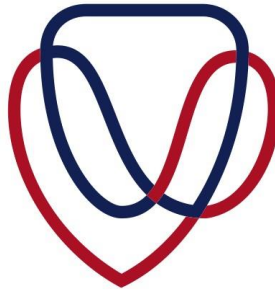


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Preparation of multiplex immunofluorescent platform for serology

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Preparation of multiplex immunofluorescent platform for serology

by

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DECLARATION

I, Nyiko Given Maswanganyi, solemnly declare that the dissertation presented for the M.Med.Sc. Virology qualification at the University of the Free State is the result of my independent effort. I affirm that this work has not been previously submitted for any qualification at another university or faculty.



Nyiko Given Maswanganyi

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To God, the Source of wisdom and guidance:

'Trust in the Lord with all your heart and lean not on your own understanding; in all your ways submit to him, and he will make your paths straight.'

-Proverbs 3:5-6:

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List of abbreviations

°C- Degrees celsius

µg – microgram

µl- Microliter

µM – micromolar

ABTS- 2,2'-azino-di-3-ethylbenzthiazoline-6-sulfonate

ARDS- acute respiratory distress syndrome

Amp- Ampicillin

ATCC- American Type Culture Collection

BBS- borate- buffered saline

BHK-21- Baby Hamster Kidney

BLAST – Basic Local Alignment Search

bp – base pair

BSL-2- biosafety level 2

BSL-4 – biosafety level 4

BSL-3- biosafety level 3

BBB- Blood brain barrier

C- Capsid

CNS- central nervous system

CSF- cerebrospinal fluid

CCHF – Crimean-Congo hemorrhagic fever

CCHFV- Crimean-Congo hemorrhagic fever virus

CHIKV- chikungunya virus

CPE- Cytopathic effects

CO₂- Carbon dioxide

Cx- Culex

DNA- Deoxyribonucleic acid

dH₂O- Distilled water

DMEM- Dulbecco's Modified Eagle Medium

DMSO – dimethyl sulfoxide

DENV- dengue virus

DNA – deoxyribonucleic acid
ED-III- Envelope domain III
E. coli – Escherichia coli
EDTA- Ethylene-diamine-tetra-acetic acid
ELISA- Enzyme-linked immunosorbent assay
E- envelope protein
eGFP- enhanced green fluorescent protein
FBS- Fetal bovine serum
FITC- Fluorescein isothiocyanate
FDA- Food and drug Administration
GFP- Green fluorescent protein
GPC- glycoprotein precursor complex
HEK-293- Human embryonic kidney-293
His- Histidine
HRPO- Horse radish peroxidase
IFA- Immunofluorescence assay
IgG- Immunoglobulin G
IgM- Immunoglobulin M
ICTV- International Committee for the Taxonomy of Viruses
IL- interleukin
JEV- Japanese encephalitis virus
kDa – kiloDaltons
KZN- KwaZulu-Natal
L – Large
LB- Luria-Bertani
L-glut- L-glutamine
MAYV- Mayaro virus
M – Medium
ml- Milliliter
mM-Millimolar
MEM- minimum essential media

MOI- multiplicity of infection
MVEV- Murray valley encephalitis virus
ng – nanogram
nm – nanometer
NP- Nucleocapsid protein
NSs- non-structural proteins
NSm- non-structural M protein
ONNV- o'nyong nyong virus
OD- Optical density
ORF – open reading frame
OTU- ovarian-tumor-like
PBS- Phosphate buffered saline
PBS-T – 1× PBS containing 1% Tween® 20
PCR – polymerase chain reaction
pen/strep- penicillin and streptomycin
PP- Percentage positive
prM- pre-membrane
PVDF – Polyvinylidene fluoride
RdRp- RNA-dependent RNA polymerase
RNA – ribonucleic acid
RPM- Revolutions Per Minute
RRV- Ross River virus
RT-PCR- Reverse transcription-polymerase chain reaction
S – Small
SFV- Semliki forest virus
SINV- Sindbis virus
SLE- St. Louis encephalitis virus
TBS – tris-buffered saline
TNF- tumor necrosis factor
YFV- yellow fever virus
USUV- Usutu virus

VBD – Vector-Borne Disease

VEEV- Venezuelan equine encephalitis virus

WNV- West Nile virus

WNF- West Nile fever

WNM- West Nile meningitis

WNP- West Nile poliomyelitis

WEEV- western equine encephalitis virus

WHO- World health Organisation

ZIKV- Zika virus

Abstract

Arboviruses are transmitted by blood-feeding insects such as mosquitoes and ticks, and pose significant global health challenges. In South Africa there are arboviruses that are known to occur causing outbreaks annually after heavy rainfall and there are arboviruses that have been detected historically but their medical and veterinary importance is unknown. Due to the unavailability of commercial assays for many arboviruses, the development of in-house assays becomes crucial. This study aimed to develop a cost-effective multiplexing technique using immunofluorescent polyvalent antigen slides for simultaneous screening of IgG antibodies against multiple arboviruses.

The nucleoprotein (NP), envelope domain III (EDIII), and capsid (C) genes for Crimean-Congo hemorrhagic fever virus (CCHFV), West Nile virus (WNV), and Sindbis virus (SINV) respectively were codon optimized and cloned into pcDNA 3.1+ plasmids. The plasmids were transfected into human embryonic kidney (HEK) 293 mammalian cells, and the expression of these genes was confirmed through immunofluorescent assay (IFA). The expressed proteins were further validated by SDS and Western blot analyses. In-house enzyme-linked immunosorbent assays (ELISAs) were developed and optimized to screen human and cattle serum samples for IgG antibodies against SINV and CCHFV. A total of 386 human serum samples and 97 cattle serum samples were screened. Polyvalent IFA slides were developed and used to screen all positive serum samples, some negative and borderline from inhouse ELISAs.

The results demonstrated successful expression of CCHFV NP, confirmed through positive IFA reactions with anti-CCHF IgG. SDS and Western blot analyses further validated the size of the expressed CCHFV. WNV showed low IFA reactivity with antihis antibody and SINV showed no expression. Hence it was decided to continue using SINV-infected cells to replace SINV transfected cells, confirmed using IFA with antiSINV sera. WNV was omitted going forward due to low yield. Among the samples positive for SINV IgG ELISA, 39 exhibited concordant positive results with the polyvalent slides. Similarly, three samples positive for CCHFV IgG antibodies with CCHFV ELISA were concordant with polyvalent slides, while eight negative serum samples tested negative using the ELISAs and IFA. Three ELISA borderline samples

for SINV ELISA tested positive for SINV suggesting increased sensitivity for IFA. Furthermore, specificity testing of IFA revealed an accuracy rate of 83.3%.

Multiplex assays offer a low-cost and fast method for simultaneous detection of IgG antibodies against multiple arboviruses on a single platform. The IFA results were concordant with the ELISA, suggesting the reliability of the developed technique. The positive reactors among the borderline samples for SINV antibody ELISA indicate that additional screening of negative samples could improve the cut-off value. Importantly, all samples tested negative with ELISAs were also negative with the multiplex IFA. This approach enables efficient serosurveillance, particularly in resource-limited regions. The accuracy and sensitivity of the multiplex assay were evident through the confirmation of IgG presence by monovalent IFA.

In conclusion, the developed cost-effective multiplexing technique using immunofluorescent polyvalent antigen slides provides a valuable tool for simultaneous screening of IgG antibodies against multiple arboviruses. The accuracy and sensitivity of the multiplex assay were validated through monovalent IFA confirmation. Subsequent research and refinement of this technique hold the potential to broaden its platform, enabling the screening of a more extensive array of viruses.

Keywords: Arboviruses, multiplexing, immunofluorescent polyvalent antigen slides, IgG antibodies, In-house assays, ELISA, CCHFV, WNV, SINV, resource-limited.

Chapter 1: Literature review

1. Literature Review

1.1. Background

Arboviruses or arthropod borne viruses are a group of pathogens transmitted to vertebrates by haematophagous arthropods such as mosquitoes, ticks, midges and sandflies during a blood meal (Weaver & Reisen, 2010). There is a wide range of known RNA taxonomical groups worldwide and the most medical important arboviruses causing human/animal diseases includes families such as *Togaviridae*, *Flaviviridae*, *Peribunyaviridae* and *Reoviridae* family (Buckner et al., 2016). Arboviral diseases constitute more than 17% of all infectious diseases each year, which results in approximately 1 million deaths worldwide per year, highlighting the public health significance of these viruses (Matthews et al., 2022). The re-occurrence and spread of arthropod-borne viruses is continual and present a potential threat to veterinary and public health and socioeconomic effect (Campbell-lendrum et al., 2015). The spread of arboviruses and potential for epidemics are not exclusive to developing countries, as developed nations are now also vulnerable to these viruses (Raulino et al., 2021). The emergence of West Nile virus (WNV) in the Americas, chikungunya virus (CHIKV) in Italy, and Usutu virus (USUV) in Austria serve as illustrations of the susceptibility of these societies to arboviruses (Lanciotti et al., 1999; Venturi et al., 2017; Weissenböck et al., 2003).

Various factors have contributed to the re-emergence and spread of arboviruses including change in viral genetics, increase in host and vector population, change in weather patterns, and migration of the vectors (Go et al., 2014). The increasing prevalence of several arboviruses in South Africa, such as Sindbis virus (SINV) and WNV infections, can be attributed to favourable climatic conditions. Previous documented records of these arboviruses reveals that the epidemics are particularly prominent during the summer months, especially in years with ample rainfall occurring between November and April (Cornel et al., 2018). These circumstances create ideal conditions for mosquito breeding, primarily during the hot and wet period spanning from January to mid-April, when mosquito populations are at their peak (Cornel et al., 2018). The presence of abundant vegetation, standing surface water, and diverse

wildlife contributes to the creation of a suitable habitat for mosquitoes that are not solely dependent on rain (Mensah & El Zowalaty, 2018).

In South Africa the most known and emerging medically important arboviruses belong to the families *Flaviviridae*, *Togaviridae*, *Peribunyaviridae* and *Nairoviridae*. This literature will focus on some of the mosquito-borne viruses and tickborne viruses such as WNV, SINV and Crimean-Congo hemorrhagic fever virus (CCHFV) belonging to each of these families known to have history of causing epidemics in South Africa. It will focus on aspects such as genomic characteristics, epidemiological patterns, diagnostic methods, available treatments, and preventive measures. Additionally, it will highlight the existing gaps in diagnostic capabilities and surveillance, underlining the need for advancements in these areas to effectively combat and mitigate the impact of these viruses on South African and global public health.

1.2. The viruses

1.2.1. Crimean-Congo hemorrhagic fever virus

Crimean-Congo hemorrhagic fever (CCHF) is a tickborne disease caused by CCHFV in humans. This virus is classified under the family *Nairoviridae* of the genus *Orthonairovirus* (Serretiello et al., 2020). According to the current International Committee for the Taxonomy of Viruses (ICTV), the family *Nairoviridae* consists of two other genera namely *Shaspivirus* and *Striwavirus* (Serretiello et al., 2020). CCHFV is the most widely distributed tickborne virus and the second most widely distributed virus causing hemorrhagic fever after dengue virus (DENV) (Al-Abri et al., 2017; Salih et al., 2023). The World Health Organisation (WHO) classifies CCHFV as a high priority pathogen because of the high fatality rate (up to 40%) and the difficulties associated with its treatment, control, and prevention (Mehand et al., 2018; Shahhosseini et al., 2021; Tajudeen et al., 2022). CCHFV has been detected in various hard tick species (Ixodidae), and the primary vector group belongs to the genus *Hyalomma* such as *H. marginatum*, *H. anatolicum*, *H. truncatum*, *H. impeltatum*, and *H. impressu* (Serretiello et al., 2020).

Humans can contract CCHFV infections via a bite of an infected tick, or through inadvertent crushing of an infected tick, or by coming into contact with the blood or bodily tissues of humans or animals that are infected (Bente et al., 2013). Human to human transmission is possible and can occur in hospital environments (Gürbüz et al.,

2009). Ticks exploit wild and domestic animals, including goats, cattle, sheep, and birds, as hosts for amplification (Hollidge et al., 2010). In contrast, humans and horses serve as dead-end reservoirs, as the virus induces low viremia in them, hindering transmission to other humans or animals (Gonzalez et al., 1992). CCHFV is a continuous emerging virus with increasing incidence and geographic range since early identification (Al-Abri et al., 2017).

1.2.1.1. CCHFV structure and genome organisation

CCHF virion has a spherical shape with a diameter of about 100 nanometers (nm). It is surrounded by a lipid bilayer envelope which is approximately 5 to 7 nm in thickness. Additionally, there are glycoprotein spikes, Gn and Gc extending from this envelope, measuring around 8 to 10 nm in length (Ö. Ergönül, 2006) (Figure 1.1A). CCHFV is a single-stranded, negative-sense RNA virus with a structured three segmented genome, named Small (S), Medium (M) and Large (L) according to the size of each segment (Ö. Ergönül, 2006) as shown in Figure 1.1B.

The S segment of CCHFV encodes for nucleoprotein (NP) and, in opposite orientation, encodes for small non-structural proteins 1 (NS1) (Hawman & Feldmann, 2023). NP is responsible for the encapsidation of the virus. It also plays a role in transcription and replication by forming ribonucleoprotein complexes with viral RNA, interacting with host heat shock proteins during intracellular viral replication, and enhancing the translation of viral mRNAs (Ozdarendeli, 2023). The M segment encodes for glycoprotein precursor complex (GPC) which undergoes proteolytic cleavage, resulting in the production of viral structural glycoproteins, namely the Gn and Gc, secreted non-structural proteins (GP160, GP85 and GP38), and non-structural M protein (NSm). Gn and Gc structural proteins form spikes on the viral surface for receptor binding and also mediate the virus entry into the host (Ahsan et al., 2020; Bertolotti-Ciarlet et al., 2005) (Figure 1.1A). NSm is responsible for promoting glycoprotein processing and the assembly of viral particles (Shanna et al., 2021). The GP160, GP85 and GP38 functions remain unknown (Freitas et al., 2020). The L segment of CCHFV encodes for viral RNA-dependent RNA polymerase (RdRp), responsible for the viral genome transcription and replication (Honig et al., 2004). The amino terminus of the L segment protein contains an ovarian-tumor-like (OTU) protease that exhibits dual functionality, encompassing de-ISGylating and deubiquitylating capabilities (Devignot et al., 2020).

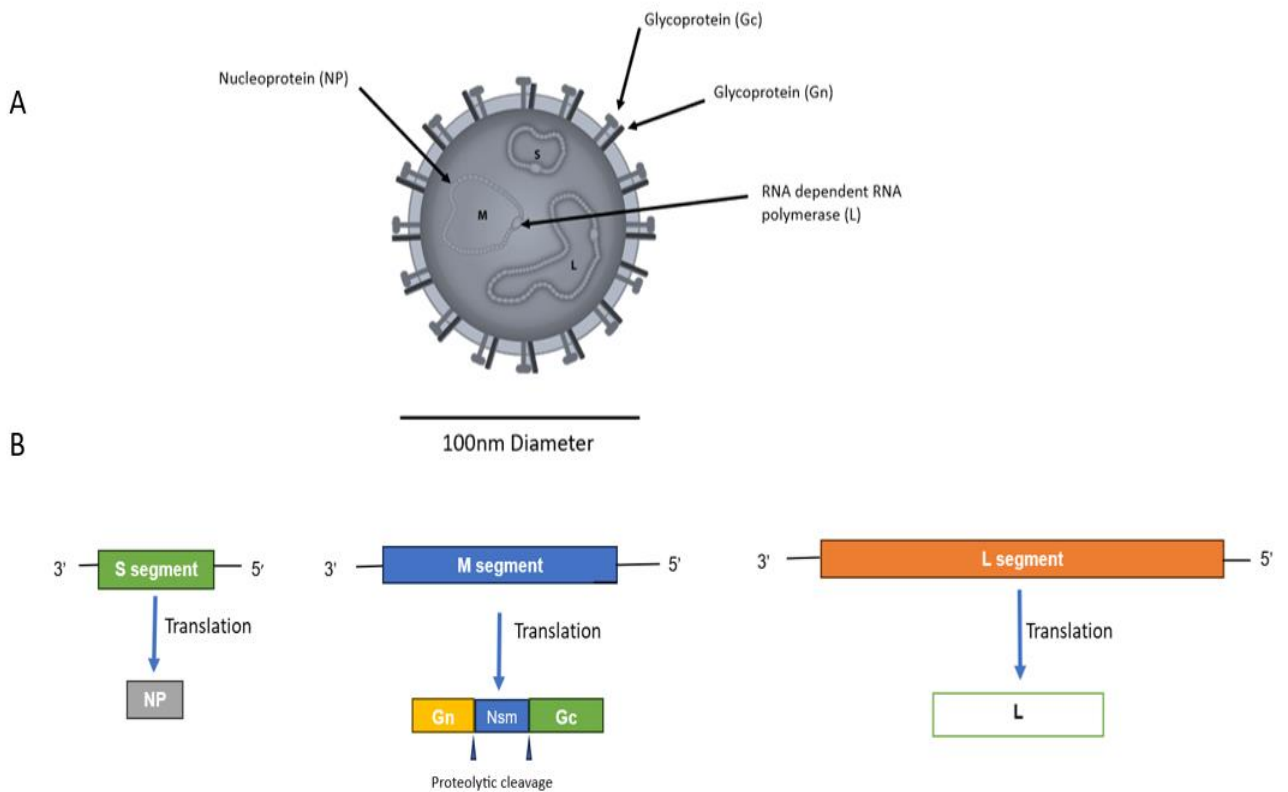


Figure 1.1: (A) CCHFV structure. (B) CCHFV genome segments. Modified from (Kamboj & Pathak, 2013; Zivcec et al., 2018)

1.2.1.2. Epidemiology and clinical manifestations of CCHF

CCHFV first occurred in Crimea in 1944 and was isolated in Congo in 1956 (Bente et al., 2013). Viral isolates that were acquired from people in Congo presented with febrile illness similar to those in Crimea and pointed towards the virus being identical. Investigation of febrile illnesses in Crimea showed that the virus responsible for these cases was antigenically identical to the cause of illnesses in the Congo (Casals, 1969). Thus, the term Crimean-Congo hemorrhagic fever was developed. CCHFV is endemic in Africa, Asia, eastern and southern Europe. Around 30 countries have reported cases of CCHF since the first case in Crimea.

In Africa, the majority of the cases are reported from South Africa (Temur et al., 2021). CCHFV was first identified in South Africa in 1981, after a child passed away following a tick bite during a camping event held at a nature reserve in the western Transvaal province now known as North West province (Swanepoel et al., 1983). Following the

identification of this case, serological investigations were conducted, affirming that the virus had existed in South Africa for years and already had a broad geographical presence throughout the country. Since then, isolated cases of CCHF have been confirmed among farm and wildlife workers in various provinces, with a reported seroprevalence of 3.9% and a seroprevalence of 74.2% in cattle (Msimang et al., 2021). CCHFV is predominantly endemic in the Free State and the Northern Cape provinces of South Africa, with *H. marginatum rufipes* and *H. truncatum* identified as the primary vectors (Vawda et al., 2018).

CCHFV infection can vary widely, ranging from a mild febrile illness with non-specific symptoms to a severe condition known as CCHF, characterized by vascular dysfunction and multi-organ failure (Hawman & Feldmann, 2023). CCHF infection is usually divided into four stages: incubation, pre-hemorrhagic, hemorrhagic and convalescence (Papa et al., 2008). The incubation phase length is influenced by the method of exposure and the viral dose (Appannanavar & Mishra, 2011). Incubation period after an infected tick bite lasts 1–3 days with a maximum of 9 days, and slightly longer when exposure occurs through contact with the blood, through tissue, or secretions of infected livestock and humans, lasting for 5-6 days with a maximum of 13 days (Hawman & Feldmann, 2023). After incubation, the pre-hemorrhagic stage lasting 4-5 days usually presents with symptoms such as photophobia, myalgia, nausea, headache, fever, chills, vomiting and, more rarely diarrhea (Hoogstraal, 1979). During this phase it is common to observe red eyes, a flushed face, a red throat, and petechiae on the palate. Additional symptoms may involve jaundice in severe cases (Aslam et al., 2016). As the disease progresses the hemorrhagic stage begins by showing symptoms such as internal nose bleeding, conjunctival hemorrhage, gums bleeding, hematemesis and melena. Severely affected patients may undergo rapid kidney deterioration, sudden liver failure, or pulmonary failure (Shayan et al., 2015). The convalescence stage begins 10 to 20 days after the onset of illness and could take as long as a year. In the convalescent stage, the patient experiences marked fatigue, weak pulse, poor appetite, poor vision, headache, dizziness, and memory loss (Gavrilova et al., 2014).

1.2.1.3. Pathogenesis of CCHFV

CCHFV is introduced into the host through a tick bite on the host's epithelial barrier or via direct contact with the blood or tissues of an infected animal (Papa et al., 2017). The virus replicates extensively at the site of inoculation encompassing epithelial cells, dendritic cells, and macrophages, supporting its spread to lymph nodes and peripheral blood-borne monocytes (Akıncı et al., 2013; Burt et al., 1997; Connolly-Andersen et al., 2009). To date, the specific receptors of CCHFV in target cells remain unknown and it is suggested that the viral glycoproteins Gn and/or Gc play a role in the initial attachment to the cell plasma membrane, particularly with Gc proposed for binding to cellular receptors (Papa et al., 2017). The virus enters cells through clathrin and clathrin pit adaptor protein-2 complex-mediated processes, which are cholesterol and pH-dependent, progressing to early endosomes and multivesicular bodies for membrane fusion using components of the endosomal sorting complex required for transport regulators (Garrison et al., 2013; Simon et al., 2009). Infection of endothelial cells and peripheral blood-borne monocytes leads to extravasation into parenchymal tissue, enabling interaction with basolateral cell receptors in target organs, supporting secondary replication and systemic spread, as observed in animal models (Akıncı et al., 2013; Bente et al., 2010; Connolly-Andersen et al., 2007). Bente et al. highlighted that STAT-1 knockout mouse model had the ability to mimic the manifestations in humans and indicated that the virus initially spread to the blood and then infects the liver and the spleen. The experimentally infected mammalian model also showed changes in the liver, spleen, and other replication sites, while the effect of the central nervous system (CNS) only appeared to be later on during the stages, possibly due to blood-borne systemic spread (Bente et al., 2010). The virus causes thrombocytopenia, vascular endothelial injury, and the dysfunction of the liver leading to a disruption in blood clotting, as it prompts platelets to clump together and release their contents, ultimately triggering the internal blood clotting system. Damage to the pulmonary vasculature after endothelial infection lead to cardiac and pulmonary complication, acute respiratory distress syndrome (ARDS) (Aktaş & Aktaş, 2019). Heart damage results in a reduced left ventricular ejection fraction, potentially accompanied by pericardial effusion, cardiac congestion, and interstitial edema (Engin et al., 2009). Patients with fatal CCHF exhibit considerably elevated levels of interleukin (IL)-6 and

tumor necrosis factor (TNF)- α compared to those with non-lethal infections highlighting the role in pathogenesis and mortality of individuals with CCHF (Awad et al., 2014).

