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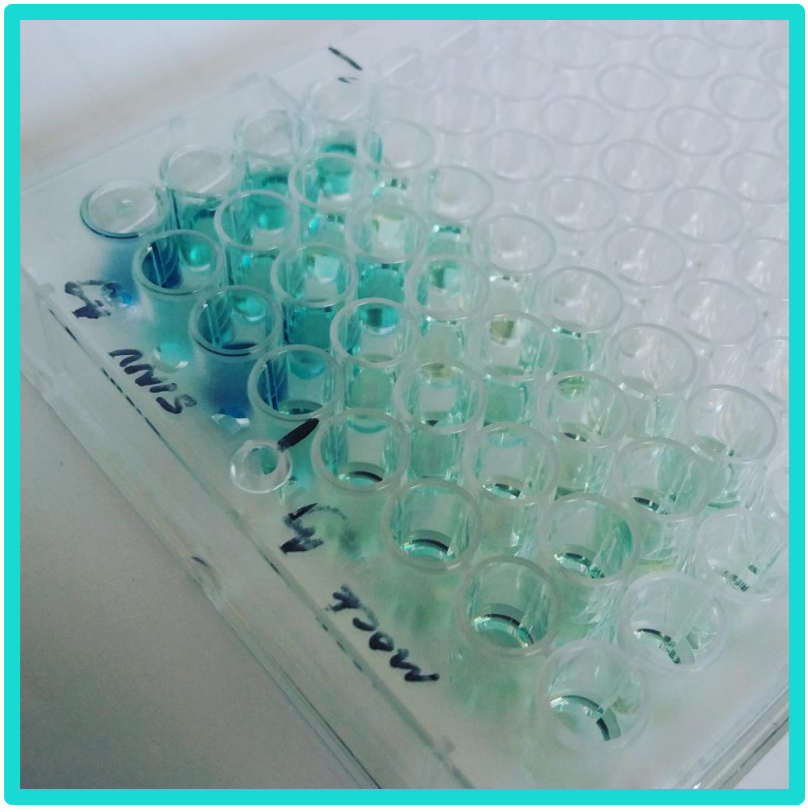
DEVELOPMENT OF IN HOUSE ASSAYS FOR DETECTION OF SINDBIS VIRUS INFECTIONS



Nicole Kennedy

February 2019

DEVELOPMENT OF IN HOUSE ASSAYS FOR DETECTION OF SINDBIS VIRUS INFECTIONS

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MMedSc (Virology)

Dissertation submitted in fulfillment of the requirements for the Master of Medical Science Virology degree completed in the Division of Virology in the Faculty of Health Sciences at the University of the Free State

Supervisor: Professor Felicity Jane Burt

Division of Virology

Faculty of Health Sciences

University of the Free State

University of the Free State, Bloemfontein

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Declarations

I, Nicole Kennedy, hereby declare that the master's research dissertation that I herewith submit at the University of the Free State, is my independent work and that I have not previously submitted it for a qualification at another institution of higher education.

I, Nicole Kennedy, hereby declare that I am aware that the copyright is vested in the University of the Free State.

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Presentations and publications

Presentations

Development of an in-house ELISA for detection of Sindbis virus antibodies. N. Kennedy and F.J. Burt. Poster Presentation at SASM. Johannesburg. South Africa. 4-7 April 2018.

Virus = Arthritis?. N. Kennedy. Oral Presentation at Three Minute Thesis. Bloemfontein. South Africa. 24 August 2018.

Prevalence of Sindbis virus infections in the Free State. N. Kennedy, F.J. Burt, D. Goedhals. Oral Presentation at 50th Faculty of Health Sciences Research Forum. Bloemfontein. South Africa. 30-31 August 2018.

Prevalence of Sindbis virus infections in the Free State. N. Kennedy, F.J. Burt, D. Goedhals. Oral Presentation at 7th Annual Free State Health Research Day. 8-9 November 2018.

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Table of contents

Abstract

Sindbis virus is a mosquito-borne virus associated with chronic arthritis and is endemic in South Africa. It is the prototype virus for the genus *Alphavirus* in the family *Togaviridae*. Sporadic outbreaks occur naturally, often related to heavy rainfall and an increase in mosquito populations. The virus causes a mild disease and hence the exact prevalence in South Africa is not known. In addition, the association of Sindbis virus disease and arthritis is not well documented in South Africa. The aim of this study was to investigate Sindbis virus prevalence and develop serological assays for detection of Sindbis virus infection. An in-house ELISA was developed and optimized. The ELISA was used to screen a total of 165 stored serum samples collected from patients attending a local arthritis clinic in Universitas Hospital, 266 stored serum samples from patients with acute febrile illness, suspected of tickbite fever and with no diagnosis, as well as 136 serum samples from high risk populations (horse and stable workers in Bainsvlei). Production of a recombinant antigen of the Sindbis virus E2 protein for use in immunofluourescence assays (IFA) was attempted. An in-house IFA, prepared with Sindbis virus infected cells, was developed. The positive samples were tested using a commercial immunofluorescence assay (IFA), a neutralisation assay and the in-house IFA. The results indicated that 31/165 samples from patients attending arthritis clinic, 13/136 samples from high risk populations, and 25/266 samples from acute febrile illness patients with no diagnosis tested positive for immunoglobulin G (IgG) Sindbis virus antibodies using the in-house ELISA. Commercial IFA results were as follows: 46/69 samples tested positive, 15/69 samples tested negative and 8/69 samples were indeterminate. A total of 65/69 samples tested positive using the neutralisation assay. Sensitivity for the ELISA and commercial IFA was determined and found to be 100% for the ELISA and 70.7% for the commercial IFA. Unfortunately, the recombinant protein could not be transiently expressed in mammalian cells and used to develop an in-house IFA. In-house antigen slides were prepared for in-house IFA tests. Using the in-house slides, a total of 50/68 samples tested positive for anti-Sindbis virus IgG antibodies and 8/56 samples tested positive for anti-Sindbis virus IgM antibodies. The ELISA and in house IFA were shown to be more sensitive than the commercial IFA. The prevalence of IgG antibody in targeted populations suggests a higher occurrence of Sindbis virus infections and that Sindbis virus infection should be considered in patients with joint pain.

Keywords: Sindbis virus, arbovirus, ELISA, viral arthritis, IFA, sensitivity.

List of figures and tables

* This list of figures and tables were compiled with WPS Writer software in Linux (Ubuntu Gnome) operating system and therefore could not be separated into two lists.

List of abbreviations

°C	Degrees celsius
µl	Microliter
µg	Microgram
ABTS	2,2'-azino-di-3-ethylbenzthiazoline-6-sulfonate
AG80	Rio Negro virus
Amp	Ampicillin
Arbovirus	Arthropod-borne virus
ATCC	American Type Culture Collection
AURAV	Aura virus
BBS	Borate buffered saline
BEBV	Bebaru virus
BF	Barmah forest
BFV	Barmah forest virus
BHK-21	Baby hamster kidney
CABV	Cabassou virus
CEF	Chicken embryo fibroblasts
CF	Complement fixation
cfu	Colony forming units
CHIKV	Chikungunya virus
CHO	Chinese hamster ovary
CMV	Cytomegalo virus
CPE	Cytopathic effects
CO_2	Carbon dioxide
DNA	Deoxyribonucleic acid

EDTA	Ethylene-diamine-tetra-acetic acid
EEE	Eastern equine encephalitis
EEEV	Eastern equine encephalitis virus
eGFP	Enhanced green fluorescent protein
EILV	Eilat virus
ER	Endoplasmic reticulum
EVEV	Everglades virus
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FMV	Fort Morgan virus
FN	False negative
FP	False positive
GETV	Getah virus
GFP	Green fluorescent protein
HAI	Haemagglutination inhibition
HEK-293	Human embryonic kidney
His	Histidine
HJV	Highlands J virus
HRPO	Horse radish peroxidase
IFA	Immunofluorescence assay
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IRS	Infection rates
KYZV	Kyzylagach virus
LB	Luria-Bertani
M	Molar
MADV	Madariaga virus

MAYV	Mayaro virus
MDPV	Mosso das Pedras virus
MEM	Minimum essential media
mg	Milligram
Mg^{2+}	Magnesium
MID	Middelburg
MIDV	Middelburg virus
min.	Minutes
ml	Milliliter
mM	Millimolar
MOI	Multiplicity of infection
MUCV	Mucambo virus
N18	Mouse neuroblastoma
NCBI	National Center for Biotechnology Information
NDUV	Ndumu virus
NHLS	National Health Laboratory Service
NICD	National Institute for Communicable Diseases
nm	Nanometer
NSAIDs	Non-steroidal anti-inflammatory drugs
ONNV	O'nyong-nyong virus
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PIXV	Pixuna virus
PP	Percentage positive
PRNT	Plaque-reduction neutralisation
RNA	Ribonucleic acid
RRV	Ross river virus

RT-PCR	Reverse transcriptase polymerase chain reaction
rpm	Revolutions per minute
SAGV	Sagiyama virus
SD	Standard deviations
SDV	Sleeping disease virus
SESV	Southern elephant seal virus
SFV	Semliki forest virus
SINV	Sindbis virus
Sn	Sensitivity
SPDV	Salmon pancreatic disease virus
TALV	Taï forest virus
$TCID_{50}$	Tissue culture infectious dose
THP-1	Human monocytic leukemia
Tn	True negatives
TONV	Tonate virus
Tp	True positives
TROV	Trocara virus
u	Units
UNAV	Una virus
UV	Ultraviolet
VEE	Venezualan equine encephalitis
VEEV	Venezualan equine encephalitis virus
VERO-76	African green monkey
WEE	Western equine encephalitis
WEEV	Western equine encephalitis virus
WHAV	Whataroa virus

Chapter 1: LITERATURE REVIEW

1.1 Introduction

An arbovirus, which is short for arthropod-borne virus, is a virus transmitted by blood-feeding arthropod vectors especially mosquitoes, to vertebrates. Arboviruses are from various virus families and genera, including a variety of informal groups and subgroups. These groups were originally derived from the immunological relationship of each virus to other viruses in that group. These relationships are essential for serological diagnosis, since the interpretation of the immune response in patients can be confounding when they have had sequential infections by two or more related viruses (McIntosh, 1986).

Sindbis virus (SINV) is the prototype virus for the genus *Alphavirus* in the family *Togaviridae* (Jost *et al.*, 2010). The *Togaviridae* family contains more than 30 virus species (Chen *et al*., 2018). SINV is the most widely distributed arthropod-borne single-stranded RNA virus (Jost *et al.*, 2010). SINV was initially isolated from a pool of *Culex pipiens* and/or *Culex univittatus* mosquitoes in the Sindbis village, in Egypt in August of 1952; When the mosquitoes were collected with light traps, no distinction between *Cx. pipiens* and *Cx. univittatus* was made, however ensuing collections proved that *Cx. pipiens* is rare during that season (mid-summer), thus the most likely mosquitoes it was isolated from is *Cx. univittatus*. The virus was isolated by inoculating triturated mosquito suspension into three-day-old mice. Thereafter the virus has been repeatedly isolated from mosquitoes (Taylor *et al*., 1988). The virus was first referred to as Coxsackie-like', since it caused fatal infection in newborn mice and not in adult mice; as well as causing myositis of the skeletal muscles (Hurlbut, 1953). Shortly thereafter, it was discovered that strain Ar-339 had other characteristics than the Coxsackie group of viruses;

and as a result named ‘Sindbis’ virus after the village in Egypt where the mosquitoes with the virus were caught (Taylor *et al*., 1988). SINV was found to be the agent causing febrile illness coupled with maculopapular rash and joint pain in humans in Africa, Eurasia and Australia (Hubálek, 2008; Jost *et al.*, 2010). Most reports of clinical infections are in Northern Europe and South Africa (Adouchief *et al*., 2016).

Alphaviruses consists of a diverse group of small, spherical, enveloped viruses containing a single-stranded, positive-sense, RNA genome, isolated from all continents except Antarctica and some of these viruses cause severe disease (Forrester *et al*., 2012). These viruses belong to the *Togaviridae* family. All alphaviruses are transmitted by mosquitoes except salmon pancreatic disease virus (SPDV), its subtype sleeping disease virus (SDV), and southern elephant seal virus (SESV) (Forrester *et al*., 2012). Both these viruses are associated with the lice, *Lepeophtheirus salmonus* for SPDV and *Lepidohthirus Macrorhini* for SESV, which suggests an arthropod-borne cycle, but the vector has not yet been identified (Forrester *et al*., 2012). Most of the pathogenic alphaviruses cause acute, febrile illness in humans and/or animals culminating in either encephalitis or arthritis. Some enzootically circulating alphaviruses are not known to cause disease. Most of these viruses were first isolated during mosquito surveillance, and for many the transmission cycles are still unknown, such as Trocara virus (TROV) and Aura virus (AURAV) (Forrester *et al.*, 2012).

By 1986, at least 22 mosquito-borne viruses had been isolated in South Africa and several of these viruses were identified as causing disease in humans including, chikungunya virus, SINV, West Nile virus, and Rift Valley fever virus (Jupp, 2005).

1.2 Classification

The *Togaviridae* family includes two genera, namely: *Alphavirus* with 40 recognised species, and *Rubivirus* with one species. The name “togaviridae” comes from the Latin “toga” meaning roman mantle or cloak, and relates to the envelope (Kuhn, 2013). The members of this family have two proteins (E1, E2, and sometimes, E3) associated with surface projections on the virus envelope, which encloses the spherical nucleocapsid with icosahedral symmetry. Maturation occurs via the preformed nucleocapsids budding through the cytoplasmic or plasma membranes (Kuhn, 2013).

Viruses belonging to the genus *Alphavirus* have a capsid with icosahedral symmetry and the envelope proteins are arranged in clusters of trimers, which forms an icosahedron with T = 4 icosahedral symmetry (Weaver & Smith, 2011). The *Alphavirus* genus contains 31 approved species that are transmitted by mosquitoes, have a wide host range of vertebrates, and cause febrile illness with rash, arthritis or encephalitis. There are six serologic complexes for the species, which is derived from their antibody reactions with E1 (hemagglutinin inhibition) and E2 (neutralization) (Kuhn, 2013). Homology studies have shown that Alphaviruses have conserved sequences at 19 nucleotides or less close to both termini of the genome as well as at the start of the subgenomic 26S RNA, especially in SINV, Highlands J virus and Semliki Forest virus (SFV) (Rice & Strauss, 1981). The Alphaviruses are divided into seven antigenic complexes, namely: Venezuelan equine encephalitis (VEE), Semliki forest (SF), eastern equine encephalitis (EEE), western equine encephalitis (WEE), Middelburg (MID), Barmah forest (BF), and recombinants of WEE. (Powers *et al.*, 2001) SINV is one of the members of the Western equine encephalomyelitis (WEE) antigenic complex of alphaviruses (Olson & Trent, 1985). The alphaviruses are summarised in Table 1.1.

Table 1.1 Antigenic complexes, abbreviations, primary vertebrate hosts and geographic distribution of the alphaviruses (Calisher *et al.*, 1980; Powers *et al.*, 2001; Schmaljohn & Mcclain, 1996; Griffin, 2013; Forrester *et al*., 2017).

Antigenic Complex	**Species**	**Abbreviation**	**Primary Vertebrate Host**	**Geographic distribution**
Barmah Forest	Barmah Forest virus	BFV	Birds	Australia
Eastern Equine Encephalitis	Eastern Equine Encephalitis virus	EEEV	Birds	North America South America Caribbean
	Madariaga virus	MADV	Rodents	South America
Middelburg	Middelburg virus	MIDV	Horses	Africa
Ndumu	Ndumu virus	NDUV	Pigs	Africa
Western Equine Encephalitis	Western Equine Encephalitis virus	WEEV	Birds Mammals	North America South America Argentina
	Aura virus	AURAV	Unknown	South America
	Sindbis virus	SINV	Birds	Northern Europe, Asia, Africa Australia Middle East
	Whataroa virus	WHAV	Birds	New Zealand Australia
	Kyzylagach virus	KYZV	Birds	Azerbaijan
Recombinants of Western Equine Encephalitis	Fort Morgan virus	FMV	Swallow bug Birds	Western North America (Colorado)
	Highlands J virus	HJV	Birds	Eastern North America
Unclassified	Trocara virus	TROV	Unknown	South America
	Southern Elephant Seal virus	SESV	Seals	Australia Antarctica
	Salmon Pancreas Disease virus	SPDV	Fish	North Atlantic
	Sleeping Disease virus	SDV	Rainbow Trout	Europe
	Eilat virus	EILV	Insects	Israel
	Taï Forest Alphavirus	TALV	Unknown	Ivory Coast

Antigenic Complex	Species	Abbreviation	Primary Vertebrate Host	Geographic distribution
Semliki Forest	Semliki Forest virus	SFV	Birds	Africa
	Chikungunya virus	CHIKV	Primates Humans	Africa Southeast Asia Philippines Indonesia
	O'nyong-nyong virus	ONNV	Primates	East Africa
	Getah virus	GETV	Mammals	Asia, Oceania
	Mayaro virus	MAYV	Primates Humans	South America Trinidad
	Bebaru virus	BEBV	Unknown	Malaysia (Asia)
	Ross River virus	RRV	Mammals Humans	Australia South Pacific Oceania
	Una virus	UNAV	Primates Humans	South America Trinidad Panama
	Sagiyama virus	SAGV	Humans Horses Pigs	Asia Japan Africa
Venezuelan Equine Encephalitis	Venezuelan Equine Encephalitis virus	VEEV	Rodents Horses Mammals	South America North America
	Everglades virus	EVEV	Rodents Mammals	Florida
	Mucambo virus	MUCV	Unknown	South America Caribbean Trinidad
	Pixuna virus	PIXV	Rodents Mammals	South America Brazil
	Tonate virus	TONV	Humans	French Guiana
	Cabassou virus	CABV	Unknown	French Guiana
	Rio Negro virus	AG80	Mammals	Argentina
	Mosso das Pedras virus	MDPV	Unknown	Brazil

1.3 Structure and Genome Organisation

SINV has the morphology of spherical shape with spikes and 6-10 nm long surface projections. The nucleocapsid has a diameter of 12-14 nm, is cubically symmetrical, and consists of 240 capsomeres in an icosahedral form (Westaway *et al*. 1985; Weaver *et al*., 2012). The envelope has an external diameter of 70 nm and is comprised of a host cell derived lipid bilayer membrane including two viral specific glycoproteins (Chen *et al*., 2018; Strauss & Strauss, 1994). These glycoproteins are the E1 and E2 proteins, which form trimer spikes consisting of three E1-E2 heterodimers twisted anti-clockwise around each other to form a stalk and then separated into a tripartite head (Weaver, *et al*., 2012; Strauss & Strauss, 1994). Clusters of 80 trimers form an icosahedral lattice with a T = 4 surface lattice symmetry. Its core is approximately 40 nm and consists of the RNA genome (Strauss *et al*., 2002; Strauss & Strauss, 1994).

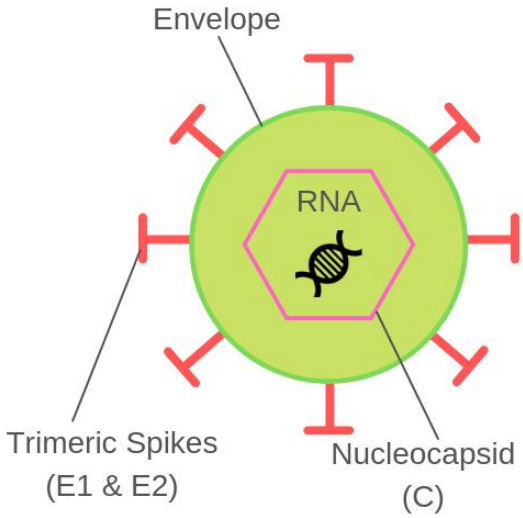


Figure 1.1 Structure of SINV with trimeric spikes embedded in the host cell derived lipid membrane and genomic RNA inside the nucleocapsid (Kennedy, N).

SINV has a positive sense, single stranded RNA genome and the core protein C inside the nucleocapsid (Weaver *et al*., 2012). The gene encodes for four non structural proteins, the capsid protein and three envelop proteins (Westaway *et al.*, 1985). A simple lipoprotein envelope with virus-encoded glycoproteins is present in SINV enclosing its icosahedral nucleocapsid (Rice & Strauss, 1981).

The genome of SINV is positive sense, single stranded RNA of approximately 11700 bases in length. It contains a 5' methylguanylate cap and the 3'-terminal polyadenylate tail as shown in figure 1.1 (Strauss & Strauss., 1994; Gorchakov *et al.*, 2005). The 5' two thirds of the genome codes for the viral non-structural proteins nsP1 to nsP4. The 3' one third of the genome encodes for the subgenomic RNA, which translates into structural proteins (Gorchakov *et al.*, 2005). The first (two thirds) part of the genome consists of the 5' terminal cap; followed by 5' untranslated nucleic acid of 59 nucleotides and 7539 nucleotides of an open reading frame except for a single nonsense termination codon. This reading frame encodes for the non-structural polypeptides. The second (one third) part of the genome consists of a junction region, followed by 3735 nucleotides of an open reading frame coding for the structural proteins, as well as 322 nucleotides of nontranslated nucleic acid and the 3' poly-A tail. The junction region separates the coding sequences of the non-structural and structural proteins and consists of 48 nontranslated nucleotides (Strauss & Strauss, 1994). The non-structural proteins and host factors form the replicase/transcriptase or RNA-dependent RNA polymerase needed for viral genome replication and transcription of the subgenomic RNA from the replicative intermediate. The viral particle consists of the genomic RNA and structural proteins (Gorchakov *et al.*, 2005).

1.4 Replication Cycle

Replication of SINV in vertebrate cells occurs very quickly and has a strong effect on essential cellular processes in cell physiology. Cellular resources are redirected toward synthesizing viral structural proteins and viral genomes after a few hours of infection, in order to assemble a large amount of viral particles (Gorchakov *et al*., 2005). Genomic RNA is released from the nucleocapsid. Thereafter translation of the genomic RNA by the cellular translational machinery to generate the viral non-structural proteins nsP1 to nsP4 takes place (Gorchakov *et al.*, 2005). The replication cycle is shown in figure 1.1.

From the genomic RNA, the non-structural proteins are directly translated into two polyprotein precursors. The first polyprotein precursor has a length of 1896 amino acids, terminates at a nonsense codon at position 1837, and undergoes further processing into three polypeptides, namely nsP1, nsP2 and nsP3 (Strauss & Strauss, 1994). The second polyprotein precursor is translated into the fourth non-structural protein, nsP4, of 2513 nucleotides in length when the nonsense codon is read through. All four of these polyprotein precursors are cleaved post-translationally (Strauss & Strauss, 1994). A replication complex containing viral specified polymerase will transcribe the positive-strand 49S RNA into complementary minus-strand 49S RNA, which will function as the template for subgenomic 26S RNA and the progeny 49S RNA (Westaway *et al.*, 1985). The subgenomic messenger RNA (26S RNA), starts at nucleotide 7598, with a length of 4106 nucleotides, excluding the poly-A tail, is co-terminal with the 3' end of the genomic RNA, and is translated into the structural proteins as a polyprotein precursor (Rice & Strauss, 1981; Strauss & Strauss, 1994). This polyprotein precursor is also cleaved into a nucleocapsid protein (C protein), two integral membrane glycoproteins and two small peptides not found in the mature virion (Strauss & Strauss, 1994; Westaway *et al.*, 1985).

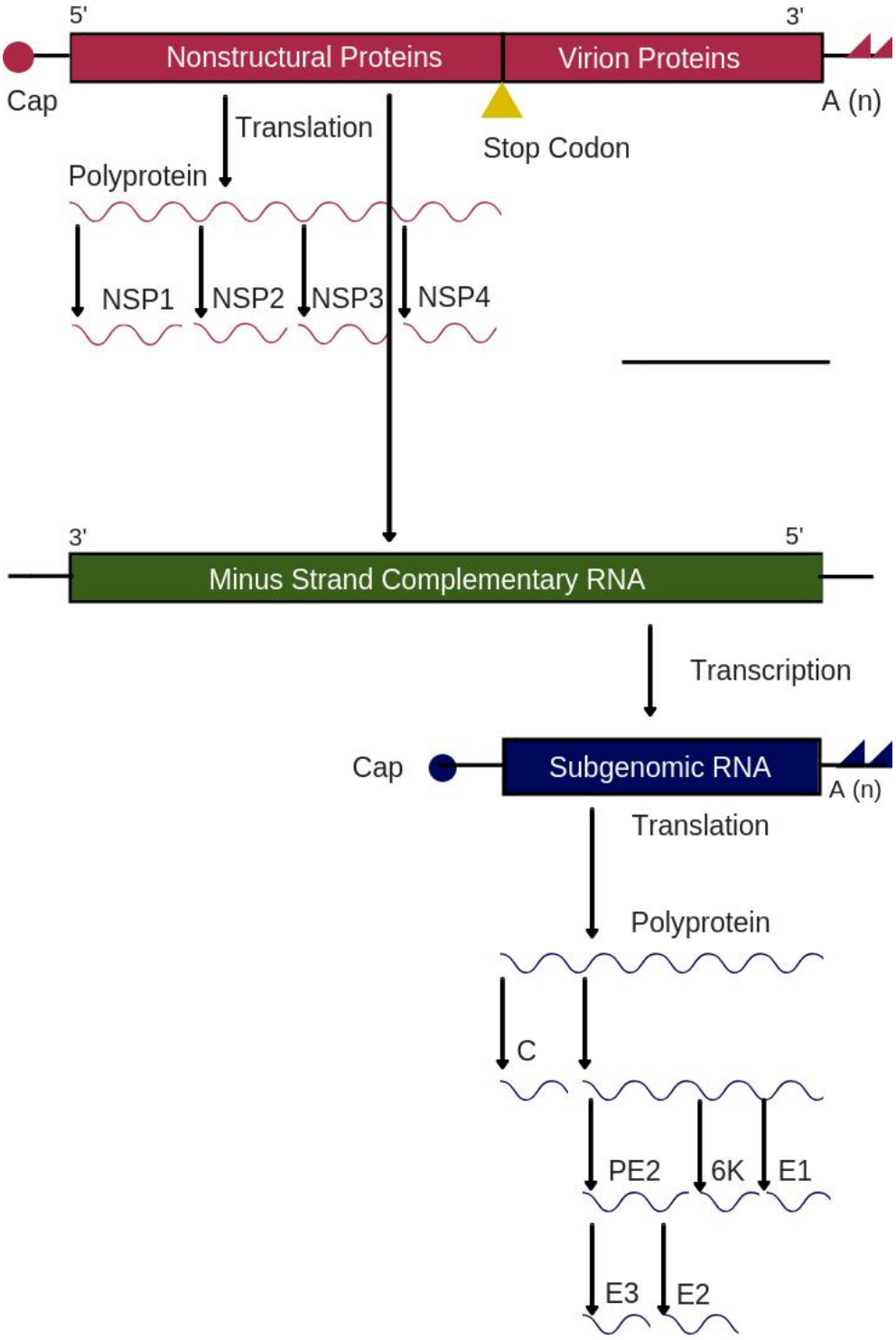


Figure 1.2 The replication cycle of SINV. First translation of the non-structural proteins into a polyprotein, which is then cleaved into four non-structural proteins (nsP1, nsP2, nsP3 and nsP4). Thereafter transcription of the complementary minus strand RNA as a template for the genomic RNA and subgenomic RNA strand. Structural proteins are translated from the subgenomic RNA into a polyprotein, which is cleaved into the structural proteins (C, E1, E2, E3 and 6K) (Kennedy, N).

The C protein is the first protein to be cleaved of the structural proteins and is 264 amino acids in length. The first 120 residues of the capsid protein are possibly involved in interaction with the virion RNA (Rice & Strauss, 1981). This protein forms the nucleocapsid core by encapsulating the genomic RNA. Its carboxyl domain is a serine protease (Kuhn, 2013).

Glycoprotein E3 is 64 amino acids in length (Rice & Strauss, 1981). It contains malleable disulfide bonds and is in close proximity to E2 throughout assembly. This implies that it could be a disulfide isomerase catalyzing proper folding and disulfide bonds formation (Parott *et al*., 2009). The first 19 residues may function as the signal sequence for the insertion of PE2 into the endoplasmic reticulum (ER) during protein synthesis. This signal will not be cleaved from PE2; however PE2 is cleaved into E2 and E3 during maturation. E3 protein is glycosylated with a complex polysaccharide chain. The site of glycosylation occurs within the signal sequence for the PE2 glycoprotein (Rice & Strauss, 1981).

