize the ELISA which was subsequently used to screen serum samples collected from 100 patients with an acute febrile illness with no diagnosis. Directly transfected SF9 cells expressed a 50kDa protein which corresponds to the predicted size of the E2 protein. Further characterization of the protein is required.

CONCLUSION: There was 100% correlation between the results from the Neutralization assay and the ELISA. Expression of a high yield of E2 protein is now required to screen the positive sera for antibodies against Sindbis E2 to determine its usefulness as a diagnostic tool.

APPENDIX 4

Title and abstract of poster presented at Virology Africa 2011, 29 November to 2 December, Cape Town, South Africa

DEVELOPMENT OF ELISA FOR DETECTION OF SINDBIS IGG ANTIBODIES IN HUMAN SERA TO DETERMINE PREVALENCE OF SINDBIS VIRUS INFECTIONS IN SOUTH AFRICA

Sindbis virus is a mosquito borne virus that belongs to the *Togaviridae* family and Alphavirus genus. Acute and persistent polyarthritis is a common symptom of disease caused by members of the Alphavirus genus. Sindbis virus occurs annually in South Africa (SA), however there is little information regarding its association with persistent arthritis. Serological assays for detecting antibody against Sindbis virus are not readily available commercially and many laboratories worldwide still use haemagglutination inhibition (HI) assays which require the use of animals for the preparation of reagents, do not distinguish between IgG and IgM and are difficult to automate for serological surveys. It is likely that many cases of Sindbis virus are undiagnosed particularly with mild infections. In addition it is frequently not considered as a differential diagnosis for patients with chronic arthritis. The aim of this study was to optimize an in house ELISA for the detection of antibodies against Sindbis to be able to determine the prevalence of the virus in SA.

MATERIALS AND METHODS: A cell lysate antigen was prepared by infecting Vero cells with Sindbis virus and harvesting the cells before appearance of cytopathic effects. Infectivity was confirmed by immunofluorescent tests. The cells were lysed, sonicated and clarified before being tested as an antigen in ELISA. Checkerboard titrations were used to optimize the ELISA using serum samples previously confirmed as positive and negative using HI. The ELISA was subsequently used to screen serum samples collected from 100 patients with an acute febrile illness and no diagnosis submitted during the arboviral season. In addition the ELISA was used to test serum samples submitted from patients presenting with joint pain following an acute illness. An *in-vitro* neutralization assay (the gold standard) was performed on positive reactors where serum samples were available.

RESULTS: A total of 35 serum samples tested weakly of strongly positive by ELISA for IgG antibody, including a sample from a patient with chronic arthritis and a positive IgM antibody against Sindbis. A total of 18 sera gave OD values below 0.25 in the ELISA but were nonetheless tested using the neutralization assay and titers of 1:4 to 1:8 were obtained for 7 samples, suggesting that low reactors should be considered unequivocal. The remaining 14 sera gave high optical density (OD) values in the ELISA and sera from 6 were

available for neutralization assays, of which 4 were positive with neutralizing antibody titers ranging from 1:4 to 1:32, 1 sample was negative and 1 sample was contaminated and a result could not be obtained.

CONCLUSION: The usefulness of the antigen for IgM detection is to be determined. The ELISA has application for testing large numbers of samples for serological surveys. The relatively high number of positive reactors in our cohort suggests that Sindbis virus likely occurs more frequently particularly in seasons with high rainfall favoring increased mosquito populations.

APPENDIX 5

Title and abstract of presentation at the Faculty of Health Sciences, Research Forum 23 to 24 August 2012, University of the Free State, Bloemfontein

QUANTITATIVE RT-PCR TO MEASURE VIRAL LOAD IN SINDBIS INFECTED MACROPHAGES

AUTHORS: Hanekom M, Burt FJ.