1.2.2. West Nile virus

WNV is a widely prevalent mosquito-borne virus capable of inducing encephalitic illness in both humans and various vertebrate species. It is classified within the *Flaviviridae* family under the *Flavivirus* genus. This genus also encompasses various other well-known viruses, including DENV, Zika virus (ZIKV), and yellow fever virus (YFV). WNV belongs to the same serocomplex as Japanese encephalitis, which includes the Japanese encephalitis virus (JEV), St. Louis encephalitis virus (SLE) and Murray valley encephalitis virus (MVEV). Different lineages of the WNV virus have been identified due to the variation of their genomic sequence (Donadieu & Couplier, 2013). WNV strains are classified into nine distinct lineages, even though only lineages I, II and V have been linked to notable human outbreak occurrences (Habarugira et al., 2020). Lineage I is distributed across Africa, Europe, the Middle East, Asia, and America. It is subdivided into two sub lineages namely 1a and 1b. Sub lineage 1a have caused major outbreaks in Europe, Middle east and Africa, while 1b the Kunjin virus caused outbreaks in Australia (Monini et al., 2010). Lineage II originates from Africa and spread to other regions such as Russia, Hungary, Italy and Greece (Bakonyi et al., 2006). This lineage has a history of causing a sporadic outbreak in South Africa. Lineage III, known as the Ravensburg virus, was discovered in the Czech Republic from *Cx. pipiens* mosquitoes and *Ae. rossicus* (Bakonyi et al., 2006). Lineages IV and V were identified in Russia and India respectively (May et al., 2011). Lineage VI was identified and found to be endemic in Australia (Bondre et al., 2007). Lineages VII isolated from *Cx. pipiens* mosquitoes in southern Spain (Virus et al., 2010). Lineage IX mostly regarded as the subset of lineage IV, has been detected in Austria in *Uranotaenia unguiculata* mosquitoes (Pachler et al., 2014). Wild birds act as amplifying hosts for WNV and play a role in its spread. WNV is transmitted among avian hosts by ornithophilic mosquitoes. The virus can amplify within both avian and mosquito populations, which can then lead to its transmission to unintended hosts, such as horses and humans (Vasić et al., 2019).

1.2.2.1. WNV structure and genome organisation

WNV is a single-stranded RNA positive-sense genome. It consists of an enveloped icosahedral capsid of approximately 50nm in size. It has three structural proteins:

capsid protein (C), the pre-membrane (prM) and envelope protein (E) (Nicholls et al., 2020) (Figure 1.2). C protein binds to the viral genomic RNA and enhance RNA encapsidation and uncoating during viral replication (Byk et al., 2016). The C protein triggers cytotoxic effects in infected cells, leading to cell cycle arrest in the G2 phase and stimulates the upregulation of caspase-9, activating the apoptosis pathway, and ultimately resulting in cell death (Oh et al., 2006; Yang et al., 2002). prM glycoprotein interacts with the E protein, acting as a chaperone to aid in the correct folding of E and prevents the premature fusion of the E proteins within the host cell membrane (Navaratnarajah et al., 2008). E protein is responsible for maintaining the integrity of the viral envelope and viral entry. The E protein is the most immunogenic part of all flaviviral proteins and consists of three domains: envelope domain (ED) I, EDII, and EDIII (Habarugira et al., 2020). The most potent neutralising antibodies against WNV have been identified to target the EDIII region of the E protein (Habarugira et al., 2020). There are also seven non-structural proteins that play a role in replication cycle of the viral RNA genome (Chambers et al., 1990). NS3 comprises an ATP-dependent helicase, which is associated with NS2B proteins for virus polyprotein processing using serine protease (Schlesinger, 2006). NS5 is an RNA-dependent RNA polymerase (NS5) (Egloff et al., 2002). NS1 glycoprotein functions intracellularly as a cofactor for viral replication, whilst on the cell surface, it acts as an immunomodulator. NS2A is responsible for the virus assembly and inhibition of IFN- β promoter activation (Leung et al., 2008). The NS4A protein plays a crucial role in the virus replication process by reorganizing the viral membrane. Furthermore, the interaction between NS4A and NS1 is essential for viral RNA synthesis (Brinton, 2002). All the nonstructural proteins are significant for efficient replication (Mackenzie et al., 1998).

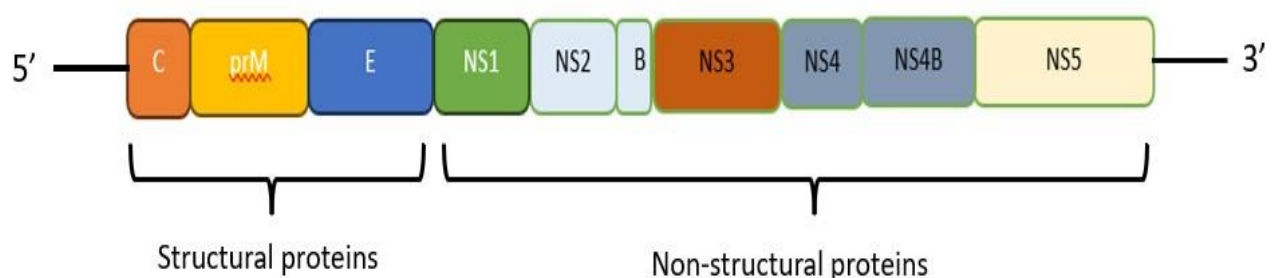


Figure 1.2: WNV genome segments modified from Habarugira et al., 2020.

1.2.2.2. Pathogenesis of WNV

The full range of understanding of WNV pathogenesis in humans remains not fully understood. There is little data on WNV with limited virulence in human infections (Samuel & Diamond, 2006). The majority of the information and knowledge about viral pathogenesis is from mammals experimentally infected for the purpose of understanding the pathway of the infection by also controlling the conditions (Rossi et al., 2023). Humans acquire WNV through a bite by the infected mosquitoes feeding on human blood. The infected mosquito transfers WNV through saliva into the blood and skin tissue of the host during the feeding process (Hudson & Bowman, Lorr, 1960; Ribeiro et al., 1985). WNV contained within the tissues infects cutaneous Langerhans cells, which then migrate to the nearby lymph nodes (David & Abraham, 2016). The virus multiplies in these tissues, resulting in a temporary, low-level viremia. Furthermore, the virus spreads to various organs, including the spleen, liver, kidneys, and CNS (Gyure, 2009). WNV accesses the CNS through various mechanisms, including direct infection that disrupts the blood-brain barrier (BBB), particularly when there's high levels of viremia (Roe et al., 2012). Infection of CNS neurons by WNV results in architectural loss, degeneration, and cell death (Lazear et al., 2011). Brain stem, hippocampal, and spinal cord injuries are observed in both humans and rodents affected (David & Abraham, 2016).

1.2.2.3. Epidemiology and clinical manifestations of WNV

In nature, WNV is sustained through a cycle involving birds and mosquitoes (Colpitts et al., 2012). While various mosquito species can participate in this cycle, *Culex* mosquitoes have the most significant role in facilitating natural transmission (Colpitts et al., 2012). Various *Culex* species, such as *Culex univittatus* in South Africa, Egypt and Israel, *Culex modestus* in France, and members of the *Culex vishnui* complex, including *Culex pseudovishnui*, *Culex tritaenorrhynchus*, and *Culex vishnui* in India and Pakistan, have been associated with the transmission of WNV (Dandawate et al., 1969; Hubálek & Halouzka, 1999; Umrigar & Pavri, 1977). Additionally, there have been sporadic findings of WNV in *Aedes* and *Anopheles* mosquitoes, as well as argasid and ixodid ticks (Gubler, 2007). WNV is primarily transmitted by a mosquito when it feeds on viremic birds. The infected mosquitoes transmit the virus to

susceptible vertebrates when they bite and feed on them, including humans (Hayes et al., 2005). Humans and horses serve as "dead-end" hosts since they are unable to transmit the virus to other hosts or vectors, as the virus levels remain below the threshold required for transmission. (Bondre et al., 2007).

WNV was first identified in Uganda in 1937 from blood collected from a woman with febrile illness (Smithburn et al., 1940). Epidemics of WNV are believed to have already occurred across a significant portion of Africa, the Middle East, and southern Asia long before clinical cases were observed in humans. This is because serosurveys conducted between 1939 and 1940 revealed widespread human seropositivity for WNV, as determined by comparing neutralization titers for WNV, SLEV, and JEV, in countries such as Uganda, Sudan, Democratic Republic of the Congo, and Kenya (Chancey et al., 2015). Seropositivity was also identified in western Nigeria, based on samples collected in 1951 and 1955 (Chancey et al., 2015). In South Africa, seropositivity for WNV was detected in humans who had not travelled, as well as in monkeys, domestic animals, and juvenile birds, through samples collected in 1954 (Kokernot et al., 1956). Therefore, it was evident that WNV had historically been present across a broad geographical expanse in Africa long before clinical infections were observed in most areas.

In South Africa, WNV is endemic, with lineage 1 and lineage 2 viruses being the most frequently identified. Lineage 2 is frequently identified in horses with neurological illnesses and Lineage 1, on the other hand, was only observed in a solitary instance, involving a horse with a fatal neurological condition and an aborted fetus, throughout an eight-year monitoring period (Venter et al., 2009, 2011, 2017). To date one large epidemic and one epizootic have been documented in South Africa. The epidemic occurred in 1973-1974 in the Karoo and Northern Cape following unusually heavy summer rains. This epidemic resulted in tens of thousands of human cases within the areas (Jupp, 2001). In 1983-1984 an epizootic occurred in the Witwatersrand-Pretoria area of the Highveld in Gauteng and was concurrent with the SINV outbreak (Jupp et al., 1986).

WNV infections are usually asymptomatic (Sejvar, 2016). WNV infection in humans can range from a mild febrile illness known as West Nile fever (WNF) to more severe neuroinvasive conditions such as meningitis, encephalitis, or acute flaccid paralysis

(Sejvar, 2014). WNF has an incubation period of about 2 to 14 days, and infected individuals present with symptoms such as fever, headache, fatigue, muscle aches, nausea and vomiting. WNF may be accompanied by a rash, typically appearing as a maculopapular, and non-itchy rash (Gorsche & Tilley, 2005). This rash can be shortlived, lasting less than 24 hours for some individuals. Though most patients ultimately experience full recovery, deaths related to WNF are more common among older individuals and those with compromised immune systems, and the fatalities are often linked to cardiopulmonary complications (Sejvar et al., 2011).

West Nile meningitis (WNM) is typically difficult to differentiate from other forms of viral meningitis, often referred to as aseptic meningitis (Sejvar, 2014). Individuals who develop WNM typically experience a sudden onset of symptoms, including fever and headache, and exhibit meningeal signs such as nuchal rigidity, Kernig's as well as photophobia or phonophobia. The headache can be severe enough to necessitate hospitalization for pain management (Sejvar et al., 2008).

WNV infection can also result in West Nile poliomyelitis (WNP) (Sejvar, 2014). In most instances, patient experience partial weakness (limb paresis) or complete loss of muscle power (paralysis). This results from the virus affecting the lower motor neurons in the spinal cord, specifically the anterior horn cells, leading to a condition known as anterior (polio) myelitis. WNP usually develops shortly after the onset of illness, typically within the first 24 to 48 hours (Kulstad & Wichter, 2003). Limb weakness may occur abruptly, sometimes causing concerns resembling stroke symptoms. Additionally, central facial weakness, often occurring on both sides of the face. Sensory loss or numbness is generally not a prominent feature, although some patients may experience intense pain in the affected limbs just before or during the onset of weakness, and this limb pain may persist (Sejvar, 2014).

1.2.3. Sindbis virus

SINV is a mosquito-borne zoonotic arbovirus within the genus *Alphavirus* of the family *Togaviridae*. Alphaviruses are divided based on their clinical disease into arthritogenic viruses known as the old-world alphavirus and encephalitic alphaviruses also known as new world alphaviruses (Suhrieb et al., 2012). Arthritogenic viruses includes viruses, such as the CHIKV, Mayaro virus (MAYV), o'nyong nyong virus (ONNV),

Ross River virus (RRV) and Semliki Forest virus (SFV) and SINV (Suhrbier et al., 2012). Encephalitic alphaviruses include, Venezuelan equine encephalitis virus (VEEV), western equine encephalitis virus (WEEV), and eastern equine encephalitis virus (EEEV), which are primarily responsible for inducing severe neuro-pathological manifestations in humans, often posing a life-threatening risk (Zacks & Paessler, 2010). SINV is grouped into six antigenically distinct genotypes based on the E2 glycoprotein gene. Sindbis genotype I circulate mainly in Europe, the Middle East, and Africa. Sindbis genotype II is mainly found in Australia and Malaysia, while Sindbis III is mainly found in Asia. Sindbis IV is mainly found in Azerbaijan and China. Sindbis V is found in New Zealand, and Sindbis VI is found mostly in southwest Australia (Adouchief et al., 2016). Sindbis virus has caused many outbreaks, specifically in South Africa, in the Northern Cape, Free State, Gauteng and KwaZulu-Natal (KZN) (Storm et al., 2014). Birds are the main reservoirs with the ability to spread viruses into different continents while migrating.

1.2.3.1. SINV structure and genome organisation

SINV is a spherical icosahedral capsid virus. It is a positive RNA single-stranded genome and consists of five structural proteins and four non-structural proteins (Rupp et al., 2015) (Figure 1.3). The structural proteins include the C protein, E (1-3) proteins and the 6K protein. E1 plays a role in the continuous icosahedral protein shell on the virion to cover the virion as well as the attachment of the virion (Jose, 2009). E2 plays a role in the binding of receptors and endocytosis (Wengler et al., 2003). The E3 protein is responsible for assembling particles to mediate viral entry into the host (Wu et al., 2008). The 6K protein is responsible for the membrane modification and formation of cation-selective ions channels in planar lipid bilayers. It is therefore classified as a viroporin, which plays a role in various processes, including the release of viral particles from cells, glycoprotein trafficking, altering membrane permeability, and initiating caspase-dependent programmed cell death (Madan et al., 2008).

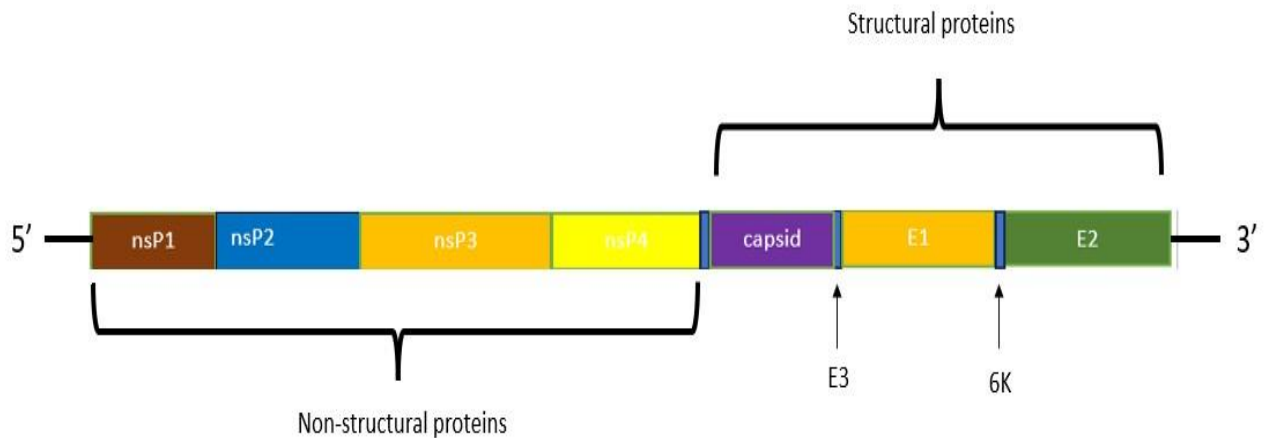


Figure 1.3: SINV genome organisation, modified from (LaPointe & Sokoloski, 2021)

1.2.3.2. Pathogenesis of SINV

SINV is transmitted to human through mosquito bite. The virus enters the cells by attaching the viral membrane with the endosomal membrane receptor on the surface of the host (Kielian et al., 2010). The virus binds to the DC-SIGN and L-SIGN, which act as attachment factors (Klimstra et al., 2003). In vitro infection of mammalian cells infects the human macrophages and uses them to replicate and protect itself from phagocytosis. The infected macrophages inhibit the activation of the TNF α , IL-6 and matrix metalloproteinases 1 and 3 used for tissue repairs during infection (Bozza & Poian, 2010). The liver and spleen are usually regarded as the primary sites for viral replication and spreading of the virus. Once the virus has spread, it reaches the bones, muscles, and joint tissues, giving rise to the acute phase of the disease, which is strongly associated with local inflammation (Habarugira et al., 2020). Several factors influence the pathogenesis of alphavirus infections in animals, including the host's age, immune system status, virus strain virulence, and the persistence of the virus (Suhrbier & Gasque, 2012). The susceptibility of mice to SINV infection appears to involve age-dependent inflammation triggered by the stress response to infection. The severity of the disease and the persistence of symptoms are linked to the extent of virus replication and the presence of inflammatory mediators in the blood of patients and specific tissues in animal models (Sane et al., 2012).

1.2.3.3. Epidemiology and clinical manifestations of SINV

SINV was first isolated in 1952 in a village called Sindbis, in Cairo, Egypt, from *Culex pipiens* and *Culex (Cx) univittatus* mosquitoes (Timimoun et al., 2022). SINV is prevalent in various regions across Africa, Eurasia, and Australia, and the majority of reported human illnesses occur in South Africa and Northern Europe (Mehand et al., 2018). In Europe, the vectors for the virus include *Cx pipiens*, *Cx torrentium*, *Culiseta morsitans*, as well as mosquitoes from the *Aedes* spp. and *Ochlerotatus* spp (Hubálek, 2008). In South Africa, the virus is maintained in a wild cycle between mosquitoes and wild birds, with infection rates suggesting that the mosquito species *Cx univittatus* serves as the predominant vector in the region (Braack et al., 2018).

In South Africa, during the West Nile virus outbreak of 1974, which was previously discussed in the WNV section, the first epidemic of SINV was reported too, in the same regions of Karoo and Northern Cape regions of South Africa, impacting thousands of people experiencing symptoms such as fever, myalgia, rash, and arthralgia. Another epidemic occurred in 1984 concurrent with the WNV and took place in the Witwatersrand - Pretoria region of South Africa. This led to numerous cases of SINV infection in humans, but no fatalities were reported (Cornel et al., 2018). In both of these outbreaks, the primary vector responsible for transmitting SINV was the *Cx univittatus* mosquito.

Examination of febrile and neurological infections in horses in South Africa has revealed the presence of numerous SINV infections. Some of these infections were found in the brains of horses, with some of the cases resulting in fatalities (van Niekerk et al., 2015). Between 2014 and 2018, a total of 1084 horses in South Africa exhibiting unexplained febrile and acute neurologic infections were examined. The study found that approximately 11(1.01%) of these horses tested positive for SINV through PCR testing (Fourie et al., 2022).

The symptoms of SINV infections are often associated with arthralgia and acute febrile illness. The initial manifestations include rash, malaise, fatigue, mild fever, headache, nausea, lymphadenopathy, and dizziness (Azar et al., 2022). After the initial symptoms, musculoskeletal symptoms can occur. Skin lesions occur in every patient and are found on the trunk and the limbs (Sane et al., 2011). Muscle aches may also develop. Severe complications are usually rare but possible (Storm et al., 2014).

Patients presenting with joint and muscle aches usually get better with time without any treatment and recover completely over time (Laine et al., 2004).

1.3. Diagnosis Methods

The diagnosis of arboviral infections plays an important role in disease surveillance and management of outbreaks. To accurately track the spread of a virus timeously an accurate diagnosis is important, efficient control and treatment measures. A range of diagnostic methods are required due to various factors such as, cross-reactivity in serological tests, particularly in regions where these viruses are endemic, similarity of clinical manifestations to other diseases, asymptomatic or mildly symptomatic cases, and limited access to specialized reference laboratories capable of conducting distinct diagnostic assessments (Chan et al., 2022; Reusken et al., 2018). Diagnostic techniques used for arboviruses include traditional techniques, such as viral isolation and serological testing, and modern molecular diagnostic assays, including polymerase chain reaction (PCR) techniques (Varghese et al., 2023). Each of these techniques have their own advantages and limitations in terms of sensitivity, specificity, cost, and availability.

1.3.1. Viral Isolation

Viral isolation is a method used for the diagnosis of viral infections (Louten, 2016). Viruses can be amplified through inoculation of suckling mice or cell culture. Isolation of CCHFV requires biosafety level 4 (BSL-4) facilities due to its high pathogenicity in humans. Various susceptible cell lines used to isolate viruses including SW-13, Vero, LLC-MK2, BHK-21, and CER.1 (O. Ergönül, 2006). Isolation of the virus can be accomplished over a period of 2–5 days; however, cell cultures tend to lack sensitivity, typically enabling the detection of only relatively elevated viraemia observed in the initial 5 days of illness (Whitehouse, 2004). Although the virus may exhibit minimal or no cytopathic effects, it can still be identified through immunofluorescence assay (IFA) tests utilizing specific monoclonal antibodies (Whitehouse, 2004).

The isolation procedures of WNV requires biosafety level 3 (BSL-3) conditions. WNV is challenging to isolate from tissues, plasma, serum, and cerebrospinal fluid (CSF) samples in cell culture (Sambri et al., 2013). Cell lines like Vero E6, RK-13, AP61, or C6/36 are used for virus isolation and cytopathic effect (CPE) can be identified in approximately seven days post- infection (Caceda & Kochel, 2007; Jayakeerthi et al.,

2006; Rossini et al., 2011; Sudeep et al., 2009). Isolating WNV strains offers added value by facilitating further studies on pathogenesis, genetic variation, evolution, epidemiology, and related research areas.

The isolation of SINV requires biosafety level 2 (BSL-2) conditions. Samples, including tissues, plasma, serum, and CSF, are collected from infected sources. Cell lines used for SINV isolation include BHK-21 (Baby Hamster Kidney) and Vero (African Green Monkey Kidney). Following inoculation onto the cell monolayer, the culture is incubated for a period ranging from 3 to 5 days, during which cytopathic effects such as cell rounding, and detachment become apparent (Akhrymuk et al., 2018). SINV isolation is confirmed through molecular techniques, immunofluorescence, or serological assays.

1.3.2. Molecular diagnostic assay

Molecular diagnostic assays can be used to detect or measure genetic material of arboviral RNA, present in a biological sample (Varghese et al., 2023). Molecular assays are more sensitive and faster to perform than virus isolation. Reverse transcription-polymerase chain reaction (RT-PCR), nested RT-PCR and quantitative RT-PCR (qRT-PCR) techniques have been used for the identification of various arboviruses (N. Johnson et al., 2012).