The E2 protein is 423 amino acids in length with two glycosylation sites. This protein has two carbohydrate components, one component is a simple oligosaccharide chain that includes only mannose and N-acetylglucosamine and the other component is a complex chain including galactose, fucose, and sialic acid. This protein has two hydrophobic regions and both regions have a fatty acid covalently attached. The first hydrophobic region is 28 amino acids in length (363-390 residues) and crosses the bilayer. The second hydrophobic region is 23 amino acids in length (396-418 residues), with a significant homology (78%) between SINV and SFV proteins, which could allow for the specific interaction between the viral nucleocapsid and the glycoproteins during budding (Rice & Strauss, 1981). This protein plays an important role in the attachment of the virus to the target host cell through binding of the cell receptor (Carleton *et al*., 1997). This protein contains the main neutralising

epitopes and binds to the receptors of a wide range of animals and cells in culture, such as chicken embryo fibroblast (CEF), mouse neuroblastoma (N18), baby hamster kidney (BHK-21), Chinese hamster ovary (CHO) and human monocytic leukemia (THP-1) cells (Kuhn, 2013).

The 6K protein is a 55 amino acid peptide which can function as or partly as the signal sequence for E1 and it also increases infectivity of the particles (Kuhn 2013).

The glycoprotein E1 is the viral hemagglutinin, and is 439 amino acids in length, which means it is the largest Sindbis glycoprotein. When E1 is grown in primary chicken cells, it is modifed into both simple and complex polysaccharide chains, however it only has complex chains when grown in hamster cell lines (Kuhn, 2013). It also has two carbohydrate chains. There are two long sequences of uncharged amino acids. The first sequence is 17 amino acids in length and not present in the spikeless particles, which implies that it is not embedded in the viral membrane (Rice & Strauss, 1981). This protein has fusin activity, and is involved in virus fusion with cell membranes (Sanz *et al*., 2003). The second sequence is highly hydrophobic and found at the COOH terminal, which implies that E1 crosses the bilayer with at least two residues on the cytoplasmic side (Rice & Strauss, 1981).

The protease that cleaves the capsid-PE2 has a chymotrypsin-like specificity. It is thought that the proteolytic activity is in the capsid protein. The cleavage site has a highly conserved region that could be essential for site-specific viral-encoded cleavage, occurring during translation and is essential for the function of PE2 glycoprotein's signal sequence (Rice & Strauss, 1981). The protease that cleaves PE2 into E3 and E2 has the same specificity as trypsin. This cleavage occurs quite late, about 20 min after PE2 is produced and is necessary for maturation of the virus. PE2 is needed to form oligomers and to export E1 from the endoplasmic reticulum (Carleton *et al*., 1997). E3 is found

in the culture medium after cleavage, therefore the plasma membrane, external or internal to the cell, or even in the Golgi's lumen is most likely the site of cleavage, soon before or simultaneously with their fusion to the plasma membrane. The 55 amino acid peptide is membrane associated or released into the endoplasmic reticulum's lumen after cleavage (Rice & Strauss, 1981).

Glycoproteins are translated on the rough ER and are glycosylated; and thereafter move to the plasma membrane through the Golgi apparatus, where modification of the carbohydrates and covalent attachment of lipids occur. The icosahedral nucleocapsid is constructed in the cytoplasm. Thereafter it diffuses to the cell surface and buds through the host cell plasmalemma to obtain a lipoprotein envelope consisting of only the above-mentioned virus-encoded glycoproteins. These glycoproteins interact more specifically with its alphavirus nucleocapsid than other enveloped viruses. The mature virions will only contain alphavirus proteins (Rice & Strauss, 1981).

1.5 Genetic and Antigenic Diversity

The prototype strain Ar-339 was first isolated from mosquitoes collected in Sindbis village in Egypt (McKnight *et al*., 1996). The first European isolate was isolated from a reed warbler (*Acrocephalus scirpaceus*) in Western Slovakia in 1971 (prototype strain R-33) (Hubálek, 2008). The Ockelbo (Edsbyn 5/82) and Karelian fever (LEIV-9298 'Karelia') strains are similar to the prototype strain and cross react serologically using complement fixation and haemagglutination inhibition and have similar polypeptide composition, however they can be differentiated with a neutralization assay (Hubálek, 2008). Particular antigenic and genetic differences have been found in SINV strains isolated from different geographic areas. (Hubálek, 2008) These four geographic areas are Paleoarctic, Ethiopian, Oriental and Australian. Plaque-reduction neutralization (PRNT) assays of SINV with polyclonal

antibody to prototype virus AR-339 were performed. Even though there was no more than a fourfold difference to the prototype strain, there was some variation. European and African isolates had similar PRNT titers, whereas the Far East and Australian virus isolates reacted at a lower titer. An enzyme-linked immunosorbent assay (ELISA) with anti-E2 monoclonal antibody was used to analyse the viruses, which revealed differences in the antigen-antibody reactivity. The Sicily isolate showed similar reactivity to the prototype strain. Two African isolates reacted less than the prototype strain. Viruses from India, the Far East and Australia did not react with significant titers against the antibody, which suggests that there are antigenic differences in the E2 epitope between these isolates and the prototype strain (Olson & Trent, 1985).

There are two genetic lineages of SINV strains, namely Paleoarctic/Ethiopian and Oriental/Australian (Jost *et al.*, 2010). The homology and zoogeographic groupings of SINV isolates, which were originally determined by stringent hybridization of SINV genomic RNAs, and were later supported by the oligonucleotide fingerprints of the Sindbis viruses. However there were lower oligonucleotide homologies than the RNA homologies of Rentier-Delrue and Young. The SINV groupings were further supported by the tryptic peptide analyses of the SINV virion proteins, which indicate a close relationship between the AR-339 prototype virus and Sindbis viruses in the Paleoarctic zoogeographic region. The results were compared and showed that Sindbis viruses are restricted to its zoogeographic region, even if the bird hosts are not restricted to them (Olson & Trent, 1985). They could not determine whether genetic drift or unique adaptations to their zoogeographic origins caused the genetic variation (Olson & Trent, 1985). The data for these two studies are presented in Table 1.2.

Table 1.2 Comparison of geographic groupings of SINV isolates based on T1 oligonucleotide and tryptic peptide homologies with RNA-RNA hybridization (Olson & Trent, 1985).

SINV groups based on T1 oligonucleotide fingerprints and tryptic peptide maps	**SINV groups based on RNA RNA hybridization***
Paleoarctic (Group I)	Paleoarctic (Group I)
AR-339 Egypt Acrocephalus Czechoslovakia Gresikova Sicily W32309 Israel	AR-339 Egypt M-1855 Israel R-33 Czechoslovakia AZ-16 U.S.S.R.
Ethiopian (Group II)	Ethiopian (Group II)
AR-18132 South Africa MP-684 Uganda	AR-86 South Africa AR-18132 South Africa AR-6071 South Africa Girdwood South Africa
India-Far Eastern (Group III)	India-Far Eastern (Group III)
A-1036 India B-322/23/24 India P-886 Philippines	A-1036 India B-322/23/24 India P-886 Philippines
Australian Far Eastern (Group IV)	Australian Far Eastern (Group IV)
MRM-18520 Australia C-377 Australia MM-2215 Malaysia	C-377 Australia CH-19470 Australia MM-2215 Malaysia

**Rentier-Delrue & Young (1980)

Currently there are six antigenically well-defined genotypes of SINV. These genotypes are based on the E2 glycoprotein gene sequence (Sigei *et al*., 2018). The genotypes and where they are located are listed in table 1.3. Bird migration routes overlap these genotypes. Strains from Northern Europe and Sub-Saharan Africa share a common ancestor with genotype 1 (Adouchief *et al*., 2016).

Table 1.3 The six antigenically defined genotypes of SINV.

Genotype	Location
SINV-I	Europe, Middle East & Africa
SINV-II	Australia & Malaysia
SINV-III	India & Philippines
SINV-IV	Azerbaijan & China
SINV-V	New Zealand
SINV-VI	South-west Australia

1.6 Epidemiology

1.6.1 Geographic Distribution

SINV is one of the most widely distributed arboviruses throughout the Old World and is regularly isolated in four of the six zoogeographic regions (Paleoarctic, Ethiopian, Oriental and Australian) of the world (Olson & Trent, 1985). Infections of humans with SINV were regarded as an insignificant medical problem before the epidemics involving hundreds of cases in South Africa in 1974 and in Northern Europe regions in the mid-1980s were reported (Sammels *et al.*, 1999).

Migratory birds play an essential role in SINV's wide geographic distribution and possibly introducing SINV into previously nonendemic areas, most recently Germany and Northern Sweden (Lundström & Pfeffer, 2010; Adouchief *et al*., 2016). Wetland ecosystems of diverse biomes are the natural hub/central point of SINV infections (Hubálek, 2008). There have been reported outbreaks of SINV infections in northern Europe during 1981 – 1982, 1988, 1995, 2002 and 2013; and in South Africa during 1963, 1974, and subsequent cases between 1983 – 1984 and 2006 – 2010 (Lwande *et al*.,

2015). Outbreaks also occurred in Malaysia, Philippines, Papua New Guinea, Kenya, Australia, and China (Lwande *et al.*, 2015). Sporadic smaller outbreaks likely occur annually in endemic regions and are undetected unless there are significant numbers.

There are widespread SINV infections in South Africa, with the highest prevalence across the central plateau, which includes Gauteng, Free State and Northern Cape provinces (Storm *et al*., 2013). There have been sporadic cases reported from North-West, Mpumalanga, Eastern Cape, Western Cape and Kwazulu-Natal provinces (Storm *et al*., 2013). Endemics occur annually, whereas minor epidemics occur periodically (McIntosh, 1986). SINV is widely distributed in South Africa as determined by antibody surveys on sera from persons residing in different regions of this country, which indicates that humans are quite often infected (McIntosh *et al*., 1964). The Free State and Gauteng provinces, as well as the parts of the surrounding areas and the length of the Orange River have a particularly high prevalence of infections. This area has favourable breeding conditions for the vector of the virus with high temperatures and irrigation. Isolation of the virus from mosquitoes and wild birds collected in the northern Natal and Highveld has also confirmed the presence of SINV in these regions of South Africa (McIntosh *et al*., 1964).

Even though SINV has been identified since 1952 and isolated in numerous parts of the world from birds and mosquitoes, it was only first recovered in 1961 from human cases when it was isolated from blood specimens taken from five sick Africans in Uganda (McIntosh *et al*., 1964). The patients showed the following signs and symptoms: fever, headache, malaise, jaundice, widespread body pains and pain in the chest and joints. The first isolate of SINV from a human in South Africa was from skin lesions of a sick person in

Johannesburg, in January 1963 (McIntosh *et al*., 1964). This patient had fever, malaise, pains in the joints and tendons, a maculopapular rash over the trunk and limbs, and vesicles on the fingers and toes. From March to April 1963 additional cases with rather similar clinical features occurred at several regions in the Gauteng, North West, Limpopo, Mpumalanga and Free State. No virus was isolated from these patients, but paired sera, collected during the acute and convalescent stages of the infection, made a diagnosis of SINV infection possible (McIntosh *et al.*, 1964).

Human SINV infections occur occasionally across the central plateau of South Africa, including the provinces of Gauteng, Free State and Northern Cape during the summer (Storm *et al.*, 2014). Cases up until 1974 were mostly from the moister areas of the former Transvaal (now Gauteng) and the Free State, even though SINV occur broadly in the Highveld and Karoo areas from antibody surveys in humans and animals (Jupp *et al*., 1986). A large epidemic of SINV infections was recorded in South Africa, in early 1974, from wide areas of the Karoo and Northern Cape Province with thousands of human infection cases (Jupp *et al.*, 1986). There was a second large epidemic of SINV infection in the Witwatersrand-Pretoria area during the summer from mid-December 1983 until mid-April 1984. This epidemic involved hundreds of human cases. There were higher infection rates for SINV in *Culex univittatus* mosquitoes collected in the Witwatersrand area in February and March than in previous years. This epidemic was due to a high level of viral activity in the feral *Cx. univittatus*-bird transmission cycle (Jupp *et al*., 1986). SINV infection was efficiently transferred from this cycle by *Cx. univittatus to humans during this epidemic.* This was the largest epidemic of SINV infections yet documented in South Africa since the 1974 Karoo epidemic (Jupp *et al.*, 1986).

The infection rates (IRs) for *Cx. univittatus* in 1984 were compared with IRs of previous years. These IRs were much higher and show that an epizootic of SINV occurred evident by the high level of viral activity in the feral maintenance cycle between birds and *Cx. univittatus*. The environmental factors of abnormally high rainfall early in the summer and above normal temperatures throughout the mosquito season were the most likely contributors of this epizootic and successive SINV infection epidemic. The high *Cx. univittatus* populations in December were due to the rainfall pattern favouring mosquito breeding early in the summer. Viral infection in the mosquito and successive transmission of the virus was favoured by the high temperatures (Jupp *et al.*, 1986).

Storm *et al.* (2014) reported the epidemiology of human SINV infections in South Africa from 1 January 2006 till 31 December 2010. A total of 3631 specimens submitted from patients with suspected arbovirus infections were analysed with the following serological screenings: haemagglutination inhibition (HAI) assay and immunoglobulin M (IgM) ELISA. Detection of anti-SINV IgM antibodies was taken as positive for recent SINV infection. During the years, 2006 till 2009, 5.4% of the specimens tested positive for SINV on the HAI screen and 1.3% of these were positive for anti-SINV IgM antibodies. There was an increase in SINV cases during 2010, 12% of the specimens tested positive for SINV on the HAI screen and in 10% of these anti-SINV IgM antibodies were detected. For the period 2006 till 2010, almost double the amount of specimens was obtained from men (64%) than women (35%). Anti-SINV IgM antibody was detected more frequently in men (7%) than in women (5%). This could probably be attributable to a higher occurrence of mosquito bites in men, since more are employed in the farming sector and spend more time outdoors. Most of the specimens were obtained from persons

between 20 and 49 years old, whereas the least amount of specimens were obtained from persons less than 10 years old and more than 70 years old. Just 7% of persons with SINV infections were less than 18 years old. The risk of becoming infected with SINV increased linearly with age and most cases occurred in patients with a mean age of 42 years. It was found that most of SINV infections were diagnosed during the months of March and April, which is the time when *Cx. univittatus* mosquitoes are in abundance in South Africa (Storm *et al*., 2014). The number of specimens submitted from each province were as follows: 32% from Gauteng, 26% from Free State, 11% from Northern Cape, 9% from Western Cape, 7% from Eastern Cape, 7% from North West, 3% from Kwazulu Natal, 3% from Mpumalanga and 0% from Limpopo. The percentage of anti-SINV IgM positive samples detected from each province were as follows: 18% from Free State, 14% from Northern Cape, 6% from Gauteng, 5% from North West, 5% from Mpumalanga, 5% from Western Cape, 3% from Eastern Cape, 3% from Kwazulu Natal and 0% from Limpopo. This study concluded that SINV infections are sporadic, but continuously occur in the provinces of the Free State, Northern Cape and Gauteng in South Africa. During 2010, there was an increase in the amount of cases. In this period there was an an increase in rainfall that provided favourable breeding grounds for mosquito vectors and samples being submitted for suspected Rift Valley fever (Storm *et al.*, 2014).

During January 2008 till December 2013, van Niekerk *et al.* (2015) investigated specimens from 623 horses that had undiagnosed febrile and neurologic infections. Old world alphaviruses were identified in 52 of the 623 horses in South Africa. Eight of the horses tested positive for SINV, of which three of the horses survived febrile illness, two horses survived neurologic disease, and three horses died from neurologic disease (van Niekerk *et al.*, 2015).

1.6.2 Reservoirs and Vectors

Various species of ornithophilic mosquitoes are the major enzootic vectors for SINV (Olson & Trent, 1985). *Culex univittatus* is the main vector of SINV and maintains the virus in a feral cycle with wild birds. This mosquito can also transfer SINV infection to humans (Jupp *et al.*, 1986). The primary vertebrate hosts are mainly wild passeriform birds (Olson & Trent, 1985). SINV uses several species of wild birds as its primary vertebrate hosts. The primary vector in the central region of South Africa is *Culex univittatus*. Whereas the primary vector in the coastal lowlands of Natal is *Culex neavei*, another bird-biting species. Humans are not able to infect mosquitoes, since they are poorly viremic. Human infection depends on the vectors acquiring infection from birds (McIntosh, 1986).

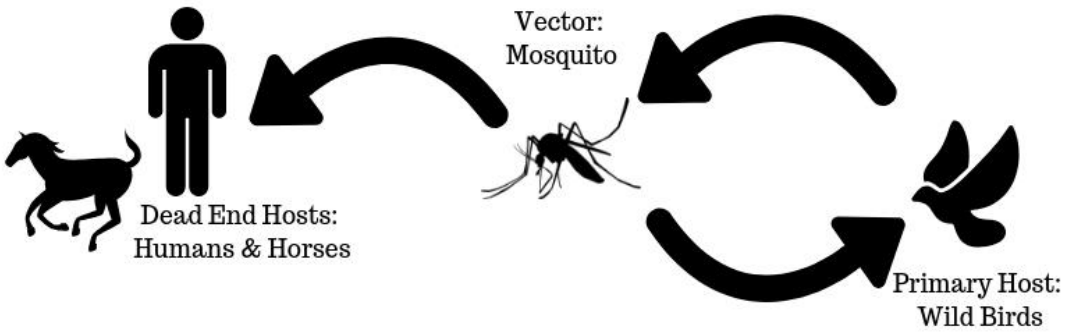


Figure 1.3 Illustration of the maintenance cycle of SINV in nature. The virus is maintained by wild passeriform birds and *Culex univittatus* mosquitoes and accidental spillover occurs to humans and horses via mosquito bite (Kennedy, N).

1.6.3 Transmission

Arboviruses replicate in both vertebrates and vectors. Viremia must be produced by the viruses in vertebrates in order to infect the vectors. The viruses need to infect the vector's salivary gland to secrete virus in the saliva when vertebrates are infected. Humans are considered as incidental hosts for these viruses and not generally involved in maintaining the viruses in nature (McIntosh, 1986). However some viruses cause humans to become highly viremic and easily infect mosquitoes. This also allows human outbreaks to ensue independently of any transmission cycles that involve animals (McIntosh, 1986).

Mosquitoes of the *Aedes* or *Culex* genera are the primary or viral maintenance vectors in South Africa (McIntosh, 1986). These mosquitoes have rather distinctive adaptations in the manner they survive unfavourable weather, such as tremendous drought or low temperatures, in which viral transmission is not achievable. *Aedes* species can pass through these periods in the egg stage, since the egg is drought-resistant and require very little humidity. While *Culex* species survive these periods during the larval or pupal stages requiring constant water collections, or even more commonly as a dormant adult female (McIntosh, 1986).

In *Aedes* species, a virus passes transoviarally and trans-stadially from one generation of females to the next (McIntosh, 1986). Whereas transovarian and trans-stadial transmission may not be required in *Culex* species, given that a female may obtain infection before becoming dormant and transmit the virus when suitable weather occurs. Aedine eggs can be a safe viral shelter during lengthy imerepidemic spells, since the eggs are able to stay viable for long periods (McIntosh, 1986).

These different survival adaptations of the mosquito hosts are revealed by the detectable activity of *Aedes* and *Culex* maintained viruses in South Africa. The activity of viruses, for example Sindbis, West Nile, Banzi and Germiston viruses is maintained by *Culex* vectors annually during the summer in South Africa. While the activity of *Aedes* maintained viruses, such as chikungunya, Rift Valley fever, Wesselsbron and numerous other viruses, are only detected periodically, and sometimes varied by some infection-free years (McIntosh, 1986).

Infections usually occur seasonally, generally from the middle of summer till autumn. Unusually heavy rainfall is required by larger outbreaks so that the vector populations are high enough (McIntosh, 1986).

The following are factros that increase transmission of SINV. Migratory birds are involved in intercontinental transport of the virus and could possibly introduce new virus strains. Vector density and weather and climate, such as high temperatures in spring and summer, concurrent summer precipitation and change in interannual precipitation. Vertical transmission of enzootic vector mosquitoes. Aggressive vector behaviour and human behaviour, such as outdoor activities, exposure to mosquito bites, low socio-economic status and living in a rural area. Human characteristics, such as HLA haplotype (DRB1*01), gender, age and rheumatic conditions (Adouchief *et al*., 2016).

The number of human cases in South Africa increased with high temperatures and precipitation. Human outbreaks usually occur in South Africa after extreme weather conditions, such as droughts and/or floodings. The most crucial risk factor is mosquito bites. Spending time outdoors increases risk of disease. Disease occurs more frequently in patients that live in rural areas and small

communities in the countryside, working on farms, and a below university educational level. Host genetic factors, such as the HLA allele DRB1*01 has been found to occur more frequently in SINV infected patients, especially patients with persistent joint symptoms three years after infection. The risk factors for prolonged symptoms are old age and female gender (Adouchief *et al*., 2016).

1.7 Signs and Symptoms

Sindbis fever has the following symptoms: mild fever, headache, malaise and maculopapular rash with pains in the joints and tendons (Adouchief *et al*., 2016). The acute disease usually lasts from three to 10 days, and the malaise and joint pains lasting for several weeks. The rash mostly affects the limbs, trunk, soles and palms; and is occasionally rather widespread (McIntosh, 1986). First macules appear which soon become papular. The papules of the rash are isolated, with a diameter of 3 mm and usually surrounded by a pale halo (Adouchief *et al*., 2016). These papules have a tendency to form vesicles, mainly on the hands and feet, with friction and can be somewhat haemorrhagic (McIntosh, 1986; McIntosh *et al.*, 1964). The lesions are seldom itchy and can disappear to reappear later to leave brown stains. Often isolating the virus from the blood is unsuccessful and seroconversion is mostly used for diagnosis; which is usually reliable, since there are no closely related viruses in South Africa infecting humans (McIntosh, 1986).

Other clinical features are low fever, not higher than 38 ºC, which only lasts for a few days. Early symptoms are fatigue and malaise on slight exertion and tend to persist until period of recovery. No rigors, however some patients experience hot and cold feelings. Occasionally muscle tenderness and deep

aches in the limbs occur. The hands and feet will be painful, but larger joints are usually affected. The extremities tend to become swollen. Sometimes pain in the extensor tendons on the dorsum of the hand and in the tendo calcaneus (Achillis) is present. Periocular pain can occur, however photophobia and conjunctivitis does not occur. Usually a mild headache occurs, however the central nervous system is not involved. Paraesthesia, for example pricking or tingling sensations, occur in few patients, especially in the hands and shooting pains in the limbs. No lymphadenopathy occurs, however there have been cases of inguinal, occipital and posterior cervical nodes had enlarged. Many patients has experienced right subcostal tenderness, however the liver could not be felt. A common symptom is anorexia, but nausea and vomiting is rare. No disruption of bowel function occurs. The respiratory system is not involved. The symptoms disappears after 10 days, however fatigue and tendon pains occur in some patients for a number of weeks (McIntosh *et al.*, 1964).

The viremia of SINV infection has a low level and is short-lived. The patient will generally only seek medical attention when the maculopapular rash occurs. This rash is a sign of initiation of antibody formation and usually prevents successful isolation of the virus. Thus the only practical method of laboratory diagnosis is an increase in antibody titre seroconversion, which depends on paired sera – acute and recuperating – obtainable from each patient (Jupp *et al*., 1986). One should obtain the first (acute) serum as early in the infection as possible as well as the second (recuperating) serum after two to three weeks or even longer (Jupp *et al.*, 1986). Table 1.4 lists the medically important alphaviruses and the symptoms of their diseases.

Table 1.4 The medically important alphaviruses and the diseases they cause (Wang *et al*, 2006).

Medically Important Alphaviruses	Disease Symptoms
Chikungunya virus	Rash Arthritis
Mayaro virus	
O'nyong-nyong virus	
Ross River virus	
SINV	
Eastern Equine Encephalitis virus	Encephalitis
Venezuelan Equine Encephalitis virus	
Western Equine Encephalitis virus	

1.8 Pathogenesis

SINV is the least pathogenic of the most comprehensively studied Alphaviruses (Gorchakov *et al*., 2005). It is able to infect an extensive range of insect and vertebrate cell lines commonly used in experimental research. SINV replication in vertebrate cell lines results in cytopathic effect and cell death within 24 to 48 hours after infection (Gorchakov *et al.*, 2005).

Joint pain and swelling of extremities is most likely due to necrobiosis and oedema of insubcutaneous, periarticular and tendinous tissues (McIntosh *et al.*, 1964). The cytopathic effect of SINV infection is the production of small nuclear inclusions in tissue culture by known arboviruses. Lesions involving the heart, fat, thymus and striated muscle occur in mice that have been inoculated with SINV. Also there was quite widespread destruction of connective tissue (Malherbe & Strickland-Cholmley, 1963). The pathogenesis of SINV infection may be affected by the low viral load found in serum during the acute infection (Sane *et al.*, 2012). SINV is able to readily infect human macrophages. The

pro-inflammatory response based on macrophages contributes towards the pathogenesis of arthritis (Assunção-Miranda *et al.*, 2010). After inoculation, it is feasible that the virus targets cells flowing through blood, for example monocytes, which promotes dissemination of virus to different tissues, such as the skin, joints and muscles; whereas the virus in serum is only present for a short period (Kurkela *et al.,* 2005).

Alphaviruses are inoculated subcutaneously by a mosquito bite and then spread through the lymph nodes and microvasculature to the liver, spleen, muscles, and connective tissues of the bones and joints (Assunção-Miranda *et al.*, 2013; Adouchief *et al*., 2016). Usually a decrease in white blood cells occurs during the acute phase of alphavirus infection, which implies that the virus replicates primarily on leukocytes. When the virus reaches the bones, muscles and articular tissues, the acute phase strongly associated with a local inflammatory process is produced (Assunção-Miranda *et al.*, 2013). These include lymphocytes, Natural Killer cells, neutrophils and particularly macrophages. Upregulation of proinflammatory cytokines and chemokines occur (Adouchief *et al*., 2016). The pathogenesis of alphavirus infection is mainly determined by the age of the host, the immune system status, virulence of the virus strain and persistence of the virus (Assunção-Miranda *et al.*, 2013).

It was found that arthritogenic alphaviruses, namely chikungunya, Mayaro, Ross River, O'nyong nyong and Barmah Forest, enter the cells by a cell adhesion molecule called Mxra8 (Zhang *et al*., 2018). It is expressed on cells that forms muscle, bone and cartilage cells and found in mammals, birds and amphibians. However SINV and Venezuelan equine encephalitis virus do not require Mxra8 to enter the cells (Zhang *et al*., 2018).

Alphavirus replication and dissemination involves articular and non-articular

cells. Alphavirus-induced arthritis experimental models propose that pathogenesis is caused by a combination of direct cellular and tissue damage from viral replication, as well as an indirect immune response activated in target tissues (Assunção-Miranda *et al.*, 2013).

1.9 Laboratory Diagnosis

Diagnosis depends on detecting SINV antibodies by HAI assay or ELISA. Recent infection is indicated by the detection of IgM antibodies or immunoglobulin G (IgG) seroconversion between paired samples taken two weeks apart (NICD-NHLS, 2014). The usual source of the virus is from blood collected as early as possible during the acute phase; however organs and skin lesions can also be used. Another blood sample is collected two to three weeks later in order to show seroconversion. One should determine whether the patient was exposed to a recent mosquito bite. The incubation period is short-lived, generally three days (McIntosh, 1986). Other tests that may be used are reverse transcription PCR and isolating the virus from a serum sample (NICD-NHLS, 2014). Currently serology methods, namely ELISA and HAI, are used in laboratory diagnosis of SINV (Sane *et al.*, 2012).

1.9.1 Viral Isolation

Samples can be taken during the acute phase and transported to a laboratory while kept at 4 ºC and inoculated into tissue cultures or suckling mice. Blood samples can be used to inoculate tissue culture to observe the cytopathic effects. SINV cannot be isolated from throat and rectal swabs. Vesicle fluid of skin lesions on the hands and feet can be swabbed and inoculated into tissue

culture tubes and observed for cytopathic changes. Some of these changes are focal degeneration and small eosinophilic inclusions in the nuclei. The vesicle fluid can also be inoculated into mice. Most of the time, the virus cannot be isolated, probably due to very little quantity of virus present in the samples (Malherbe & Strickland-Cholmley, 1963).

1.9.2 Immunological Methods

HAI assays are the classical serological asays that are still used in many laboratories. Sera taken from both the acute and convalescent phases should be tested. Neutralization assays can also be performed (Malherbe & Strickland-Cholmley, 1963). ELISA can also be used to detect SINV antibodies. When a patient is infected with SINV, IgM antibodies or IgG seroconversion is detected between paired samples taken two weeks apart. Sometimes IgM testing has to be repeated, since only 60% of patients will have detectable IgM antibodies during the first week of infection. (NICD-NHLS, 2014).