PRESENTER: Hanekom M

Sindbis virus is a mosquito borne virus that belongs to the *Togaviridae* family and *Alphavirus* genus. Acute and persistent polyarthritis is a common symptom of disease caused by members of the *Alphavirus* genus. The aim of this study was to develop and optimize a quantitative real time PCR assay for studying virus replication in infected mammalian cells.

MATERIALS AND METHODS: Novel primers and a detection probe were designed to amplify a 181 bp region of the NSP2 gene. A real time PCR assay for detection of viral loads was optimized using in-vitro transcribed RNA. A method for differentiating peripheral blood mononuclear cells (PBMC) was optimized. PBMC were isolated from whole blood, differentiated into mature macrophages and infected with Sindbis virus at a multiplicity of infection of 0.1. Samples were taken at two hourly intervals for in-vitro testing and as well as quantitative real time PCR analysis.

RESULTS: Novel primers amplified a 181 bp region of the gene encoding the nsp2 protein of Sindbis virus. The amplicon was cloned into the pGEM T-easy cloning vector. A RNA control was prepared by linearizing the vector with a restriction enzyme and in-vitro transcription with MEGAscript T7. The quantitative real time PCR was optimized using serial dilutions of the transcribed RNA. The assay was optimized to detect viral loads ranging from 2×10^{7} - 2×10^{12} copies of RNA/µl.

CONCLUSION: The novel primers and probe were able to detect and quantify Sindbis viral nucleic acid within the required range. The assay will be used to determine viral loads at intervals after infection to determine if the virus replicates in human macrophages.

APPENDIX 6

Sequence alignment of SINV sequences for nsp2 gene (5' - 3')

NSP2SAAR86 - U38305
NSP2WHOLESINDBIS1 - NC_001547
NSP2WHOLESINDBIS2 - J02363
Forward primer
Probe
Reverse primer

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GACAGTATATCGTTGTCTCGCCGATCTCTGTGCTGAAGAACGCTAAACTCGCACCAGCAC
GACAGTATATCGTTGTCTCGCCAAACTCTGTGCTGAAGAATGCCAAACTCGCACCAGCGC
GACAGTATATCGTTGTCTCGCCAAACTCTGTGCTGAAGAATGCCAAACTCGCACCAGCGC
 ********************* * ***************  ** ************** *

ACCCGCTAGCAGACCAGGTTAAGATCATAACGCACTCCGGAAGATCAGGAAGGTATGCAG
ACCCGCTAGCAGATCAGGTTAAGATCATAACACACTCCGGAAGATCAGGAAGGTACGCGG
ACCCGCTAGCAGATCAGGTTAAGATCATAACACACTCCGGAAGATCAGGAAGGTACGCGG
************** ***************** ************************ ** *

   TCGAACCATACGACGCTAAAGTACTGATGCCAGCAGGAAGTGCCGTACCATGGCCAGAAT
TCGAACCATACGACGCTAAAGTACTGATGCCAGCAGGAGGTGCCGTACCATGGCCAGAAT
TCGAACCATACGACGCTAAAGTACTGATGCCAGCAGGAGGTGCCGTACCATGGCCAGAAT
************************************** ***********************

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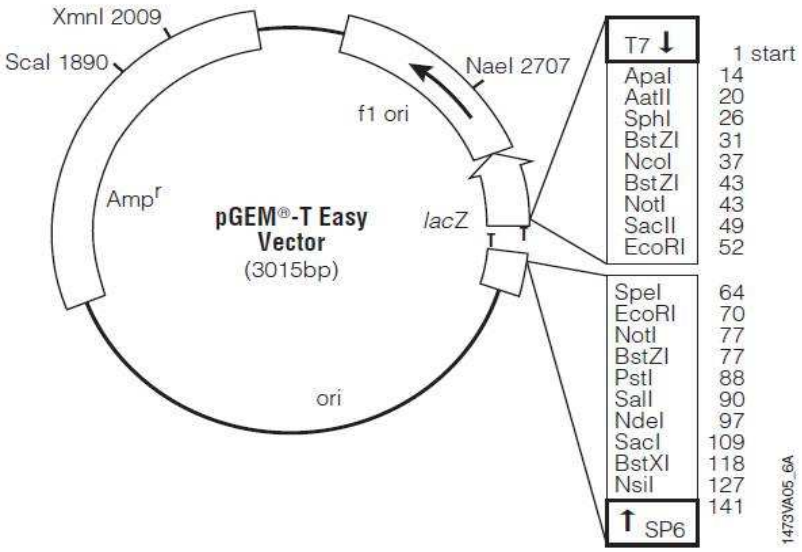
APPENDIX 7