CCHFV is primarily identified through RT-PCR due to its high sensitivity in detecting active infections (Mazzola & Kelly-cirino, 2019). RT-PCR assays commonly target the nucleoprotein gene region within the S segment, a conserved genome region across various geographic isolates of CCHFV (Burt et al., 1998; Drosten et al., 2002; Rodriguez et al., 1997). The presence of CCHFV RNA peaks within the initial week of symptom onset and remains detectable for up to three weeks (Mazzola & Kelly-Cirino, 2019). Variability in viral load among CCHF patients can indicate disease severity. Quantitative RT-PCR (qRT-PCR) outperforms conventional RT-PCR or nested RT-PCR, offering lower contamination rates, heightened sensitivity and specificity, and improved time efficiency (Escadafal et al., 2012; Fernandez-Garcia et al., 2014).

For WNV detection, RT-PCR is employed, with sensitivity relying on the chosen target sequence. C and prM gene are the most targeted regions (Sambri et al., 2013). Nested RT-PCR techniques can enhance the RNA detection threshold by up to 10-fold but with drawbacks such as elevated risk of contamination and longer duration. In recent

years, qRT-PCR has become the standard method, providing higher sensitivity and specificity with a rapid turnaround time. However, RT-PCR faces limitations in WNV due to the virus's low level and short-lived viremia (Boonham et al., 2014; Lustig et al., 2018). RT-PCR assays for SINV commonly target nsP1 and nsP2 regions as these are highly conserved regions. qRT-PCR offers advantages over conventional RT-PCR methods for SINV detection. It provides higher sensitivity, specificity, and a faster turnaround time, making it a preferred choice in clinical laboratories.

1.3.3. Antigen Detection

The presence of arboviral antigens can be identified using techniques such as ELISA, reverse passive hemagglutination assay and lateral flow assay (A. J. Johnson et al., 2000). While these methods offer rapid and straight forward applicability, they tend to have lower sensitivity when compared to virus isolation and RT-PCR. This technique demonstrates usefulness in diagnosing infection during the acute phase of the illness. Detecting arbovirus antigen can be particularly valuable for quickly diagnosing acute infections, especially in cases with high viremia levels that may lead to fatal outcomes (Burrell et al., 2020). Nevertheless, in non-fatal cases and in patients with detectable antibody responses, it's crucial to consider the potential limitations in sensitivity associated with antigen detection methods. Antigen testing offers the advantage of providing rapid results compared to viral isolation and requires less specialized equipment than nucleic acid amplification testing.

1.3.4. Antibody detection

Confirmation of recent or ongoing arboviral infection relies on the demonstration of seroconversion, which involves an increase in IgG antibody titers in sera, or the presence of IgM antibody in a single specimen (Kaslow et al., 2014). Serologic tests such as complement fixation, hemagglutination inhibition, and reversed passive hemagglutination inhibition were used in the past to detect antibodies. However, these methods were less sensitive (Atmar, 2014; Davis et al., 2008). Presently, ELISA and indirect immunofluorescence (IF) assays are the most commonly used serologic tests for diagnosing most arboviral infections. The CCHFV nucleocapsid protein (NP) is known to be highly immunogenic and is the predominant antigen expressed early after infection (Karaaslan et al., 2021). E glycoproteins are also highly immunogenic in WNV and SINV (Martina et al., 2008). These proteins play a significant role in detection of the antibody for each virus.

1.4. Prevention of animal and human outbreaks

Animals and human outbreaks can also be prevented by doing mosquito control such as use of bed nets and repellents in human populations and insecticide application. Making people aware about the signs and symptoms of the viruses could also assist in preventing mortality. Then specifically for tick-borne viruses (CCHFV), pesticides for killing the tick could be used to control the infection and reduce effect of on livestock, which in turn would protect farmers from getting exposed to the ticks (Goswami et al., 2014). Wearing protective clothing would avoid tick attachment to the legs and arms when working with livestock. Doctors and nurses treating an infected individual should wear protective gloves and gowns to reduce the risk of getting exposed to the blood or tissue of patients infected by the virus (Bajpai & Nadkar, 2011).

1.5. Treatment and vaccines

CCHF has no specific treatment, but ribavirin has been developed over the past years and has been recommended to use for treatment. Ribavirin has an inhibitory effect on the replication of CCHF (Dai et al., 2021). To date, there is no internationally licenced vaccine for CCHFV. The Bulgarian vaccine is recognised and only used in Bulgaria. (Papa et al., 2011). Currently there is no Food and Drug Administration (FDA) approved medication or vaccine for CCHF. The use of ribavirin for CCHF treatment has primarily relied on in vitro sensitivity testing and efficacy studies conducted on animals, with only limited studies and anecdotal experiences in human cases (Rusnak, 2011). Informal accounts from small groups of CCHF patients have suggested a potential increase in survival when ribavirin is administered within 72 hours of the onset of illness (Agravat et al., 2014).

According to WHO, ribavirin is the antiviral medication recommended for treating CCHF. The prescribed regimen consists of an initial dose of 30mg/kg, followed by 15mg/kg for four days, and then 7.5mg/kg for six days, amounting to a total treatment duration of 10 days (Appannanavar & Mishra, 2011). Nevertheless, the use of ribavirin remains a subject of debate, and there is ongoing discussion about whether it truly enhances patient outcomes (Ergonul, 2009; Ergonul et al., 2006). Comprehensive meta-analyses have pointed out that the data supporting the effectiveness of ribavirin against CCHFV is weak due to complicating factors present in the reported datasets

(Ascioglu et al., 2011; S. Johnson et al., 2018). Furthermore, findings from animal studies have provided inconsistent results regarding the efficacy of ribavirin against CCHFV infection. While ribavirin proved effective in lethally infected Stat1^{-/-} mice and Stat2^{-/-} hamsters, two studies involving lethally infected Ifnar^{-/-} mice showed no protection and led to mortality (Hawman et al., 2018; Oestereich et al., 2014). When considering the collective evidence from both human cases of CCHFV infection and animal models, it suggests that ribavirin's efficacy against CCHFV is limited, and any potential benefit likely requires rapid treatment following a known exposure and achieving such prompt treatment is challenging outside of recognized laboratory or healthcare settings (Hawman et al., 2018; Oestereich et al., 2014).

WNV-infected patients have limited treatment options. There is no specific drug or therapy recommended for this infection. There is no FDA licensed vaccine to combat West Nile disease in humans, but there are vaccines that can be used in horses (Alessandro Sinigaglia Elektra Peta & Barzon, 2020). Treatment for humans primarily focuses on supportive and symptomatic measures, such as pain management for headaches, antiemetic therapy and rehydration for nausea and vomiting, monitoring of intracranial pressure, and addressing seizures if they occur (Debiasi & Tyler, 2006). Various research approaches using tissue culture and animal models are under investigation to advance effective preventive and therapeutic methods. Ribavirin has shown inhibitory effects against WNV in cell culture when administered at high doses, but it increased mortality in hamsters (Jordan et al., 2000; Morrey et al., 2004). Consequently, ribavirin is not considered an ideal candidate for treating WNV infection.

SINV has no specific recommended treatment. In the meantime, care is focused mainly on alleviating the pain by using of non-steroidal anti-inflammatory drugs. Several experimental treatments have undergone in vitro testing. One such treatment involves a dioxanebased antiviral agent that has been created to hinder SINV replication in baby hamster kidney cells (Burdeinick-Kerr & Griffin, 2005; Kim et al., 2007). Another approach involves the use of gamma interferon (IFN- γ), which enhances virus clearance in a neural cell culture derived from rodents. It is theorized that IFN- γ suppresses viral RNA synthesis and reactivates cellular protein synthesis to facilitate the clearance of the virus. So no vaccine or treatment is available to combat SINV. The infection can be avoided by using protective clothing to reduce the infection from mosquito bites (Adouchief et al., 2016).

1.6. Importance of Multiplex antigen platform

We live in the era of pandemics, where various pathogens can emerge and affect humans. The population is continuously increasing, meaning that the number of breeding sites for arboviruses is as well increasing. There is increased international movements and travels around the world, and this could favour the spread of arboviruses between countries. Many other factors may result in the spread of arboviruses, such as change in land use due to increased population and the increased cargo transportation of goods. Change in climate with increased rainfall can also increase the number of vectors, such as mosquitoes and ticks to spread the virus. So multiplex assays would be great for surveillance during the time of increased viral infection, hence the importance of the study.

1.7. Problem statement

In South Africa the mosquito-borne viruses such as SINV and WNV, along with tickborne viruses like CCHFV, are known to cause sporadic outbreaks annually. These outbreaks mostly occur after heavy rainfall as it favours vector breeding. However, between large epidemics the prevalence and circulation of these viruses is largely unknown. Surveillance of arboviruses in human and wildlife populations plays a crucial role in effectively assessing the risk of infectious disease outbreaks in humans and/or animals. It also assists healthcare providers in considering circulating pathogens in the differential diagnosis for patients presenting with symptoms. The need for reliable diagnostic tools is evident, especially given the challenges posed by the co-circulation of arboviruses and the shared clinical manifestations.

Molecular diagnostic methods are known to have a high specificity, but they tend to be effective mainly during the initial stages of infection and have challenges in areas with limited resources due to high expenses and lack of facilities. Therefore, serology, which plays a vital role in diagnosis and surveillance strategies for arboviruses, becomes crucial (Kerkhof et al., 2020; Ward et al., 2022). The identification of IgG immunoglobulin offers an extended timeframe for diagnosis since antibodies can remain detectable in the bloodstream for longer durations. Although not easily automated for surveillance, immunofluorescence assays (IFAs) do have a role in smaller surveillance studies and diagnosis. IFAs accurately detect these antibodies, serving as markers of viral activity and providing valuable information for surveillance. The lack of commercially available kits specifically designed for screening serum

samples for lesser-known arboviruses presents a significant challenge in the field of diagnostics highlighting the necessity of developing our own in-house IFA. IFA can be developed using infected or transfected cells to make antigen slides (Ikegami et al., 2002). Recognizing the complexity of co-circulating arboviruses there is a need for the development of multiplex IFAs that will utilize more than one antigen to screen samples, enabling the simultaneous detection of antibodies against multiple arboviruses in a single test to reduce time, reagents, and effort in screening samples for multiple viruses for emerging viruses. In this study, the integration of molecular constructs for level 4 pathogens and infected cells for laboratory-cultured viruses exemplified a safer and more controlled approach to diagnostic tool development.

1.8. Aim

The aim of this study was to prepare polyvalent antigen slides for screening samples for IgG antibody against arboviruses.

1.9. Objectives

- To prepare multiple constructs expressing arbovirus proteins from selected viruses known to occur in South Africa, including CCHFV, SINV, and WNV.
- To transfect a mammalian cell line using expression plasmids carrying marker for antibiotic selection and use cells transiently expressing proteins to prepare polyvalent antigen slides.
- To characterize expressed proteins and evaluate transfected cells for detection of IgG antibody against the viruses.
- To infect a suitable cell line with Sindbis virus to prepare cells for antigen slides.
- To screen serum samples using in house developed multiplex assay for IgG against each selected virus.

Chapter 2: Preparation of antigen slides using transfected cells.

2.1. Introduction

Increase in occurrence of emerging and re-emerging arboviral diseases, present significant risks to global public health (Tajudeen et al., 2022). The ability of these arboviruses to adapt to new vectors has the potential to greatly influence their spread to new geographical areas. Various factors contribute to the emergence of arboviruses, such as genetic changes within the viruses themselves, climate changes, adaptation to new reservoir or amplification hosts, the global increase in human populations and the rise in migration (Ciota & Kramer, 2010). These factors indicate the significance of arboviruses in the field of medicine and emphasize the need for early and accurate diagnosis.

Molecular tests are limited to the early stages of infection (Musso & Desprès, 2020). Serological tests offer a wider timeframe for diagnosis compared to direct methods and are applicable for detecting both recent and previous infections targeting IgM and IgG respectively. These tests are useful for disease monitoring and surveillance. The commonly used tests include enzyme-linked immunosorbent assays like (ELISA) and immunofluorescence assays (IFA) (Ernst et al., 2021).

Several studies have reported that IFA is sensitive and a specific test for screening and/or confirming IgM and IgG in serum (Lederer et al., 2013; Niedrig et al., 2008). An IFA is based on antigen-antibody reactivity and used to detect and visualize specific antibodies within a biological sample or antigens within cells or tissues (Im et al., 2019). The specificity of the immunoassay derives from the antibody–antigen interaction (Sztefko, 2002). Indirect IFA is commonly used to detect antibodies to infectious agents as it is easy to use, sensitive and specific (Betterle & Zanchetta, 2012).

Commercially available IFA assays are not readily accessible for all arbovirus infections, hence the need to develop in-house IFA methods. Commercial assays can be expensive to produce, hence manufacturers prioritize producing and distributing assays for more prevalent or high-profile diseases, leaving less prevalent assays with lower accessibility. Resource-limited areas are unable to access the assays due to the high cost. Antigen slides can be prepared from infected or transfected cells.

The production of recombinant proteins plays a crucial role in biotherapeutic and biomedical research (Khan et al., 2016). It involves manipulating DNA molecules through recombinant DNA technology. For expression of selected viral proteins, this process includes inserting genes that encode for viral proteins of interest, into a suitable vector. The organism's genome can be manipulated by introducing new genes and regulatory elements or by blocking the expression of existing genes through gene recombination (Khan et al., 2016). The vector containing the gene is then introduced into a host organism, which is grown to produce multiple copies of the DNA fragment. This plasmid can then be used to transfect mammalian cells. Recombinant DNA technology offers means of producing antigens for diagnostic tests that are cost-effective to produce and safer to generate compared to viral cultivation for pathogens that require high biocontainment (Fox & Klass, 1989). The synthetic genes offer the advantage of flexibility, as it can be designed to produce specific antigens that might not be commercially available (Khan et al., 2016).

Traditionally, recombinant proteins have been produced in mammalian cells, insect cells, bacteria, and yeast (Ahn et al., 2008). Mammalian cell lines, despite lower yields, are considered the gold standard due to their similarity to human cells (Zou et al., 2022). Mammalian cells are preferred for producing complex glycosylated proteins because they allow for proper folding and post-translational modification (Khan, 2013). There is a wide range of mammalian cell lines available from different tissues and species with suitable growth properties, making them an ideal expression system (Tripathi & Shrivastava, 2019). Mammalian cell expression has demonstrated the capability to produce proteins that closely resemble the target viruses (Gray, 1997).

CCHFV, SINV, and WNV have been detected in South Africa and are medically important pathogens that are likely underdiagnosed. Patients present with similar clinical symptoms and the viruses circulate in overlapping regions hence it was decided to target these viruses in a multiplex IFA using constructs expressing arbovirus proteins of CCHFV nucleoprotein (NP), WNV envelope protein domain III (EDIII) and SINV capsid (C).

2.2. Methods and Materials

2.2.1. Preparation of plasmid constructs for expression of viral proteins

Synthetic genes expressing selected viral proteins of CCHFV, WNV and SINV inserted in pcDNA 3.1 were designed and obtained from GenScript.

2.2.1.1. CCHFV NP expression construct

The gene encoding the NP of CCHFV, cloned into a mammalian expression plasmid pcDNA3.1 was available from a previous study. The NP from the South African CCHFV isolate 92/ 01, (GenBank Accession number KC880335.1), was codon optimised and cloned into a mammalian expression plasmid using NotI and BamHI restriction sites to flank the gene. The gene (1492 bp) was constructed to include a Kozak sequence and his tag (Figure 2.1). The pcDNA3.1 (+), contains essential components T7 and BGH priming sites, a CMV promoter, an ampicillin resistance gene, and a neomycin resistance gene.

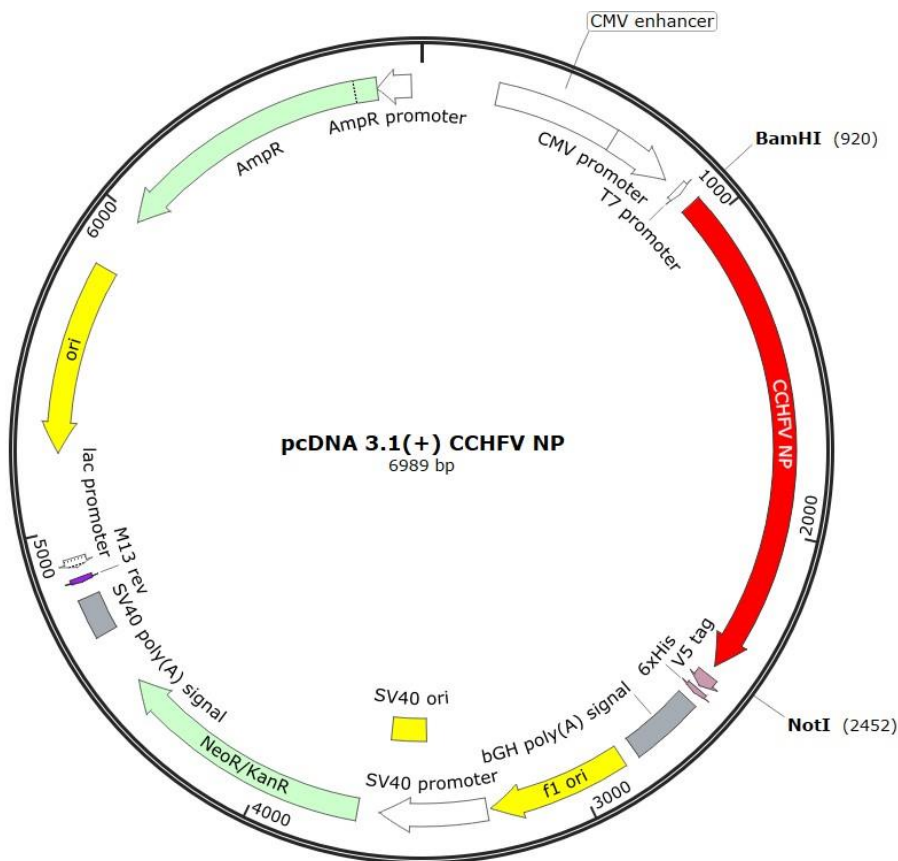


Figure 2.1: Vector map for pcDNA 3.1 CCHFV NP drawn using snap gene version 7.0.1.

2.2.1.2. WNV EDIII expression vector

The 319 bp gene encoding the EDIII of WNV isolate SA93/01, (Genbank Accession number EF429198) was codon optimised, synthesised and cloned into pcDNA 3.1 expression plasmid by GenScript. The 5' and 3' ends modified for cloning and expression by adding a kozak sequence, start codon, his tag and stop codon. The modified genes were cloned into a pcDNA 3.1 plasmid using NotI and BamHI restriction sites. The vector map is shown in Figure 2.2.

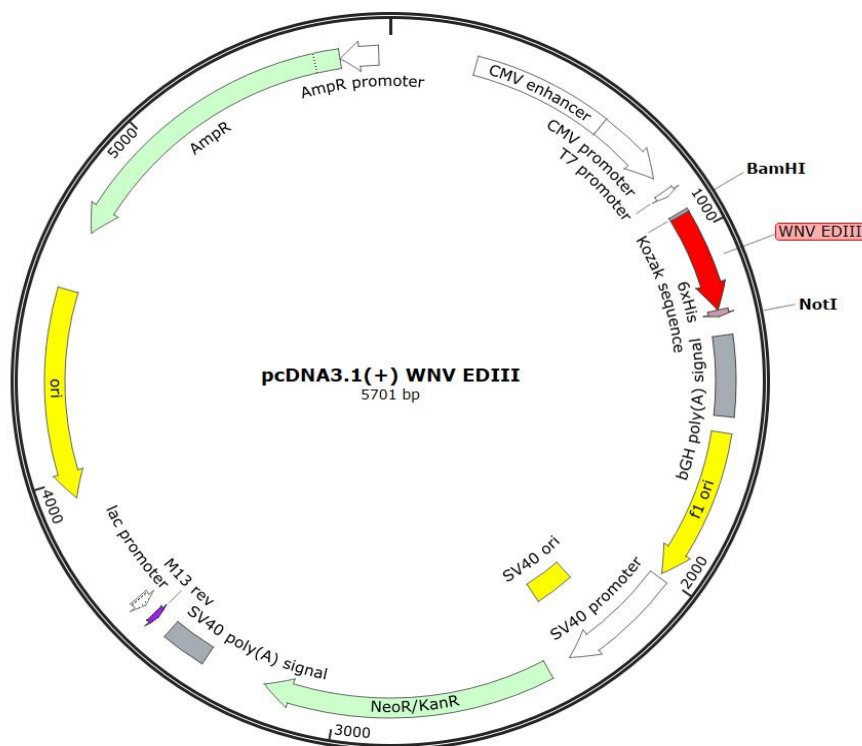


Figure 2.2: Vector map for WNV_EDIII in pcDNA 3.1 drawn using snap gene version 7.0.1. The diagram shows essential components, T7 and BGH priming sites, a CMV promoter, an ampicillin resistance gene, and a neomycin resistance gene.

2.2.1.3. SINV-C Expression vector

The gene encoding the C of SINV strain Girdwood, (Genbank Accession number MF459683) was codon optimised, synthesised and cloned into pcDNA 3.1 expression plasmid by GenScript. The 814 bp gene was modified at the 5' and 3' ends of the gene for cloning and expression by adding a kozak sequence, start codon, his tag and stop codon. The modified genes were cloned into a pcDNA 3.1 plasmid using NotI and BamHI restriction sites. The vector map is shown in Figure 2.3.

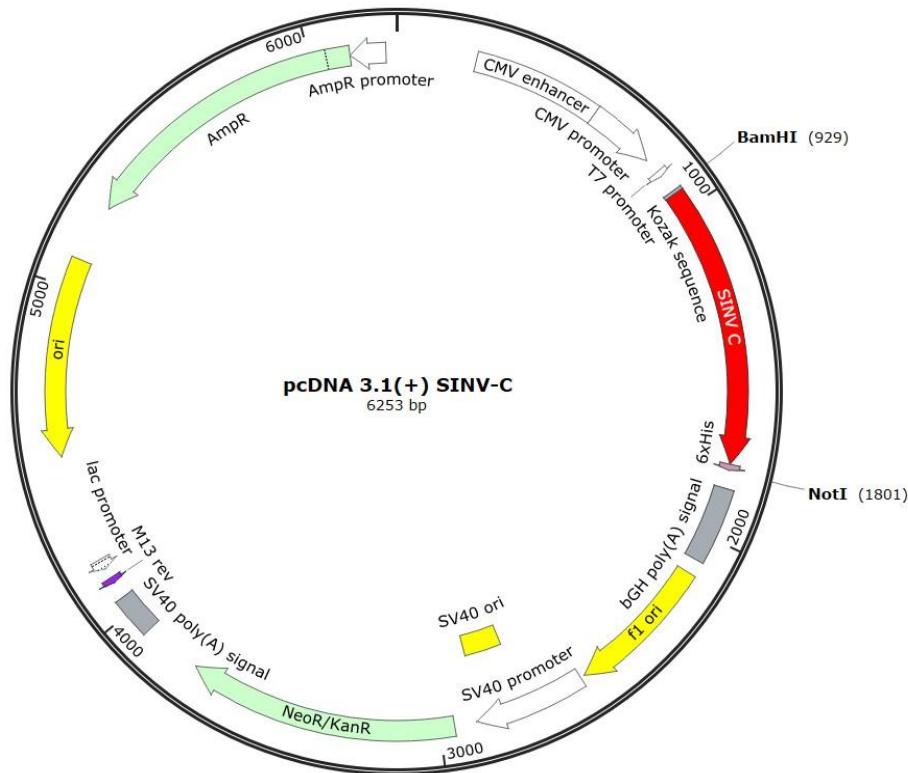


Figure 2.3: Vector map for SINV capsid in pcDNA 3.1 drawn using snap gene version 7.0.1. The diagram includes components such as, T7 and BGH priming sites, a CMV promoter, an ampicillin resistance gene, and a neomycin resistance gene.