Enzyme-linked immunosorbent assays (ELISA) is a method that uses antibodies or antigens coupled to an enzyme that creates a colourimetric reaction when in contact with its substrate. This technique is performed in plates to detect and quantify peptides, proteins, antibodies and hormones. In an ELISA, an antigen is immobilized to a solid surface and then bound to an antibody that is linked to an enzyme. The conjugated enzyme is incubated with a substrate and detected by measuring the product produced. The interaction between the antibody and antigen must be highly specific. It is frequently used to test for specific IgG or IgM antibody. There are four different types of ELISA; namely direct, indirect, sandwich and competitive. Direct ELISA involves an antigen which is coated to a multi-well plate and detected by an antibody directly conjugated to an enzyme. Indirect ELISA consists of an antigen coated

a multi-well plate which is detected in two steps or layers. In the first step, an unlabelled primary antibody specific for the antigen is added and binds to the antigen. In the second step, an enzyme-labeled secondary antibody is added and binds to the first antibody. Sandwich ELISA uses matched antibody pairs. Each antibody is specific for a distinct epitope of the antigen. The capture antibody is the first antibody and is coated to the wells. Then the sample solution containing the target protein is added to the well. Thereafter the detection antibody is added. Competitive ELISA involves a competitive reaction between the sample antigen and antigen bound to the microtiter plate's wells with the primary antibody. It can also be known as inhibition ELISA. Firstly the primary antibody is incubated with the sample antigen. These resulting antibody-antigen complexes are added to wells coated with the same antigen and incubated. Thereafter any unbound antibodies are washed off. The more antigens are present in the sample, the more primary antibodies will be bound to the sample antigens. Therefore a signal reduction will occur, i.e. The colour is lighter, due to the smaller amount of primary antibodies available to bind to the antigen coated to the well (Bosterbio.com, n.d.**)**.

1.9.3 Molecular Techniques

Molecular assays, such as conventional PCR and real-time RT-PCR, can be used during the acute phase of illness. However its application is limited due to the low level of viremia and the short duration of viremia (Sane *et al.,* 2012). Acute SINV infection has a narrow viremic window and a low level of viremia. It is estimated that the level of SINV RNA in serum from patients with acute illness is less than 103 RNA copies/ml (Adouchief *et al*., 2016). Hence most patients present after the window period and hence molecular assays have

limited application in diagnostics compared to detection of specific IgM antibody or increasing titers of IgG.

1.10 Treatment

There is currently no specific antiviral treatment available for Sindbis fever. Treatment is symptomatic, and includes antihistamines for pruritic rashes and non-steroidal anti-inflammatory drugs (NSAIDs) for joint symptoms (NICD-NHLS, 2014).

1.11 Sindbis as an arthritogenic alphavirus in Europe

Fennoscandia, which consists of Finland, Norway, Sweden, Murmansk Oblast, most of Karelia and Northern Leningrag Oblast (northwest area of Russia); has had outbreaks approximately every seven years, namely 1974, 1981, 1988, 1995 and 2002 (Hubálek, 2008). Hundreds of cases have occurred during these outbreaks. North Karelia of Finland had the highest incidence of 25.7 cases per 100 000 population, with a mean seroprevalence of 5.2%. Sweden had the highest incidence of 2.9 per 100 000 population during 1981 till 1988 (Hubálek, 2008). Sweden had 47 cases and Finland had 1352 cases during 1995 (Hubálek, 2008). A study in Finland found that 50% of patients that were infected with SINV during the 2002 outbreak, had joint symptoms one year later. A follow-up study was done three years after infection to investigate the prognosis and joint symptoms of those patients. Serum samples were also analysed for the presence of SINV IgM antibodies. The study found that 4.1% of patients had arthritis with swelling and tenderness in the joint, 14.3% of

patients had tenderness in palpation or in joint movement and 10.2% of patients complained of joint pain. The small and peripheral joints were mainly affected. The study also found that SINV IgM was detected in 6.8% of samples and borderline IgM was detected in an extra 6.8% of samples (Kurkela *et al*., 2008). This study proves that chronic arthritis can be caused by SINV, however only a small amount of patients experience this. During 1985, 24% of patients in Sweden had joint pain three to four years after infection. During 2000, 8% of patients in Finland had arthritis and 42% had osteoarthritis, fibromyalgia or occasional joint pain two and a half years later (Kurkela *et al*., 2008). During 2013 in Sweden, 29% of patients with rash, joint pain and fever tested positive for SINV-specific IgM and IgG. Of those patients, 39% had persistent joint and muscle pain six to eight months after initial infection (Gylfe *et al*., 2018)

1.12 Problem Identification, Aims and Objectives

SINV is a mosquito-borne virus associated with chronic arthritis and is endemic in South Africa. The exact prevalence in South Africa is not currently known and the association of SINV disease and arthritis is not well documented in South Africa. We believe that more cases of arthritis are caused by SINV than currently acknowledged. Currently there is only one commercial assay available for SINV. This assay is expensive and not suitable for surveillance studies since only a small amount of samples can be tested. Therefore there is a need for the development of more suitable and cost-effective assays.

The aim of this study was to investigate SINV prevalence and develop serological assays for detection of SINV infection.

The objectives of this study were as follows:

1. To compare various serological assays for detection of antibody against SINV.

2. To screen stored serum samples and serum samples collected from patients attending arthritis clinics and high risk populations in South Africa using commercial assays and in-house assays for antibody against SINV.

3. To develop in-house assays for detecting antibody against SINV and to use positive samples identified in the serological survey to validate the assays

Chapter 2: SEROLOGICAL ASSAYS FOR DETECTION OF SINV

2.1 Introduction

Numerous emerging or reemerging infectious diseases are caused by medically important arthropod-borne viruses (arboviruses) (Johnson *et al*., 2000; Martin *et al, 2000)*. Detection of the virus, viral antigens or antibodies forms the basis for laboratory diagnosis of arboviral infections. The preferred specimen is serum taken at different points in time after onset of illness (Cleton *et al*., 2012). Incubation period duration, specific pattern of viremia and development of antibody are factors that determine the preferred diagnostic method (Cleton *et al*., 2012). Arboviral diagnostic serology is complex, due to wide variation in viruses (Martin *et al.*, 2000). It is usually difficult to clinically diagnose alphaviral infections due to developing illness' nonspecific characteristics as well as other viruses causing similar diseases that may be present (Wang *et al*., 2006). It is important that laboratories have rapid and adaptable serological methods for diagnosing arboviruses and for performing surveillance studies (Johnson *et al*., 2000). SINV has a wide geographic distribution and causes outbreaks. Therefore appropriate/suitable serodiagnostic methods are required for clinical, epidemiologic and virologic studies (Calisher *et al.*, 1986).

The gold standard to confirm the aetiologic agent for an arboviral infection is virus isolation, however detection of specific immunoglobulin G (IgG) and immunoglobulin M (IgM) antibody responses also play an important role due to the short period of viremia (Cleton *et al*., 2012). Virus isolation by inoculation of cell cultures or mice, serological testing to detect IgM antibodies or IgG seroconversion, and nucleic acid amplification are assays commonly used to

detect alphavirus infections, but have restrictions. Isolation of the virus is time consuming, expensive and succeeding diagnostic steps is needed for identification of the virus (Wang *et al*., 2006). Virus isolation is typically used to diagnose alphaviruses, such as SINV, but requires human sera within three days of onset and incubation of one to two days, which delays diagnosis. Also biosafety level 3 facilities and procedures may be required for some of the alphaviruses and these are not available in public health settings and developing countries. The most rapid and subtype specific IgM antibody response only develops four to five days after infection at earliest. (Wang *et al*., 2006) However as most patients with alphavirus infection only seek medical advice after several days, serology is an important assay for diagnosis.

Classical serological assays, such as haemagglutination inhibition (HAI), complement fixation (CF), and neutralisation tests have been used to measure antibody for alphaviruses and are traditionally used to diagnose patients with illnesses caused by alphaviruses. These tests have some disadvantages, such as time consumption, sensitivity, specificity, rapidity, expense, and unreliable results (Calisher *et al.,* 1986; Frazier & Shope, 1979). Also it is just retrospectively diagnostic as these assays do not differentiate between IgG and IgM and therefore paired acute and convalescent phase sera are needed for determination of seroconversion or an increase in antibody titer (Wang *et al*., 2006; Martin *et al*., 2000). However neutralisation assays, although not suitable for routine use, remain the gold standard for specificity.

Enzyme-linked immunosorbent assay (ELISA) can be widely used for detecting antibody responses and are extremely sensitive, reliable, quick, inexpensive and readily automated. Minimal sophisticated equipment, small amounts of serum and antigen, as well as readily obtained reagents with a

long shelf life are needed for ELISA. A pure coating antigen can be prepared in order for the ELISA to be successfully used. If the antigen should be used in the field, it should be inactivated for safety (Frazier & Shope, 1979). Sensitivity and specificity in detecting virus-specific antibody is increased with ELISA and is simple to interpret (Wang *et al*., 2006; Roehrig, 1982). However false positive results can be caused by cross reaction of IgM and IgG antibodies within serogroups when ELISA or immunofluorescence assay (IFA) are used. If confirmation for a specific viral infection is needed, a neutralisation assay is still the most reliable (Cleton *et al.*, 2012).

ELISA has been shown to have an increased sensitivity over standard assays (Roehrig, 1982). Therefore a more rapid and specific serodiagnosis of alphavirus infections in humans can be achieved with ELISA, instead of the complex methods used in conventional serodiagnosis (Calisher *et al*., 1986; Roehrig, 1982). Large numbers of serum samples can be tested rapidly with significant results, especially during an epidemic (Johnson *et al.*, 2000). Although commercial assays are available, they are expensive and therefore in-house assays are more affordable for prevalence studies.

The aim of the chapter was to develop an in-house ELISA to detect antibodies against SINV in patient sera, to compare its performance against a commercial assay, and to screen select populations for evidence of previous infection.

2.2 Materials and Methods

2.2.1 Human serum samples

Ethics approval was obtained from the University of the Free State Health

Sciences Research Ethics Committee and informed consent was obtained. A total of 165 serum samples submitted to the National Health Laboratory Service (NHLS), Division of Virology from patients that attended the arthritis clinic in Universitas Hospital between 2013 and 2017 were tested retrospectively and anonymously. These samples were numbered ARC 1 to ARC 165. A total of 136 serum samples were collected for an unrelated study from horse and stable workers and residents of Bainsvlei, Bloemfontein during 2016 and 2017. Bainsvlei is considered a high risk area since the primary vector for SINV is found there and cases have been identified previously (unpublished data). These samples were numbered HSW 1 to HSW 138 (total of 136 excluding two samples from the cohort that were not available). A total of 267 stored serum samples submitted between 2008 and 2010 from patients suffering from acute febrile illness with no confirmed diagnosis were tested retrospectively and anonymously. These samples were numbered QWF 2 to QWF 201 and TB 1 to TB 84 (total of 267 excluding 17 samples not available for testing). All of these samples were tested for immunoglobulin G (IgG) antibody against SINV with the in-house ELISA. The panel of negative controls were stored serum samples from an unrelated study with informed consent and confirmed SINV IgG negative using HAI and commercial IFA. A positive control was obtained from a patient with a confirmed infection using HAI and IgM ELISA (NICD) from a previous study and used to determine the optimal concentration of reagents for the in-house ELISA. The negative controls were used to determine the cut off. Each test sample was tested once per assay and not in duplicate.

2.2.2 Enzyme-linked immunoassay

2.2.2.1 Cell lysate antigen preparation

A cell lysate antigen (coating antigen) was prepared in a previous study

(Hanekom, 2013) using SINV infected Vero 76 (Vero) cell cultures American Type Culture Collection (ATCC) number CRL-1587. Confluent Vero cells in ten T150 flasks were infected with SINV SAAR386 strain at a multiplicity of infection (MOI) of 0.1 and adsorbed for 60 minutes. Cells were maintained in minimum essential media (MEM) (Invitrogen, USA) containing 2 % fetal bovine serum (FBS) (BioWhittaker™, Switzerland), 100 u/ml penicillin (BioWhittaker™, Switzerland), 0.1 mg/ml streptomycin (BioWhittaker, Switzerland) and 2 mM L-glutamine (BioWhittaker, Switzerland), incubated at 37 ℃ and monitored daily for cytopathic effects (CPE).

When the initial signs of CPE occurred, the cells were harvested and clarified for 20 minutes at 12 500 x g. The cells were lysed with 1% Triton X100 in borate buffered saline (BBS) and sonicated on ice at five minute intervals. In order to remove the cell debris, the cells were centrifuged at 10000 x g for 20 minutes at 4 ℃ and the supernatant fluid was stored for use as antigen at -80 ℃. Mock antigens were prepared similarly, but using uninfected cells. Briefly, uninfected Vero cells were grown to confluency, lysed and processed used the same method as described for the cell lysate antigen.

2.2.2.2 Indirect ELISA for detection of IgG antibodies

The ELISA was performed in 96 well Polysorb plates (Nunc-Immuno Plate, Denmark). The optimal concentration of reagents, namely coating antigen and conjugate antibody were determined by checkerboard titrations (Figure 2.1). Throughout the assay, reagent volumes of 100 µl were used unless specified otherwise, the diluent for reagents was phosphate buffered saline (PBS) pH 7.4 (Sigma, USA) containing 2% skimmed milk powder, incubations were performed for 1 hour at 37 ℃, 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.

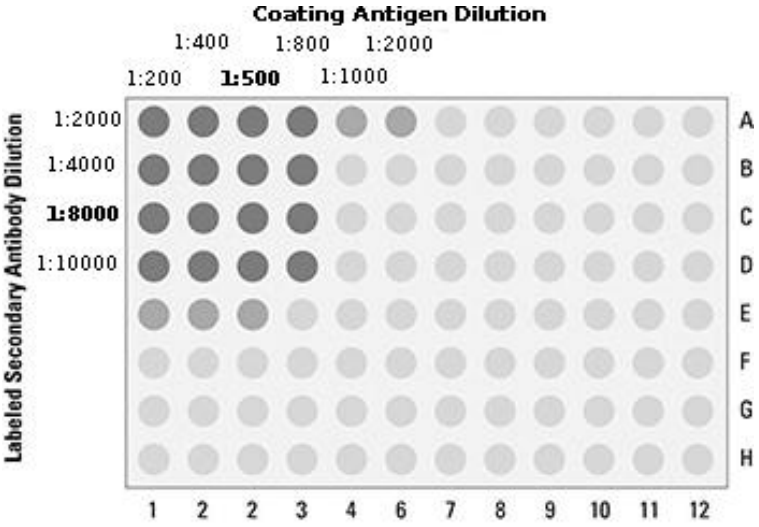


Figure 2.1 Plate layout for checkerboard titrations, with the cell lysate antigen as the coating antigen and the anti-human IgG horse radish peroxidase as the labelled secondary antibody.

Antigen dilutions ranging from 1:200 to 1:1000 were tested and an optimal dilution of 1:500 was selected. The optimal dilution was based on the optical density values obtained using a positive and negative control. For optimal reactivity an optical density value of less than 0.2 is required and the positive control at least greater than the cut off value but preferably .0.5. Briefly, a 96 well microtiter Polysorb plate was coated overnight at 4 ℃ with SINV cell lysate antigen diluted 1:500. Plates were coated similarly with mock antigen diluted 1:500. 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. Figure 2.2 indicates negative controls one to six and figure 2.3 indicates negative controls seven to 11.

The plates were incubated, washed and anti-human IgG horse radish peroxidise (HRPO) (SeraCare Life Sciences Inc., USA) diluted 1:8000 was added to each well. After further incubation and washing, positive reactors

were visualized using the substrate 2,2'-azino-di-3-ethylbenzthiazoline-6-sulfonate (ABTS) (SeraCare Life Sciences Inc., USA). The plates were incubated at room temperature 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 was determined by subtracting the OD for each sample with mock antigen from the OD with SINV cell lysate antigen.

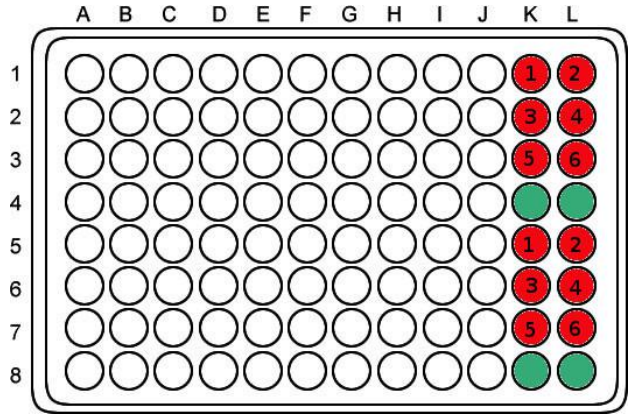


Figure 2.2 Layout of the plates where wells were coated with SINV cell lysate antigen in rows 1 to 4 and the mock antigen in rows 5 to 8. The positive control is indicated in green and the negative controls, which are numbered 1 to 6, in red.

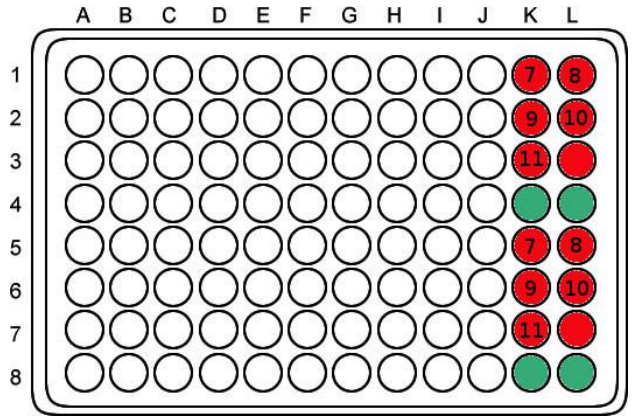


Figure 2.3 The layout of the plates where wells were coated with SINV in rows 1 to 4 and the mock antigen in rows 5 to 8. The positive control is indicated in green and the negative controls, which are numbered 7 to 11, in red.

2.2.2.3 Selection of cut-off values

A panel of eleven negative control sera was used to determine the cut off value to separate the positive results from the negative results. For each control the net OD was determined by subtraction of OD recorded on SINV antigen from OD recorded on mock antigen. The cut off value was then determined by the mean net OD of the eleven sera from the negative control panel plus 2 standard deviations (SD).

2.2.2.4 Statistical analysis of data (Normalisation 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 net OD was calculated by subtracting the OD of the wells with the mock antigen from the OD of the wells with the SINV antigen. The percent positivity (PP) of each sample was calculated by the following formula:

Percent Positivity = mean net OD of sample/mean net OD of positive control x 100

The Coefficient of Variance (CV) was calculated to establish the coating efficacy with the following formula:

Coefficient of Variance = standard deviation/mean net OD of positive control repeated on 15 plates x 100

Hypothesis testing to compare two population proportions (Z test) was used to determine if there was any significant difference between the different groups of samples.

2.2.3 Commercial Assay

A commercial immunofluorescent assay (IFA) (Euroimmun, Germany) was used to screen the samples found to be positive with the in-house ELISA for anti-SINV IgG antibodies according to manufacturer's instructions, and using antihuman IgG fluorescein isothiocyanate (FITC) (Zymed Laboratories, UK). This assay makes use of the TITERPLANE™ technique, in which samples or reagents are placed onto reaction fields of a reagent tray. Then the BIOCHIP slides are applied onto the reagent tray, in order for the BIOCHIPs to reach the sample or reagent fluids. A BIOCHIP consists of a coating of a biological substrate, cut into millimetre-sized fragments on a cover plate. An example of a BIOCHIP is illustrated in Figure 2.4. The substrates for this particular assay are cells infected with SINV and uninfected cells, and therefore there are two BIOCHIPs per field. In each field, one BIOCHIP contains SINV infected cells and the other BIOCHIP contains cells that have not been infected. However the strain of the virus is not known and provided by the supplier. With this assay, a moist chamber is not needed, since the fluids are contained in an enclosed area.

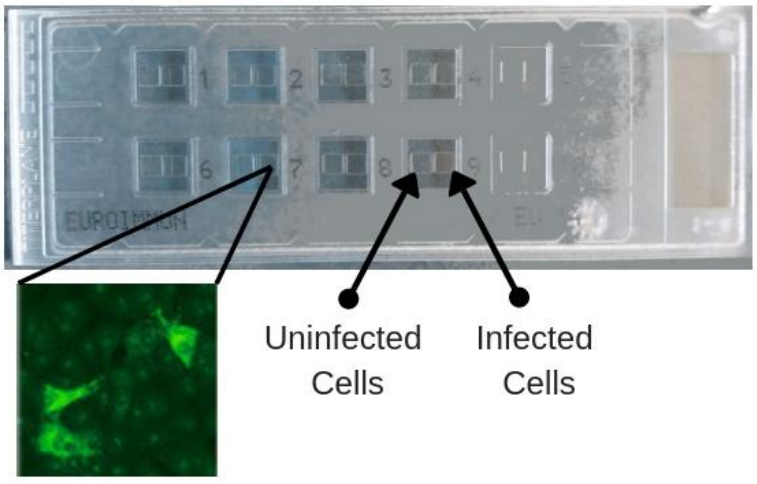


Figure 2.4 Anti-SINV IgG EuroImmun slide with uninfected cells in the left hand BIOCHIP and SINV-infected cells in the right hand BIOCHIP of a field.

Briefly, serum samples, diluted 1:10 in PBS, were loaded onto reaction fields of the reaction trays without air bubbles, BIOCHIP slides were placed onto corresponding recesses of the reaction trays and incubated at room temperature for 30 minutes. Thereafter the slides were washed in PBS-Tween20 for 5 minutes on a rotary shaker, blotted dry to remove excess wash buffer and incubated with 1:20 dilution of antihuman IgG FITC in filtered Evan's Blue for 30 minutes in the dark at room temperature. Slides were washed again in PBS-Tween for 5 minutes on a rotary shaker, blotted dry to remove excess wash buffer and cover glasses were mounted onto the slides. The slides were viewed under a fluorescence microscope (Nikon, Japan).

2.2.4 Neutralisation Assay

Neutralisation assays remain the gold standard for serological assays, hence a neutralisation assay was performed on all positive serum samples for confirmation of an antibody response to SINV infection and to validate the ELISA.

2.2.4.1 End-point *in-vitro* neutralization assay

The endpoint is the dilution at which a certain proportion of infected cells show CPE. It is generally accepted that 50% endpoints are computed as there are interfering factors that interfere with use of 100% endpoint. Hence estimates are calculated using either the Reed-Muench or Kärber methods (Lennette, 1995). The tissue culture infectious dose $_{50}$ ($TCID_{50}$) represents the dose that will cause cytopathic changes in ≥50% of the infected cells.

2.2.4.2 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}. The assay was performed using a 96 well tissue culture plate. Briefly, each well was seeded with Vero cells at 1 x 10^4 cells/50 µl per well using growth media (DMEM, 5% FBS, 1% L-glutamine, 1% Pen/Strep). 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 was added to each well to a final volume of 150 µl. The plate was then incubated at 37^0C in a carbon dioxide (CO_2) incubator and CPE read daily for 5 days. The $TCID_{50}$ was calculated according to the Reed and Muench method (Lennette, 1995). The formula is:

$\log_{10}$ 50% end point dilution = $\log_{10}$ of dilution showing CPE next above 50% - (difference of logarithms × logarithm of dilution factor).
Difference of logarithms = [(CPE at dilution next above 50%)-50%]/[(CPE next above 50%)-(CPE next below 50%)]

2.2.4.3 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 diluted two-fold in serum free growth media from 1:10 - 1:640.

Briefly, a 50µl aliquot of each dilution of sera was added to each well in rows A-G, and two fold dilutions were performed from row B to G. A 50µl aliquot of virus diluted to contain 100$TCID_{50}$ per 50 µl was added per well. Row H was a control row with 100 µl growth media and no sera or virus. The plate was incubated at 37^{o}C 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 (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 5 days. The neutralization titer was determined on the last day as the reciprocal of the highest dilution of test sera at which CPE was inhibited.

2.3 Results

2.3.1 ELISA

An in house ELISA was developed and used to screen a total of 568 serum samples. A panel of eleven negative serum samples from volunteers was used to determine the cut off value. The mean net OD plus two SD was used to determine the cut off value as 0.253 (Table 2.1). The mean net OD of sample divided by the mean net OD of positive control times a 100 was used to determine the PP value as 21.88%. The CV was determined to be 0.21%. Since this is less than 10%, it confirms the acceptability of the coating efficacy. The in house ELISA used stored cell lysate reagents and newly purchased commercial reagents for detection. Hence it was optimized using checkerboard titrations for all reagents as described in the methodology including the antigen.

Table 2.1 Cut off value calculated from negative control panel.

Serum	Number of replicates	Mean	SD	Cut Off Value	
				OD Value	PP
Negative Panel	83*	0.057	0.098	0.253	21.88%

*6 negative samples tested in 8 replicates and 5 negative samples tested in 7 replicates

It was found that 31/165 samples from patients attending arthritis clinic, 13/136 samples from high risk populations, and 25/267 samples from acute febrile illness patients with no diagnosis tested positive for SINV IgG antibody using the in house ELISA. Approximately 45% of the samples that tested positive were from the patients attending the arthritis clinic. Using Z test, it was determined that there was no statistical difference between the samples from the arthritis clinic and the high risk populations, as well as between the samples from the arthritis clinic and the febrile illness patients. However there is a statistical difference between the samples from the high risk populations and the febrile illness patients at 0.05 level. The results are summarised in table 2.2 and illustrated in figures 2.5 to 2.11. Graphs illustrating the OD values of the positive results are in Appendix D.

Table 2.2 Summary of samples that tested positive for SINV antibodies using ELISA.

Serum Samples	Positive Samples	Percentage Positive Samples	Year Received Samples
Patients attending arthritis clinic	31/165	19%	2013 - 2017
High risk populations	13/136	10%	2016 - 2017
Febrile illness patients without diagnosis	25/267	9%	2007 - 2010
Total	69/568	12%	

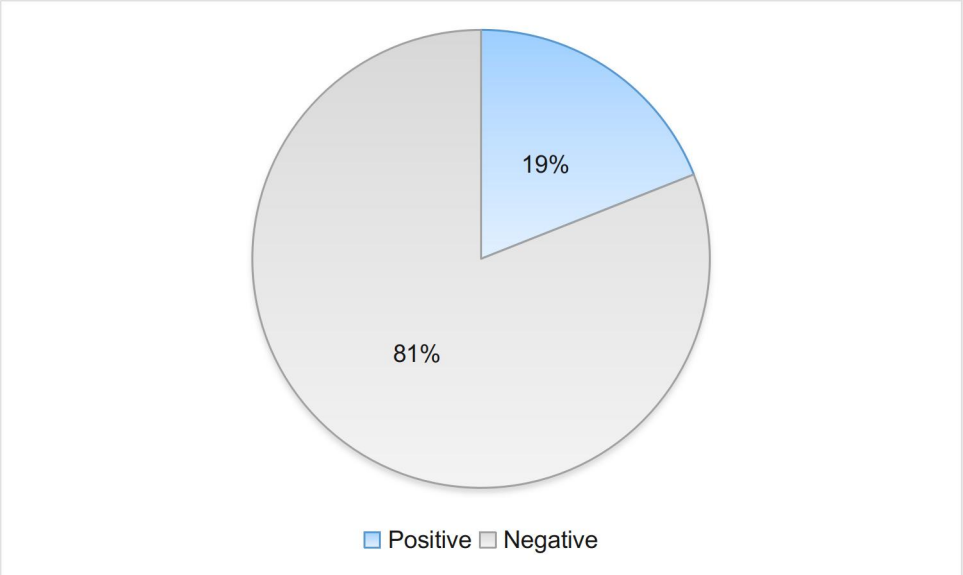


Figure 2.5 In-house ELISA results from samples obtained from arthritis clinic patients tested for IgG antibody to SINV.