pGEM® T-Easy

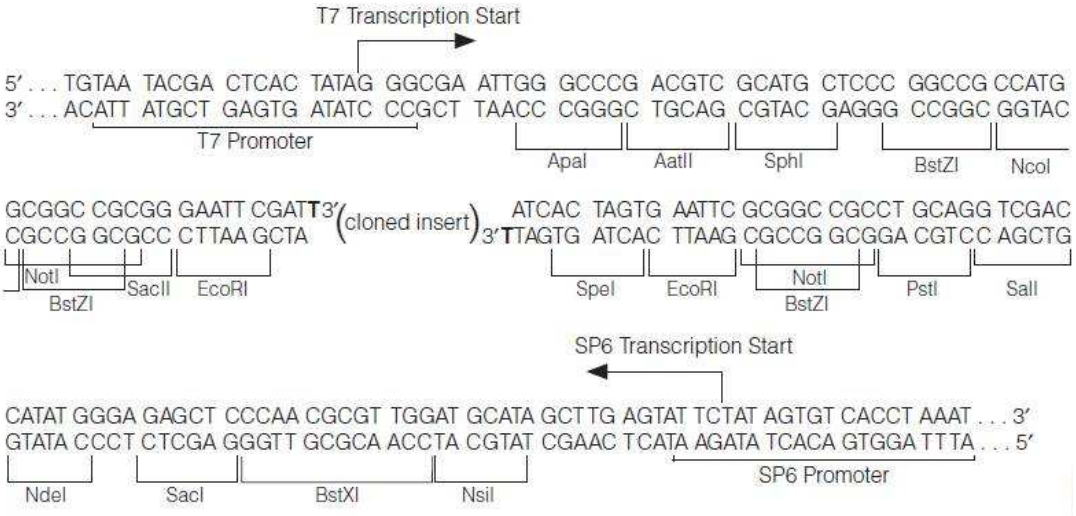
Vector map of pGEM® T-Easy vector



The promoter and multiple cloning sequence of the pGEM®-T- Easy Vector

T7 RNA polymerase transcription initiation site	1
multiple cloning region	10–128
SP6 RNA polymerase promoter (–17 to +3)	139–158
SP6 RNA polymerase transcription initiation site	141
pUC/M13 Reverse Sequencing Primer binding site	176–197
*lac*Z start codon	180
lac operator	200–216
β-lactamase coding region	1337–2197
phage f1 region	2380–2835
lac operon sequences	2836–2996, 166–395
pUC/M13 Forward Sequencing Primer binding site	2949–2972
T7 RNA polymerase promoter (–17 to +3)	2999–3