2.2.2. Modification of constructs for expression of WNV and SINV

An error in the design of the synthetic gene caused a downstream sequence frame shift in the genes for WNV and SINV. An additional base in the kozak sequence (gccRcc**ATGG**) was responsible for this frameshift. In order to avoid having to reorder new constructs the existing constructs were amplified using an inverse PCR to delete the unwanted base.

Two primers were designed to perform an inverse PCR (Table 2-1). The reverse primer targeted a region of the Kozak sequence and BamHI restriction site, and forward primers were designed targeting the 5' end WNV and SINV gene inserts EDIII and C respectively (Figure 2.4). Inverse PCR was performed using Q5® High-Fidelity DNA Polymerase (New England BioLabs, USA) according to the manufacturer's recommendations as shown in table 2-2. The PCR reaction was done on Proflex™ PCR system (ThermoScientific, USA) using the following cycling conditions: Initial denaturation 98°C 30 seconds one cycle, followed by 30 cycles of denaturation at 98°C

for 10 seconds, annealing at 72°C for 30 seconds, elongation at 72°C for 3 minutes and followed by one final elongation at 72°C for 2 minutes (Figure 2.5).

Table 2-1: Forward and reverse primer nucleotide sequences designed.

Primers	Nucleotide sequence	Forward/Reverse	G/C content	Tm
UniRev	GGT GGC GGA TCC GAG CTC	Reverse	72.2%	61.1°C
Wnile	ATG GCC TTC AAG TTT GTG GGA AC	Forward	47.8%	58.2°C
SinF	ATG AAC CGA GGA TTC TTT AAT ATG CTG G	Forward	39.3%	57°C

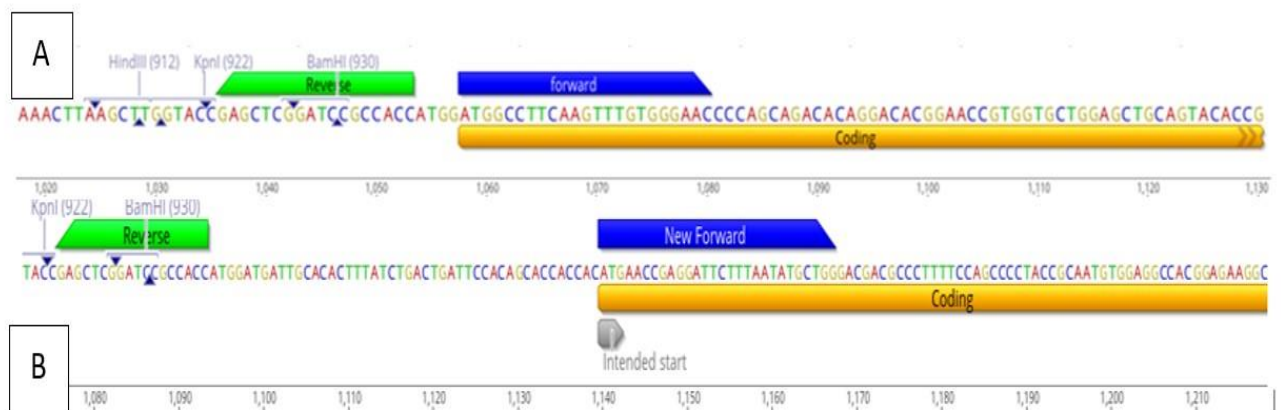


Figure 2.4: A: shows the forward and reverse primers used to remove the extra four bases of pcDNA WNV EDIII designed using Geneious Prime (Version 11.0.18+10). B: shows the forward and reverse primer designed to remove the non-coding region of pcDNA SINV-C (Geneious Prime).

Table 2-2: Reagents for preparation of PCR using Q5® Hot Start High-Fidelity DNA Polymerase

Component	Volume	Final Concentration
5X Q5 Reaction Buffer	10 µl	1X
10 mM dNTPs	1 µl	200 µM
10 µM Forward Primer	2.5 µl	0.5 µM
10 µM Reverse Primer	2.5 µl	0.5 µM

Template DNA	1 μ l	\leq 1,0 ng
Q5 Hot Start High-Fidelity DNA Polymerase	0.5 μ l	0.02 U/ μ l
Nuclease-Free Water	32.5 μ l	
Total	50 μ l	

Initial denaturation	98 °C 30 seconds	}	1 cycle
Denaturation	98 °C 10 seconds		
Annealing	72 °C 30 seconds	}	30 cycles
Elongation	72 °C 3 minutes		
Final elongation	72 °C 2 minutes		
		}	1 cycle

Figure 2.5: Cycling Conditions

The PCR products were purified using Promega Wizard®SV Gel and PCR Clean-up System (Promega, USA) according to the manufacturer's instructions. The purified PCR product was dried using a DNA concentrator (Eppendorf AG, Germany) and stored at 4°C until required. The PCR was digested using DpnI to remove methylated DNA. The reaction mix was prepared according to Table 2-3 and the reaction incubated overnight at 37°C. The products were purified using Promega Wizard®SV Gel and PCR Clean-up System (Promega, USA) according to the manufacturer's instructions. The purified product was phosphorylated and ligated simultaneously as shown in Table 2-4. The reaction was incubated at room temperature for 3 hrs then at 4°C overnight. The ligated products (plasmids) were transformed into NEB® 10-beta Competent E. coli (High Efficiency). The ligated plasmids were designated pcDNA 3.1 WNV EDIII and pcDNA 3.1 SINVC.

Table 2-3: DpnI digestion reaction mixture

Component	Volume
DpnI (10 U/ μ L)	1 μ l

Tango Buffer (10X)	2 μ l
Nuclease-free water	17 μ l
Total	20 μ l

Table 2-4: Phosphorylation/ ligation reaction mixture

Component	Volume
10X T4 DNA Ligase buffer	2 μ l
ATP, 10 mM	2 μ l
T4 Polynucleotide Kinase (10 U/ μ L)	1.5 μ l
T4 DNA Ligase (1 U/ μ L)	1.5 μ l
Nuclease- free water	13 μ l
Total	20 μ l

2.2.3 Control plasmid

The plasmid pcDNA3.1 GFP, which contains the enhanced green fluorescent protein (eGFP), prepared from a previous study was available in the lab as glycerol stocks. The pcDNA 3.1 GFP plasmid was transformed together with other constructs and was used as a positive control for transfection experiments to confirm expression of cells.

2.2.4. Transformation of plasmids into *Escherichia coli* cells

NEB® 10-beta Competent *E. coli* (High Efficiency) cells were transformed with pcDNA 3.1 SINV-C and pcDNA 3.1 WNV_EDIII plasmids using a heat shock method according to the manufacturer's instructions. Briefly, 200 ng of pcDNA 3.1 SINV-C and pcDNA 3.1_WNV EDIII respectively were each added to a 50 μ l aliquot of competent cells on ice for 30 min. The cells were subsequently heat shocked for 30 seconds at 42 °C in a water bath and returned to ice for an additional 5 min. NEB 10beta/stable outgrowth medium was added into the mixtures to a final volume of 1ml each and the reaction mix incubated for 1 hour at 37 °C while shaking. The cells were plated onto Luria-Bertani (LB) agar plates overnight containing 0.1 mg/ml ampicillin (LB/amp). LB/amp plates were prepared as follows: 2,5g tryptone, 1.25g yeast extract, 2.5g sodium chloride, 3.75g agar and distilled water to a final volume of 250 ml, autoclaved at 121 °C for 30 minutes, then allowed to cool and 0.1 mg/ml ampicillin added. The

following day single colonies were selected for identification of positively transformed cells. Each colony was 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. A 1ml aliquot of the overnight culture was transferred to a 25 ml LB/amp to prepare a large yield of plasmid. The plasmid was purified using QIAGEN® Plasmid Plus Midi Kit according to the manufacturer's instructions.

Glycerol stocks of pcDNA 3.1 CCHFV NP and pcDNA 3.1 GFP stored at -80°C were retrieved and inoculated into a 5ml LB/amp broth containing 0.1 mg/ml of ampicillin and incubated overnight at 37 °C on a shaker. The plasmid DNA was purified from overnight cultures using the PureYield™ Plasmid miniprep system (Promega, USA) according to manufacturers' instructions. Briefly, 1.5 ml bacterial cultures were centrifuged at 14000 rpm for 1 minute (min). The pellets were resuspended in 600 µl ultrapure water. A 100 µl aliquot of lysis buffer supplied in the kit was added to lyse the cells and then neutralised with a 350 µl aliquot of neutralization solution. The mixtures were centrifuged at 14000 rpm for 3 min. The supernatant was transferred to the PureYield™ Minicolumn and centrifuged for 30 seconds at 14000 rpm. Endotoxin removal wash was added to each column and centrifuged. The columns were then washed with column wash solution containing ethanol and centrifuged for 30 seconds. DNA was eluted in 30µl of nuclease free water. A Nanodrop™ 2000 (Thermo Fisher Scientific, USA) was used to determine the DNA concentration. The plasmids were transformed into NEB® 10-beta Competent E. coli (High Efficiency) using the heat shock method according to the manufacturer's instructions and the colonies were 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. A 1ml aliquot of each culture was subcultured into 25 ml LB/amp overnight, the plasmid was purified using the QIAGEN® Plasmid Plus Midi Kit and transformation confirmed using restriction digestion.

2.2.5. Purification of plasmids

A 25ml culture was prepared and purified using the QIAGEN® Plasmid Plus Midi Kit according to manufacturer's instructions. The bacterial culture was harvested by centrifugation at 14000 rpm for 15 minutes at 4 °C. The supernatant was discarded, and pellet re-suspended in 4ml of Buffer P1. A 4ml of Buffer P2 was added to lyse the bacterial cells and incubated for 3 minutes at room temperature. Buffer S3 was added

and mixed to the lysate to stop cell lysis and incubated for 10 minutes. The cell lysate was filtered through a QIAfilter cartridge and passed through a QIAGEN Plasmid Plus spin column with Buffer BB. Buffer ETR was added to the column to remove endotoxins from the DNA. Buffer PE was added to wash the DNA. DNA was eluted by adding 100 µl of Buffer EB and incubated for 1 minute. The column was centrifuged for 1 minute at 14000 rpm. The DNA concentration and purity were determined using NanoDrop2000 spectrophotometer and aliquots were stored at -20 °C. Confirmation of positive transformants was done using restriction digest with restriction enzymes that cut HindIII and BamHI that flank the insert.

2.2.6. Confirmation of positive transformants using restriction digestion

A restriction digestion was performed on the purified plasmids to confirm the presence of the genes of interests (Table 2-5). Restriction enzymes NotI and BamHI (Promega), Buffer D (Promega), and nuclease free water were added to pcDNA 3.1 CCHFV NP, pcDNA 3.1 SINV-C, pcDNA 3.1 WNV_EDIII plasmid DNA and incubated at 37 °C in a water bath for two hours. The products were separated using electrophoresis and a 1% SeaKem® LE Agarose gel at 80V for 60 minutes and postained using GelRed™ Nucleic Acid Gel Stain 10000X solution (Biotium, USA) for 45 min and visualised under UV light.

Table 2-5: Restriction enzyme by double digestion using BamHI and NotI

Component	Volume
Buffer D 10X Buffer	2 µl
BamHI 10u/µl	1 µl
NotI 10u/µl	1 µl
DNA 1µg/µl	3 µl
Nuclease free water	13 µl
Total	20 µl

2.2.7. DNA Sanger sequencing of pcDNA 3.1 SINV-C and pcDNA 3.1 WNV EDIII.

To confirm that the inverse PCR had correctly modified the plasmids to remove the four bases that caused the frameshift, each plasmid was sequenced using primer sites

upstream of the inserted gene pcDNA 3.1 EDIII and pcDNA 3.1 SINV-C and Big Dye Terminator ready reaction kit (Applied Biosystems, USA) according to manufacturer's instructions. Sequencing was performed using the T7 forward primer and BGH reverse primer located on the plasmid, T7 upstream from the inserted gene and BGH downstream from the insert gene. The sequencing reaction was prepared as shown in Table 2-6. The sequence data was analysed using Geneious Prime software (Version 11.0.18+10).

The sequence data was aligned with the reference sequence from GenScript using Geneious Prime (Version 11.0.18+10). The sequence data was further translated into protein sequence using ExPASy (<https://web.expasy.org/translate/>). The protein sequence was blasted using BLASTp (<https://blast.ncbi.nlm.nih.gov/>) to determine if the gene sequence encoded for the correct protein.

Table 2-6: Sequencing reaction mixture preparation

Reaction component	Volume
Terminator Ready Reaction	1 µl
Sequencing primers: forward/reverse (0.8 picomolar/ primer)	4 µl
Dilution Buffer	2 µl
Template DNA	2 µl
Nuclease Free water	1 µl
Total	10 µl

The sequencing reaction mixture was run on a Proflex™ PCR system (ThermoScientific, USA) by using the following conditions: initial denaturation at a temperature of 96 °C for a duration of 1 minute. Then, this was followed by 25 cycles, each consisting of denaturation at 96 °C for 10 seconds, annealing at 50 °C for 5 seconds, and elongation at 60 °C for 4 minutes. After the cycles were completed, the samples were kept at 4 °C until the EDTA/ethanol precipitation method was performed. The sequencing reaction was cleaned up using the ethylene-diamine-tetra-acetic acid (EDTA)/ethanol precipitation method. A 5 µl of 125mM EDTA was added to 60 µl absolute ethanol in a 1.5ml tube. The volume was adjusted to 20 µl by adding nuclease

free water. The sequencing mixture was then added to EDTA/absolute ethanol, vortexed and incubated for 15 minutes at room temperature. After incubation, the mixture was centrifuged at 14000 rpm for 20 minutes at 4°C and the supernatant was aspirated. The pellet was washed using 500 µl of 70% ethanol and centrifuged again at 14000 x g for 10 minutes at 4 °C. The pellet was dried at 37 °C overnight. To prepare for sequencing the pellet was resuspended in 10 µl of Hi-Di and transferred into a 96 well plate and incubated at 95 °C for 6 min and cooled on ice.

2.2.8. Optimization of transfection methods of mammalian cells

Human embryonic kidney (HEK-293) cells (ATCC CRL-1573) were transfected with pcDNA 3.1 CCHFV NP, pcDNA WNV EDIII, pcDNA SINV-C and pcDNA GFP plasmids. HEK-293 cells were cultivated in a T25 flasks (Corning, USA). The cells were grown on Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco™), 0.1mg/ml penicillin/streptomycin (Life Technologies, USA) and 2mM L- glutamine (Life Technologies, USA) and grown at 37°C, 8% CO₂. When confluent the cells were trypsinised using 500 µl of 0.25% trypsin EDTA for 3 min and 5 ml of DMEM was added to stop trypsin. Cell were counted and seeded at 2x10⁵ to 3x10⁵ cells per well in a 24 well plate and incubated at 37 °C until 70-90% confluent. Lipofectamine™ 3000 reagent (Thermo Fisher Scientific, USA) and X-tremeGENE™ 360 Transfection Reagent (Sigma-Aldrich, USA) were used to transfect the plasmids. Different volumes of Lipofectamine™ 3000 and X-tremeGENE™ 360 were added to the DNA. Lipofectamine™ 3000 ratios used to optimize pcDNA 3.1 CCHFV NP were 1 and 1.5 to 1 µg of DNA and X-tremeGENE™ 360 1, 2 and 3 µl to 1 µg of DNA respectively. pcDNA WNV EDIII was optimized using Lipofectamine™ 3000 amounts of 0.75, 1, 1.5 to 1 µg and 2 µg DNA and X-tremeGENE™ 360 1, 2 and 3 µl to 1 µg of DNA respectively. pcDNA SINV-C was optimized using Lipofectamine™ 3000 amounts 0.75, 1, 1.5 to 1 µg and 2 µg DNA. The optimization parameters are shown in Table 2.7. These complexes were mixed and incubated for 15-20 minutes at room temperature. Transfection media was added to the wells and the complexes were added to the cells. Plasmid pcDNA3.1 GFP was used as positive control. Cells treated with complexes without DNA were used as a negative control. The cells were incubated for 72 hours at 37 °C. IFA were performed to confirm the expression of the viral proteins.

Table 2.7: Optimization of DNA plasmids in HEK-293 cells

Seeding rate of cells per well	Plasmid (concentration of DNA ng/ μ l)	Transfection reagent and DNA concentration (μ g) /amount of reagent (μ l)	Incubation Time (min)	
2X10 ⁵ - 3X10 ⁵	pcDNA3.1 CCHFV NP (6582.8 ng/ μ l)	X-tremeGENE™ 360	20	
		1 μ g / 1 μ l		
		1 μ g / 2 μ l		
		1 μ g / 3 μ l	Lipofectamine™ 3000	15
		1 μ g / 1 μ l		
		1 μ g / 1.5 μ l		
		2X10 ⁵ - 3X10 ⁵	pcDNA3.1 SINV (3714.2 ng/ μ l)	Lipofectamine™ 3000
1 μ g / 0.75 μ l				
1 μ g / 1 μ l				
1 μ g / 1.5 μ l				
2 μ g / 1 μ l				
2 μ g / 1.5 μ l				
2X10 ⁵ - 3X10 ⁵	pcDNA3.1 WNV EDIII (10673.3 ng/ μ l)	Lipofectamine™ 3000	20	
		1 μ g / 0.75 μ l		
		1 μ g / 1 μ l		
		1 μ g / 1.5 μ l		
		2 μ g / 1 μ l		
		2 μ g / 1.5 μ l		
		X-tremeGENE™ 360		
		1 μ g / 1 μ l		
		1 μ g / 2 μ l		
		1 μ g / 3 μ l		

2.2.9. IFA for confirmation of transfected cells

Expression of viral proteins was confirmed using IFA. Transfected cells were fixed 72 hours post transfection and IFA were conducted to detect viral antigen in the HEK-293 cells. Cells were fixed with methanol: acetone in a 1:1 ratio for 20 minutes at -20 °C. Subsequently, the cells were blocked using a blocking buffer (10% sucrose, 0.5% Triton X-100 and PBS) for 20 minutes at room temperature. Protein expression was confirmed using both mouse anti-histidine antibody (Roche, Switzerland) and specific human anti-virus IgG. Each gene has a 6xHIS tag on the 3'end for detection. For detection using mouse anti-his (Roche, Switzerland), the mouse anti-his antibody was diluted 1:200 in blocking buffer, incubated at 37 °C for 90 minutes. The slides were washed four times with 1% PBS-Tween20 for 30 seconds. A secondary antibody, goat anti-mouse IgG labeled with fluorescein isothiocyanate (FITC) (Sigma-Aldrich, USA), diluted 1:20 in 0.1% Evan's Blue, was applied and incubated at 37 °C for 30 minutes. The slides were washed four times with 1% PBST for 30 seconds, then dried and mounted using mounting media. The slides were viewed under a fluorescence Leica DM1000 microscope (Leica Microsystems, Germany). For confirmation of expression using specific positive serum, human anti-CCHFV or anti-SINV or anti-WNV IgG was diluted 1:10 in PBS and added to the slides and incubated for 30 minutes at 37 °C. The slides were washed thrice with 1% PBS for 3 minutes. A secondary goat antihuman IgG FITC (Invitrogen, California, USA), diluted 1:20 in 0.1% Evan's Blue, was added, and incubated at 37 °C for 30 minutes. The slides were then washed 3 times with PBS for 3 minutes, air dried and mounted using mounting media. The slides were viewed under a fluorescence microscope (Leica Microsystems, Germany). The control plasmid expressing GFP was used to monitor transfection experiments.

2.2.10. Large scale preparation and characterisation of the CCHFV NP for antigen slides

2.2.10.1. Transfection of pcDNA 3.1 CCHFV NP in HEK- 293 cells for high yield of transfected cells.

HEK 293 cells were transfected using the pcDNA 3.1 CCHFV NP plasmid. The cells were seeded at a rate of at 7 to 8 x10⁶ cells in two T75 flasks (Corning, USA). The cells were grown as described previously in section 2.2.8. The cells were transfected with pcDNA 3.1 CCHFV NP plasmid using Lipofectamine 3000. Lipofectamine 3000 complexes were prepared by diluting 40 µl of lipofectamine 3000 in 938 µl of Opti PRO™ SFM (Gibco™, USA) and DNA dilution complexes were prepared by diluting 75ug of pcDNA 3.1 CCHFV NP in 938 µl of Opti PRO™ SFM and finally 78 µl of P3000 reagent was added to the diluted DNA complex. The complexes were mixed and incubated for 15 minutes at room temperature to facilitate the formation of DNA/Lipofectamine complexes. The cells were washed twice with 1X PBS. Cells were then overlaid with transfection medium (advanced DMEM medium supplemented with 10% FBS and 1% L-glutamine). Thereafter, the transfection complexes were added to the T75 flask and incubated for 72 hours at 37°C, 8% CO₂. The transfected HEK 293 cells were harvested 72 hours post-transfection by trypsinisation. Briefly, the transfection medium was removed and the cells were washed with PBS. A 1ml aliquot of 0.25% trypsin-EDTA (Gibco, Paisley, UK) was added to each T75 flask and incubated for 3 minutes at 37°C. Trypsinisation was stopped by resuspending the cells in 5 ml DMEM growth media. The cells were clarified at 6000 rpm and the pellet was washed in 4 ml of PBS, centrifuged and the supernatant was discarded. The cells were lysed with 5 ml of the lysis buffer at a 1:5 ratio composed of 0.1 ml 100x protease inhibitor, 1.5ml 1M NaCl (pH 8), 0.5 ml 50 mM Tris, 0.1 ml NP40 and dH₂O to a total volume of 10 ml. The cells were lysed at 37°C for 45 minutes while shaking. The lysis was followed by three cycles of freezing and thawing at -80 °C to complete cell lysis. Finally, the cells were centrifuged at 6000 rpm for 30 min. The supernatant was collected and filtered sterilised with 0,22 µm filter and stored at 4°C until purification by affinity chromatography.