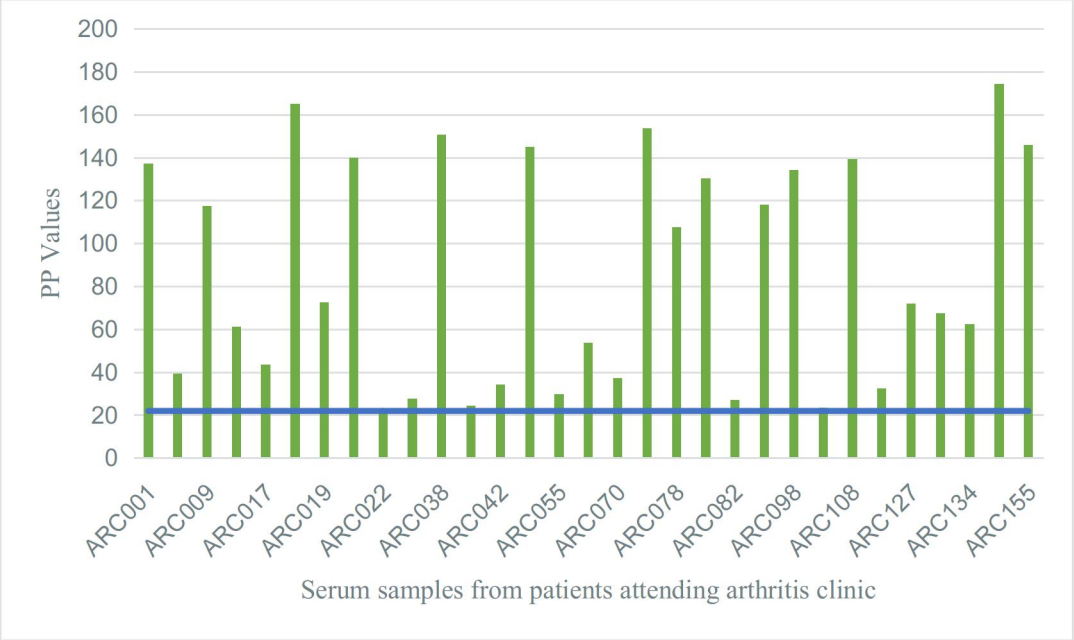


Figure 2.6 PP values calculated for IgG positive serum samples from patients attending arthritis clinic. A cut off of 21.88% was used to differentiate positive from negative samples.

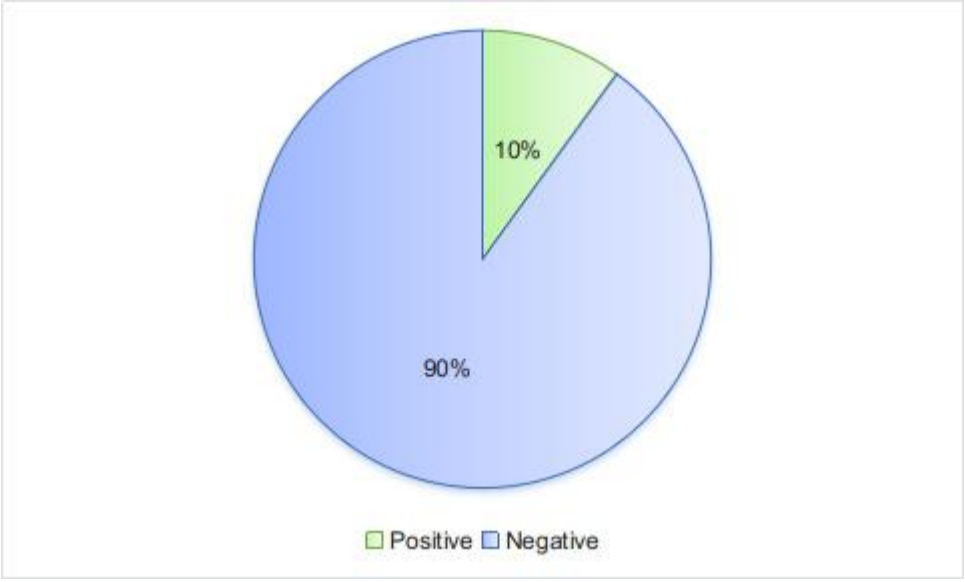


Figure 2.7 Number of IgG positive and negative samples from high risk populations.

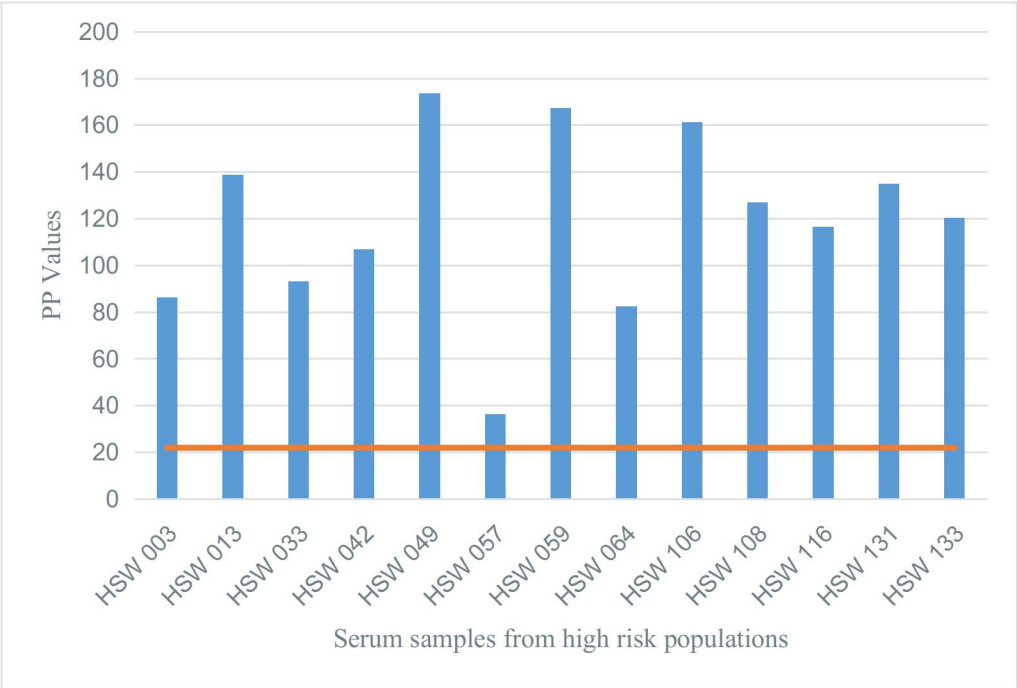


Figure 2.8 PP values calculated for IgG positive serum samples from high risk populations. A cut off of 21.88% was used to differentiate positive from negative samples.

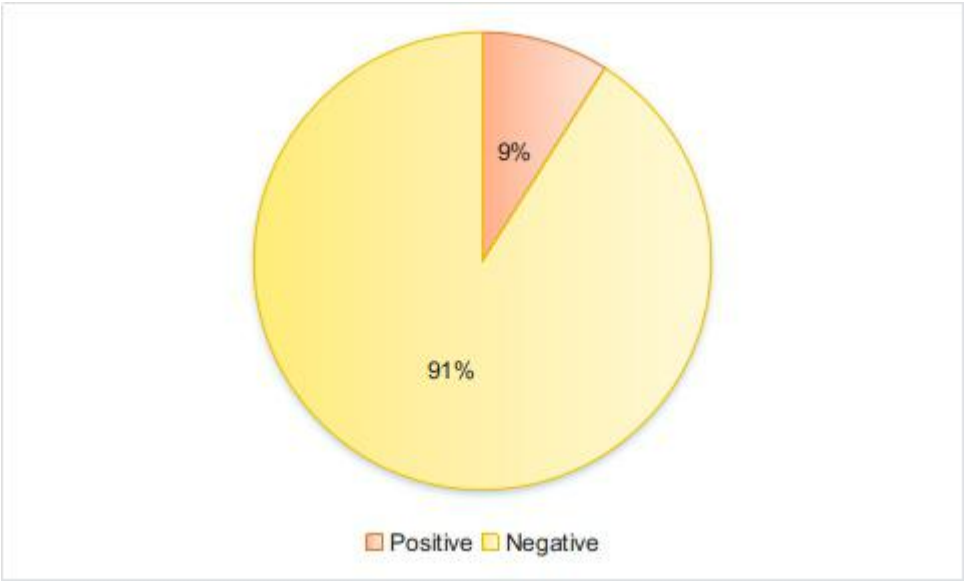


Figure 2.9 Number of IgG positive and negative samples from febrile illness patients with no diagnosis.

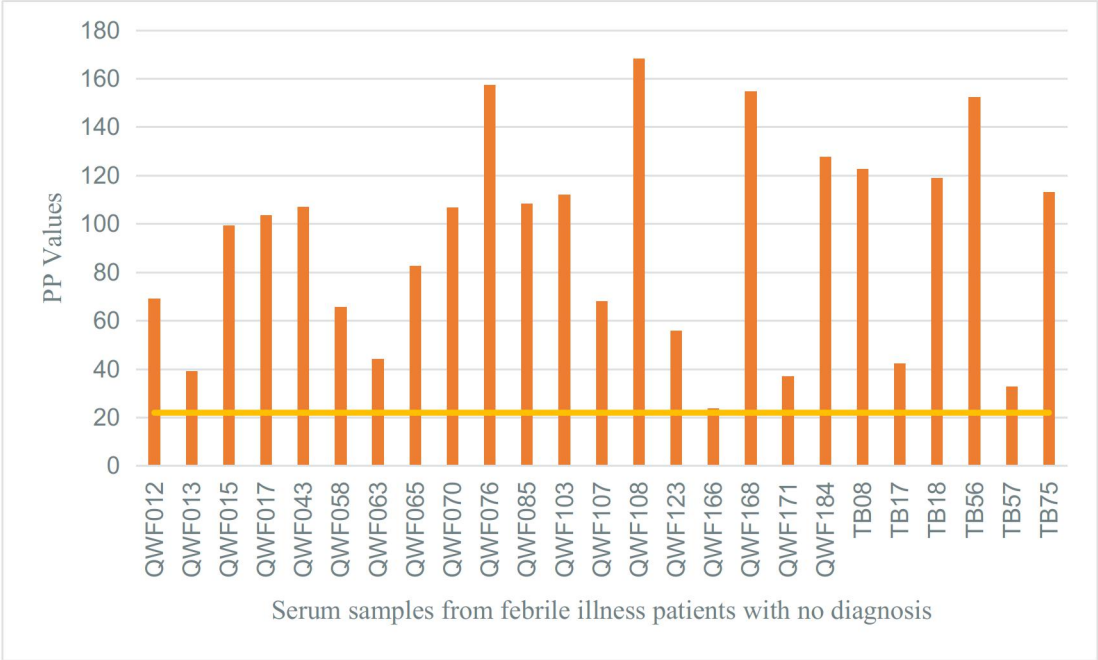


Figure 2.10 PP values calculated for IgG positive serum samples from patients with febrile illness. A cut off of 21.88% was used to differentiate positive from negative samples.

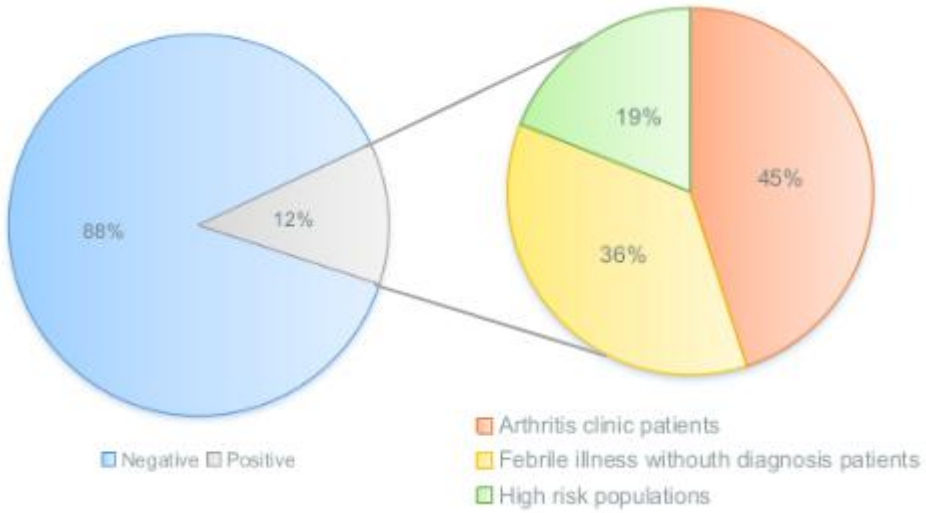


Figure 2.11 Total number of positive and negative samples.

2.3.2 Commercial Assay

Samples that were found to be positive with the in-house ELISA were screened using a commercial immunofluorescent assay. The results from the commercial assay were as follows: 46/69 samples tested positive, 15/69 samples tested negative and 8/69 samples were indeterminate. Due to non specific reactivity, some samples were difficult to read and therefore described as indeterminate. The results are illustrated in figure 2.12.

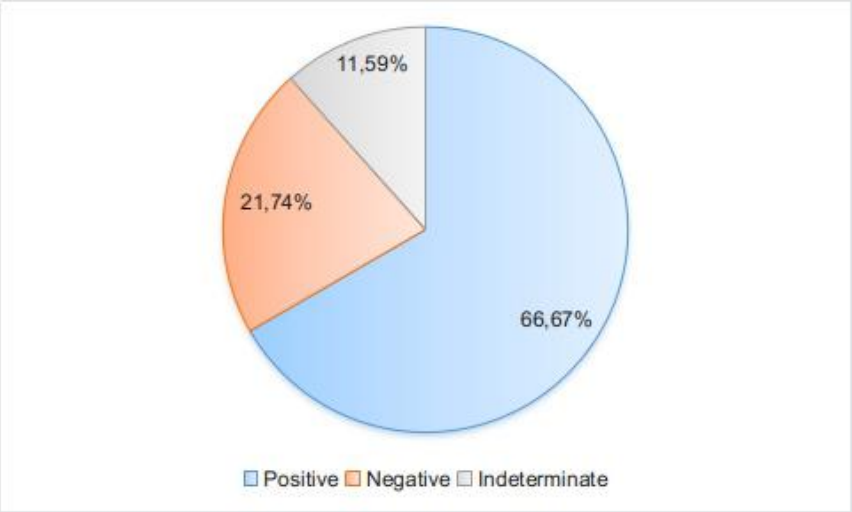


Figure 2.12 Number of positive, negative and indeterminate results from commercial assay of the samples determined to be positive from the in-house ELISA.

2.3.3 Neutralisation Assay

The true positives, i.e. the samples found to be positive from the in-house ELISA, were confirmed using a neutralisation assay. Antibody titers of 20 or higher were considered as positive, and an antibody titer of 10 were

considered as indeterminate. No CPE was considered as negative. The results from the neutralisation assay were as follows: 65/69 tested as positive, one sample was negative and three samples were indeterminate. No CPE was observed in all the negative controls.The results are illustrated in figures 2.13 and 2.14.

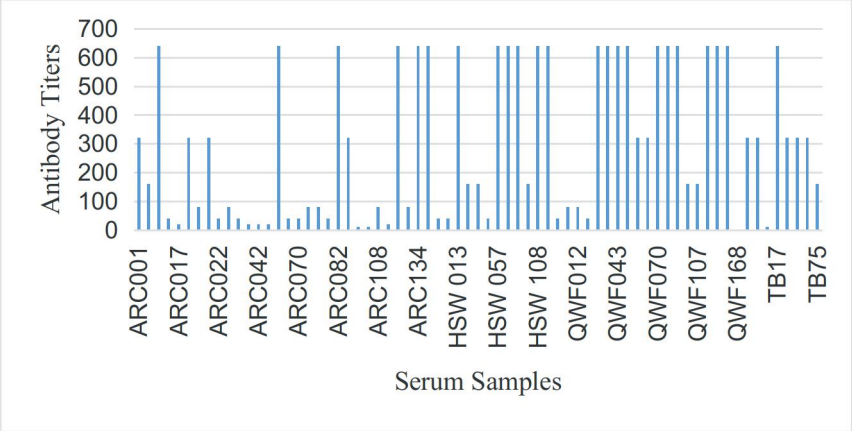


Figure 2.13 Antibody titers for 69 samples that were tested with the neutralisation assay.

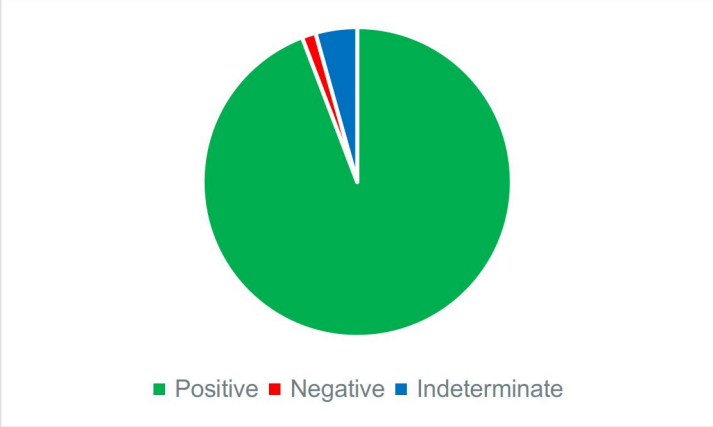


Figure 2.14 Number of positive, negative and indeterminate results from neutralisation assay.

2.3.4 Comparison of Assays

Three assays, namely the in house ELISA, the commercial IFA, and the neutralisation assay were compared.

In summary there were 69 samples that were considered positive using ELISA, and 46/69 were found to be positive with the commercial IFA, as shown in table 2.3. In table 2.4 the commercial IFA was compared with the neutralisation assay, and one of the samples, found to be positive with the commercial IFA, was determined to be negative with the neutralisation assay. Three of the samples, with high net OD values for the ELISA, was found to be positive with the commercial IFA, and were determined to be indeterminate with the neutralisation assay. One of the samples, had a net OD close to the cut off value of the ELISA, was found to be negative with the commercial IFA, and was determined to be indeterminate with the neutralisation assay. The other samples that was close to the cut off value for the ELISA, was found to be negative or indeterminate with the commercial assay, but positive with the neutralisation assay. Table 2.5 compares the population groups' positive results using each of the assays, ELISA, neutralisation assay and commercial IFA. Overall the commercial IFA had less positive results than the other two assays. For both the arthritis clinic patients and the febrile illness patients without diagnosis, only two less positive results with the neutralisation assay than the ELISA for each population group detected. For the high risk populations, there was no difference in positive results between the ELISA and neutralisation assay.

Table 2.3 Comparison of positive samples from ELISA with the results of the commercial IFA.

		In House ELISA
		Positive
Commercial IFA	Positive	46
	Negative	15
	Total	69

Table 2.4 Comparison of commercial assay and neutralisation assay.

		Neutralisation Assay		
		Positive	**Negative**	**Indeterminate**
Commercial IFA	**Positive**	43	1	2
	Negative	14	0	1
	Indeterminate	8	0	0
Total		65	1	3

Table 2.5 Comparison of samples found positive by ELISA, neutralisation assay and the commercial assay.

	ELISA	**Neutralisation Assay**	**Commercial IFA**
Arthritis Clinic Patients	31	29	21
Febrile illness patients without diagnosis	25	23	14
High Risk Populations	13	13	11
Total	69	65	46

2.3.5 Sensitivity of in house ELISA and commercial IFA

The sensitivity of the in house ELISA and commercial IFA were compared with that of the gold standard neutralisation assay for detection of IgG antibodies against SINV. A 2 × 2 contingency table was used to illustrate the sensitivity of the ELISA. Samples from the neutralisation assay are considered as true positives (TP). Data from the validation study that agree with the known positive and negative status of the sera in question are classified as true positive (TP) and true negative (TN), respectively. Data from the validation study that do not agree with the known positive and known negative status of the sera in question are classified as false negative (FN) and false positive (FP),respectively (Simmons, 2008). The sensitivity (Sn) of the assays was determined using the formula:

$$Sn = \frac{TP}{TP + FN}$$

	Neutralisation antibody positive	**Neutralisation antibody negative**
IFA IgG positive	65 TP	0 FP
IFA IgG negative	46 FN	0 TN

Figure 2.15 A 2 x 2 contingency table illustrating assessment of the IFA.

	Neutralisation antibody positive	**Neutralisation antibody negative**
ELISA IgG positive	65 TP	4 FP
ELISA IgG negative	0 FN	0 TN

Figure 2.16 A 2 x 2 contingency table illustrating assessment of the ELISA.

Sensitivity =true positive/true positives+false negativesx100

IFA:

Sensitivity =46/65 x100 = 70.7%

ELISA:

Sensitivity =65/65 x 100=100%

In the absence of testing for true negatives the specificity of the assay could not be determined. The detection of four false positives using the ELISA just suggest that further validation of the specificity of the assay would be required using samples that test negative using the neutralization assay.

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 568 patient samples for antibodies against SINV. The samples were submitted from 165 patients that attended an arthritis clinic, 267 patients with acute febrile disease and no confirmed diagnosis and 136 horse and stable workers in Bainsvlei. A total of 69 out of 568 samples tested positive for anti-SINV antibodies. Majority of the samples that tested positive were from the patients that attended the arthritis clinic.

Since the amount of total samples was so large, and due to the cost of the commercial IFA, only samples that were positive using the ELISA were tested for IgG using the commercial IFA. Therefore only the positive samples were tested with the commercial IFA and gold standard for the comparative analysis. Possibly a limitation of the study is that the samples that were negative using ELISA could have been confirmed using the neutralization assay. In total 69 samples were tested in the ELISA, commercial assay and the gold standard *in-vitro* neutralization assay for comparison. A total of 46 out of 69 samples tested positive for anti-SINV IgG antibodies with the commercial IFA. Compared with the commercial assay the ELISA was shown to be more sensitive. This in-house ELISA is also more cost effective, easier to automate for large amount of samples, easier to read and able to test 5 times more samples on one plate than the commercial IFA, which can only test 8 samples on one slide. The IFA slides were difficult to read and a portion of the samples were recorded as indiscriminate. In comparison, using the ELISA, positive and negative samples were readily differentiated using the cut off value. A total of 65 out of 69 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 ELISA would be suitable as a tool for diagnosis and surveillance.

Chapter 3: EXPRESSION OF RECOMBINANT ANTIGEN AND DEVELOPMENT OF IMMUNOFLUORESCENCE ASSAYS

3.1 Introduction

There is an urgent demand for development of sensitive diagnostic immunoassays that can be used for endemic and emerging infectious diseases, which can be useful during outbreaks, especially in developing countries (Ricks *et al*., 2018; Voorhees *et al*., 2019). Viral isolation is time consuming and RT-PCR is expensive and have a potential risk for DNA/RNA contamination (Gao *et al*., 2018).

Immunoassays are preferably used to confirm diagnosis of diseases, due to their reliability, robustness and accessibility to diagnostic laboratories. These assays detect protein-based antigens and antibodies. Monoclonal antibodies in antigen detection must be highly specific whereas the antigens' structure in antibody detection must be precise. Surface glycoproteins are usually used as the viral antigen target of interest, since it generates strong antibody responses (Ricks *et al*., 2018). Direct immunoassays, which uses inactivated whole virus or lysates from infected cells, are generally used to detect antiviral glycoprotein antibody responses during serosurveillance and clinical diagnosis. The disadvantages of using inactivated whole viruses are that these assays must be performed in high biosafety containment levels and inactivation can destroy viral epitopes (Ricks *et al*., 2018; Saasa *et al*., 2018).

For the last 30 years, recombinant technology has developed as an important technique (Baldi *et al*., 2007). Recombinant proteins can be expressed using a bacterial system and bacterial cells such as *Escherichia coli*, yeasts such as

Saccharomyces cervisiae and *Pichia pastoris*, mammalian cells, baculovirus, genetically modified animals and transgenic plants. Mammalian cells are frequently used for expression of recombinant proteins, because they express large proteins, proteins requiring glycosylation, S-S rich proteins, and proteins requiring post-translational modifications (Demain & Vaishnav, 2009). Transient gene expression of recombinant proteins is suitable for short term production of recombinant proteins (Baldi *et al*., 2007). Firstly the gene of interest is cloned and then the protein is amplified within an expression system. In choosing the correct expression system to produce recombinant proteins, the following factors must be taken into consideration: quality of the protein, protein function, as well as speed and yield of production. Fatty acid chains can be added and tyrosine, threonine and serine hydroxyl groups can be phosphorylated in mammalian cells. There is a high productivity when using mammalian cells, namely 20-60 pg/cell/day. The disadvantages of mammalian cells are poor secretion, and costly to produce recombinant proteins (Demain & Vaishnav, 2009). However transfected cells can be a simple method to prepare antigen slides for IFA without the use of infectious virus. ELISA have advantages that they can be readily automated however IFA can be performed very rapidly with a result available within 2-3 hours. Hence both ELISA and IFA are frequently used for detection of IgG and/or IgN antibody and are important diagnostic or detection tools.

Recombinant proteins are an alternative to using infectious virus which may require high biosafety level containment facilities for handling (Ricks *et al*., 2018; Saasa *et al*., 2018).. Recombinant antigens have the advantage of being safe to use and can be prepared without the need to culture virus. Genes can be synthesized using sequence data from databases and hence this is not even the requirement for a viral isolate.

A recombinant nucleocapsid protein-based indirect immunofluorescent antibody assay (IFA) was successfully developed to diagnose Crimean-Congo haemorrhagic fever virus infection and deemed as safe, specific and sensitive (Saasa *et al*., 2018). Immunfluorescence assays have the following advantages over viral isolation, ELISA and RT-PCR: simple operation, low cost and less time consuming (Gao *et al*., 2018). However, cell culture facilities are needed for IFA slide preparation. When screening a small amount of sera, IFA slides are quite useful (Saasa *et al*., 2018).

The E2 protein is conserved in alphaviruses, and has been used as an antigen to develop diagnostic or subunit vaccines against Chikungunya virus infections. It can also be used to detect antibodies against Chikungunya virus in serum samples. A recombinant E2 protein of Chikungunya virus together with a compatible adjuvant was found to be highly immunogenic and high titers of antibodies was produced by the humoral response (Tripathi *et al*., 2014). Recombinant E2 proteins of Chikungunya virus have been expressed in *Escherichia coli* cells, *Drosophila* cells, and insect cells (Bréhin *et al*., 2008; Weber *et al*., 2015; Kumar *et al*., 2012; Cho *et al*., 2008). Expression of E2 alphavirus protein in mammalian cells has not been reported.

Hence in this study attempts were made to use recombinant antigens, namely the E2 protein due to its immunogenicity, to prepare IFA slides. Briefly, it was anticipated that transiently transfected mammalian cells could be used to prepare antigen slides for IFA assays. Although SINV can be readily cultured within biosafety level 2 facilities, it was deemed a suitable virus to show a proof of concept that a recombinant antigen could have application in preparation of antigen slides. In comparison antigen slides were also prepared using SINV infected cells.

3.2 Materials and Methods

3.2.1 Construction of plasmid containing SINV E2 gene

A plasmid for the expression of the E2 protein of SINV in mammalian cells, designated pcDNA3.1 SinE2, was prepared. The complete sequence for the gene coding for E2 protein was retrieved from the Sindbis-like virus isolate Girdwood S.A. (Genbank Accession number U38304.1) from the online database NCBI (https://www.ncbi.nlm.nih.gov/). The gene was synthesised by GenScript®. The 5' and 3' ends of the gene were modified for cloning and expression purposes as follows. A kozak translation initiation sequence (GCCACCATG) was added to the 5' end of the gene upstream of the start codon. The G after the ATG in the consensus sequence of gccRccATGG, would have caused a downstream sequence frame shift and therefore was not added. A His tag was added at the 3' end of the sequence before the stop codon to facilitate detection of expressed protein. The sequence was codon optimized with OptimumGene™ for expression in baby hamster kidney cells. The modified gene was supplied by GenScript cloned into a pcDNA™ 3.1 plasmid between the *Hind*III and *BamH*I restriction sites. A map of the gene is attached in Appendix G.

3.2.2 Transformation of plasmid into *Escherichia coli* cells

The plasmid was received, freeze dried and reconstituted in nuclease free water as per the manufacturers' instructions. The recombinant vector was transformed into TOP10 *Escherichia coli* cells (Invitrogen, USA) (transformation efficiency: $1x10^9$ cfu/µg plasmid DNA) using a heat shock method according to the manufacturer's instructions. The heat shock method

relies on the principle that when cells are made competent, their membranes are modified to facilitate the uptake of the DNA plasmid during heat shock. Briefly, an aliquot of the reconstituted plasmid was added to a 50µl aliquot of chemically competent cells, gently mixed and kept on ice for 20 min. The cells were heat shocked for 50 seconds at 42 ºC in an incubator, and returned to ice for an additional two min incubation. SOC medium was added to a final volume of 1ml and the reaction mix incubated for 1.5 hours at 37 ºC with shaking at approximately 150rpm. SOC medium was prepared as follows: 2g Bacto® tryptone, 0.5g Bacto®- yeast extract, 1ml 1M NaCl and 0.25ml 1M KCl in a final volume of 98ml distilled water. The mixture was autoclaved at 121 ºC. After cooling, a 1ml aliquot of each of 2M stock solution of Mg^{2+} and 2M stock solution of glucose were added. The final solution was filter sterilized with a pH of 7.0.

The cells were grown overnight on Luria-Bertani (LB) agar plates containing 0.1 mg/ml ampicillin (LB/amp). 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 l liter, autoclaved at 121 ºC for 30 minutes, cooled to approximately 50 ºC and 100µg/ml ampicillin added. The following day colonies were selected for identification of positively transformed cells. A colony was selected and inoculated into 5 ml LB/amp broth containing 0.1 mg/ml of ampicillin and incubated overnight at 37 °C on a shaker at 200 rpm. To prepare sufficient plasmid suitable for transfections a larger volume was prepared using an aliquot and subcultured for 10 hours.