Sequence reference points of pGEM® T Easy vector



Opsomming

SINV is ’n lid van die *Alphavirus* genus en is deel van die *Togaviridae* familie. Die virus het ’n positiewe sin RNS genoom van ongeveer 11700 basis pare wat vir strukturele en nie strukurele protiëne enkodeer. Infeksie word gewoonlik gediagnoseer gebaseer op kliniese, epidemiologiese en laboratorium kriteria. Laboratorium bevestiging is baie belangrik omdat SINV infeksies van ander soortgelyke kondisies ondersky moet word. Die mees algemeen gebruikste metodes vir identifikasie is haemagglutinasie inhibisie, ensiem-gekoppelde immunosorbent toets (ELISA), plak reduksie neutralisasie toets sowel as konvensionele *in-vitro* neutralisasie toets. Serologiese toetse vir die opsporing van SINV is nie algemeen kommersieel beskikbaar nie en as gevolg van die nie spesifieke simptome wat deur SINV infeksie veroorsaak word kan die hoeveelheid infeksies wat jaarliks waargeneem word minder wees as die werklike hoeveelheid. Die doel van hierdie studie was om serologiese toetse soos ELISA en *in-vitro* neutralisasie toetse wat in serologiese ondersoeke vir die opsporing van IgG teenliggame teen SINV te ontwikkel. Dit was ook uitgeset om toetse te ontwikkel was gebruik kon word om die vlak van virale replikasie in soogdier selle vir die karakterisering van infeksie in soogdier selle sowel as om die effek van interferon op virale replikasie te ondersoek en te kyk vir bewyse van apoptose wat deur SINV infeksie veroorsaak is.

’n In-huise ELISA was ontwikkel en gebruik om 146 serum monsters te toets vir IgG teenliggame teen SINV. Die *in-vitro* neutralisasie toets is die goue standaard vir serologie en 43 monsters was getoets in beide die ELISA en die *in-vitro* neutralisasie toets. Analise en vergelyking van die resultate verky in die in-huise ELISA en die *in-vitro* neutralisasie toets het gewys dat die ELISA ’n sensitiwiteit van 68.9% gehad het met spesifisiteite tussen 78.57 - 85.71% afhangende of PP of OD gebruik was om positiewe van negatiewe waardes te onderskei. Vorentoe en agtertoe peilstukke was ontwerp vir die amplifisering van n konserveerde 181 bp deel van die nsp2 geen wat vir die nsp2 protien van SINV enkodeer sowel as n TaqMan hidrolise peil wat in ’n "real time" PCR gebruik was. Die infeksie van soogdier selle soos HeLa en menslike makrofages was ondersoek en toenames in virale lading was bepaal deur die gebruik van "real time" kwantitatiewe RT-PCR. Twee stamme van SINV was gebruik in pogings om makrofages te infekteer, ’n stam van Egipte sowel as Suid Afrika. Klein vermeerderings in die virale lading het voorgestel dat daar lae vlakke van virale replisering is, maar dit was onvoldoende om verder te ondersoek.

Supernatante vloeistof monsters vanaf makrofaag infeksies was getoets vir die sekresie van interferon gamma wat moontlik virale replikasie kon inhibeer. Daar was n nege tot vyftien-fout toename in die interferon gamma konsentrasie tussen tyd 0h en 24h. HeLa selle was behandel met soortgelyke

konsentrasies interferon gamma by verskillende tye. Selle behandel met interferon gamma voor of tydens infeksie het verminderde vlakke van virale lading gewys in vergelyking met selle wat na infeksie of glad nie behandel was met interferon gamma nie. Dit was daarom voorgestel dat die interferon gamma ’n rol gespeel het in hoekom SINV virale ladings nie toegeneem het in menslike makrofages nie. DNS was geekstraheer vanuit HeLa selle wat infekteer was met SINV en die DNS fragmente was geskei deur agarose gel elektroforese. Daar was verskeie bande sigbaar in die infektiewe monsters in teenstelling met die negatiewe kontrole waar daar net n enkele groot band teenwoordig was wat genomiese DNS van die HeLa voorgestel het. Die teenwoordigheid van verskeie DNS bande in infektiewe selle en die afwesigheid van hulle in on-infekteerde sells het voorgestel dat virus induseerde apoptose plaasgevind het.

Key terms

Sindbis virus

ELISA

IgG

In-vitro neutralization assay

Real time quantitative PCR

Infection

Viral load

Macrophages

HeLa cells

Interferon gamma

Apoptosis