2.2.10.2. Purification of recombinant CCHFV NP using affinity chromatography

The CCHFV NP protein was purified using affinity chromatography. A Ni-NTA agarose column was assembled, and 6 ml of Ni-NTA resin (ThermoFisher Scientific, Illinois, USA) was added to the column. The column was equilibrated with 15 ml of His binding buffer (50 mM Tris-HCl; pH 8.0, 5 mM imidazole, 100 mM NaCl) and allowed to flow by gravity. After equilibration, the supernatant (or cleared lysate) was added to the column for binding and allowed to flow through. The column was washed with 200 ml of wash buffer (50 mM Tris-HCl; pH 8.0, 300 mM NaCl, 20 mM Imidazole). The protein was eluted using the elution buffer (50 mM Tris-HCl; pH 8.0, 150 mM NaCl, 300 mM Imidazole) and concentrated using a 10kDa Millipore centrifugal filter (Sigma-Aldrich, USA) by centrifuging at 4500 rpm for 45 minutes. To remove imidazole, PBS was added to the 10kda Millipore centrifugal filter and centrifuged for 30 minutes, this step was repeated four times. The concentration was measured using the nanodrop and stored at -20 freezer.

2.2.10.3. Characterization of expressed CCHFV NP protein

NuPAGE Bis-Tris 4–12% polyacrylamide precast protein gels (Thermo Fisher Scientific, USA) were used for the separation of proteins according to their molecular weight. Protein samples were diluted 1:5 with the Lane Marker Reducing Sample Buffer (Thermofisher Scientific, USA) and heated at 95°C for 5 min. The PageRuler Pre-Stained Ladder (Thermofisher Scientific, USA) was used to determine the size of the protein. The protein samples were separated at 80 V for 90 minutes and then stained with GelCode Blue Safe Protein Stain (Thermo Scientific, USA) for 90 minutes. The gel was destained using distilled water until the bands became visible. The gel image was captured with the Molecular Imager® Gel Doc™ XR+ (Bio-rad, USA).

2.2.10.4. Characterization of recombinant protein using Western blot

Western blot was performed to detect the CCHFV NP antigen by transferring proteins from the gel onto the polyvinylidene difluoride (PVDF) transfer membrane (Thermo Scientific, USA). To transfer the proteins, PVDF membrane was first activated by soaking it in 100% methanol for 5 minutes, then soaked in 1X Tris- buffered saline, 0.1 % Tween 20 (TBST) with the thick blot paper and the gel for 10 minutes. The proteins were transferred from the gel to the PVDF membrane using the Trans-Blot Turbo Transfer System (Bio-Rad, California, USA) at 25V for 30 minutes. After the transfer,

the PVDF membrane was washed with TBS for 5 minutes and then blocked with 5% skimmed milk-TBS for 1 hour. The membrane was rinsed with TBS for 5 minutes. Anti CCHFV serum was used as the primary antibody by diluting the serum in 3% skimmed milk-TBS, 0.1% Tween 20 (TBST) at a 1:200 dilution and incubated for two hours. After the incubation, the membrane was washed twice with 3% skimmed milk-TBST for 10 minutes and rinsed for 5 minutes with TBS. The secondary antibody, goat anti-human IgG alkaline phosphatase (AP), was diluted 1:5000 in 3% skimmed milk-TBST and the membrane exposed for 60 minutes. The membrane was washed subsequently with TBS for 10 min, then 3% skimmed milk TBST for 30 minutes and a final wash with TBS for 10 minutes. The protein bands were visualized using 1-Step NBT/BCIP Substrate Solution (Thermofisher Scientific, USA).

2.3. Results

2.3.1. Modification of plasmids

Two plasmids were modified to correct a design error prior to transformation. The pcDNA SINV-C and pcDNA WNV EDIII plasmids were amplified using the primer pairs described in the Materials and Methods section (2.2.2). Inverse PCR products, were analyzed on a 1% agarose gel electrophoresis as shown in Figure 2.6 A and B. The remaining PCR products were digested with DpnI, followed by phospholigation.

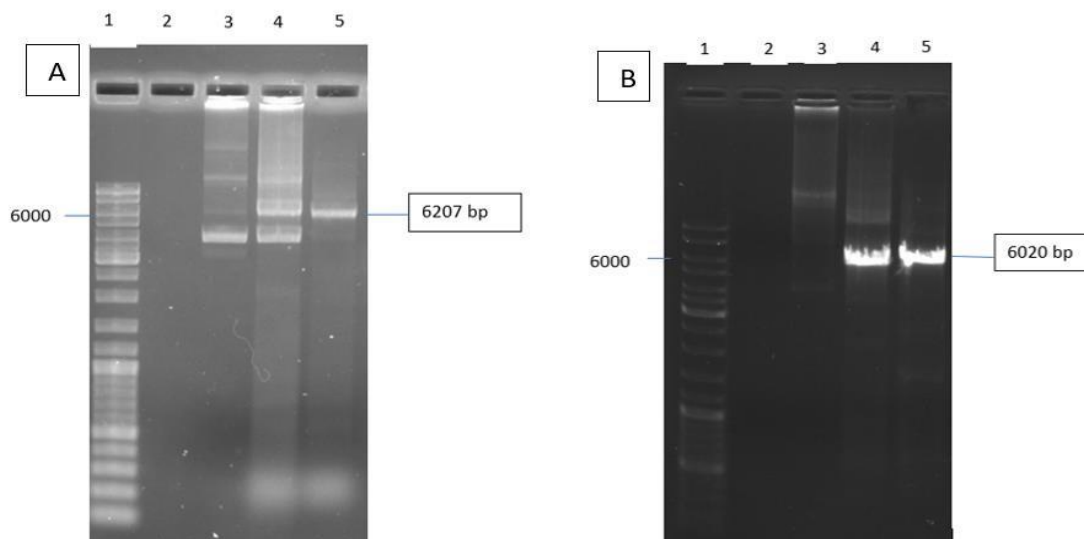


Figure 2.6: A. Agarose gel electrophoresis PCR products using the forward and reverse primers for pcDNA SINV-C. Lane 1: DNA marker (O'GeneRuler™ DNA Ladder Mix, #SM0333). Lane 2: negative control, no DNA. Lane 3: negative control, DNA without primers. Lane 4: PCR product of undiluted DNA and lane 5 is PCR product with diluted DNA 1:1000. B. Agarose gel electrophoresis PCR product using forward

and reverse primers for pcDNA WNV EDIII. Lane 1: DNA marker. Lane 2: empty. Lane 3: primers with no DNA used as negative control Lane 4: PCR product of undiluted DNA and lane 5: DNA diluted 1:1000.

2.3.2. Purification of plasmids

The plasmids pcDNA 3.1 SINV-C, pcDNA 3.1 WNV EDIII, pcDNA 3.1 CCHFV NP, and pcDNA 3.1 GFP, were successfully transformed into NEB® 10-beta Competent E. coli (High Efficiency) cells, and positive transformants were selected on LB/amp agar plates. Plasmids were purified using a mini prep kit, and their measured concentrations were determined to be 320.9 ng/μl, 218.2 ng/μl, 172.3 ng/μl, and 381.8 ng/μl respectively. The remaining culture was subcultured in 25 ml LB/amp and purified using the QIAGEN® Plasmid Plus Midi Kit to prepare a high yield of plasmid for transfection. After purification, the concentrations of pcDNA 3.1 CCHFV NP, pcDNA 3.1 WNV EDIII, pcDNA 3.1 SINV-C and pcDNA 3.1 GFP were 6582.8 ng/μl, 10673.3 ng/μl, 3714.2 ng/μl, and 9866.9 ng/μl respectively.

2.3.3. Restriction enzyme digestion to confirm the presence of gene in plasmid

The pcDNA 3.1 plasmids containing CCHFV NP, WNV EDIII and SINV C genes were double digested using Not 1 and BamHI restriction enzymes. The digestion reactions were separated by electrophoresis using a 1% agarose gel at 80V for 60 minutes and post-stained using GelRed™ Nucleic Acid Gel Stain 10000X solution (Biotium, USA) for 45 min and visualized under a UV transilluminator. Digestion products of estimated 1492 bp, 319 bp, and 814 bp for genes CCHFV NP, WNV EDIII and SINV C were identified (Figure 2.7 A-C).

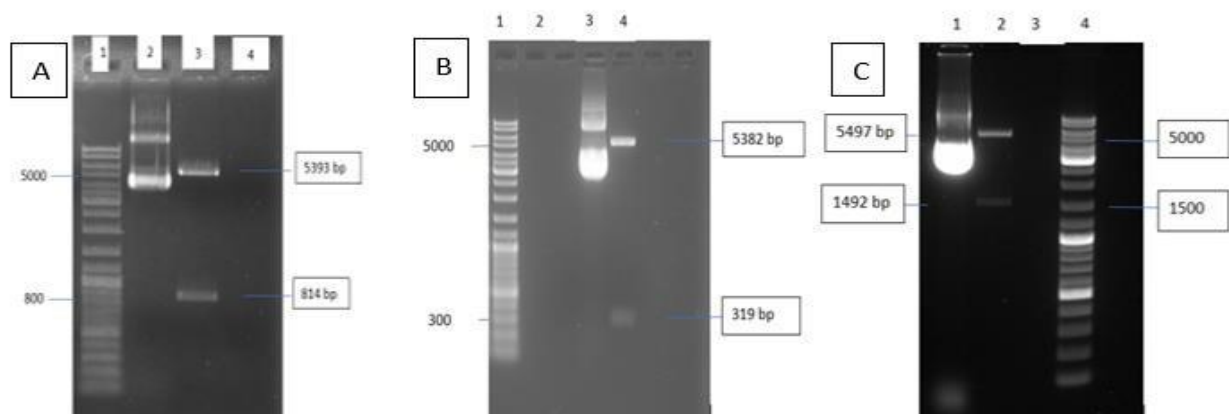


Figure 2.7: A. restriction digestion of pcDNA SINV-C. Lane 1 DNA marker (O'GeneRuler™ DNA Ladder Mix, ready to use, #SM0333). Lane 2 undigested plasmid, lane 3 double digested plasmid and lane 4 negative control (No DNA). B. shows restriction digestion of pcDNA 3.1 WNV EDIII. Lane 1 DNA marker, lane 2 negative control, lane 3 undigested plasmid and lane 4 double digested plasmid. C. restriction digestion of pcDNA 3.1 CCHFV NP. Lane 1 undigested plasmid, lane 2 double digested plasmid, lane 3 negative control and lane 4 DNA marker.

2.3.4. Nucleotide sequence analysis

The nucleotide sequences of pcDNA 3.1 SINV-C and pcDNA 3.1 WNV EDIII were modified using inverse PCR, and the modifications were confirmed by Sanger sequencing. Geneious Prime software was used to align nucleotide sequence data from sequencing results with the data obtained from Genscript. The alignments are shown in Figure 2.8.a and 2.9a. In addition, the nucleotide sequence data was translated using ExPASy (<https://web.expasy.org/translate/>) to confirm that the nucleotide sequence coded for the correct protein (Figure 2.8.A and 2.9.B). The translated protein sequences corresponding to the nucleotide sequences are shown in Figure 2.8.A and 2.8.B.

Genscript WNV EDIII sequence	-----GGATCCGCCACCATGGATGGCCTCAAG	28
WNVF1_c01_2023-02-03-07-58-18 (2).ab1	CMYMSKCYCCGAMRCGGCKCGTTAACTTAAGCTTGGTAACCGAGCTCGGATCCGCCACCATGG----CCTTCAAG	71
Genscript WNV EDIII sequence	TTTGTGGGAACCCAGCAGACACAGGACACGGAAACCGTGGTGTGGAGCTGCAGTACACCGGCACAGACGGCCCA	103
WNVF1_c01_2023-02-03-07-58-18 (2).ab1	TTTGTGGGAACCCAGCAGACACAGGACACGGAAACCGTGGTGTGGAGCTGCAGTACACCGGCACAGACGGCCCA	146
Genscript WNV EDIII sequence	TGCAAGGTGCCCATCAGCTCCGTGGCCAGCCTGAACGATCTGACACCTGTGGGCGGCTGGTGACCGTGAACCCA	178
WNVF1_c01_2023-02-03-07-58-18 (2).ab1	TGCAAGGTGCCCATCAGCTCCGTGGCCAGCCTGAACGATCTGACACCTGTGGGCGGCTGGTGACCGTGAACCCA	221
Genscript WNV EDIII sequence	TTCTGTCTGTGGCCACAGCCAATAGCAAGGTGCTGATCGAGCTGGAGCCCCCTTTTGGCGATTCCCTATATCGTG	253
WNVF1_c01_2023-02-03-07-58-18 (2).ab1	TTCTGTCTGTGGCCACAGCCAATAGCAAGGTGCTGATCGAGCTGGAGCCCCCTTTTGGCGATTCCCTATATCGTG	296
Genscript WNV EDIII sequence	GTGGGCAGAGGCGAGCAGCAGATCAATCACCCTGGCACAAGTCCACCACCACCACCACCACTGAGCGGCGCG	328
WNVF1_c01_2023-02-03-07-58-18 (2).ab1	GTGGGCAGAGGCGAGCAGCAGATCAATCACCCTGGCACAAGTCCACCACCACCACCACCACCACTGAGCGGCGCG	371
Genscript WNV EDIII sequence	-----	403
WNVF1_c01_2023-02-03-07-58-18 (2).ab1	CGAGTCTAGAGGGCCCGTTTAAACCCGCTGATCAGCCTCGACTGTGCCTTCTAGTTGCCAGCCATCTGTGTGTTG	446

Figure 2.8: A: Alignment of the pcDNA 3.1 WNV EDIII sequencing data with the EDIII sequence data provided by GenScript. The removed ATGG nucleotides are highlighted in red.

5' Frame 1

MAFKFVGT PADTGHGTVVLELQYTGTDGPCKVPISSVASLNDLTPVGRVLTVNPFVSVATANSKVLIELEPPFGDSYIVVGRGEQQINHHWHKSHHH

HHH-

Figure 2.8: B: Analysis of WNV ED III protein by ExPASy showing that it is in frame.

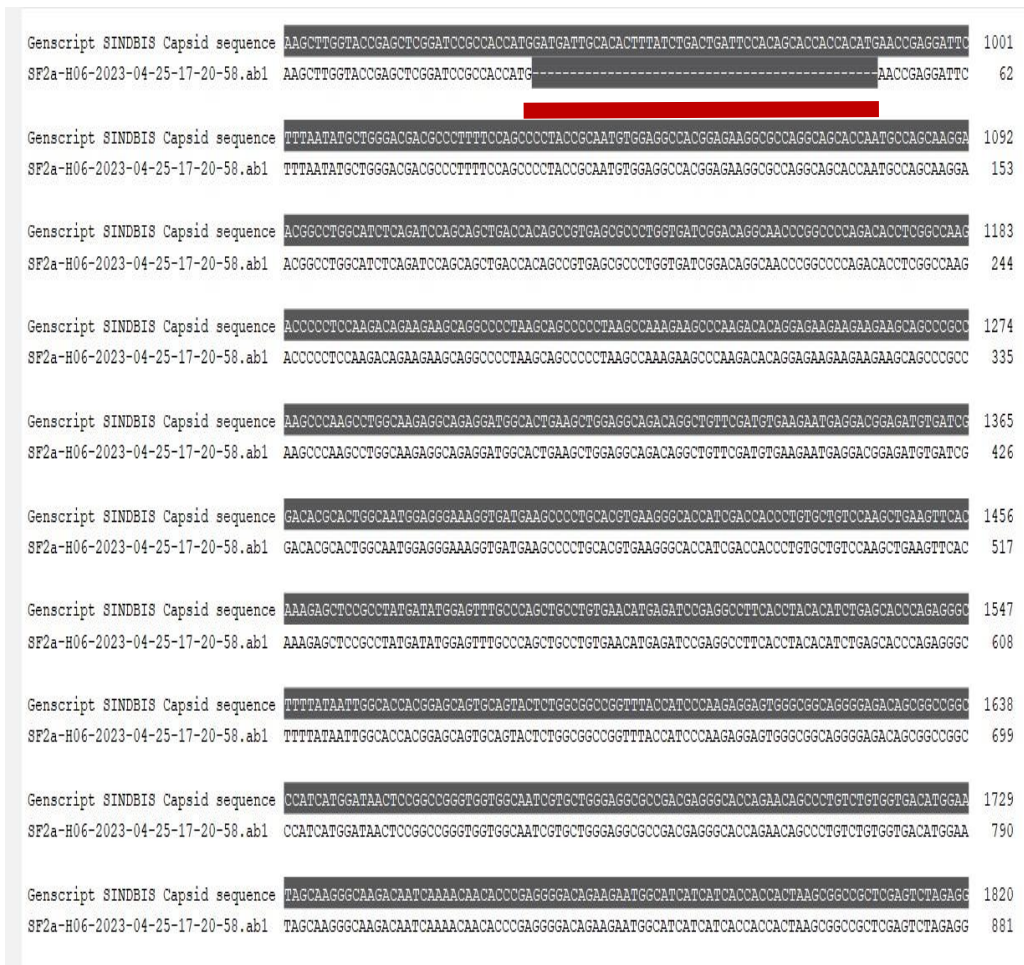


Figure 2.9: A. Alignment of the sequencing data with the pcDNA 3.1 SINV-C sequence data provided by GenScript. The removed ATGG and non-coding region are highlighted in red.



Figure 2.9: B. Analysis of SINV C protein by ExPASy showing that it is in frame.

2.3.5. Protein expression in mammalian cells

HEK-293 cells were transfected with pcDNA 3.1 CCHFV NP, pcDNA 3.1 WNV_EDIII, pcDNA 3.1 SINV-C and pcDNA 3.1 GFP. Transfection of the plasmids were optimized using various concentrations of X-tremeGENE and lipofectamine 3000 transfection

reagents to the DNA. The results demonstrated that the optimal transient expression of NP and EDIII protein was achieved when using 2 µg of DNA to 1 µl Lipofectamine 3000 and 2 µl of P3000 reagent respectively. Cells transfected with pcDNA CCHF NP showed a transfection efficiency of > 50% when reacted against both mouse anti-HIS IgG (Figure 2.10.B) or human anti CCHF IgG (Figure 2.10.C). pcDNA 3.1 SINV-C showed no expression despite the optimisations. Cells transfected with pcDNA 3.1 WNV EDIII reacted with mouse anti-HIS IgG (Figure 2.10.D) showed a low transfection efficiency of less than <20%.

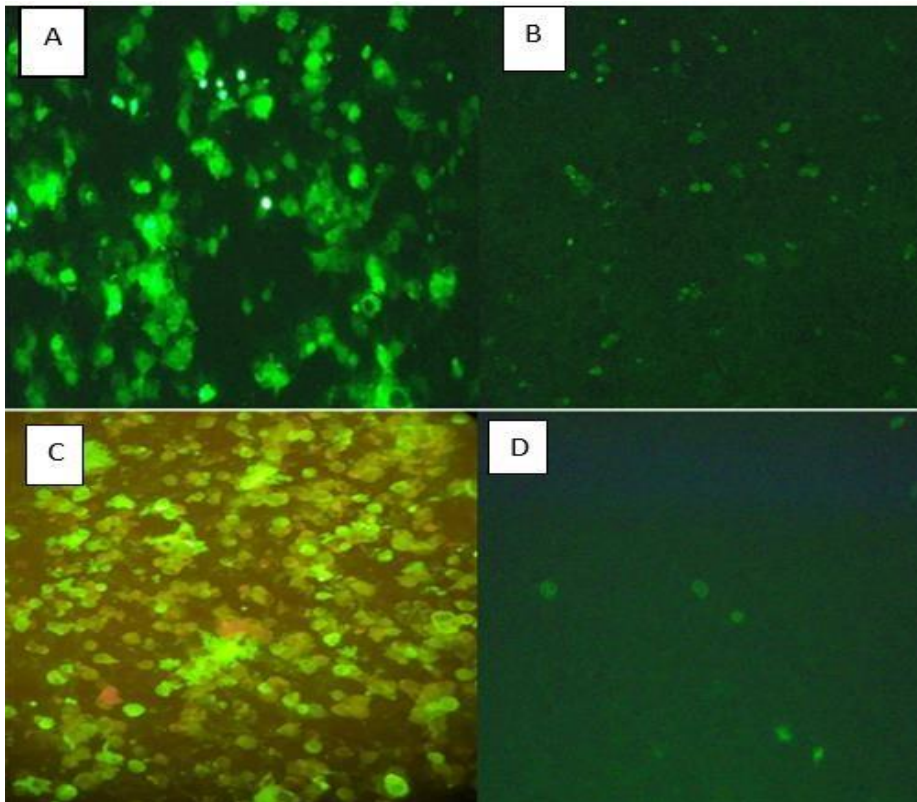


Figure 2.10: A. IFA of pcDNA3.1 GFP transfected HEK- 293 cells. B. Confirmation of CCHFV NP expression in HEK- 293 cells using anti-his antibody. C. Confirmation of CCHFV NP expression in HEK- 293 cells using anti-CCHF IgG human serum. D. Confirmation of WNV EDIII expression in HEK- 293 cells using anti-his antibody. Cells were visualised using Leica Microsystems microscope at 20X magnification.

Following transfection, the HEK 293 transfected cells were collected and stored for future use. The transfected cells were trypsinized and resuspended in a cryoprotective medium comprising of 70% DMEM, 20% FBS, and 10% DMSO and stored at a -80°C.

2.3.6. Characterization of expressed CCHFV NP

The expressed proteins were purified using affinity chromatography. Analysis on NuPAGE Bis-Tris precast polyacrylamide gels confirmed the predicted size of 57 kiloDaltons (kDa) for the recombinant CCHFV NP protein as shown in Figure 2.11. Purified proteins were further characterised using Western blot analysis.

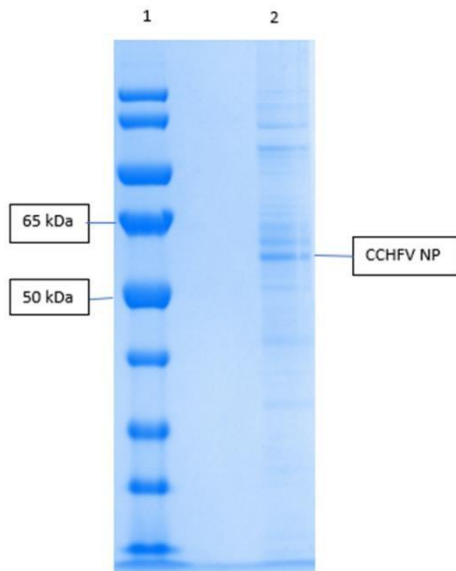


Figure 2.11: SDS-PAGE analysis of CCHFV NP protein. Lane 1 PageRuler™ plus prestained protein marker (Thermo Fisher Scientific, USA). Lane 2 purified NP protein by affinity chromatography.

To further confirm the expression of the CCHFV NP protein, Western blot analysis was performed. The protein was detected using human anti-CCHF antibody confirming expression of 57 kDa proteins (Figure 2.12).