3.2.3 Purification of plasmid

The plasmid was purified from the culture using the QIAGEN® Plasmid Plus

Midi Kit according to manufacturer's instructions. The overnight bacterial culture was harvested by centrifugation for 15 minutes at 6000 x g. The pelleted bacteria were re-suspended in Buffer P1 and incubated with Buffer P2 for 3 minutes at room temperature. Buffer S3 was added to the lysate, mixed, transferrred to a QIAfilter Cartridge and incubated for 10 minutes at room temperature. The cell lysate was collected. Buffer BB was added to the cleared lysate, mixed and transferred to a QIAGEN Plasmid *Plus* spin column under vacuum. Buffer ETR was applied to the column to wash the DNA. The DNA was further washed by addition of Buffer PE. The residual wash buffer was removed by centrifugation of the column at 10000 x g for 1 minute. The DNA was eluted by incubating with nuclease free water for 1 minute and centrifugation for 1 minute at 10000 x g. The NanoDrop2000 spectrophotometer was used to determine the concentration and purity of the DNA. The purified plasmid DNA was stored at -20 °C. An aliquot of the overnight culture was stored at -80 °C as a 15% glycerol stock. Confirmation of positive transformants was done using restriction digest with restriction enzymes that cut *Hind*III and *BamH*I that flank the insert.

3.2.4 Restriction Digestion

A restriction digestion was performed on the purified plasmid to confirm the presence of gene of interest. *Hind*III, *BamH*I, Buffer E and nuclease free water were added to plasmid DNA and incubated at 37 °C in a waterbath for two hours. The plasmid was also incubated with Buffer E, nuclease free water and only *BamH*I or *Hind*III as controls. A double digestion was done to excise the gene SINV E2 encoding protein and a single digestion was done to linearise the plasmid and confirm the size of the plasmid enzyme activity. The products were separated by electrophoresis on a 1% SeaKem® LE Agarose gel (pH 8.0)

at 80V for 60 minutes and visualised under UV light.

Table 3.1 Composition of double restriction digestion reaction mixture for linearisation of pcDNA3.1 SinE2.

Components	Plasmid (µl)	*BamH*I control reaction (µl)	*Hind*III control reaction (µl)
Buffer E	4	4	4
DNA (679.9 ng/µl)	1	1	1
*BamH*I (10 U/µl)	2	2	-
*Hind*III (10U/µl)	2	-	2
Nuclease free water	11	13	13
Total	20	20	20

3.2.5 Control GFP Plasmid

A plasmid expressing the enhanced green fluorescent protein (eGFP), designated pcDNA3.1 GFP, prepared from a previous study (Viljoen, 2014) was used as a positive control. A map of the gene is attached in Appendix I. Glycerol stock was inoculated into LB/amp, grown overnight and subcultured for 10 hours. The same purification method used to purify pcDNA3.1 SinE2 was used to purify this plasmid.

3.2.6 Protein expression in mammalian cells

Two different transfection reagents, lipofectamine and TransIT-X2, and two different cell lines were used in the attempt to achieve expression of the SINV E2 protein. The SinE2 protein consists of 1299 bp and the eGFP gene consists

of 729 bp. Various conditions were used for optimisation of plasmid size for transfections.

3.2.6.1 Transfection of plasmid in BHK-21 cells

Baby hamster kidney (BHK-21) cells (ATCC C13) were transfected with the purified recombinant plasmid according to the protocol for that cell line. Briefly, trypsinised cells were counted and the number of cells per volume were adjusted to obtain a final seeding rate of 1-3 $x10^5$ cells per well of a 24 well plate and incubated at 37 ℃ until 70-90% confluent. Each well contained a cover slip for retrieval of transfected cells for IFA. Plasmid-lipid complexes were prepared as follows. For complex 1, pcDNA3.1 SinE2 plasmid, 50 µl of Opti-MEM and 2 µl of P3000 reagent (Invitrogen, USA) were added and mixed well. For the other complex, 25 µl of Opti-MEM and 1.5 µl of 3000 Reagent (Invitrogen, USA) was added and mixed well. The complexes were added together, incubated for 15 minutes while the cells were washed with PBS (pH 7.4) (Gibco®, USA) twice. Transfection media was added to the wells and the lipid-DNA complexes were added to the cells. One well was transfected with pcDNA3.1 GFP plasmid as a positive control. One well was transfected with complexes without DNA as a negative control. The cells were incubated for 48 hours at 37 ℃.

Similarly, trypsinised cells were counted and the number of cells per volume were adjusted to obtain a final seeding rate of 1 to $3x10^5$ cells per well of a 24 well plate and incubated at 37 ℃ until 80% confluent. TransIT-X2 was warmed to room temperature and vortexed gently. Placed 250µl of Opti-MEM I Reduced-Serum Medium in a sterile tube, added pcDNA3.1 SinE2 plasmid

DNA, and pipetted gently to mix thoroughly. A 3 µl aliquot of TransIT-X2 was added to the diluted DNA mixture, mixed gently and incubated at room temperature for 15–30 minutes, while cells were washed twice with PBS (pH 7.4) (Gibco®, USA). Transfection media was added to the wells and the complexes were added to the wells and gently rocked to ensure even distribution to cells. One well was also transfected with pcDNA GFP plasmid as a positive control. One well was transfected with complexes without DNA as a negative control. The plates were incubated for 48 hours at 37 ℃.

Table 3.2 Summary of transfection experiments in BHK-21 cells.

Amount of cells per well	Concentration of DNA (ng/µl)	Volume of DNA (µl)/Final DNA concentration (µg)	Amount of Reagent (µl)	Incubation Time (min)
$1X10^5$	pcDNA3.1 GFP (1049.8)	2/2	Lipofectamine 0.75	15
$1x10^5$	pcDNA3.1 GFP (561.9)	3.6/2	Lipofectamine 1.5	15
	pcDNA3.1 SinE2 (499.6)	4/2		
$1x10^5$	pcDNA3.1 GFP (561.9)	3.6/2	Lipofectamine 0.75 1.5	15
	pcDNA3.1 SinE2 (679.9)	2.94/2		
$3x10^5$	pcDNA3.1 GFP (561.9)	3.56/2	TransIT-X2 3	15
	pcDNA3.1 SinE2 (679.9)	2.94/2		
$3x10^5$	pcDNA3.1 GFP (561.9)	5.3/3	TransIT-X2 6	15
				30
	pcDNA3.1 SinE2 (679.9)	4.4/3		15
				30
$3x10^5$	pcDNA3.1 GFP (561.9)	3.56/2	TransIT-X2 6	15
	pcDNA3.1 SinE2 (679.9)	4.4/3		
$1x10^5$	pcDNA3.1 GFP (561.9)	3.56/2	Lipofectamine 1.5	15
	pcDNA3.1 SinE2 (679.9)	5.9/4		
$3x10^5$	pcDNA3.1 GFP (561.9)	3.56/2	TransIT-X2 3	15
	pcDNA3.1 SinE2 (679.9)	5.9/4		

3.2.6.2 Transfection of plasmid in HEK-293 cells

Human embryonic kidney (HEK-293) cells (ATCC CRL-1573) were transfected with the purified recombinant plasmid according to the protocol for that cell line. Trypsinised cells were counted and the number of cells per volume were adjusted to obtain a final seeding rate of $2x10^5$ cells per well of a 24 well plate and incubated at 37 °C until 70-90% confluent. Plasmid-lipid complexes were prepared as follows. For complex 1, pcDNA3.1 SinE2 plasmid, 50 µl of Opti-MEM and 2 µl of P3000 reagent (Invitrogen, USA) was added and mixed well. For the other complex, 25 µl of Opti-MEM and 1.5 µl of 3000 Reagent (Invitrogen, USA) was added and mixed well. The complexes were added together, incubated for 15 minutes while the cells were washed with PBS (pH 7.4) (Gibco®, USA) twice. Transfection media was added to the wells and the lipid-DNA complexes were added to the cells. One well was transfected with pcDNA3.1 GFP plasmid as a positive control. One well was transfected with complexes without DNA as a negative control. The cells were incubated for 48 hours at 37 °C.

Similarly for TransIT-X2 transfections, trypsinised cells were counted and the number of cells per volume were adjusted to obtain a final seeding rate of $2x10^5$ cells per well of a 24 well plate and incubated at 37 °C until 80% confluent. TransIT-X2 was warmed to room temperature and vortexed gently. Placed 250 µl of Opti-MEM I Reduced-Serum Medium in a sterile tube, added pcDNA3.1 SinE2 plasmid DNA, and pipetted gently to mix thoroughly. Added 3 µl TransIT-X2 to the diluted DNA mixture, mixed gently and incubated at room temperature for 15–30 minutes, while cells were washed twice with PBS (pH 7.4) (Gibco®, USA). Transfection media was added to the wells and the complexes were added to the wells and gently rocked to ensure even distribution to cells. One well was also transfected with pcDNA GFP plasmid as

a positive control. One well was transfected with complexes without DNA as a negative control. The plates were incubated for 48 hours at 37 °C.

3.2.6.3 Immunofluorescence assay (IFA) of transfected cells

An immunofluorescence assay was performed to detect expression of protein in the BHK-21 cells. Briefly, cells were fixed in a 1:1 methanol:acetone solution for 20 minutes at -20 °C, coverslips containing fixed cells were placed onto microscope slides, and blocked with blocking buffer (10% sucrose, 0.5% Triton X-100 and PBS) for 20 minutes at room temperature. Mouse anti-histidine antibody (Roche, Switzerland) diluted 1:100 in blocking buffer was added, incubated at 37 °C for 90 minutes, and washed thrice with 1% PBS-Tween20 for 30-60 seconds. A secondary anti-mouse IgG fluorescein isothyocyanate labeled antibody (SeraCare Life Sciences, USA), diluted 1:20 in 0.1% Evan's Blue, was added, and incubated at 37 °C for 30 minutes. The slides were washed thrice with 1% PBST for 30-60 seconds, air died and mounted using mounting media. The slides were viewed under a fluorescence microscope (Nikon, Japan) and stored at 4 °C.

3.2.7 Sequence determination of pcDNA3.1 SinE2 plasmid

The nucleotide sequence of the pcDNA3.1 SinE2 plasmid was provided by GenScript as the gene was synthesized. However the nucleotide sequence was determined in the laboratory to confirm the sequence.

Briefly, the nucleotide sequence of the pcDNA3.1 SinE2 plasmid was determined using the Big Dye Terminator sequencing ready reaction kit (Applied Biosystems, USA) according to manufacturer's instructions. The components of the sequencing reaction are shown in Appendix J. Bidirectional sequencing was performed using the T7 and BGH primers located on the

plasmid upstream 5' and downstream 3' of the inserted gene respectively.

The following cycling conditions were performed for the reactions. Initial denaturation at 96 ℃ for one minute for one cycle. Thereafter 25 cycles of denaturation at 96 ℃ for 10 seconds, annealing at 50 °C for five seconds and extension at 60 °C for four minutes. Finally samples were held at 4 °C until clean up.

The Ethylene-Diamine-Tetra-Acetic acid (EDTA)/ethanol precipitation method was used to cleanup the sequencing reaction amplicons. The sequencing cleanup was added to the sequencing reaction, vortexed and incubated for 15 minutes at room temperature. The components of the sequencing cleanup is shown in Appendix J. The reaction was then centrifuged at 20000 xg for 20 minutes at 4 ℃. The supernatant was aspirated. The pellet was resuspended in 500 µl of 70% ethanol and centrifuged at 20000 xg for 10 minutes at 4 ℃. The supernatant was completely aspirated, the pellet was dried in the Speedy vac until dry and stored in the dark at 4 ℃. The samples were submitted to the Department of Microbial, Biochemical and Food Biotechnology of the University of the Free State for electrophoresis.

The sequence data was aligned with the sequence data provided by GenScript using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). The sequence was translated into the amino acid sequence using ExPASy (https://web.expasy.org/translate/) and then analysed with BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.2.8 In-house immunfluorescence assay (IFA)

3.2.8.1 Preparation of antigen slides using SINV infected cells

A confluent T25 flask of Vero 76 (Vero) cell cultures (ATCC number CRL-1587) was infected with a 5µl aliquot of seed virus Sindbis SAAR 86. All infectious work was performed in the biosafety level 2 laboratory. Cells were maintained in minimum essential media (MEM) (Invitrogen, USA) containing 2 % fetal bovine serum (FBS) (BioWhittaker™, Switzerland), 100 u/ml penicillin (BioWhittaker™, Switzerland), 0.1 mg/ml streptomycin (BioWhittaker, Switzerland) and 2 mM L-glutamine (BioWhittaker, Switzerland), incubated at 37 ℃ and monitored daily for CPE. When the initial signs of CPE occurred, the cells were harvested using trypsin and clarified for 20 minutes at 1000 x g. the cells were resuspended in 1ml of 10% fetal bovine serum in PBS. Antigen slides were prepared using 10 µl of cells per well on 10 well slides. The antigen was dried at 37 °C overnight and the cells fixed and virus inactivated by placing slides in cold acetone for 2 hours. An IFA was performed using a known positive serum sample to confirm that the cells were positive for SINV.

3.2.8.2 Immunofluorescence assay (IFA) of SINV-infected cells

A total of 68 Serum samples that were found to be positive in the previous chapter using neutralization assays and the in house ELISA were screened using an immunfluorescence assay to detect antibody against SINV. Serum sample ARC065 could not be used due to unavailability. Serum samples were placed onto prepared SINV-infected antigen slides and incubated in a moist chamber at 37 °C for 30 minutes. The slides were washed twice in 1% PBST

and air dried. A secondary anti-human IgG fluorescein isothyocyanate (FITC) labeled antibody (SeraCare Life Sciences, USA), diluted 1:20 in 0.1% Evan's Blue, was added, and incubated at 37 °C 30 minutes. The slides were washed twice with 1% PBST, air dried, mounted. The slides were viewed under a fluorescence microscope (Nikon, Japan) and stored at 4 °C.

3.3 Results

3.3.1 Purification of pcDNA3.1 SinE2 and pcDNA3.1 GFP

The pcDNA3.1 SinE2 plasmid DNA was transformed into TOP10 *E. coli* cells and successful transformants were selected on LB/amp agar. A colony was grown overnight in LB/amp medium, subcultured and purified. The pcDNA3.1 SinE2 plasmid was purified on multiple occasions and the DNA concentration ranged from 499.6 to 778.7 ng/µl.

The pcDNA3.1 GFP *JM109* transformed cells were grown in LB/amp medium overnight, subcultured and purified. The pcDNA3.1 GFP plasmid was purified on multiple occasions and the DNA concentration ranged from 561.9 to 938.2 ng/µl.

3.3.2 Confirmation of presence of gene in plasmid

The pcDNA3.1 SinE2 DNA was linearised by restriction digestion, after each purification, using *BamH*I and *Hind*III restriction endonucleases to confirm the gene of interest's presence. Single digestions were performed as a control of the restriction endonucleases' function. Figure 3.1 shows that restriction

endonucleases were actively functioning and the linearised DNA correlates to approximately 1299 bp.

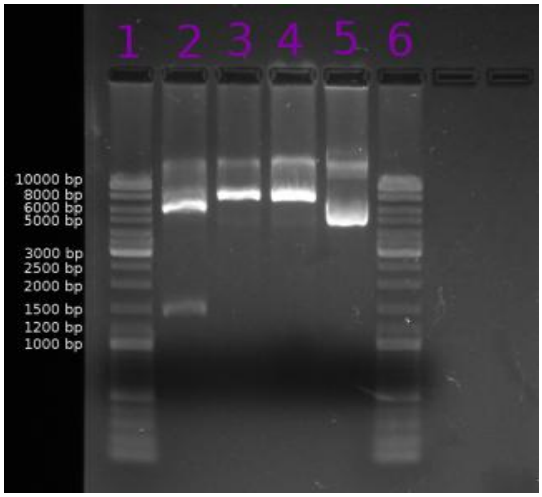


Figure 3.1 1% gel of restriction digest. Lanes 1 and 6 contains the marker (M), lane 2 contains the plasmid cut by both restriction enzymes (C), lane 3 contains the plasmid cut by only *BamH*I (B), lane 4 contains the plasmid cut by only *Hind*III (H), and lane 5 contains the uncut plasmid (U).

3.3.3 Protein expression in mammalian cell line

BHK-21 cells were transfected with the pcDNA3.1 SinE2 and pcDNA3.1 GFP. To confirm expression of the E2 protein, an IFA was performed using anti-His monoclonal antibody. GFP expression was determined by visualization using a fluorescent microscope.

Table 3.3 Summary of transfection results.

DNA	Seeding Rate	Total DNA added (µg)	Amount of Reagent (µl)	Percentage Fluorescence(%)
pcDNA3.1 GFP (561.9 ng/µl)	$1x10^5$	2	Lipofectamine (0.75-1.5)	100
	$3x10^5$	2	Transit X2 (3)	100
pcDNA3.1 SinE2 (679.9 ng/µl)	$1x10^5$	2	Lipofectamine (0.75-1.5)	0
		3		0
		4		0
	$1x10^5$	2	Transit X2 (3)	0
		3		0
		4		Sparse

The positive control confirmed expression of GFP, as shown in Figure 3.2. Irrespective of the transfection reagent used it was possible to confirm transient expression of the GFP. However expression of the E2 protein could not be confirmed, as shown in Figure 3.3. In some experiments sparsely fluorescing cells (Figure 3.4) were noted, however overall transient expression was not confirmed. HEK-293 cells were also transfected with the pcDNA3. SinE2 and pcDNA3.1 GFP to confirm expression of the E2 protein and GFP respectively. The expression of the E2 protein could still not be confirmed. The positive control also confirmed expression of GFP in this cell line.

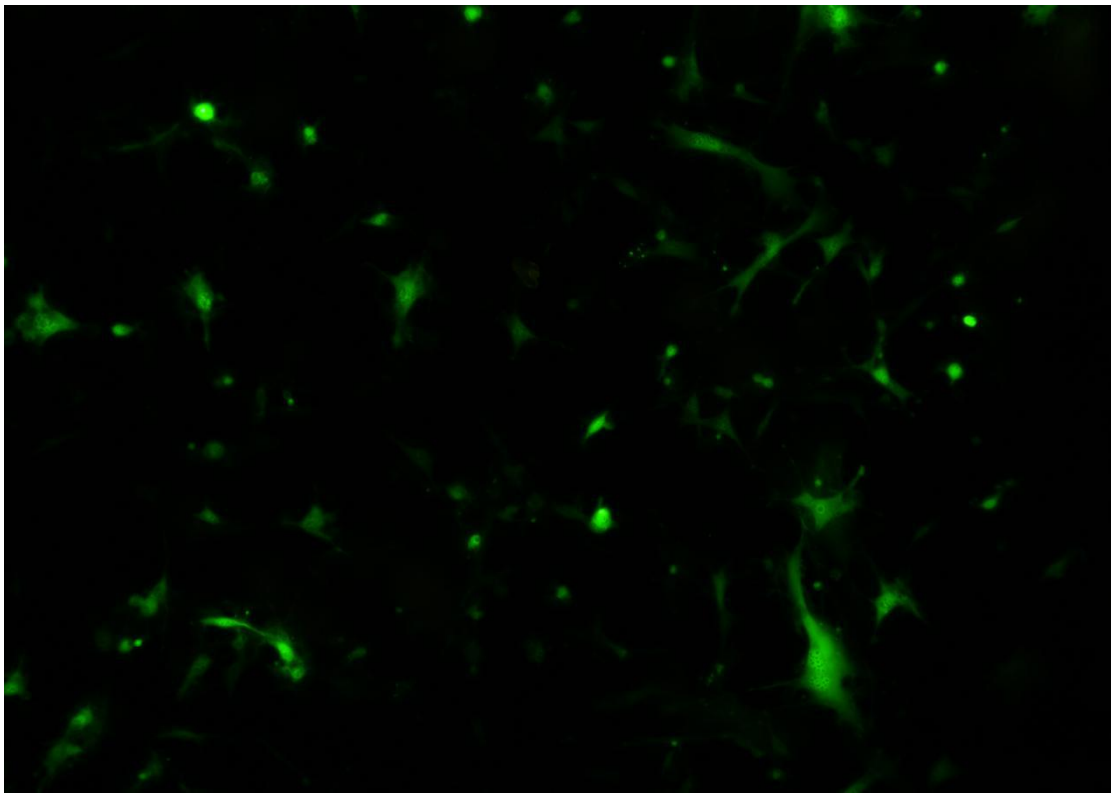


Figure 3.2 IFA of pcDNA3.1 GFP transfected BHK-21 cells

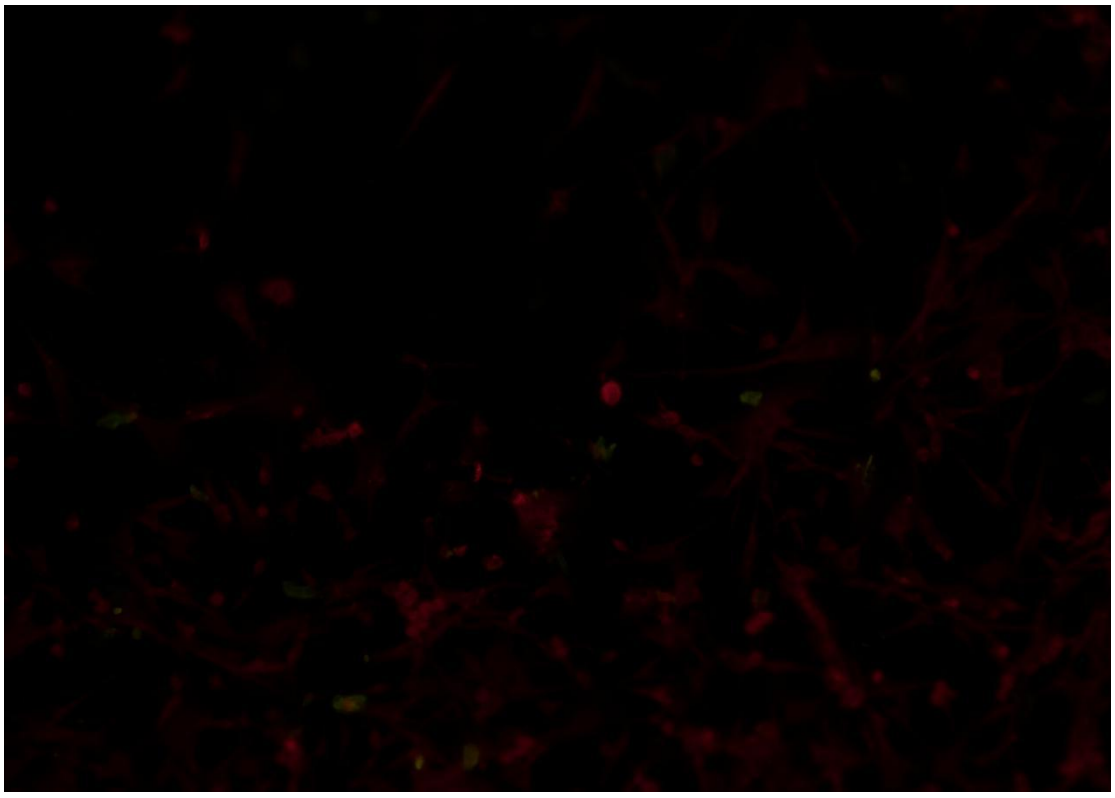


Figure 3.3 IFA of pcDNA3.1 SinE2 transfected cells.

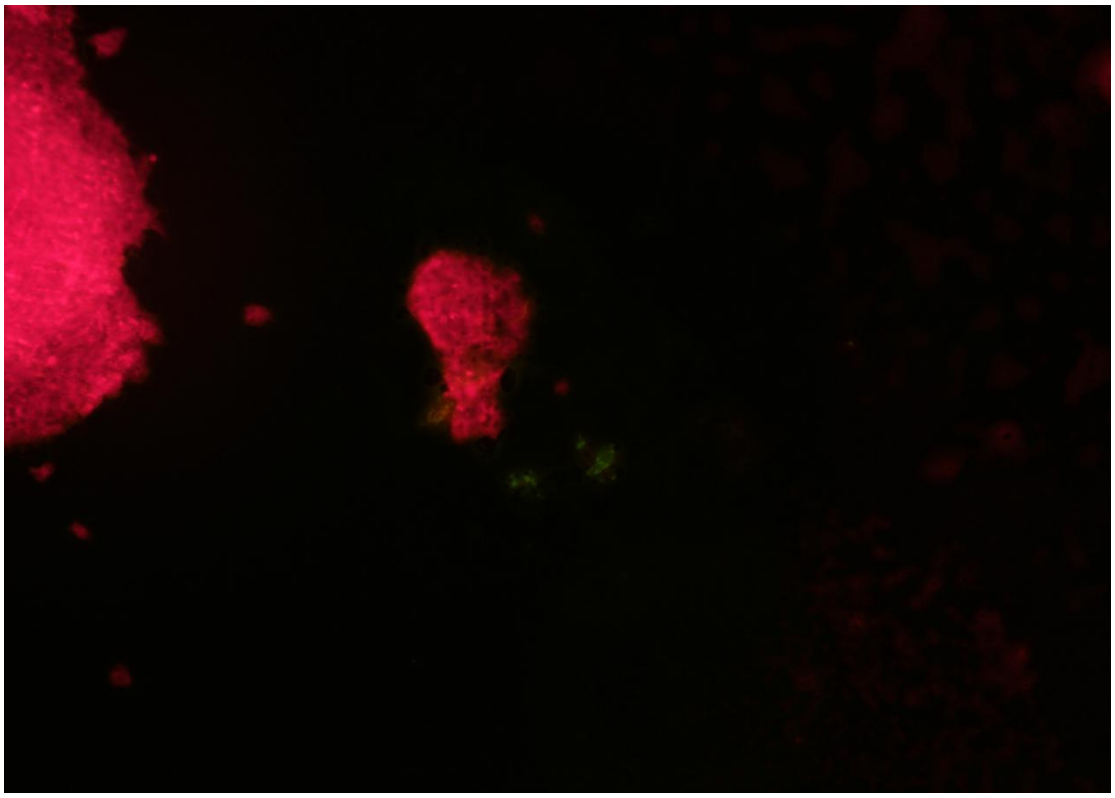


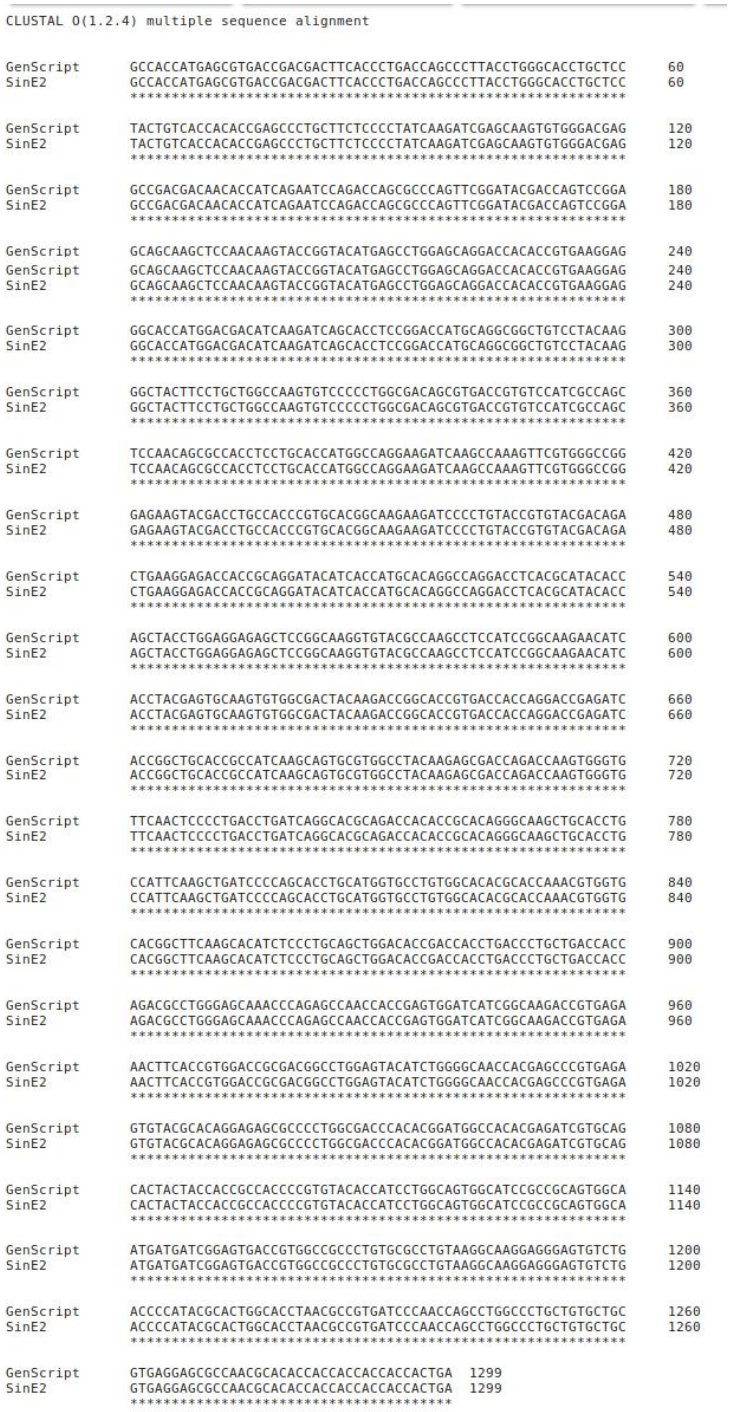
Figure 3.4 IFA of pcDNA3.1 SinE2 transfected cells showing the sparsely fluorescing cells.