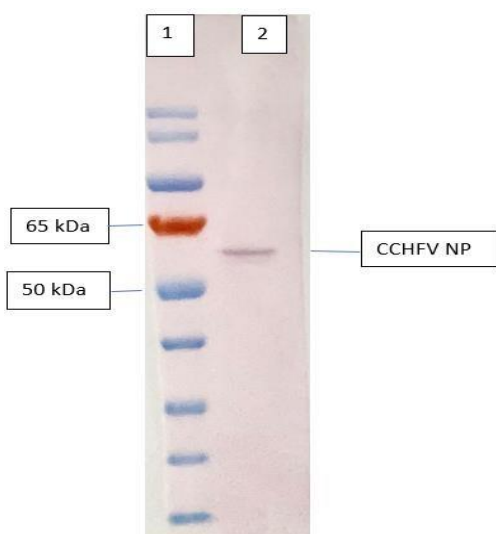


Figure 2.12: Western blot analysis of the recombinant NP of CCHFV. Lane 1: PageRuler™ plus prestained protein marker (Thermo Fisher Scientific, USA). Lane 2: purified CCHF NP protein.

2.4. Summary

HEK293 cell lines are suitable cells for mammalian expression systems for recombinant antigens because of their post-translational modifications which are absent in bacterial and baculovirus expression systems (Tan et al., 2021). These modifications are essential for antigen functionality and antigen-antibody interactions, making them important in the development of in-house IFA. The ability to easily transfect the cells and relatively high protein production rate have made the cell line a preferred option for generating recombinant proteins on a small scale for scientific research purposes (Tan et al., 2021).

Preparation of polyvalent antigen slides required transfected cells expressing recombinant viral proteins of interest. In this chapter the aim was to develop and optimise the methods required to obtain cells expressing recombinant antigens and characterise any successfully expressing arbovirus protein prior to inclusion in the polyvalent antigen slides.

The choice of using recombinant proteins in this study was to mitigate the inherent risks associated with handling infectious agents, particularly those requiring Biosafety Level 4 (BSL-4) containment and also allow for a more focused exploration of specific viral components, to reduce challenges such as cross reactivity. The inclusion of CCHF NP was due to its classification as a BSL-4 agent requiring BSL-4 facilities to culture the virus. WNV EDIII was selected due to its demonstrated reduced serological cross-reactivity with other flaviviruses (Holbrook et al., 2004; Mathengtheng & Burt, 2014). Similarly, SINV C was selected to limit serological cross-reactivity of alphaviruses (Priya et al., 2014; Tuekprakhon et al., 2018). SINV allowed for a more flexible experimental approach because of its classification (BSL-2), allowing the continuation of experiments with infected cells even after unsuccessful transfections. WNV was excluded due to the significant serological cross reactivity against whole viral antigens. The choices of CCHF NP, WNV EDIII, and SINV C were made with consideration of safety requirements, serological cross-reactivity, and the demands of our experimental workflow.

The genes encoding NP, EDIII and C of CCHFV, WNV and SINV were codon optimized and cloned into a pcDNA 3.1. The pcDNA SINV-C and pcDNA WNV EDIII constructs, underwent necessary modifications to address design errors before transformation. This was done by reverse amplification of plasmids, subsequent digestion with DpnI and phospholigation.

pcDNA 3.1 CCHFV NP, pcDNA 3.1 SINV and pcDNA 3.1 WNV EDIII plasmids were transfected respectively into HEK 293 cells and expressions were confirmed using anti his antibody and serum samples from survivors against each specific virus. There was evidence of expression of CCHFV NP with anti-his and anti CCHFV serum samples. WNV EDIII had limited IFA expression level <20% and insufficient yield to include in the polyvalent slides. CCHFV NP was purified using affinity chromatography and protein characterised using SDS and Western blot to confirm the correct size of the expressed protein. Despite correcting the design errors and altering experimental conditions such as transfection reagents and incubation times, experiments failed to yield recombinant SINV C and WNV at sufficient levels for detection on an IFA slide. However, SINV is a biosafety level 2 pathogen and hence an alternative method to produce antigens for serology was through infected cells with whole virus to prepare antigen slides for serology. It was decided to only include SINV infected cells and not continue using WNV infected cells due to the serological cross reactivity among flaviviruses.

Chapter 3: Design of multiplex antigen slides for the detection of IgG against Crimean-Congo haemorrhagic fever and Sindbis virus

3.1 Introduction

The growing geographical expansion and increasing frequency of occurrence of arboviral (arthropod-borne virus) infections pose a challenge to public health (Fauci et al., 2016). Similarity in signs and symptoms often leads to misdiagnoses of these arboviruses, highlighting the critical need for precise diagnostic tools for laboratory confirmation and provide early alerts about potential outbreaks (Mejía et al., 2023). Effectively predicting virus activity in vector populations within human or animal hosts is crucial for implementing control, treatment strategies, and initiating vector management or vaccination programs (Hall et al., 2012). Arboviruses emerge or reemerge and cause epidemics due to various factors such as changes in land use caused by population growth or urbanization, climate change patterns such as increased rainfall, increased population of vectors like mosquitoes and ticks, mutation in the viruses, changes in host and increased cargo transportation (Liang et al., 2015). Given the increased geographic co-circulation of these arboviruses worldwide, it is imperative to develop ideal diagnostic tests capable of detecting various arboviruses.

The traditional serological methods used for diagnosing arboviral diseases includes enzyme-linked immunosorbent assays (ELISA), and immunofluorescence assays (IFA)., ELISAs and IFAs are mostly used as screening tools for arboviral antibodies in humans and animal sera. ELISA operates on the fundamental immunology principle of an antigen binding specifically to its corresponding antibody that generates a signal in the presence of the antibody. ELISA technique is designed to measure antibodies that specifically bind to viral proteins (Sakamoto et al., 2018). ELISA is exceptionally precise and capable of detecting antigens even at extremely low concentrations. The distinctive challenge in using ELISA is the need to utilize the 'optimal' antigen, which should be pure and in its natural conformation, without compromising binding specificity, for instance, through coating techniques (Sakamoto et al., 2018).

IFA uses the same principle of antigen-antibody interaction as ELISA and has been employed as a rapid method for virus detection. IFA uses conjugated antibodies to detect viral proteins in virus-infected cells (Atiya-Nasagi et al., 2022). IFA allow the possibility of multiplexing to reduce time and costs. Multiplexing capability will provide a faster, easier, time-saving and inexpensive technique making it more suitable for use

in endemic resource-limited countries and surveillance (Kerkhof et al., 2020). The subsequent sections of this chapter detail the preparation of antigen slides and the screening process using multiplex/polyvalent and monovalent slides expressing the CCHFV and SINV antigen viruses as confirmatory tests. The emphasis will be on demonstrating multiplex immunofluorescence assay's ability to readily detect antibody against multiple viruses in one test with equally comparable results to ELISA. In the absence of transfected cells expressing recombinant SINV protein for use in the IFA, Vero infected cells were prepared.

This chapter specifically focuses on the application of in-house serological tests, enzyme-linked immunosorbent assays (ELISA), and immunofluorescence assay (IFA), to investigate two prominent viruses, SINV and CCHFV, by detection of immunoglobulin G (IgG) antibodies in serum samples. Multiplex assays can be influenced by interference from multiple antigens or the risk of non-specific reactivity. The primary objective was to show the ability to develop multiplex IFA for detection of more than one arbovirus without interference from multiple antigens being included in the assay and to use ELISA to show concordance between the results from the multiplex assay and ELISA.

3.2. Materials and Methods

3.2.1. Ethical approval

The study was approved by the Environmental and Biosafety Research Ethics Committee (UFS-ESD2022/0119/22/3) of the University of the Free State to culture SINV S.A.AR86 in biosafety level 2 (BSL 2) laboratory. Additionally, approval to work with stored human and animal serum samples was granted by the Health Science Research Ethics Committee (UFS-HSD2022/1278/2510-0002) and the Animal Research Ethics Committee (UFS-AED2023/0040), respectively. Ethics approval letters are attached in Appendices A, B and C.

3.2.2. Serum samples

3.2.2.1. Control samples

Human: A panel of 11 negative human serum samples, designated laboratory numbers VBD 41/13 - 51/13, were collected in a previous unrelated study, from volunteers with

no history of CCHFV and SINV infection. Volunteers provided informed consent. Samples were used as negative controls to optimise the in-house ELISA and establish an appropriate cut-off value. VBD 43/13 was selected as a negative control for IFA. Two serum samples, VBD 17/19 and 18/19, previously tested positive for SINV IgG antibodies using a commercial assay and confirmed with a neutralisation assay, were used as positive controls for the optimization of in house SINV ELISA using a cell lysate antigen and confirmation of in-house IFA using slides prepared with SINV infected cells. Two serum samples, VBD 51/10 and 52/10 from laboratory confirmed CCHF survivors, were used to optimize CCHFV ELISA and confirmation of in-house IFA using slides prepared with transfected cells.

Bovine: Bovine anti-CCHF positive (C++) SPU 21003021 and bovine anti-CCHF negative control (C--) SPU 21007021 (supplied by Professor Paweska, National Institute for Communicable Diseases) were used to optimize the CCHFV ELISA for screening cattle samples.

3.2.2.2. Samples for screening

Human: A total of 386 human serum samples collected from healthy volunteers in Bloemfontein between 2016 and 2017 for an unrelated study on seroepidemiology of CCHFV and other vector-borne diseases were available (HSREC 34/2016). These samples were screened for IgG antibodies against both SINV and CCHFV using inhouse ELISAs.

Bovine: A total of 97 stored cattle serum samples collected between 2022 and 2023 in Bloemfontein from the University of the Free State Paradys Farm, for other unrelated studies were used in the study. The sera were screened for IgG antibodies against SINV and CCHFV using in-house ELISAs.

3.2.2. Virus strain

SINV S.A.AR86 (ex 6123 MBe#6 vero#1) strain was used in the study (supplied by Professor Paweska, NICD).

3.2.3. Preparation of Sindbis virus infected cells for antigen slides

Vero cells (Vero 76, ATCC number CRL- 1587) were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal bovine serum (FBS)

(Gibco™, UK), 0.1mg/ml penicillin/streptomycin (P/S) (Life Technologies, USA) and 2mM L-glutamine (Life Technologies, USA) in a T25 flask (Corning, New York, USA). The Vero cells were allowed to grow and reach 80-90% confluency before passaging. Cells were detached from the flask using 0.25% trypsin-EDTA (Gibco, UK) and were resuspended in 3 ml DMEM. The cells were seeded by dividing at a 1:3 ratio of cells to media in a T25 flask (Corning, USA) and expected to reach 90-100% confluency the following day for infection. On the day of infection, the media was discarded and cells were washed twice with 3 ml PBS, and a 10 µl aliquot of the SINV S.A.AR86 (ex 6123 MBe#6 vero#1) stock was added. Infected cells were incubated at 37°C in a humidified 5% CO₂ incubator for 30 minutes. A 5 ml aliquot of DMEM supplemented with 2% FBS, 0.1mg/ml P/S and 2mM L-glutamine was added to the infected cells. The flask was incubated in 5% CO₂ at 37°C and monitored for cytopathic effects (CPE). Cells were harvested after initial signs of CPE by trypsinizing the cells and resuspending in DMEM supplemented with 10% FBS, 0.1mg/ml P/S and 2mM L-glutamine. Thereafter cells were centrifuged at 6000 rpm for 5 minutes at 4 °C. Supernatant was discarded and cells were resuspended in 2 ml PBS (MilliporeSigma, USA) and 10 µl aliquots spotted per well on 10 well slides. Slides were allowed to dry and then fixed in cold acetone (Sigma-Aldrich, USA) overnight at -20 °C while remaining cells were frozen with freezing medium and stored at -80 °C.

To confirm SINV infection of cells, an IFA was performed. The 10 well slide with fixed cells was allowed to dry. Two SINV IgG positive serum samples were diluted 1:10 in PBS and a 10 µl aliquot was added to each well of the slide followed by incubation for 30 minutes at 37 °C. The slide was washed three times for three minutes with PBS and air-dried. A 10 µl aliquot of goat anti-human IgG FITC secondary antibody (Invitrogen, USA) diluted 1:20 in 0.1% Evans blue was added to the slide and incubated for 30 minutes at 37 °C. Slide was washed again with PBS. Mounting medium (Euroimmun, Germany) was carefully applied and a coverslip applied, and the slide was viewed under a fluorescence microscope using x20 lens (Leica Microsystems, Germany).

3.2.4. SINV ELISA for human serum samples

3.2.4.1. Cell lysate antigen and mock antigen preparation

Stored SINV cell lysate and control mock antigens used in the ELISA were obtained from a previous study (Hanekom, 2013). Briefly, cell lysate antigen was previously

prepared by infecting Vero 76 cells with a SINV S.A.AR86 isolate at a multiplicity of infection (MOI) of 0.1, allowing for a 60-minute adsorption period. Infected cells were maintained in minimum essential media (MEM) (Invitrogen, USA) supplemented with 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), and incubated at 37 °C. Cells were monitored daily for cytopathic effects (CPE). Upon the onset of initial CPE, cells were harvested and centrifuged for 20-minute at 12,500 rpm. After centrifugation, supernatant was discarded and cells lysed with 1% Triton X100 in borate-buffered saline (BBS) and were sonicated on ice at five-minute intervals. To eliminate cellular debris, the lysed cells were centrifuged at 10,000 rpm for 20 minutes at 4 °C, and resulting supernatant fluid was stored as cell lysate antigen at -80 °C. Mock antigen was prepared in a similar way, using uninfected cells processed using the same method outlined for the cell lysate antigen. Mock antigen employed for the ELISA was derived from uninfected Vero cells.

3.2.4.2. SINV ELISA for human serum samples

An indirect in-house ELISA was developed for detection of IgG antibody against SINV in human serum samples. Stored SINV cell lysate and control mock antigens were used to optimize an in-house SINV ELISA using a checkerboard titration of reagents to identify optimal antigen concentration for coating SINV ELISA plate based on the reactivity of known positive and negative controls. Various dilutions of secondary antibody were used to determine an optimal dilution for minimizing background and readings were taken at different intervals to determine an ideal reaction time post substrate addition (Figure 3.1).

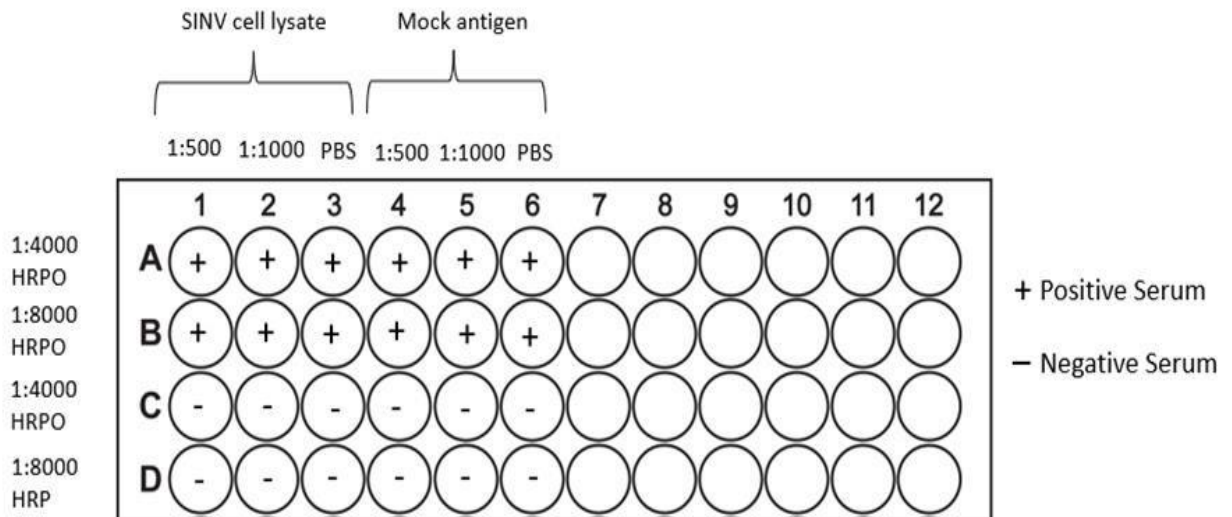


Figure 3.1: Plate layout for optimization of SINV ELISAs

Briefly, a ELISA 96 well polysorb plate (Nalgen Nunc International Corporation, Rochester, USA) was coated with SINV cell lysate antigen and control mock antigen diluted 1:500 and 1:1000 and incubated at 4 °C overnight (Figure 3.1). Subsequently, plates were washed three times with 0.1 % Tween 20 (Promega, USA) in PBS (0.1% PBST) with 15 second soaks and then blocked with a 10% skimmed milk (SigmaAldrich, USA) diluted in PBS solution for one hour. All subsequent washes were performed using 15 second soaks. After blocking, plates were washed three times with 0.1% PBST. Positive control (VBD 17/19 and 18/19) and negative control serum (VBD 43/13) samples were added to the plates, diluted 1:100 in a 2% skimmed milk/PBS solution, and incubated for one hour. Plates were washed six times with 0.1% PBST and goat anti-human IgG horse radish peroxidase (HRPO) (SeraCare Life Sciences Inc., USA) was added at dilutions of 1:4000 and 1:8000 in a 2% skimmed milk/PBST. Plates were washed six times with 0.1% PBST and a volume of 100 µl ABTS (2,2'-Azino-bis-3ethylbenzothiazoline-6-sulfonate acid) (ABTS) (SeraCare Life Sciences Inc., USA) substrate was added, and incubated at room temperature in the dark and readings taken at intervals of five minutes for a duration of 20 minutes. Optical density (OD) of plates was read at 405nm. Net OD was determined by subtracting OD values of the SINV mock antigen from SINV cell lysate antigen-coated plate.

3.2.4.3. SINV ELISA cut-off for human serum samples

To determine the cut-off value to differentiate positive from negative samples, 11 negative control sera were tested in duplicate and the cut-off value was calculated by determining the mean net OD value plus three standard deviations (SD) (Cut off = mean net OD + 3*SD). OD values were normalized by determining percentage positive (PP) values for each sample. The formula for PP was as follows:

PP = (mean net OD of human test sample / mean net OD of positive control) X 100.

3.2.5. SINV ELISA for cattle serum samples

3.2.5.1. Cell lysate antigen and mock antigen preparation

To develop a SINV ELISA to test cattle sera, SINV cell lysate and control mock antigens prepared as described in section 3.4.2.1 were used.

3.2.5.2. SINV ELISA for cattle serum samples

SINV ELISA was performed as described in section 3.4.2.2 (Figure 3.1) using bovine control samples and goat anti-bovine IgG HRPO (SeraCare Life Sciences Inc., USA).

3.2.5.3. SINV ELISA cut-off for cattle serum samples

Two negative bovine serum samples were tested together with negative human serum samples to determine if there was a similarity in the OD values between the negative human controls and the two negative control bovine samples. The unavailability of multiple SINV IgG negative bovine samples was a limitation, and the similarity in OD for human and bovine samples justified use of the cut off determined for human samples for bovine sera (section 3.2.4.3). OD values were normalized by determining PP values for each sample. PP was calculated as described in 3.2.4.3 by using mean net OD of test cattle serum samples divided by mean net OD of the positive control and multiply by 100.

3.2.6. CCHFV ELISA for human serum samples

3.2.6.1. Preparation of recombinant CCHF Gc antigen

A plasmid expressing a soluble form of Gc protein of CCHFV prepared previously in an unrelated study was used (Makoah et al, unpublished manuscript). The stored recombinant protein available was expressed from the CCHFV Gc gene from the South African isolate SPU187/90 which had been codon-optimized, synthesized and cloned into pcDNA3.1+ plasmid (GenScript).

3.2.6.2. CCHFV ELISA for human serum samples

An indirect ELISA was performed using an in house method as described previously (Litabe et al., 2022) and modified for detection of IgG antibody against CCHFV in human serum samples. Recombinant CCHFV Gc, PBS and control sera were used to confirm the dilutions for the in-house CCHFV ELISA. CCHFV Gc was used at a concentration of 0.25 µg/ml and 0.5 µg/ml, with secondary HRPO conjugated antibody dilutions of 1:4000 and 1:8000 (Figure 3.2). PBS was used as control antigen for CCHFV ELISA.

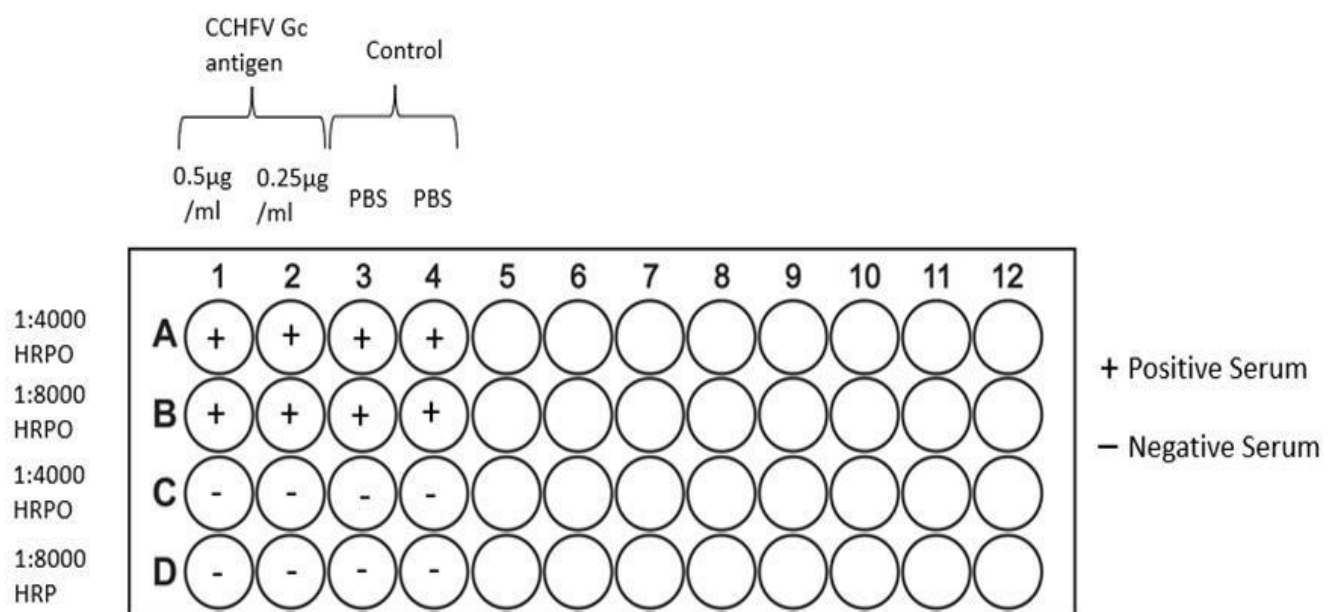


Figure 3.2: Plate layout for optimization of CCHFV ELISA

Briefly, a 96 well polysorb plate was coated with 100 µl/well CCHFV Gc antigen in PBS and incubated at 4 °C overnight. Subsequently, plates were washed three times with 0.1% PBST with 15 second soaks. Plates were blocked with a 10% skimmed milk/PBS solution for one hour. After blocking, plates were washed three times with 0.1% PBST, then positive control serum (51/10 and 52/10) and negative control serum (VBD 43/13) samples diluted 1:100 in 2% skimmed milk/PBS solution were added and incubated for one hour. Plates were washed six times with 0.1% PBST and goat anti-human IgG (HRPO) was added at 1:4000 and 1:8000 in 2% skimmed milk/PBST. Plates were washed six times with 0.1% PBST and 100 µl ABTS substrate was added and incubated at room temperature in the dark for 30 minutes. Readings were taken at

five-minute intervals from five minutes to 30 minutes. OD of the plates was read at 405 nm and net OD was determined by subtracting OD values of PBS coated wells from the CCHFV Gc antigen coated wells.

3.2.6.3. CCHFV ELISA cut-off for human serum samples

To determine the cut-off of in-house CCHFV ELISA for testing human serum samples, 11 negative control sera were tested in duplicate and a cut-off value was calculated by determining mean net OD value plus three SD for negative control sera. OD values were normalized by determining PP values for each sample. PP values were calculated using the formula described in 3.2.4.3. PP values were calculated for each human serum sample.

3.2.7. CCHFV ELISA for cattle serum samples

Recombinant CCHF Gc antigen described in 3.2.6.1. was used to perform a CCHFV ELISA for testing cattle serum samples and PBS was used as control coating antigen.

3.2.7.1. CCHFV ELISA testing cattle serum samples

CCHFV ELISA was modified for detection of IgG antibody against CCHFV in cattle serum samples. ELISA plates were coated with CCHFV Gc antigen and PBS as mock and blocked as described in 3.2.6.2. After blocking, plates were washed three times with 0.1% PBST. Cattle anti CCHF positive (C++) and cattle anti-CCHF negative control (C--) were added and incubated for one hour. Plates were washed six times with 0.1% PBST and goat bovine-human IgG (HRPO) was added at dilutions of 1:4000 and 1:8000 in 2% skimmed milk/PBST. Plates were washed six times with 0.1% PBST and ABTS substrate was added and incubated at room temperature in the dark for 30 minutes. Readings were taken at intervals of five minutes for 30 minutes. OD of the plates was read at 405 nm and net OD was determined by subtracting OD values of the PBS coated wells from the CCHFV Gc antigen coated wells.

3.2.7.2. CCHFV ELISA cut-off for cattle serum samples

Cut-off of CCHFV ELISA for testing cattle serum samples was calculated as described in 3.2.6.3. OD values were normalized by determining PP values for each cattle serum sample.

3.2.8. Preparation of antigen slides

Polyvalent/multiplex and monovalent antigen slides were prepared to screen serum samples. Polyvalent antigen slides were designed for simultaneous screening of more than one viral antigen, SINV and CCHFV, and monovalent antigen slides were prepared to confirm virus-specific reactivity observed in the polyvalent screening. Polyvalent slides were prepared by combining SINV-infected Vero cells with CCHFV transfected HEK 293 cells. CCHFV-transfected HEK 293 cells expressing CCHF nucleoprotein (NP) were prepared and stored as described previously in section 2.3.4.

Before preparation of the polyvalent antigen slides, optimization of monovalent slides was performed. SINV infected cells and CCHFV transfected cells were each diluted with uninfected Vero cells at various ratios. A combination of infected, or transfected cells, with uninfected cells facilitates differentiation between true positive and nonspecific reactivity. SINV cells were mixed with uninfected cells using ratios of 1:1, 1:2, and 1:4, and were tested for an optimal SINV:uninfected Vero cell ratio to facilitate reading of the fluorescence. Similarly, for CCHFV transfected cells, the cells were mixed with uninfected cells using ratios of 2:1, 1:1, and 1:2. A polyvalent slide was subsequently prepared by combining SINV infected and CCHFV transfected cell based on the outcome from the monovalent results. All prepared slides were fixed overnight using acetone at -20°C, ensuring the preservation of antigenic integrity for subsequent serological analyses. Prepared polyvalent and monovalent slides were used for screening and confirming presence of IgG antibodies against both SINV and CCHFV in serum samples.

Polyvalent and monovalent antigen slides were used to screen samples that tested positive using in-house ELISAs together with ELISA borderline positive samples. In addition, some ELISA negative samples were selected to test for nonspecific reactivity. Serum samples were diluted with PBS at 1:10 and screened as described in section 3.2.3.

3.2.9 Specificity tests

A panel of eight human serum samples collected from previous studies were used to test for the specificity of the assays to other viruses. Serum samples included three samples from volunteers with a history of vaccination against yellow fever virus (YFV) and previously confirmed to have anti-YFV IgG (VBD 5/12, VBD 46/09 and 19/09), one

serum sample from a patient with a laboratory confirmed history of CHIKV infection and positive for IgG against CHIKV (VBD28/09), anti WNV IgG (VBD 32/09), anti-Mayaro virus IgG (VBD 05/17), and two anti-CCHFV IgG serum samples (VBD 53/10, VBD 29/10). Polyvalent slides were used to screen these serum samples for cross reactivity IgG antibody against SINV and CCHFV. Specificity was calculated using the formula: $\text{Specificity} = (\text{True Negative (TN)} / (\text{True Negative} + \text{False Positive (FP)}) \times 100$, where TN represented samples correctly identified as negative, and FP represented samples incorrectly identified as positive.

3.3. Results

3.3.1. Confirmation of infection of Vero cells with SINV by IFA

Prior to preparation of the slides, the SINV infected Vero cells were tested using IFA. Figure 3.3 illustrates the fluorescence observed in Vero cells, confirming viral infection and replication within Vero cell culture.

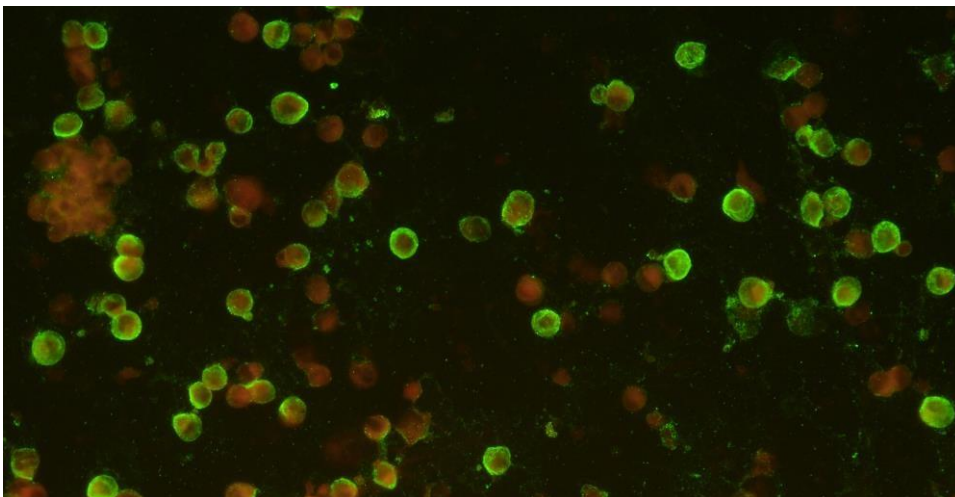


Figure 3.3: IFA of SINV-infected Vero cells demonstrating green fluorescence under the fluorescent microscope 20X.

3.3.2. ELISA

3.3.2.1. In house SINV ELISA for human serum samples

Optimal dilutions of SINV antigen and anti-human HRPO were determined as 1: 1000 and 1: 8000 respectively, and plates were read after 20 minutes.

A cut-off value for SINV ELISA was determined using 22 replicates of 11 negative samples. The mean OD value plus 3SD was calculated as the cut-off to differentiate between positive and negative samples. SINV ELISA cut off was calculated to be 0.25

(Table 3.1 and Figure 3.4). The PP value was obtained by dividing the mean net OD of the sample by the mean net OD of the positive control and multiplying the result by 100. Net PP value for SINV ELISA was calculated as 24,38% (Table 3.1 and Figure 3.4 A). (Refer to appendix D for raw data). Comparable results were obtained using net OD values and using the normalised data as shown in Figure 3.4 A and B

Table 3.1: Cut-off value calculated from the negative control panel and calculated PP value for SINV ELISA

	Mean optical density (OD405)	Standard deviation	Cut-off	PP value %
SINV ELISA	0,062	0,064	0,254	24,38

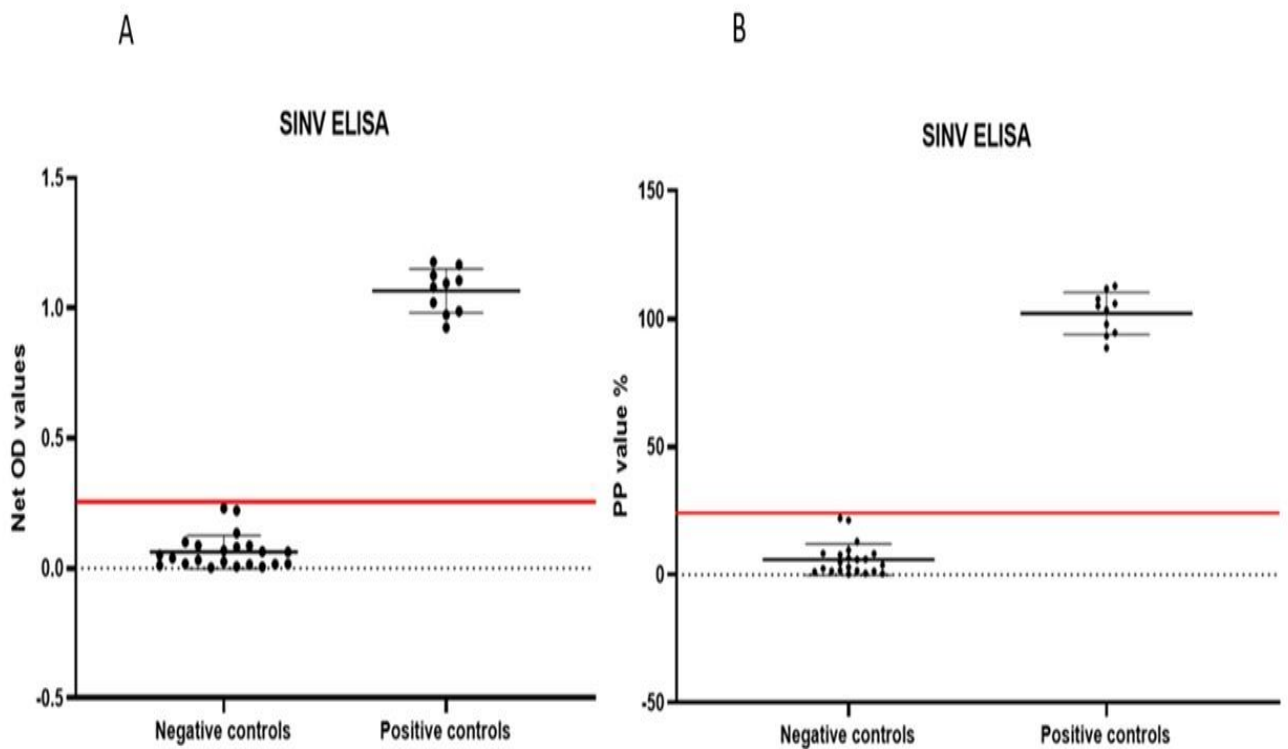


Figure 3.4: SINV ELISA cut off and normalized PP values cut off. A: 22 replicates of the negative panel were used to determine cut-off value. Red line indicates cut off value calculated from the mean OD value plus 3SD. B: 22 replicates of the negative panel and 10 replicates of the positive controls were used to determine net PP value. Red line indicates the Net PP value separating negative from positive.

Human serum samples were screened using the in house SINV ELISA and a total of 39/386 human serum samples were above the cut-off value and considered positive for SINV IgG antibodies as shown in Figure 3.5 A. Additionally, a total of three serum samples, exhibited borderline results.

The PP values for each human serum sample was obtained by dividing the net OD of the sample by the mean net OD of the positive control and multiplying by 100. Overall, 39/386 serum samples for SINV ELISA were above the PP value and three were considered borderline (Figure 3.5 B).

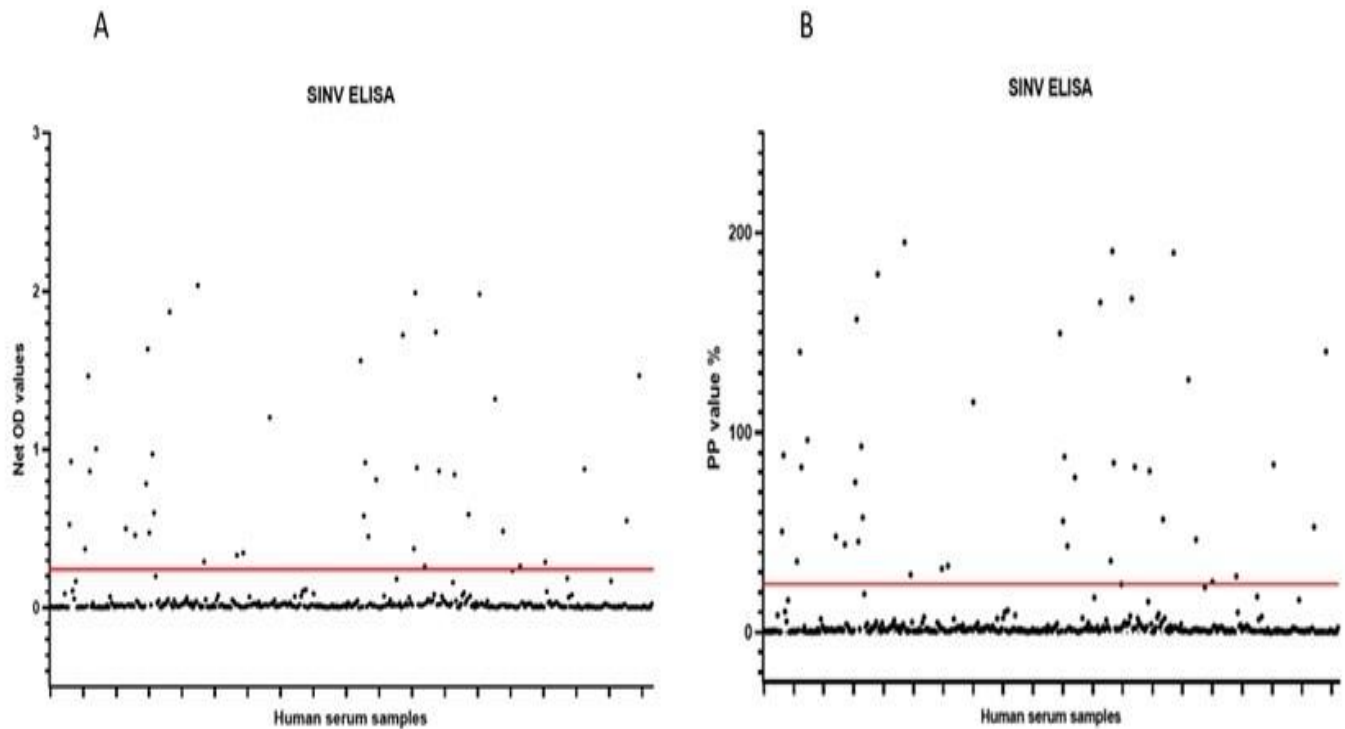


Figure 3.5: SINV ELISA and normalized data. A: The net OD for the human serum samples are shown and the red line indicates the cut-off value. B: The net PP values for SINV ELISA on human sera samples. Net PP values for SINV and the red line indicates the cut-off PP value.

3.3.2.2. In house SINV ELISA for bovine serum samples

Optimal dilutions of SINV antigen and anti-bovine HRPO were determined as 1: 1000 and 1: 8000 respectively, and plates were read after 20 minutes.

A total of 97 cattle serum samples collected for an unrelated study were screened using the in house SINV ELISA. Net mean OD cut-off used for cattle serum SINV ELISA was the same for SINV ELISA obtained from human serum samples, due to unavailability of negative cattle serum samples in the laboratory. All the cattle serum samples were below the net OD cut-off testing negative for SINV IgG antibodies (Figure 3.6A).

PP values for each cattle serum sample were calculated by dividing the net OD of the sample by the mean net OD of the positive control and multiplying the result by 100.

Overall, none of the cattle sera had detectable IgG antibody against SINV (Figure 3.6B). Comparable results were obtained using net OD values and using the normalised data.

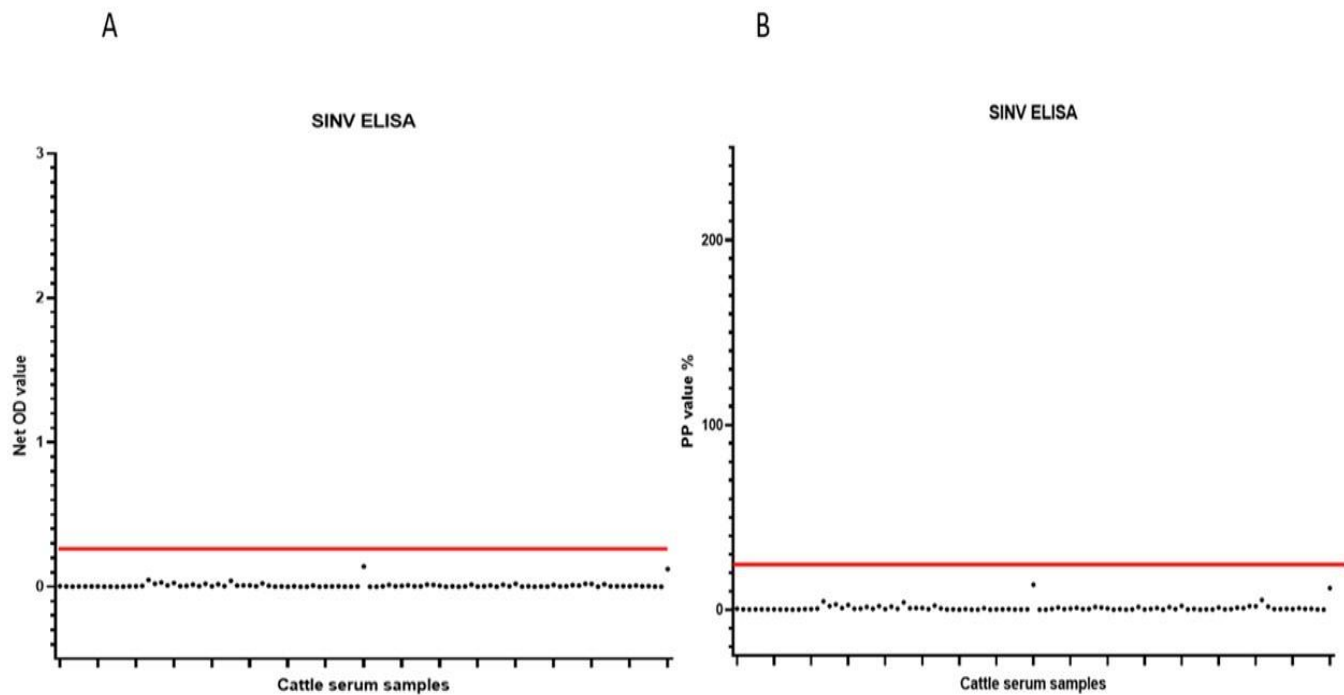


Figure 3.6: SINV ELISA and normalized data for cattle sera. A: The net OD for the cattle serum samples and the red line indicating cut-off value. B: The net PP values for SINV ELISA on cattle sera samples. Net PP values for each cattle serum sample and the red line indicates the cut-off PP value.

3.3.2.3. In house CCHFV ELISA on human serum samples

A total of 386 human serum samples were screened using CCHFV in-house ELISA. A cut-off value for CCHFV ELISA was determined using 11 negative samples tested in duplicates. Cut off was calculated as mean OD value plus 3SD. CCHFV ELISA cut off was calculated to be 0.21 (Table 3.2 and Figure 3.7A). The PP value was obtained by dividing the mean net OD of the sample by the mean net OD of the positive control and multiplying the result by 100. PP values for CCHFV ELISA was calculated as 16.53% (Table 3.2 and Figure 3.7B).

Table 3.2: Cut-off value calculated from the negative control panel and calculated PP value for CCHFV ELISA for human serum samples.

	Mean optical density (OD405)	Standard deviation	Cut-off	PP value %
CCHFV ELISA	0,144	0,022	0,21	16,53

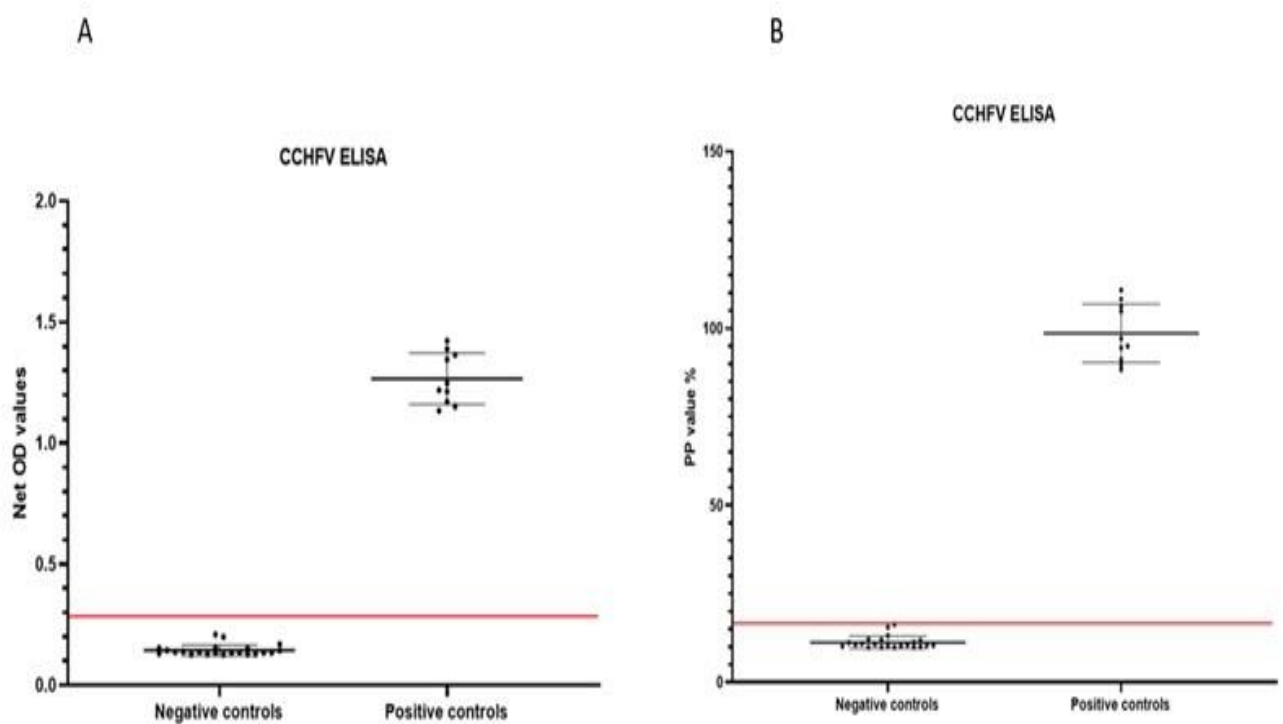


Figure 3.7: CCHFV ELISA cut off and normalized PP value cut off. CCHFV ELISA cutoff graph: 11 negative samples tested in duplicates were used to determine cut-off value. Red line indicates cut off value of calculated as mean OD value plus 3 SD. CCHFV ELISA PP value: 22 replicates of the negative panel and 10 positive controls were used to determine net PP value. Red line indicates the Net PP value separating negative from positive.