3.3.4 Confirmation of sequence of plasmid pcDNA 3.1 SinE2

Multiple attempts to optimize the expression of SinE2 protein were attempted. Most attempts were unsuccessful with only sparsely transfected cells identified with high quantities of DNA. Hence although the sequence was provided and guaranteed by the supplier, the nucleotide sequence was determined to confirm the presence of the Kozak sequence, the SINV E2 coding sequence and the HIS tag in frame with the gene of interest.

Sequence data was obtained using primers T7 and BGH. The sequence data was aligned with data obtained from GenScript for the codon optimized E2 gene. The alignment is shown in figure 3.5. In addition, the nucleotide sequence data was translated using ExPASy (https://web.expasy.org/translate/)

to confirm that the nucleotide sequence codes for the SINV E2 protein (Figure 3.6). The SinE2 protein showed a 100% similarity with the E2 structural polyprotein of the Girdwood S.A. isolate (GenBank Acession number U38304.1) and 99.53% similarity with E2 structural polyprotein of the Sindbis-like virus isolate S.A.AR86 (Genbank Accession number U38305.1) (Figure 3.7). Two mismatches between N amino acid of the S.A.AR86 strain and K amino acid of the SinE2 protein, as well as S amino acid of the S.A.AR86 strain and L amino acid of the SinE2 proteins.



```
CLUSTAL O(1.2.4) multiple sequence alignment

GenScript       GCCACCATGAGCGTGACCGACGACTTCACCCTGACCAGCCCTTACCTGGGCACCTGCTCC     60
SinE2           GCCACCATGAGCGTGACCGACGACTTCACCCTGACCAGCCCTTACCTGGGCACCTGCTCC     60
                ************************************************************

GenScript       TACTGTCACCACACCGAGCCCTGCTTCTCCCCTATCAAGATCGAGCAAGTGTGGGACGAG     120
SinE2           TACTGTCACCACACCGAGCCCTGCTTCTCCCCTATCAAGATCGAGCAAGTGTGGGACGAG     120
                ************************************************************

GenScript       GCCGACGACAACACCATCAGAATCCAGACCAGCGCCCAGTTCGGATACGACCAGTCCGGA     180
SinE2           GCCGACGACAACACCATCAGAATCCAGACCAGCGCCCAGTTCGGATACGACCAGTCCGGA     180
                ************************************************************

GenScript       GCAGCAAGCTCCAACAAGTACCGGTACATGAGCCTGGAGCAGGACCACACCGTGAAGGAG     240
GenScript       GCAGCAAGCTCCAACAAGTACCGGTACATGAGCCTGGAGCAGGACCACACCGTGAAGGAG     240
SinE2           GCAGCAAGCTCCAACAAGTACCGGTACATGAGCCTGGAGCAGGACCACACCGTGAAGGAG     240
                ************************************************************

GenScript       GGCACCATGGACGACATCAAGATCAGCACCTCCGGACCATGCAGGCGGCTGTCCTACAAG     300
SinE2           GGCACCATGGACGACATCAAGATCAGCACCTCCGGACCATGCAGGCGGCTGTCCTACAAG     300
                ************************************************************

GenScript       GGCTACTTCCTGCTGGCCAAGTGTCCCCCTGGCGACAGCGTGACCGTGTCCATCGCCAGC     360
SinE2           GGCTACTTCCTGCTGGCCAAGTGTCCCCCTGGCGACAGCGTGACCGTGTCCATCGCCAGC     360
                ************************************************************

GenScript       TCCAACAGCGCCACCTCCTGCACCATGGCCAGGAAGATCAAGCCAAAGTTCGTGGGCCGG     420
SinE2           TCCAACAGCGCCACCTCCTGCACCATGGCCAGGAAGATCAAGCCAAAGTTCGTGGGCCGG     420
                ************************************************************

GenScript       GAGAAGTACGACCTGCCACCCGTGCACGGCAAGAAGATCCCCTGTACCGTGTACGACAGA     480
SinE2           GAGAAGTACGACCTGCCACCCGTGCACGGCAAGAAGATCCCCTGTACCGTGTACGACAGA     480
                ************************************************************

GenScript       CTGAAGGAGACCACCGCAGGATACATCACCATGCACAGGCCAGGACCTCACGCATACACC     540
SinE2           CTGAAGGAGACCACCGCAGGATACATCACCATGCACAGGCCAGGACCTCACGCATACACC     540
                ************************************************************

GenScript       AGCTACCTGGAGGAGAGCTCCGGCAAGGTGTACGCCAAGCCTCCATCCGGCAAGAACATC     600
SinE2           AGCTACCTGGAGGAGAGCTCCGGCAAGGTGTACGCCAAGCCTCCATCCGGCAAGAACATC     600
                ************************************************************

GenScript       ACCTACGAGTGCAAGTGTGGCGACTACAAGACCGGCACCGTGACCACCAGGACCGAGATC     660
SinE2           ACCTACGAGTGCAAGTGTGGCGACTACAAGACCGGCACCGTGACCACCAGGACCGAGATC     660
                ************************************************************

GenScript       ACCGGCTGCACCGCCATCAAGCAGTGCGTGGCCTACAAGAGCGACCAGACCAAGTGGGTG     720
SinE2           ACCGGCTGCACCGCCATCAAGCAGTGCGTGGCCTACAAGAGCGACCAGACCAAGTGGGTG     720
                ************************************************************

GenScript       TTCAACTCCCCTGACCTGATCAGGCACGCAGACCACACCGCACAGGGCAAGCTGCACCTG     780
SinE2           TTCAACTCCCCTGACCTGATCAGGCACGCAGACCACACCGCACAGGGCAAGCTGCACCTG     780
                ************************************************************

GenScript       CCATTCAAGCTGATCCCCAGCACCTGCATGGTGCCTGTGGCACACGCACCAAACGTGGTG     840
SinE2           CCATTCAAGCTGATCCCCAGCACCTGCATGGTGCCTGTGGCACACGCACCAAACGTGGTG     840
                ************************************************************

GenScript       CACGGCTTCAAGCACATCTCCCTGCAGCTGGACACCGACCACCTGACCCTGCTGACCACC     900
SinE2           CACGGCTTCAAGCACATCTCCCTGCAGCTGGACACCGACCACCTGACCCTGCTGACCACC     900
                ************************************************************

GenScript       AGACGCCTGGGAGCAAACCCAGAGCCAACCACCGAGTGGATCATCGGCAAGACCGTGAGA     960
SinE2           AGACGCCTGGGAGCAAACCCAGAGCCAACCACCGAGTGGATCATCGGCAAGACCGTGAGA     960
                ************************************************************

GenScript       AACTTCACCGTGGACCGCGACGGCCTGGAGTACATCTGGGGCAACCACGAGCCCGTGAGA    1020
SinE2           AACTTCACCGTGGACCGCGACGGCCTGGAGTACATCTGGGGCAACCACGAGCCCGTGAGA    1020
                ************************************************************

GenScript       GTGTACGCACAGGAGAGCGCCCCTGGCGACCCACACGGATGGCCACACGAGATCGTGCAG    1080
SinE2           GTGTACGCACAGGAGAGCGCCCCTGGCGACCCACACGGATGGCCACACGAGATCGTGCAG    1080
                ************************************************************

GenScript       CACTACTACCACCGCCACCCCGTGTACACCATCCTGGCAGTGGCATCCGCCGCAGTGGCA    1140
SinE2           CACTACTACCACCGCCACCCCGTGTACACCATCCTGGCAGTGGCATCCGCCGCAGTGGCA    1140
                ************************************************************

GenScript       ATGATGATCGGAGTGACCGTGGCCGCCCTGTGCGCCTGTAAGGCAAGGAGGGAGTGTCTG    1200
SinE2           ATGATGATCGGAGTGACCGTGGCCGCCCTGTGCGCCTGTAAGGCAAGGAGGGAGTGTCTG    1200
                ************************************************************

GenScript       ACCCCATACGCACTGGCACCTAACGCCGTGATCCCAACCAGCCTGGCCCTGCTGTGCTGC    1260
SinE2           ACCCCATACGCACTGGCACCTAACGCCGTGATCCCAACCAGCCTGGCCCTGCTGTGCTGC    1260
                ************************************************************

GenScript       GTGAGGAGCGCCAACGCACACCACCACCACCACCACTGA  1299
SinE2           GTGAGGAGCGCCAACGCACACCACCACCACCACCACTGA  1299
                ****************************************
```

Figure 3.5 Alignment of the sequenced plasmid data with the data provided by GenScript.

Figure 3.6 Analysis of SinE2 protein by ExPASy showing that it is in frame.

```
CLUSTAL O(1.2.4) multiple sequence alignment


S.A.AR86        -SVTDDFTLTSPYLGTCSYCHHTEPCFSPIKIEQVWDEADDNTIRIQTSAQFGYDQSGAA      59
SinE2           MSVTDDFTLTSPYLGTCSYCHHTEPCFSPIKIEQVWDEADDNTIRIQTSAQFGYDQSGAA      60
                 ************************************************************

S.A.AR86        SSNKYRYMSLEQDHTVKEGTMDDIKISTSGPCRRLSYKGYFLLAKCPPGDSVTVSIASSN      119
SinE2           SSNKYRYMSLEQDHTVKEGTMDDIKISTSGPCRRLSYKGYFLLAKCPPGDSVTVSIASSN      120
                ************************************************************

S.A.AR86        SATSCTMARKIKPKFVGREKYDLPPVHGKKIPCTVYDRLKETTAGYITMHRPGPHAYTSY      179
SinE2           SATSCTMARKIKPKFVGREKYDLPPVHGKKIPCTVYDRLKETTAGYITMHRPGPHAYTSY      180
                ************************************************************

S.A.AR86        LEESSGKVYAKPPSGKNITYECKCGDYKTGTVTTRTEITGCTAIKQCVAYKSDQTKWVFN      239
SinE2           LEESSGKVYAKPPSGKNITYECKCGDYKTGTVTTRTEITGCTAIKQCVAYKSDQTKWVFN      240
                ************************************************************

S.A.AR86        SPDSIRHADHTAQGKLHLPFKLIPSTCMVPVAHAPNVVHGFKHISLQLDTDHLTLLTTRR      299
SinE2           SPDLIRHADHTAQGKLHLPFKLIPSTCMVPVAHAPNVVHGFKHISLQLDTDHLTLLTTRR      300
                *** ********************************************************

S.A.AR86        LGANPEPTTEWIIGNTVRNFTVDRDGLEYIWGNHEPVRVYAQESAPGDPHGWPHEIVQHY      359
SinE2           LGANPEPTTEWIIGKTVRNFTVDRDGLEYIWGNHEPVRVYAQESAPGDPHGWPHEIVQHY      360
                **************:*********************************************

S.A.AR86        YHRHPVYTILAVASAAVAMMIGVTVAALCACKARRECLTPYALAPNAVIPTSLALLCCVR      419
SinE2           YHRHPVYTILAVASAAVAMMIGVTVAALCACKARRECLTPYALAPNAVIPTSLALLCCVR      420
                ************************************************************

S.A.AR86        SANA------        423
SinE2           SANAHHHHHH        430
                ****
```

Figure 3.7 Alignment of SinE2 protein sequence with S.A.AR86 E2 protein sequence.

After confirming the correct sequence of the construct for expression of the E2 protein and the presence of the HIS tag in frame with the E2 protein further attempts to express the protein were abandoned.

3.3.5 In-house antigen slides as a rapid immunofluorescent assay for detection of IgG and IgM antibody against SINV

An in-house rapid immunofluorescent assay was developed and used to screen serum samples for anti-SINV IgG and IgM antibodies. A total of 68 samples were available for screening for IgG. Insufficient slides were available to screen all the samples for IgM, however our focus for IgM were the patients from the arthritis clinic. The results are listed in table 3.4. Briefly there were 50/68 positive for IgG and 8/56 positive for IgM. There were 12/68 for IgG and 4/56 for IgM described as indeterminate, 3/68 that were bright green for IgG and 1/56 that were bright green for IgM, and 13 were unavailable for testing as the sample was finished. As for the commercial assay, the IFA slides were not always easy to read with no clear differentiation between positive reactors when there was non-specific fluorescence. However overall the in-house IFA was more sensitive than the commercial IFA. More samples were found to be positive as well as less negative and indeterminate samples with the in-house IFA than the commercial assay as shown in figures 3.8 and 3.9. A total of 8/56 Were positive for IgM antibody.

Table 3.4 Results for IgG and IgM in house IFA.

Serum Sample	Results	
	IgG IFA	**IgM IFA**
ARC001	Positive	Negative
ARC007	Positive	Negative
ARC009	Positive	Negative
ARC011	Bright Green**	Negative
ARC017	Positive	Negative
ARC018	Positive	Negative
ARC019	Positive	**Positive**
ARC021	Positive	Negative
ARC022	Positive	**Positive**
ARC035	Indeterminate*	Bright Green
ARC038	Positive	Negative
ARC041	Positive	Negative
ARC042	Positive	Negative
ARC050	Positive	**Positive**
ARC055	Bright Green	Indeterminate
ARC065	Unavailable	Unavailable
ARC070	Positive	**Positive**
ARC074	Positive	Negative
ARC078	Positive	Negative
ARC081	Positive	**Positive**
ARC082	Positive	Negative
ARC092	Positive	Negative
ARC098	Positive	Negative
ARC100	Positive	Negative
ARC108	Indeterminate	Negative
ARC111	Positive	Negative
ARC127	Positive	Negative
ARC133	Positive	Negative
ARC134	Positive	Indeterminate
ARC143	Positive	Negative
ARC155	Positive	Negative
HSW 003	Indeterminate	Negative
HSW 013	Positive	Negative
HSW 033	Negative	Negative
HSW 042	Positive	Negative
HSW 049	Positive	Negative
HSW 057	Positive	Negative
HSW 059	Positive	Negative

Serum Sample	Results	
	IgG IFA	IgM IFA
HSW 064	Positive	Negative
HSW 106	Positive	Negative
HSW 108	Indeterminate	Negative
HSW 116	Positive	**Positive**
HSW 131	Positive	**Positive**
HSW 133	Positive	**Positive**
QWF012	Indeterminate	Negative
QWF013	Indeterminate	Indeterminate
QWF015	Positive	Indeterminate
QWF017	Positive	Negative
QWF043	Positive	Negative
QWF058	Positive	Negative
QWF063	Indeterminate	Negative
QWF065	Indeterminate	Negative
QWF070	Indeterminate	Negative
QWF076	Positive	Negative
QWF085	Bright Green	Negative
QWF103	Positive	Negative
QWF107	Negative	Negative
QWF108	Positive	NT***
QWF123	Indeterminate	NT
QWF166	Positive	NT
QWF168	Positive	NT
QWF171	Negative	NT
QWF184	Positive	NT
TB08	Positive	NT
TB17	Indeterminate	NT
TB18	Positive	NT
TB56	Positive	NT
TB57	Indeterminate	NT
TB75	Positive	NT
Total	50	8

* indeterminate: the results could not be read likely due to non specific reactivity

** bright green: non specific fluorescence

***NT: not tested due to insufficient slides

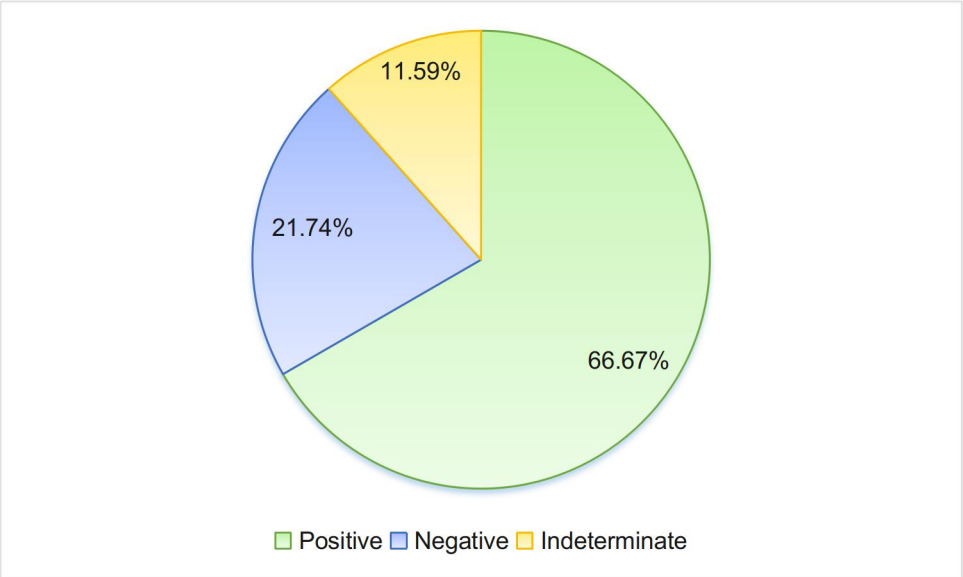


Figure 3.8 Percentage of positive, negative and indeterminate results from commercial assay.

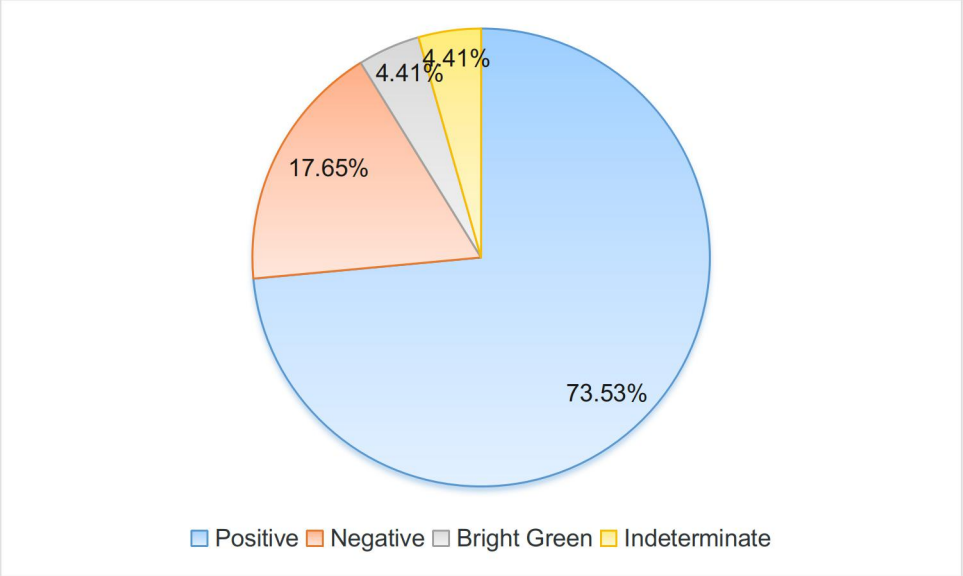


Figure 3.9 Percentage of positive, negative, bright green and indeterminate results from the in-house IgG IFA.

3.4 Summary

This chapter described the attempt at development of a recombinant antigen for use in immunofluorescence assays. Even though different conditions were

used for the transfections, expression of the SinE2 protein was unsuccessful. The presence of the gene in the plasmid was confirmed. The correct gene and protein sequence with Kozak translation sequence and His tag was also confirmed. The protein sequence was confirmed to be in frame and coding for the correct protein. Expression of the positive control plasmid was successful. Therefore, the transfection method did not appear to be a problem and the fault likely lies with the plasmid. As we were unable to ascertain a reason for non-expression, attempts to transfect cells were abandoned.

The following are possible reasons for the unsuccessful transfections. The plasmid might be especially sensitive to proteases or genetically silenced in the cells. The plasmid might not have entered the cells. If the protein was expressed, it might have been degraded by the cell if it blocked the cell's viability or physiology. The components of the media may have interfered with expression.

An in-house immunofluorescence assay using SINV infected cells was also developed and used to screen serum samples for anti-SINV IgG and IgM antibodies. It is possible to use this assay as a rapid assay to detect antibodies against SINV in human sera.

This assay can be useful for surveillance, diagnosis during outbreaks and screening small amount of samples. More advantages are that it is simple to operate, less time consuming and cheaper. The disadvantages of this assay are that the slides have to be prepared in special cell culture laboratories, the viral epitopes may be destroyed with inactivation of the virus, and may be difficult to read due to non-specific binding.

The in-house IFA was shown to be more sensitive and cheaper than the commercial assay. The commercial IFA costs approximately R320.00 per slide and since there are 10 BIOCHIPs on each slide, hence R32.00 per sample. Once all the consumables have been accumulated, it would cost

approximately R10.00 per sample for the in-house assays. The in-house IFA also has less non-specific fluorescence than the commercial IFA and is easier to differentiate between positive and negative samples.

Chapter 4: DISCUSSION

SINV is an arthritogenic alphavirus and is endemic to South Africa (Zhang *et al*., 2018; Storm *et al*., 2013) Studies in Europe have shown that SINV is associated with chronic arthritis in a small amount of cases (Kurkela *et al*., 2008). The exact prevalence is known in Europe, however in South Africa it is not currently known and the association of SINV disease and arthritis is not well documented in South Africa. The study aimed to develop serological assays for detection of SINV infection and to investigate this prevalence in arthritis patients and at risk populations.

Serological assays, in which the virus, viral antigens or antibodies are detected, are used to diagnose arboviral infections, since clinical diagnosis of alphavirus infections is difficult (Cleton *et al*., 2012; Wang *et al*., 2006). Serum is generally used in the diagnosis of arboviral infections (Cleton *et al*., 2012). There are various serological assays that can be used for diagnosis and surveillance of arbovirus infections, such as SINV, including virus isolation, detection of imunoglobulin M (IgM) and immunoglobulin G (IgG) antibodies or seroconversion, molecular assays such as nucleic acid amplification, haemagglutination inhibition (HAI), complement fixation (CF), neutralisation assays, and enzyme-linked immunosorbent assay (ELISA) (Cleton *et al*., 2012; Wang *et al*., 2006; Calisher *et al.,* 1986; Frazier & Shope, 1979).

The factors that affect gene expression include laboratory facilities, lack of expertise, type of protein, whether the protein is toxic or not, carbohydrate or other modification requirements, how much yield is needed, purification of protein, cost of production, any safety requirements, and what the protein will be used for. A good transient expression system has the following characteristics: a very short time-frame to generate product, i.e. days; ability to

be used in a wide range of host cell lines; genetic stability and consistency; suitability for processing multiple times; allowance for many genes or mutants to be studied simultaneously; and ease to construct expression vector (Kahn, 2013).

Mammalian expression can be enhanced by properly designing the vector with the following: a strong promoter, such as cytomegalovirus (CMV), for high level messenger RNA transcription; a proper signal peptide accelerates mRNA processing and enhances secretion; selected introns; codon optimization for cell type to be used; and transcription control areas (Kahn, 2013).

The cell type chosen is crucial for expression of some recombinant proteins. It has been found that baby hamster kidney (BHK) cells and rat hepatoma cells produces a functional protein from the gene coding for the blood clotting Factor IX. However, fibroblasts do not produce a functional protein from this gene, since it lacks the enzymes needed for an important modification that affects the protein's function (Bebbington & Hentschel, 1985). Some recombinant proteins have been proven to be toxic to the cells. Regulation of gene expression can be used to control production of cytotoxic proteins in mammalian cells. Some proteins are not properly secreted from mammalian cells, such as the human Factor VIII associates with the endoplasmic reticulum (ER) protein GRP 78. Due to this association, Factor VIII is retained in the ER and poorly secreted. If expression of GRP 78 is down-regulated, then the recombinant protein will have higher secretion (Yarranton, 1990).

The E2 protein was selected for recombinant expression, to be used for the development of an immunofluorescence assay, since it is the most immunogenic surface glycoprotein for SINV. However this recombinant antigen was unable to express in mammalian cells. The presence of the gene insert was confirmed by restriction digestion. The correct gene and protein sequence, orientation of the gene in the plasmid, as well as Kozak translation sequence

and His tag was confirmed by sequencing and analysis. The analysis showed that the protein sequence was in frame and coding for the right protein. Various conditions and reagents for transfection and two cell lines were used to express the protein.

Here are possible reasons that could explain why the protein was not expressed. It is possible that the plasmid did not enter the cells. The protein might have been cytotoxic and destroyed by the cells. The protein may have been degraded by proteases. The gene coding for the protein could have been silenced. The media components could have affected or inhibited protein expression. Harmful lactate buildup from glucose feeding as well as increasing ammonium levels can inhibit protein production and alter glycosylation additives could alter osmolality of the cells and therefore affect productivities and protein quality. Iron (III) citrate and dextran sulfate inhibits polyethylenimin-mediated transfection, however if added at a later stage it can increase protein production. Other components, such as N,N demethylacetamide, lithium acetate, caffeine and dimethyl sulfoxide, can assist transient transfection efficiencies and thereby improve protein production levels. Post-translational modifications can also be affected by media components. Glycan distributon is affected by various nutrients, sugar precursors, amino acids, trace elements and hormones. Glycosylation of aspargaine residues is decreased by glucose starvation. The types and amount of glycosylation can be altered by supplementing sugar galactose in the presence of manganese chloride (Hunter *et al*., 2019). The cell lines used might not have the enzymes needed to produce the protein. Poor secretion of the protein may have occurred.

The following methods can be used for future studies. Try to utilise a different chemical transfection method to transfer the plasmid into the cells or try using electroporation methods. Gene expression can be regulated to prevent a

possible cytotoxic protein from being destroyed by cells. The concentration and timing of additives must be empirically ascertained to prevent cellular toxicity and halting of cell growth (Hunter *et al*., 2019). Different cell lines than the ones used in this study can be used. DNA demethylation using glycosylases can be considered if gene silencing was the cause. Whereby the DNA glycosylases cleaves the bond between the 5-methylcytosine base and the deoxyribose moiety in DNA, followed by abasic site repair by replacing the 5-methylcytosine with an unmethylated cytosine. Addition of histone deacetylase inhibitors, such as sodium butyrate and valproic acid, can enhance protein expression by modifying histone acetylation and methylation patterns of the host and therefore increase the cell's levels of transcription and improve protein production (Hunter *et al*., 2019). Methods to increase secretion can be tried.

An in-house ELISA to detect anti-SINV IgG antibody was developed and optimized. This assay can be useful as a diagnostic tool and for surveillance. A total of 568 serum samples from select populations (i.e. patients attending arthritis clinics, high risk populations and patients presenting with febrile illness, suspected of tickbite fever and no diagnosis) were screened for antibody against SINV with the in house ELISA and 69 of those samples were found to be positive for the antibody. However not all 568 samples could be screened for with the commercial assay, since the IFA was quite expensive. Therefore only the 69 samples found to be positive with the ELISA were used for screening with the commercial assay and neutralisation assay for comparison. The commercial IFA could only detect 46 out of 69 samples as positive for IgG antibody against SINV, hence the ELISA was more sensitive. The commercial IFA is only able to screen eight samples on one slide and difficult to read due to non-specific reactivity, whereas the ELISA is able to screen up to 96 samples per plate (including controls) and was easier to read with a clear differentiation

between positive and negative samples based on the cut off value determined from replicate testing of known negative samples.

Table 4.1 The advantages and disadvantages of ELISA and IFA (Frazier & Shope, 1979; Roehrig, 1982; Calisher *et al*., 1986; Johnson *et al*., 2000; Cleton *et al*., 2012).

ELISA		**IFA**	
Advantages	**Disadvantages**	**Advantages**	**Disadvantages**
Small amounts of serum and antigen Reliable	Cross reaction within serogroups: False positive results	Small amounts of serum and antigen	Cross reaction within serogroups: False positive results
In house: Inexpensive	Commercial: Expensive	In house: cheaper	Commercial: Expensive
Easily automated		Rapid	Difficult automation
Simple to interpret Higher sensitivity		Simple to operate	Difficult to read due to nonspecific background
Minimal sophisticated equipment Reagents: ● Readily obtained ● Long shelf life		Reagents: ● Long shelf life ● Can be frozen	Preparation require specialised laboratories for culture of virus
Large numbers of samples can be tested		Screen small amount of samples	Small amount of samples can be tested
Suitable to use during an epidemic			

The true positives were confirmed by screening of the 69 positive samples using a neutralisation assay. Of those samples 65 out of 69 was found to be true positive, one sample was negative and three samples were indeterminate.

From these results, the sensitivity of the ELISA was determined to be 100%, however specificity needs further validation using known negative samples and samples from infections caused by related viruses with potential for serological cross reactivity. Therefore the in house ELISA is quite accurate in the diagnosis of SINV infections. There is no commercial ELISA available to compare the in-house ELISA with. More positive clinical samples would be required to validate the results for diagnostic purposes as well as an in-house ELISA that detects IgM would be required.

The results from the ELISA shows that 31 out of 165 (18.7%) samples from patients attending arthritis clinic, 13 out of 136 (9.5%) samples from high risk populations, and 25 out of 267 (9.3%) samples from acute febrile illness patients with no diagnosis, tested positive for IgG antibody against SINV. Majority (45%) of the samples that tested positive were from patients attending the arthritis clinic. This study indicates that there is a high prevalence of IgG antibody positive patients attending the arthritis clinic. More importantly five of the samples were positive for IgM suggesting a recent infection. Alternatively IgM antibody has been shown to persist in patients that develop a chronic arthritis following alphavirus infections. No history was available for the three patients with IgM antibody from the cohort of at risk population hence it was not possible to determine if they have a recent acute illness or history of acute arthritic problems. This proves that further investigation is needed into the association between SINV and chronic arthritis. Since the mosquito vector for SINV occurs in Bainsvlei, detection of positive cases was not unexpected. The amount of positive cases for the patients with febrile illness, shows that arboviruses should be considered as a differential diagnosis more frequently. Due to the IgG antibody prevalence found in these targeted populations, there may be more frequent undiagnosed SINV infections in South Africa.

The developed in-house IFA was found to be more sensitive and cheaper than the commercial IFA. The commercial IFA contained SINV infected cells, however there are no details available regarding which cells were used to prepare the slides or which strain of SINV was used. Vero cells was infected with the S.A.AR86 strain to develop the in-house IFA. However, antigenically there should be serological cross reactivity between cells and strains.

Storm *et al*., 2014 investigated the incidence of SINV infections during the 2006-2010 outbreak in South Africa. They found 5.4% (87/1606) positive cases with 21/87 positive for anti-SINV IgM antibodies during 2006-2009 and 12% (243/2025) positive cases with 208/2025 positive for anti-SINV IgM antibodies. Northern Sweden had an outbreak during 2013 with 29% (50/172) positive for anti-SINV IgM and IgG antibodies (Gylfe *et al*., 2018). Sweden has a prevalence of 0.1-3.6% of antibodies and Finland has a prevalence of 4.7% antibodies and 41% in Eastern Finland (Laine *et al*., 2004). In South Africa more men (64%) were affected than women (35%), while in Sweden and Finland more women (62%) than men (38%) were affected (Storm *et al*., 2014; Gylfe *et al*., 2018). This is most likely due to men in South Africa being bitten by mosquitoes more due to working outdoors and on farms, while in Europe women take walks in the countryside and are therefore more exposed to mosquito bites. The average age of infected persons in South Africa is 42 years and the average age of infected persons in Finland is 41 years (Storm *et al*., 2014). There is definitely a correlation of SINV with arthritis in Sweden and Finland (Kurkela *et al*., 2008). This is the first study investigating the prevalence of SINV infection in arthritic patients in South Africa. Using the neutralisation assay, 17.6% (29/165) of the arthritis clinic patients was found to be positive for SINV infection. This indicates an overall higher prevalence of anti-SINV antibodies in South African patients than in Northern Europe.

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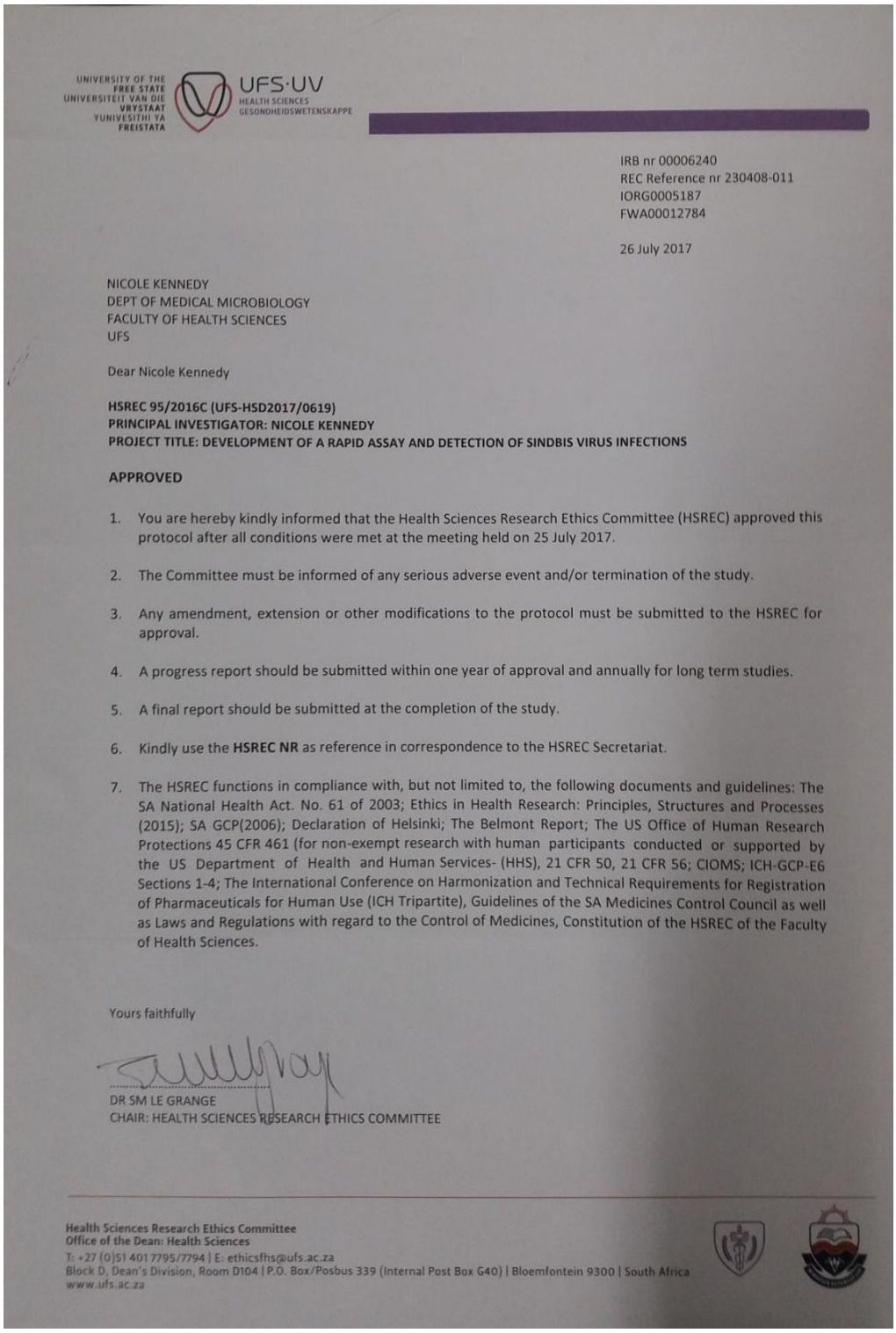
Appendices

Appendix A: Ethics Approval



UNIVERSITY OF THE FREE STATE
UNIVERSITEIT VAN DIE VRYSTAAT
YUNIVESITHI YA FREISTATA

UFS·UV
HEALTH SCIENCES
GESONDHEIDSWETENSKAPPE

IRB nr 00006240
REC Reference nr 230408-011
IORG0005187
FWA00012784

26 July 2017

NICOLE KENNEDY
DEPT OF MEDICAL MICROBIOLOGY
FACULTY OF HEALTH SCIENCES
UFS

Dear Nicole Kennedy

HSREC 95/2016C (UFS-HSD2017/0619)
PRINCIPAL INVESTIGATOR: NICOLE KENNEDY
PROJECT TITLE: DEVELOPMENT OF A RAPID ASSAY AND DETECTION OF SINDBIS VIRUS INFECTIONS

APPROVED

1. You are hereby kindly informed that the Health Sciences Research Ethics Committee (HSREC) approved this protocol after all conditions were met at the meeting held on 25 July 2017.
2. The Committee must be informed of any serious adverse event and/or termination of the study.
3. Any amendment, extension or other modifications to the protocol must be submitted to the HSREC for approval.
4. A progress report should be submitted within one year of approval and annually for long term studies.
5. A final report should be submitted at the completion of the study.
6. Kindly use the **HSREC NR** as reference in correspondence to the HSREC Secretariat.
7. The HSREC functions in compliance with, but not limited to, the following documents and guidelines: The SA National Health Act. No. 61 of 2003; Ethics in Health Research: Principles, Structures and Processes (2015); SA GCP(2006); Declaration of Helsinki; The Belmont Report; The US Office of Human Research Protections 45 CFR 461 (for non-exempt research with human participants conducted or supported by the US Department of Health and Human Services- (HHS), 21 CFR 50, 21 CFR 56; CIOMS; ICH-GCP-E6 Sections 1-4; The International Conference on Harmonization and Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH Tripartite), Guidelines of the SA Medicines Control Council as well as Laws and Regulations with regard to the Control of Medicines, Constitution of the HSREC of the Faculty of Health Sciences.

Yours faithfully

DR SM LE GRANGE
CHAIR: HEALTH SCIENCES RESEARCH ETHICS COMMITTEE

Health Sciences Research Ethics Committee
Office of the Dean: Health Sciences
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HEALTH SCIENCES
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IRB nr 00006240
REC Reference nr 230408-011
IORG0005187
FWA00012784

30 August 2017

NICOLE KENNEDY
DEPT OF MEDICAL MICROBIOLOGY
FACULTY OF HEALTH SCIENCES
UFS

Dear Nicole Kennedy

HSREC 95/2016C
PROJECT TITLE: THE DEVELOPMENT OF A RAPID ASSAY AND SEROLOGICAL DETECTION OF SINDBIS VIRUS INFECTION

1. You are hereby kindly informed that the Health Sciences Research Ethics Committee (HSREC) took note and approved the following at the meeting held on 29 August 2017:

   - *Change title from "Development of a rapid assay and detection of Sindbis virus infections" to "The development of a rapid assay and serological detection of Sindbis virus infection"*

2. Kindly use the **HSREC NR** as reference in correspondence to HSREC Administration.

3. The HSREC functions in compliance with, but not limited to, the following documents and guidelines: The SA National Health Act. No. 61 of 2003; Ethics in Health Research: Principles, Structures and Processes (2015); SA GCP(2006); Declaration of Helsinki; The Belmont Report; The US Office of Human Research Protections 45 CFR 461 (for non-exempt research with human participants conducted or supported by the US Department of Health and Human Services- (HHS), 21 CFR 50, 21 CFR 56; CIOMS; ICH-GCP-E6 Sections 1-4; The International Conference on Harmonization and Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH Tripartite), Guidelines of the SA Medicines Control Council as well as Laws and Regulations with regard to the Control of Medicines, Constitution of the Ethics Committee of the Faculty of Health Sciences.

Yours faithfully

.....
DR SM LE GRANGE
CHAIR: HEALTH SCIENCES RESEARCH ETHICS COMMITTEE

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[redacted]

Health Sciences Research Ethics Committee

28-Feb-2019

Dear **Miss Nicole Kennedy**

Ethics Number: UFS-HSD2017/0619

Ethics Clearance: **Development of in house assays for detection of Sindbis virus infection**

Principal Investigator: **Miss Nicole Kennedy**

Department: **Medical Microbiology Department (Bloemfontein Campus)**

SUBSEQUENT SUBMISSION APPROVED

With reference to your recent submission for ethical clearance from the Health Sciences Research Ethics Committee. I am pleased to inform you on behalf of the HSREC that you have been granted ethical clearance for your request as stipulated below:

Minor Amendment and title change from *Development of a rapid assay and detection of Sindbis virus infections* to *Development of in house assays for detection of Sindbis virus infection.*

The HSREC functions in compliance with, but not limited to, the following documents and guidelines: The SA National Health Act. No. 61 of 2003; Ethics in Health Research: Principles, Structures and Processes (2015); SA GCP(2006); Declaration of Helsinki; The Belmont Report; The US Office of Human Research Protections 45 CFR 461 (for non-exempt research with human participants conducted or supported by the US Department of Health and Human Services- (HHS), 21 CFR 50, 21 CFR 56; CIOMS; ICH-GCP-E6 Sections 1-4; The International Conference on Harmonization and Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH Tripartite), Guidelines of the SA Medicines Control Council as well as Laws and Regulations with regard to the Control of Medicines, Constitution of the HSREC of the Faculty of Health Sciences.

For any questions or concerns, please feel free to contact HSREC Administration: 051-4017794/5 or email EthicsFHS@ufs.ac.za.

Thank you for submitting this request for ethical clearance and we wish you continued success with your research.

Yours Sincerely

Dr. SM Le Grange
Chair : Health Sciences Research Ethics Committee

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Appendix B: Raw data for ELISA:

Plate

1.1

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	1.708	0.312	0.218	0.147	0.161	0.153	0.559	1.490	0.249	1.041	0.102	0.253
B	0.154	0.165	0.116	0.200	0.269	0.536	1.949	0.876	0.283	1.654	0.071	0.085
C	0.311	0.185	0.161	0.188	0.211	0.222	0.139	0.149	0.126	0.141	0.096	0.052
D	0.139	0.087	0.357	0.071	0.125	1.771	0.144	0.114	0.314	0.428	0.697	0.809
E	0.121	0.218	0.166	0.129	0.105	0.112	0.106	0.130	0.096	0.332	0.048	0.068
F	0.032	0.035	0.027	0.035	0.034	0.033	0.040	0.037	0.043	0.035	0.071	0.065
G	0.048	0.027	0.026	0.026	0.030	0.028	0.040	0.038	0.043	0.037	0.095	0.055
H	0.041	0.035	0.037	0.031	0.032	0.026	0.030	0.033	0.032	0.031	0.116	0.133

Plate

1.2

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	0.067	0.052	0.205	0.419	2.009	0.149	0.183	0.595	0.070	0.082	0.307	0.116
B	0.224	0.127	0.195	0.197	0.055	0.104	0.691	0.110	0.081	0.505	0.110	0.055
C	0.166	0.161	0.435	1.869	0.070	0.120	0.077	1.403	0.091	0.176	1.557	0.186
D	1.643	0.485	0.098	0.094	0.492	0.095	0.076	0.109	0.068	0.326	1.397	1.189
E	0.115	0.116	0.169	0.212	0.333	0.101	0.175	0.252	0.050	0.067	0.083	0.112
F	0.229	0.114	0.210	0.063	0.096	0.119	0.069	0.182	0.087	0.076	0.058	0.082
G	0.116	0.178	0.265	0.090	0.128	0.141	0.130	0.159	0.102	0.219	0.128	0.305
H	0.134	0.173	0.123	0.140	0.504	0.151	0.168	0.144	0.110	0.281	0.090	0.100

Plate

2.1

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	1.4950	0.4030	0.1760	0.2460	0.2880	1.6440	0.0910	0.3720	0.0920	0.1020	0.1080	0.2760
B	0.4020	0.2300	0.1330	1.6980	0.1050	0.0940	0.4840	0.2710	0.2080	0.0730	0.0580	0.0880
C	0.2720	0.1900	0.1550	0.0890	0.1070	0.0860	0.1590	0.1220	0.0820	0.2010	0.0960	0.0620
D	0.2400	0.9530	0.0860	0.2210	0.1040	0.1130	0.8570	0.9360	0.1070	0.1170	0.8940	0.9520
E	0.1300	0.1790	0.0960	0.1440	0.1680	0.0900	0.0540	0.1010	0.0590	0.0750	0.0620	0.1010
F	0.3680	0.1500	0.0940	0.0860	0.0750	0.1060	0.1090	0.3660	0.0960	0.0610	0.0340	0.0550
G	0.1480	0.1330	0.1590	0.0720	0.0820	0.0740	0.1450	0.1300	0.0570	0.1230	0.0770	0.0480
H	0.1910	0.1200	0.1040	0.1360	0.1140	0.0700	0.0780	0.2130	0.0860	0.1340	0.1080	0.1370

Plate

2.2

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	0.0970	0.2580	0.1760	0.1870	0.2310	0.2200	2.1330	0.2210	0.2170	0.2390	0.2080	0.1200
B	0.1320	0.2230	0.3440	0.3550	0.1550	1.8340	0.1740	0.1500	0.1360	0.1390	0.1160	0.0760
C	0.1530	0.1200	0.1750	0.1240	0.1290	0.1170	0.0970	0.1350	0.0800	0.2220	1.3280	0.0910
D	0.1160	0.1340	0.2600	0.1730	0.2730	0.0240	0.0240	0.0210	0.0190	0.0210	1.1900	1.1460
E	0.0990	0.1840	0.1230	0.1680	0.1540	0.1340	0.1150	0.1410	0.1210	0.1300	0.0700	0.0960
F	0.1070	0.1340	0.1740	0.1660	0.0700	0.1450	0.1030	0.0870	0.0660	0.0880	0.0440	0.0750
G	0.1870	0.1110	0.1070	0.1050	0.1060	0.0960	0.0920	0.1580	0.0780	0.2540	0.0640	0.1010
H	0.0960	0.1250	0.1480	0.1160	0.2100	0.1700	0.1060	0.1870	0.1280	0.1280	0.1690	0.1830

Plate

3.1

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	0.2460	0.2400	0.1880	0.1120	0.1540	0.2450	0.3200	1.0550	0.3440	0.1450	0.1780	0.5180
B	0.1080	0.1540	0.1750	0.1500	0.1130	0.1020	0.1970	1.6810	0.2880	0.0480	0.0550	0.2350
C	0.3800	0.1200	0.1270	0.2140	0.2240	0.1070	0.1870	0.2480	0.2490	0.2770	0.2810	0.1670
D	0.2080	0.2310	0.2890	0.1190	0.3460	0.3180	0.2170	1.1520	0.3600	0.2610	1.4890	1.5340
E	0.4150	0.1300	0.1320	0.1110	0.1060	0.1380	0.1170	0.0560	0.1430	0.1120	0.0710	0.1520
F	0.0800	0.0680	0.0660	0.1140	0.1120	0.0770	0.0720	0.0750	0.1260	0.0380	0.0670	0.1030
G	0.1780	0.0960	0.1050	0.1230	0.1640	0.0960	0.1230	0.1250	0.1130	0.1530	0.1650	0.0700
H	0.1360	0.1470	0.1370	0.0910	0.1240	0.0880	0.1120	0.0760	0.3400	0.1350	0.1040	0.1020

Plate

3.2

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	0.0650	0.1620	0.1940	0.1660	0.1090	0.1380	1.3310	0.1140	0.1580	0.1510	0.2730	0.1310
B	0.1210	0.0960	0.1560	2.1100	0.1160	0.1470	0.1040	0.1060	0.2350	0.1090	0.1720	0.1130
C	0.2510	0.5370	0.2060	2.0300	0.2010	0.2460	0.1840	0.0950	1.0410	0.1580	1.3800	0.2660
D	0.0840	0.1990	0.1820	0.2730	0.1900	0.1310	0.1460	0.1230	0.1030	0.1600	1.1010	1.1750
E	0.0880	0.0980	0.1590	0.0890	0.1080	0.0980	0.0950	0.0750	0.1010	0.0690	0.0970	0.0850
F	0.0900	0.0930	0.0630	0.1010	0.0920	0.0860	0.0840	0.0620	0.1750	0.0620	0.0500	0.0630
G	0.1020	0.1190	0.0860	0.0940	0.1340	0.1010	0.0980	0.0570	0.0880	0.0620	0.0850	0.1960
H	0.1030	0.0900	0.1390	0.0950	0.0960	0.0710	0.1660	0.0780	0.0780	0.0600	0.0860	0.0700

Plate4

.1

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	0.0890	0.0650	0.0580	0.0580	0.0800	0.0680	0.0500	0.0770	0.0950	0.0650	0.0500	0.2930
B	0.0760	0.1190	0.1040	0.0560	0.0740	0.0560	0.0490	0.0660	0.0530	0.0540	0.0460	0.0700
C	0.0930	0.0510	0.0710	0.1100	0.0540	0.0770	0.0540	0.1060	0.0850	0.0600	0.1130	0.0570
D	2.0150	0.0760	1.5210	0.0580	0.1050	0.0890	0.0390	0.0810	0.0560	1.4270	1.3000	1.2600
E	0.1520	0.0940	0.0630	0.0800	0.0790	0.1130	0.0870	0.0890	0.0550	0.0530	0.0520	0.0930
F	0.1250	0.1970	0.1800	0.0710	0.0940	0.0730	0.0570	0.0740	0.0780	0.0960	0.0390	0.0900
G	0.1400	0.0550	0.0870	0.1260	0.0560	0.1730	0.0640	0.1010	0.1220	0.0560	0.1240	0.0500
H	0.1480	0.0790	0.0520	0.0500	0.0640	0.1080	0.0450	0.0920	0.0500	0.0780	0.0570	0.0650

Plate

4.2

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	0.0610	0.0780	0.0850	0.0770	0.0890	0.0640	0.2260	0.1600	0.0980	0.1340	0.1980	0.0730
B	0.1040	0.0480	0.0650	0.0600	1.7020	0.0490	1.4530	0.0630	0.0610	0.0590	0.1040	0.0680
C	0.1230	0.0650	0.0780	0.1020	0.1640	0.0730	0.0830	0.1470	0.0930	0.1320	1.5170	0.2010
D	0.1620	0.8880	0.5070	0.1050	1.2410	0.0630	1.2640	0.0960	0.3190	0.1060	1.1300	1.2260
E	0.0640	0.0820	0.0630	0.0850	0.0930	0.0520	0.1870	0.0860	0.1060	0.0680	0.0760	0.0700
F	0.1430	0.0580	0.0770	0.0550	0.1400	0.0420	0.0610	0.0560	0.0610	0.0530	0.0470	0.0560
G	0.1370	0.0650	0.0920	0.1270	0.1170	0.0620	0.0940	0.0930	0.1110	0.1160	0.0660	0.2190
H	0.1610	0.0880	0.0540	0.1450	0.0910	0.0560	0.0650	0.0820	0.4480	0.1260	0.0750	0.0730

Plate

5.1

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	0.1820	0.1140	0.1660	0.1380	0.1410	0.0570	0.1650	0.1100	0.1160	0.1320	0.0640	0.3890
B	0.0900	0.0990	0.3000	0.0730	0.0470	0.1080	0.0550	1.3020	0.1250	0.0640	0.0520	0.0890
C	0.0680	0.0720	0.1140	0.0770	0.0670	0.0690	0.0640	0.1160	0.0490	0.0910	0.1370	0.0570
D	0.0850	0.1340	0.8770	0.2240	0.0380	0.0640	0.0510	0.6200	0.1240	1.0710	1.2190	1.3560
E	0.0720	0.0710	0.1240	0.1190	0.1010	0.0700	0.0730	0.0730	0.1120	0.1350	0.0710	0.1110
F	0.0920	0.0970	0.0670	0.0800	0.0550	0.0600	0.0530	0.0650	0.1710	0.0930	0.0450	0.0860
G	0.0570	0.0780	0.1090	0.0550	0.0800	0.0690	0.0640	0.1060	0.0470	0.1010	0.1440	0.0660
H	0.0500	0.1570	0.1170	0.1490	0.0430	0.0640	0.0510	0.1080	0.0790	0.1150	0.0750	0.1070

Plate

5.2

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	0.0810	0.0840	0.1080	0.1040	1.2970	0.1080	0.0700	0.1340	0.1430	0.1220	0.2350	0.0710
B	1.9070	0.1360	0.1780	0.0720	0.1190	0.0630	0.1260	0.0620	1.3250	0.0590	0.1070	0.0530
C	0.0770	0.0730	0.0530	0.1050	0.1310	0.0480	0.0700	0.0400	0.0510	0.0520	1.2030	0.2930
D	0.1270	0.1130	0.1160	0.0700	0.0940	1.3740	0.0910	0.1140	0.0670	0.8210	1.2260	1.5380
E	0.0750	0.0880	0.0920	0.0800	0.0630	0.1050	0.0550	0.1010	0.1150	0.0780	0.1020	0.0810
F	0.0860	0.1450	0.1460	0.0880	0.1100	0.0790	0.1150	0.0540	0.0720	0.1030	0.0480	0.0640
G	0.0790	0.0680	0.0650	0.1200	0.1360	0.0510	0.0570	0.0500	0.0590	0.1090	0.0670	0.3980
H	0.1170	0.1010	0.1160	0.0870	0.1120	0.0790	0.0880	0.1420	0.0870	0.0340	0.0760	0.0830

Plate

6.1

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	2.0830	0.1670	0.1460	0.1150	0.1850	0.1330	0.1220	0.1100	0.3230	0.0690	0.1550	0.3490
B	0.0380	0.2710	0.1820	0.1690	0.7790	0.1930	0.2560	0.1460	0.2080	0.1610	0.0910	0.1900
C	0.1700	0.2240	0.1840	0.2020	0.1370	0.1530	0.1580	0.1850	0.2130	0.2260	0.2590	0.1050
D	0.2050	0.2370	0.1240	0.1300	0.1020	0.1670	0.1530	0.1520	0.1170	0.0990	1.3780	1.4700
E	0.1350	0.0870	0.1210	0.0750	0.1900	0.0810	0.1040	0.0930	0.2290	0.0610	0.0590	0.1380
F	0.0400	0.1130	0.1330	0.0950	0.1320	0.3200	0.2610	0.1150	0.1020	0.1190	0.0540	0.1070
G	0.1240	0.1500	0.1120	0.0920	0.1590	0.1510	0.1400	0.1210	0.1300	0.1660	0.1160	0.0680
H	0.1700	0.2800	0.1090	0.1000	0.0960	0.1490	0.1490	0.1480	0.1120	0.1400	0.0890	0.1090

Plate

6.2

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	0.1930	0.1210	0.1990	0.2270	0.1990	0.0440	0.0560	0.1400	0.1160	0.1310	0.3200	0.1350
B	0.2960	0.1120	0.3310	0.1150	1.9740	0.1460	0.2340	0.5580	0.0590	0.1410	0.1750	0.1490
C	0.1400	0.1400	0.1320	0.2410	0.1200	0.1500	0.1620	0.2380	0.1220	0.1340	1.8600	0.5960
D	1.7290	0.1930	0.1260	0.1890	0.3830	0.1730	0.2670	0.2880	0.0220	0.0360	1.6430	1.7410
E	0.1700	0.1460	0.3120	0.2120	0.1730	0.0610	0.0500	0.0920	0.1120	0.1120	0.1060	0.1100
F	0.2710	0.0690	0.0580	0.0710	0.1840	0.0980	0.0620	0.1300	0.0600	0.1140	0.0620	0.0720
G	0.1180	0.1120	0.0770	0.2770	0.1090	0.1310	0.1420	0.1540	0.1690	0.0700	0.0860	0.3790
H	0.2530	0.1990	0.1360	0.1670	0.2610	0.1750	0.1120	0.1340	0.0190	0.0460	0.0950	0.0010

Plate

7.1

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	0.1270	0.1960	0.0830	0.2070	0.1520	0.0440	0.0950	0.0890	0.1520	0.0740	0.0530	0.4310
B	0.1090	0.0960	0.1000	0.0660	0.1060	1.5200	0.1100	0.0630	0.1360	0.1000	0.0610	0.1200
C	0.1030	0.0680	0.0870	0.5710	1.5040	0.0570	0.0930	0.0790	0.1170	0.0790	0.1190	0.0770
D	0.0730	0.0520	0.1370	0.0720	0.0650	0.0980	0.0900	0.2130	0.1040	0.0910	1.4400	1.4010
E	0.0950	0.1440	0.0630	0.1880	0.0990	0.0430	0.0770	0.1040	0.0700	0.0730	0.0700	0.1490
F	0.1030	0.0780	0.1110	0.0480	0.0970	0.1020	0.0770	0.0780	0.0920	0.0970	0.0650	0.1090
G	0.1120	0.0910	0.1510	0.0830	0.1280	0.0420	0.1230	0.0740	0.0770	0.0800	0.1920	0.0960
H	0.2240	0.0680	0.1880	0.0820	0.0790	0.1970	0.1420	0.1030	0.0910	0.1490	0.1100	0.1160

Plate

7.2

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	0.0710	0.0830	0.0600	0.0790	0.1660	0.0620	0.0430	0.0460	0.0530	0.0750	0.1300	0.0890
B	0.0530	0.0660	0.0530	0.0750	0.0570	0.0870	0.0510	0.0330	0.0750	0.0540	0.0950	0.0600
C	1.8640	0.5070	0.0550	0.0700	0.0600	0.0730	0.0540	0.0530	0.0760	0.0990	1.4690	0.1260
D	0.0690	0.0380	0.0580	0.1090	0.0970	0.0530	0.0580	0.0570	1.3930	0.0510	1.0510	1.3570
E	0.0790	0.0960	0.0790	0.1030	0.2430	0.1130	0.0440	0.0540	0.1060	0.0930	0.0930	0.0850
F	0.0440	0.0690	0.0750	0.0720	0.0550	0.0890	0.0780	0.0410	0.0800	0.0830	0.0500	0.0760
G	0.1010	0.1280	0.0730	0.0730	0.0460	0.0890	0.0790	0.0630	0.0960	0.1730	0.0830	0.2190
H	0.1670	0.0470	0.1040	0.1610	0.1350	0.0730	0.1080	0.0940	0.0850	0.0930	0.1050	0.0910

Plate
8

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	0.1120	0.1030	0.0930	0.1060	0.0820	0.0910	0.1470	0.1000	0.0310	0.0200	0.0610	0.5550
B	0.0280	0.0270	0.0280	0.0270	0.0280	0.0260	0.0270	0.0240	0.0250	0.0250	0.0530	0.0930
C	0.0230	0.0280	0.0230	0.0250	0.0250	0.0220	0.0210	0.0220	0.0230	0.0190	0.1390	0.0670
D	0.0250	0.0250	0.0240	0.0250	0.0330	0.0350	0.0290	0.0240	0.0270	0.0220	1.2620	1.0360
E	0.1890	0.0510	0.1390	0.1370	0.0980	0.0830	0.1580	0.1560	0.0200	0.0190	0.0530	0.1200
F	0.0280	0.0270	0.0370	0.0410	0.0360	0.0320	0.0260	0.0260	0.0220	0.0200	0.0510	0.0970
G	0.0320	0.0240	0.0230	0.0240	0.0270	0.0270	0.0220	0.0270	0.0230	0.0190	0.1150	0.0620
H	0.0490	0.0310	0.0230	0.0230	0.0210	0.0200	0.0190	0.0210	0.0170	0.0210	0.0810	0.0860

Appendix C: Composition of media, buffers and solutions used

1. ELISA Wash Buffer

- 2 L Phosphate buffered saline (PBS) pH 7.4
- 2 ml Tween 20

2. ELISA Diluent Buffer

- 0.2 g Skim milk powder
- 10 ml PBS

3. ELISA Blocking Buffer

- 1 g Skim milk powder
- 10 ml PBS

4. Super Optimal broth with catabolite repression (SOC) (1L)

- 20g Bacto-Tryptone
- 5g Bacto-Yeast extract
- 0.5g NaCl
- 2.5ml 1 M KCl
- 900ml deionised water
- Adjust pH to 7.0 with 10 M NaOH (~100 µL)
- Adjust volume to 970ml with H_2O
- Add 10ml 1M $MgCl_2$ and 20ml 1 M glucose before use a
- Store at 4°C

5. Luria Bertani broth (1L) with ampicillin

- 10g Bacto-Tryptone
- 5g Bacto-Yeast extract
- 10g NaCl
- 900ml dH_2O
- Adjust pH to 7 .0 with 10 M NaOH (app. 200 µL)
- Adjust volume to 1 liter with deionized H_2O.
- Sterilize by autoclaving and allow cooling down
- Add 100mg/ml of ampicillin
- Store at 4°C.

6. Luria Bertani agar plates (1L) with ampicillin

- 15g Bacteriological-agar
- 1L Liquid media
- Sterilization by autoclaving
- Allow to cool
- Add ampicillin
- Pour into sterile plates
- Store in an inverted position at 4°C after solidification

7. 50 X Tris-acetate-EDTA (TAE) stock

- Tris-base: 242g
- Acetate (100% acetic acid): 57.1ml
- EDTA: 100ml (0.5M sodium EDTA)

- Add dH_2O to one liter.

8. 1X TAE (pH 8.0)

- Dilute 20ml of 50X stock into 980ml dH 2 O

9. 1% Agarose gel

- 1 g Seakem® LE agarose powder (Lonza, Maine, USA)
- 100ml of 1XTAE buffer pH 8
- Heat mixture in a microwave oven until dissolved
- Wait until cool

10. 1% PBSTween20

- 1 ml Tween20
- 100 ml PBS

11. IFA Blocking Buffer

- 50 ml 1x PBS
- 50g saccharose
- 250µl triton X-100
- Mix thoroughly

Appendix D: Net OD values of positive samples

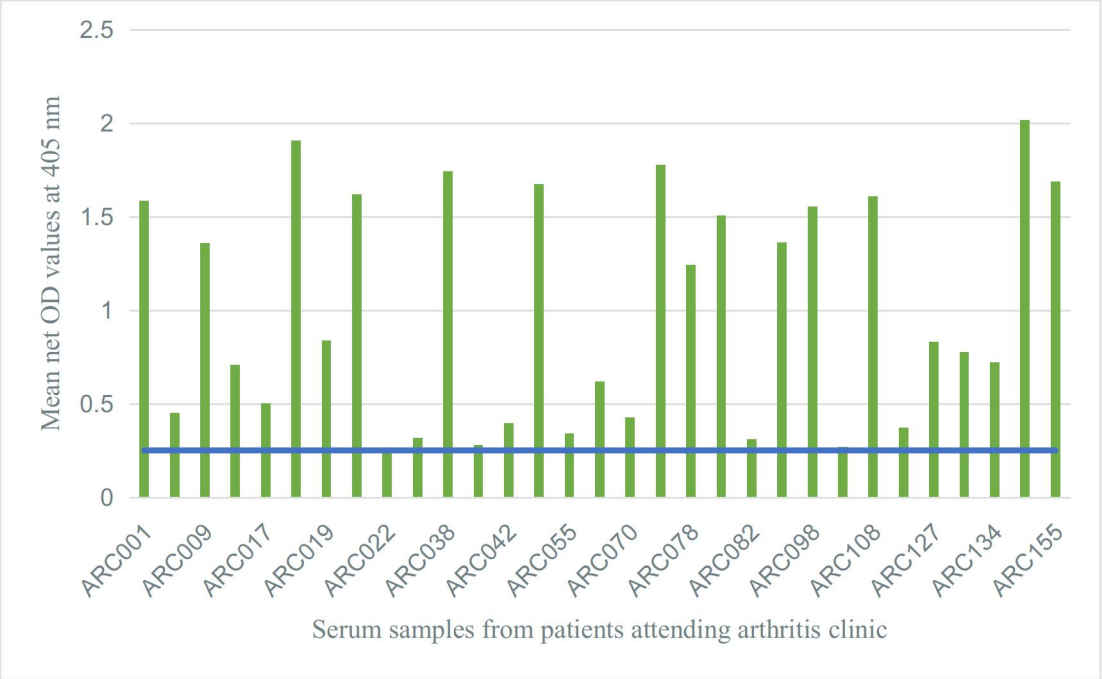


Figure D1 Net OD values calculated for IgG positive serum samples from patients attending arthritis clinic. A cut off of 0.253 was used to differentiate positive from negative samples.

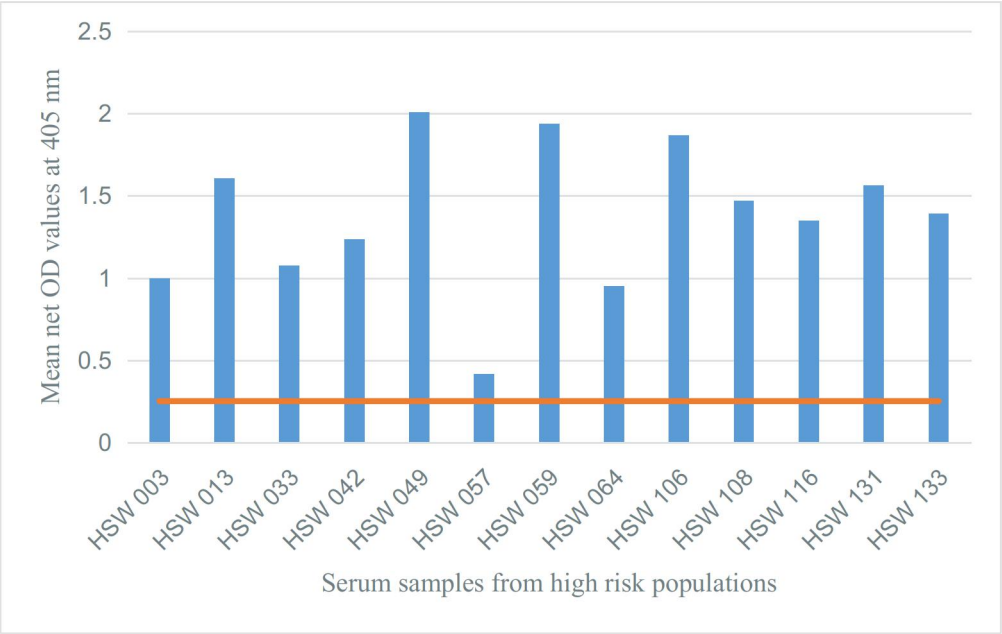


Figure D2 Net OD values calculated for IgG positive serum samples from high risk populations. A cut off of 0.253 was used to differentiate positive from negative samples.

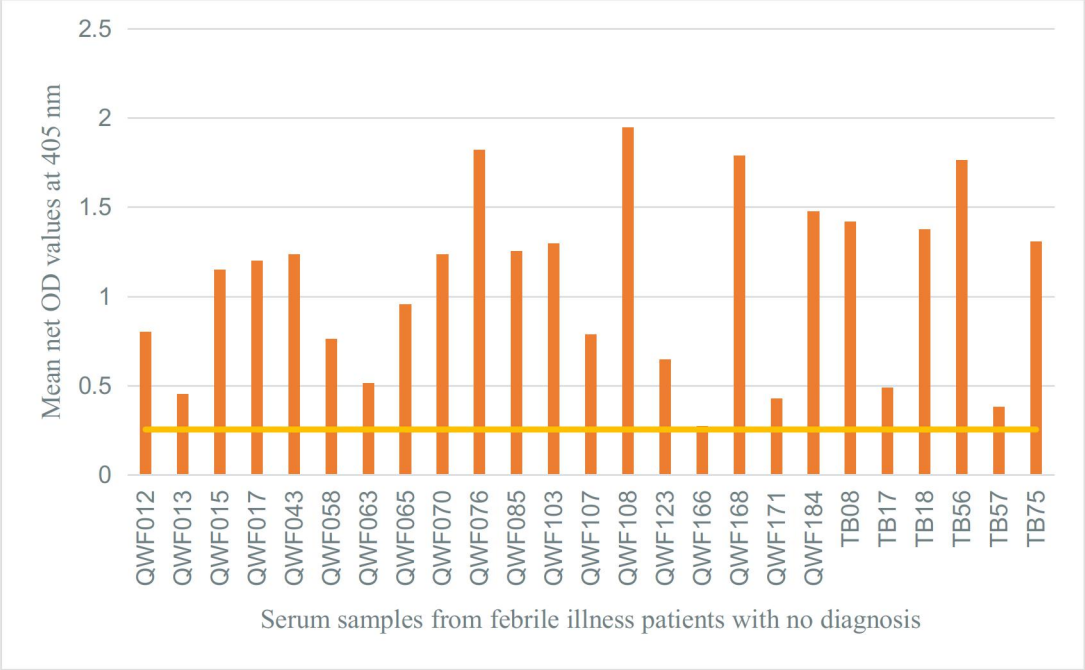


Figure 2.12 Net OD values calculated for IgG positive serum samples from patients with febrile illness. A cut off of 0.253 was used to differentiate positive from negative samples.

Appendix E: Tables showing summaries of results for ELISA, commercial assay and neutralisationa ssay

Table E1 Summary of the results of all assays for the arthritis clinic samples. Negative and indeterminate results are highlighted in yellow.

Sample Number	Net OD Value	Percent Positivity	Commercial Assay	Antibody Titer
ARC001	1,587	137,15%	Positive	320
ARC007	0,453	39,15%	Positive	160
ARC009	1,36	117,53%	Positive	≥ 640
ARC011	0,709	61,27%	Negative	40
ARC017	0,503	43,47%	Negative	20
ARC018	1,909	164,97%	Positive	320
ARC019	0,839	72,51%	Positive	80
ARC021	1,619	139,91%	Negative	320
ARC022	0,263	22,73%	Negative	40
ARC035	0,32	27,65%	Positive	80
ARC038	1,745	150,80%	Positive	40
ARC041	0,282	24,37%	Negative	20
ARC042	0,397	34,31%	Positive	20
ARC050	1,676	144,84%	Positive	20
ARC055	0,343	29,64%	Negative	≥ 640
ARC065	0,622	53,75%	Positive	40
ARC070	0,429	37,07%	Positive	40
ARC074	1,779	153,74%	Positive	80
ARC078	1,244	107,50%	Positive	80
ARC081	1,509	130,41%	Positive	40
ARC082	0,312	26,96%	Positive	≥ 640

Sample Number	Net OD Value	Percent Positivity	Commercial Assay	Antibody Titer
ARC092	1,365	117,96%	Positive	320
ARC098	1,554	134,29%	Positive	Indeterminate
ARC100	0,271	23,42%	Negative	Indeterminate
ARC108	1,612	139,31%	Positive	80
ARC111	0,375	32,41%	Positive	20
ARC127	0,833	71,99%	Positive	≥ 640
ARC133	0,779	67,32%	Negative	80
ARC134	0,723	62,48%	Negative	≥ 640
ARC143	2,018	174,39%	Positive	≥ 640
ARC155	1,689	145,96%	Negative	40

Table E2 Summary of the results of all assays for the high risk population samples. Negative and indeterminate results are highlighted in yellow.

Sample Number	Net OD Value	Percent Positivity	Commercial Assay	Antibody Titer
HSW 003	0,999	86,33%	Negative	40
HSW 013	1,606	138,79%	Positive	≥ 640
HSW 033	1,076	92,99%	Positive	160
HSW 042	1,236	106,81%	Indeterminate	160
HSW 049	2,009	173,61%	Positive	40
HSW 057	0,418	36,12%	Positive	≥ 640
HSW 059	1,936	167,31%	Positive	≥ 640
HSW 064	0,953	82,36%	Positive	≥ 640
HSW 106	1,867	161,34%	Positive	160
HSW 108	1,469	126,95%	Positive	≥ 640
HSW 116	1,349	116,58%	Positive	≥ 640
HSW 131	1,562	134,99%	Positive	40
HSW 133	1,392	120,29%	Positive	80

Table E3 Summary of results of all assays for febrile illness without diagnosis samples. Negative and indeterminate results are highlighted in yellow.

Sample Number	Net OD Value	Percent Positivity	Commercial Assay	Antibody Titer
QWF012	0,8	69,13%	Indeterminate	80
QWF013	0,453	39,15%	Positive	40
QWF015	1,15	99,38%	Positive	≥ 640
QWF017	1,199	103,62%	Positive	≥ 640
QWF043	1,237	106,90%	Positive	≥ 640
QWF058	0,76	65,68%	Indeterminate	≥ 640
QWF063	0,512	44,25%	Indeterminate	320
QWF065	0,956	82,62%	Indeterminate	320
QWF070	1,234	106,64%	Indeterminate	≥ 640
QWF076	1,821	157,37%	Positive	≥ 640
QWF085	1,253	108,28%	Positive	≥ 640
QWF103	1,295	111,91%	Positive	160
QWF107	0,787	68,01%	Negative	160
QWF108	1,948	168,34%	Negative	≥ 640
QWF123	0,647	55,91%	Positive	≥ 640
QWF166	0,273	23,59%	Negative	≥ 640
QWF168	1,79	154,69%	Positive	Negative
QWF171	0,428	36,99%	Negative	320
QWF184	1,476	127,55%	Positive	320
TB08	1,418	122,54%	Positive	Indeterminate
TB17	0,488	42,17%	Indeterminate	≥ 640
TB18	1,376	118,91%	Positive	320
TB56	1,763	152,36%	Positive	320
TB57	0,379	32,75%	Indeterminate	320
TB75	1,308	113,03%	Positive	160

Appendix F: Figures showing the net OD values and PP values for commercial assay and neutralisation assay results

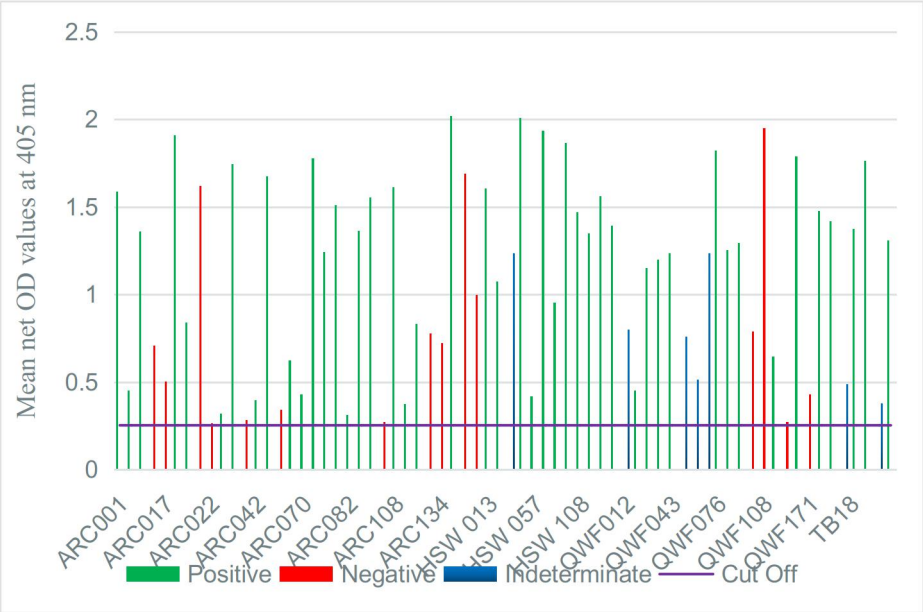


Figure F1 Net OD values for positive serum samples that were tested with the commercial assay with the cut off of 0.253.

Figure F2 PP values for positive serum samples that were tested with the commercial assay with the cut off of 21.88%.

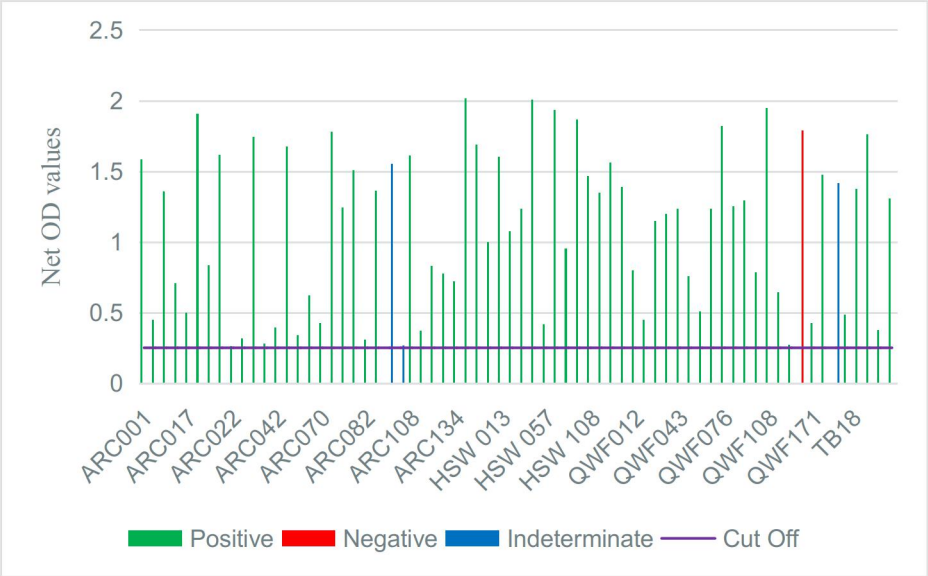


Figure F3 Net OD values for positive serum samples that were tested with the neutralisation assay with the cut off of 0.253.

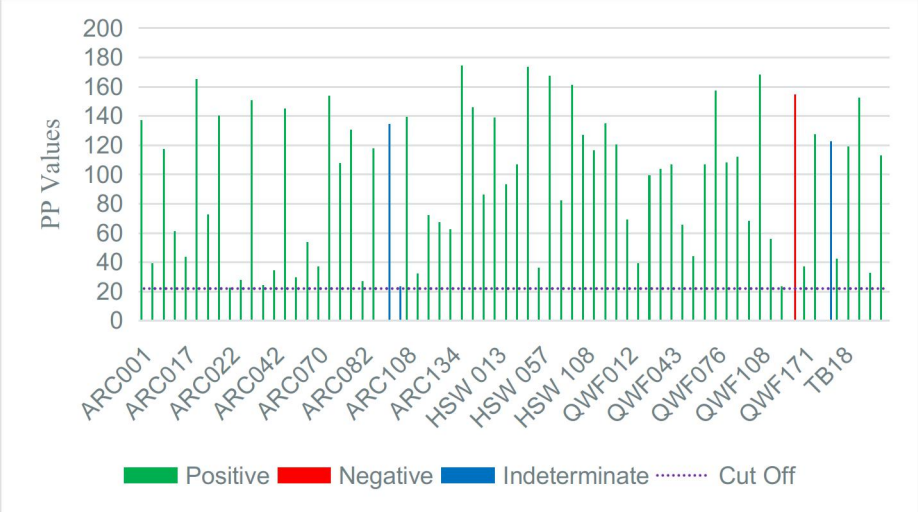
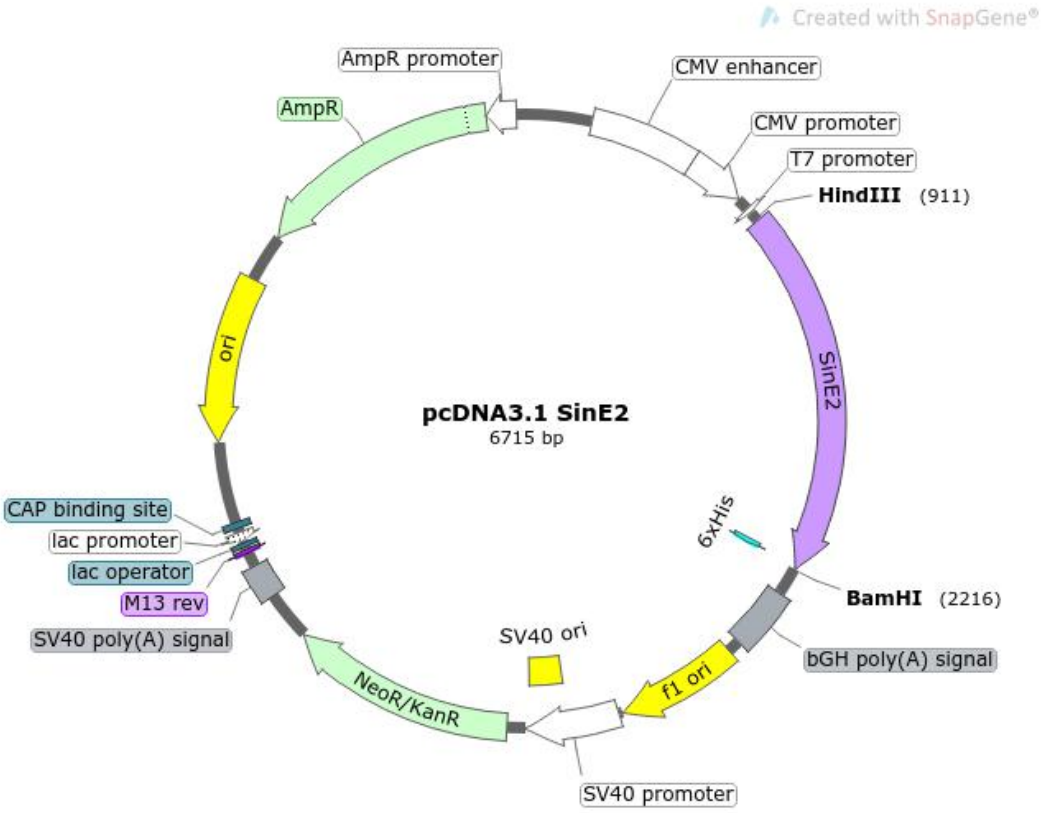


Figure F4 PP values for positive serum samples that were tested with the neutralisation assay with the cut off of 21.88%.

Appendix G: Vector Map of pcDNA3.1 SinE2 plasmid



Appendix H: Sequence of pcDNA3.1 SinE2 plasmid

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*Hind*III restriction site

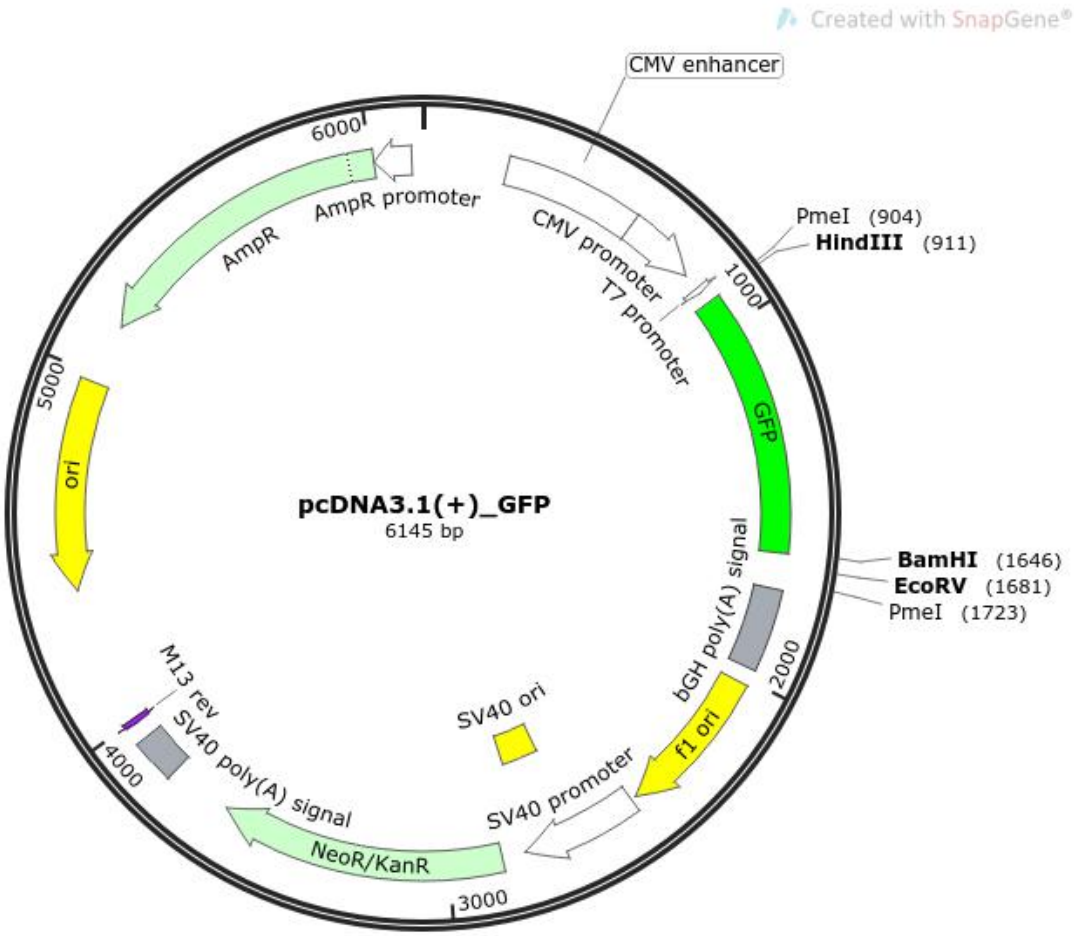
Kozak translation sequence

His tag

Stop codon

*BamH*I restriction site

Appendix I: Vector Map of pcDNA3.1 GFP plasmid



Appendix J

Table J1 Components of sequencing reaction.

Components	Volume (µl)
Ready reaction	1
Sequencing primer (0.8 pmol/µl)	4
Dilution buffer	2
Nuclease free water	1
Template DNA (679.9 ng/µl)	2
Total	10

Table J2 Components of the sequencing cleanup.

Components	Volume (µl)
Nuclease free water	10
125 mM EDTA	5
Absolute ethanol	60
Total	75