CCHFV ELISA was used to screen 386 human serum samples and a total of 3 out of 386 human serum samples were above the cut-off value and considered positive for CCHFV IgG antibodies (Figure 3.8A). The net OD of each serum sample was

calculated by subtracting OD of PBS plate from OD of the CCHFV Gc coated plate. A total of 22 serum samples exhibited borderline results.

PP values of each human serum sample was calculated by dividing the net OD of the sample by the mean net OD of the positive control multiplied by 100. Overall 3/386 serum samples for CCHFV ELISA were above the calculated net PP value (Figure 3.8B). A total of 22 serum samples were considered border line. These samples were included in the test using IFA. Comparable results were obtained using net OD values and using the normalised data.

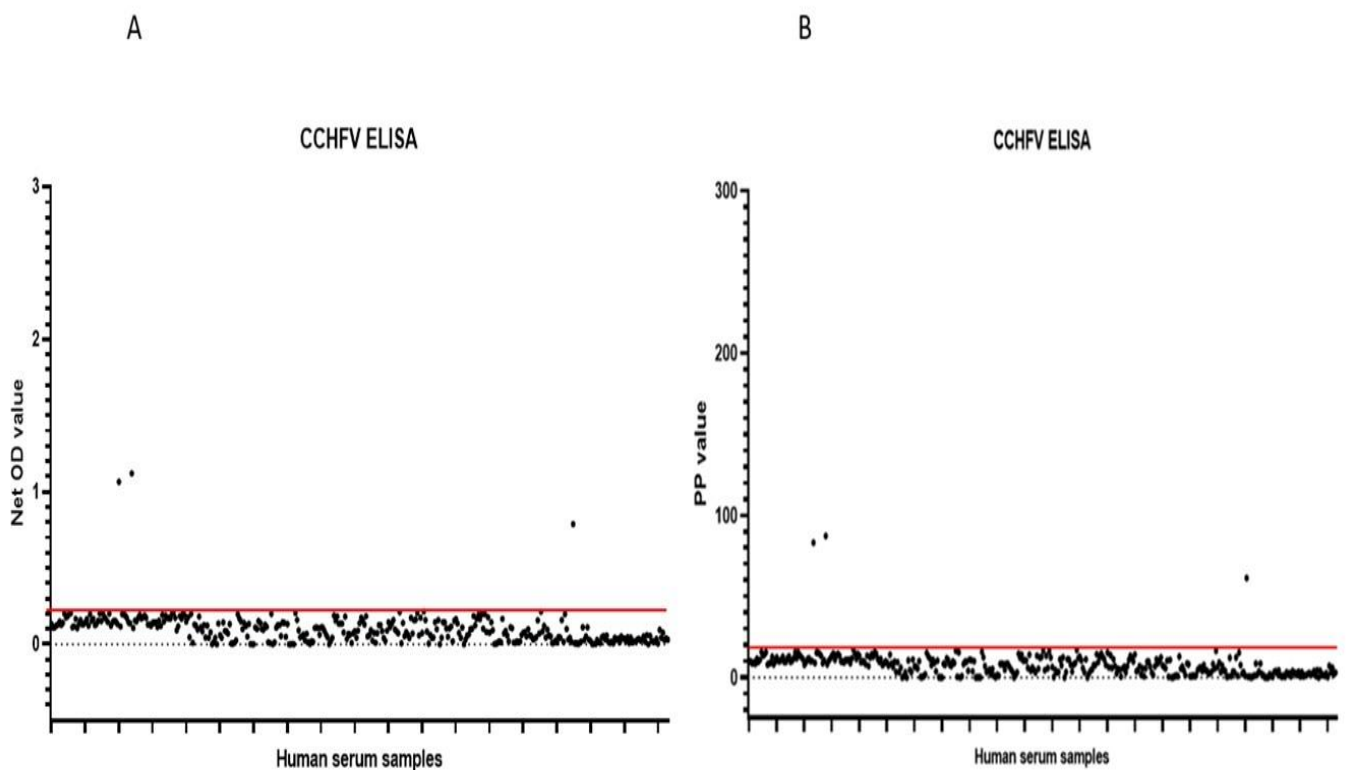


Figure 3.8: CCHFV ELISA and normalized PP value graph for human serum samples. **A:** The net OD for the human serum samples are shown and the red line indicates the cut-off value. **B:** The net PP values on human sera samples and the red line indicates the cut-off PP value.

3.3.2.4. In house CCHFV ELISA on cattle serum samples

A total of 97 cattle serum samples collected for an unrelated study were screened for CCHFV IgG antibodies using developed CCHFV in-house ELISA. Net mean OD cutoff used for cattle serum CCHFV ELISA was the same for CCHFV ELISA for human

serum samples since there was no known negative cattle serum samples in the laboratory. None of the cattle serum samples were above the net OD cut-off testing negative for CCHFV IgG antibodies (Figure 3.9A).

PP values of each cattle serum sample was calculated. None of cattle serum samples for CCHFV ELISA was above the PP value (Figure 3.9B). Comparable results were obtained using net OD values and using the normalised data.

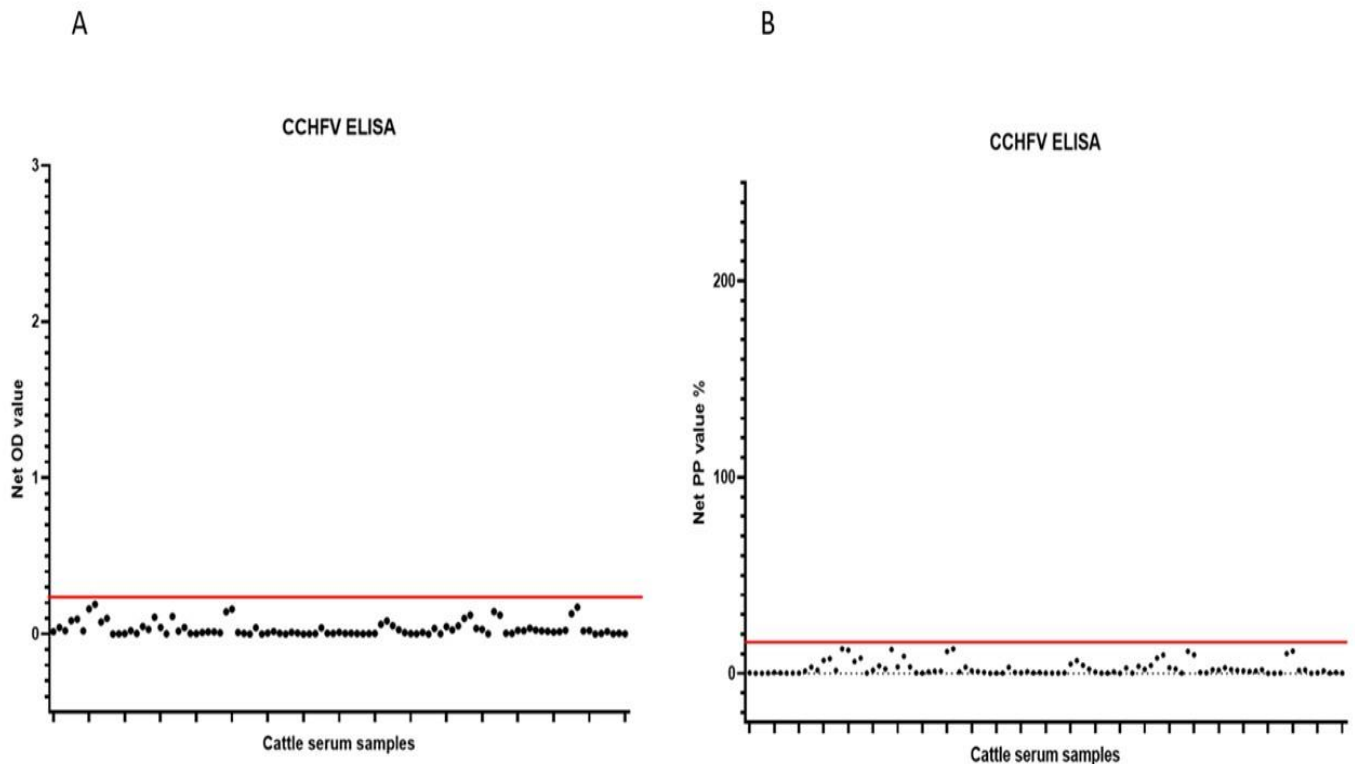


Figure 3.9: CCHFV ELISA OD graph and net PP values for CCHFV ELISA on cattle sera samples. A: The net OD for the cattle serum samples and the red line indicating cut-off value. B: Net PP values for each cattle serum sample and the red line indicates the cut-off PP value.

3.3.3 Immunofluorescent assays using monovalent and polyvalent antigen slides

Different dilutions and ratios of SINV infected cells, CCHFV transfected cells and SINV uninfected cells were tested to determine the most optimal conditions for preparing antigen slides for IFA. For SINV infected cells a 1:4 ratio was identified as the optimal condition for the detection of SINV-specific antibodies based on the proportion of

positive to negative cells facilitating differentiation between non-specific and specific reactivity (Figure 3.10A). For CCHFV NP antigen, a 2:1 ratio was determined as the most effective for CCHFV-specific antibody detection (Figure 3.10B). Following the optimization of the monovalent antigen slides, a polyvalent slide was developed for simultaneous screening for IgG antibody against both SINV and CCHFV NP. The optimized SINV and CCHFV NP were combined at a specific ratio of 2 CCHFV NP:1 SINV. IFA tests using positive control serum confirmed the reactivity of the antigen slides (Figures 3.10 C, D and E).

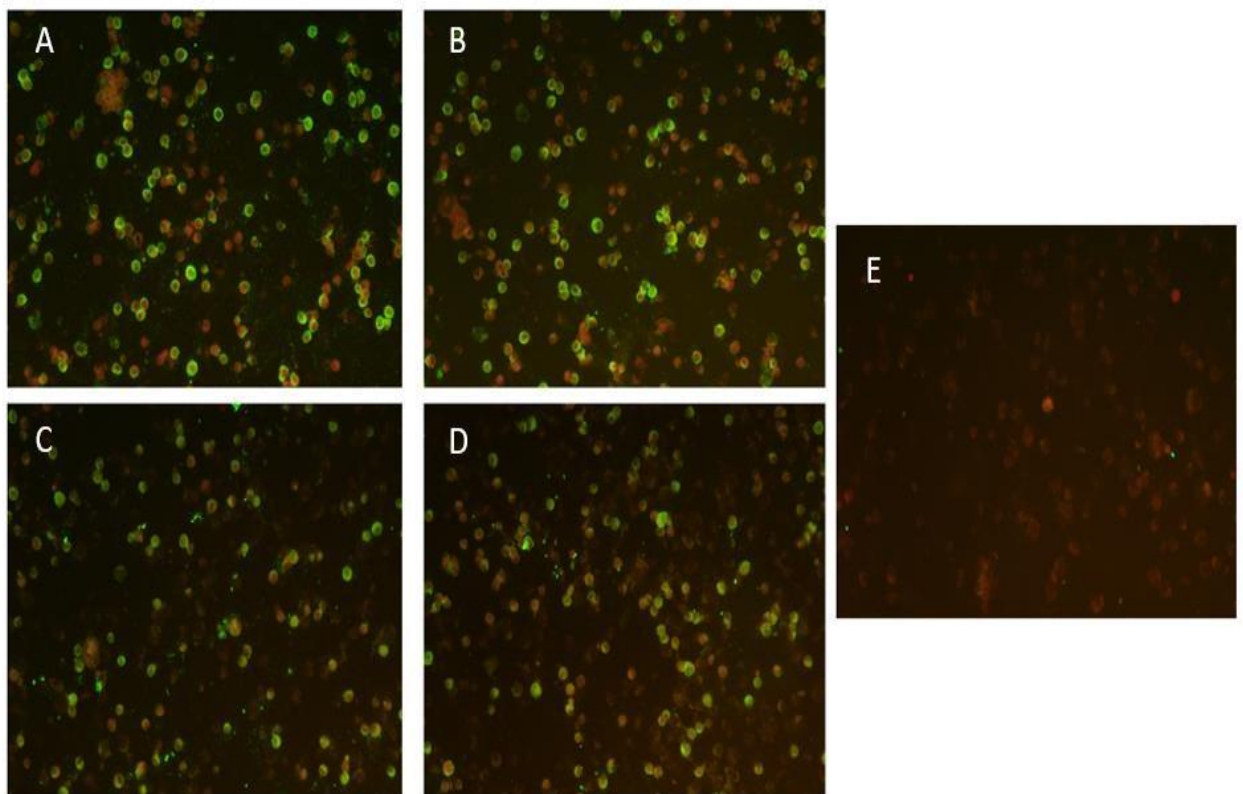


Figure 3.10: IFA using SINV infected cells and CCHFV NP transfected cells on monovalent and polyvalent slides. A. SINV monovalent slide at a ratio of 1:4 to Vero uninfected cells. B. CCHFV NP monovalent slide at a ratio of 2:1 to Vero uninfected cells. C. IFA with a polyvalent slide using IgG antibody positive SINV serum. D. IFA with a polyvalent slide using IgG antibody positive CCHFV serum. E. IFA with a polyvalent slide using IgG SINV and CCHFV negative serum sample. The images were captured at a 20X magnification.

3.3.4. Screening sera

As there was a limited number of antigen slides available, only positive samples were tested and a selection of eight negative samples were tested to check for nonspecific reactivity.

A total of 75 human serum samples were screened using polyvalent and monovalent slides for SINV and CCHFV NP IgG antibodies. This included 39 human serum samples positive for anti-SINV IgG using ELISA. All 39 samples had detectable IgG antibody against SINV using the polyvalent and SINV monovalent slides. A total of 3 human serum samples had detectable IgG antibody against CCHFV Gc and were positive using IFA on the polyvalent and CCHFV NP monovalent slides.

An additional 22 human serum samples that were considered borderline with CCHFV Gc ELISA and 3 samples considered borderline with SINV ELISA were screened using polyvalent and monovalent slides for SINV and CCHFV NP IgG antibodies. From the borderline samples 3/25 reacted positively on the polyvalent slides and were anti-SINV IgG positive using monovalent slides (Table 3.4). To determine non-specific reactivity, 8 samples were tested (as described in Section 3.2.8). The net OD values for each sample and the detection of fluorescence are presented in Table 3.4. There was no evidence of non-specific reactivity using the antigen slides.

Table 3.3: Net OD of ELISA corresponding to specific IFA and the fluorescent activity for the samples on the polyvalent and monovalent slides.

VBD no	Net OD	Polyvalent IFA	Monovalent SINV IFA	Monovalent CCHFV NP IFA
26/16/A8	0.64	Positive	Positive	Negative
26/16/A9	1.024	Positive	Positive	Negative
26/16/A18	0.487	Positive	Positive	Negative
26/16/A20	1.579	Positive	Positive	Negative
26/16/A21	0.985	Positive	Positive	Negative
26/16/A25	1.106	Positive	Positive	Negative
26/16/B17	0.619	Positive	Positive	Negative
26/16/B23	0.569	Positive	Positive	Negative

26/16/B30	0.888	Positive	Positive	Negative
26/16/B31	1.743	Positive	Positive	Negative
26/16/B32	0.585	Positive	Positive	Negative

26/16/B34	1.068	Positive	Positive	Negative
27/16/G1	0.714	Positive	Positive	Negative
42/16/3	1.978	Positive	Positive	Negative
43/16/3	2.142	Positive	Positive	Negative
43/16/7	0.386	Positive	Positive	Negative
43/16/28	0.426	Positive	Positive	Negative
43/16/32	0.442	Positive	Positive	Negative
47/16/K5	1.305	Positive	Positive	Negative
49/16/H27	1.699	Positive	Positive	Negative
49/16/H29	0.681	Positive	Positive	Negative
50/16/1	1.03	Positive	Positive	Negative
50/16/3	0.545	Positive	Positive	Negative
50/16/8	0.951	Positive	Positive	Negative
50/16/25	1.827	Positive	Positive	Negative
50/16/32	0.508	Positive	Positive	Negative
50/16/33	2.115	Positive	Positive	Negative
50/16/34	0.995	Positive	Positive	Negative
57/16/46	1.901	Positive	Positive	Negative
57/16/48	0.955	Positive	Positive	Negative
02/17/58	0.939	Positive	Positive	Negative
02/17/67	0.721	Positive	Positive	Negative
02/17/74	2.128	Positive	Positive	Negative
02/17/84	1.409	Positive	Positive	Negative
02/17/90	0.593	Positive	Positive	Negative
04/17/118	0.399	Positive	Positive	Negative
26/16/B11	0.954	Positive	Negative	Positive
26/16/B19	1.004	Positive	Negative	Positive
04/17/128	0.787	Positive	Negative	Positive

10/16	0,017	Negative	Negative	Negative
11/16	0,02	Negative	Negative	Negative
12/16	0,048	Negative	Negative	Negative
43/16/1	0,024	Negative	Negative	Negative
43/16/13	0,019	Negative	Negative	Negative
47/16/K19	0,014	Negative	Negative	Negative
49/16/H13	0,003	Negative	Negative	Negative
49/16/H14	0,005	Negative	Negative	Negative

Table 3.4: Borderline samples from SINV and CCHFV ELISA for humans. Net OD and the fluorescent activity for the samples on the polyvalent and monovalent slides.

SINV ELISA	Net OD	Polyvalent	SINV Monovalent	CCHFV Monovalent
50/16/39	0.252	Positive	Positive	Negative
04/17/102	0.254	Positive	Positive	Negative
42/16/3	0,208	Positive	Positive	Negative
CCHFV ELISA				
26/16/A8	0,202	Negative	Negative	Negative
26/16/A11	0,2	Negative	Negative	Negative
26/16/A12	0,204	Negative	Negative	Negative
26/16/A20	0,196	Negative	Negative	Negative
26/16/A26	0,201	Negative	Negative	Negative
26/16/A28	0,2	Negative	Negative	Negative
26/16/B4	0,201	Negative	Negative	Negative
26/16/B13	0,198	Negative	Negative	Negative
26/16/B14	0,195	Negative	Negative	Negative
42/16/2	0,199	Negative	Negative	Negative
42/16/9	0,201	Negative	Negative	Negative
42/16/14	0,199	Negative	Negative	Negative
43/16/25	0,196	Negative	Negative	Negative
47/16/K2	0,199	Negative	Negative	Negative
47/16/K17	0,205	Negative	Negative	Negative
50/16/17	0,21	Negative	Negative	Negative
50/16/28	0,206	Negative	Negative	Negative
50/16/32	0,205	Negative	Negative	Negative

02/17/68	0,194	Negative	Negative	Negative
02/17/70	0,197	Negative	Negative	Negative
04/17/107	0,202	Negative	Negative	Negative
04/17/122	0,198	Negative	Negative	Negative

3.3.5. Specificity of polyvalent IFA slides

A total of six samples were tested for specificity using the polyvalent slides. Specificity was calculated as $\text{Specificity} = (\text{True Negative (TN)} / (\text{True Negative} + \text{False Positive (FP)}) \times 100$, where TN represented samples correctly identified as negative, and FP represented samples incorrectly identified as positive. $\text{Specificity} = (5) / (5+1) \times 100 = 83.33\%$

Table 3.5: Samples testing specificity of the assay

IgG positive		Polyvalent
YFV	VBD 5/12	Negative
	VBD 46/09	Negative
	VBD 19/09	Negative
CHIKV	VBD 28/09	Positive
WNV	VBD 32/09	Negative
Mayaro	VBD 05/17	Negative

3.4. Summary

Continual development of serological techniques plays an important role in advancing our understanding of arboviruses. Quick and sensitive diagnostic tests are crucial in assessments of arboviruses to provide early alerts about potential outbreaks to implement control measures. Other variants such as chemiluminescence, or electrochemiluminescence are usually too expensive. Hence, an easily operated, cost-effective, and rapid assay such as multiplex IFA would have a useful role for testing a sample for multiple pathogens. In this chapter the objectives were to infect a suitable cell line with SINV to prepare cells for antigen slides and then prepare polyvalent IFA slides for screening serum samples for SINV IgG. Serum samples were subsequently screened using ELISA and IFAs for comparison of results.

The study commenced by confirming the infection of Vero cells with SINV by using IFA, since it's a BSL-2 virus. In-house ELISAs were developed and optimized using checkerboard titrations to determine the optimal dilutions for the antigens for both human and cattle. The in house ELISAs and calculation of cut off values were performed as previously described in our laboratory for detection of IgG against SARS-CoV-2 and modified for this study (Matefo et al., 2022). Cut off was calculated as mean net OD of the negative serum samples +3 SD (Lunn et al., 2012). The SINV ELISA for human serum samples established optimal dilutions and a cut-off value of 0.25, leading to the identification of 39 positive samples for SINV IgG antibodies, with three exhibiting borderline results. The ELISA was performed as previously described in our laboratory (Kennedy et al., 2022). Similar procedures were applied to the CCHFV Gc ELISA, resulting in a cut-off value of 0.21 and the identification of three positive samples for CCHFV Gc IgG antibodies, along with 22 borderline results.

Monovalent and polyvalent IFA antigen slides were then developed and optimized, confirming the reactivity of the antigens with positive control sera. Antigen slides were utilized to screen human serum samples, showing concordant results with ELISAs. The specificity of the polyvalent IFA slides was calculated at 83.33%. The findings collectively suggested the efficacy of the developed multiplex IFA for efficient serosurveillance, acknowledging limitations such as sample size constraints and the need for expanded virus inclusion in future studies.

Chapter 4: Discussion

The emergence and re-emergence of arboviruses across the globe pose a significant public health concern with increased risk of infection to humans and animals (Tajudeen et al., 2021). Over 500 arboviruses have been identified and 25% of these arboviruses are capable of causing disease in humans and animals (Gubler, 2002). The emergence and re-emergence of these viruses are influenced by various factors, including changes in land use, climate change, increased human and mosquito populations, vector competence, migration, and increased global trade (Lindahl et al., 2015). Early detection or early-warning surveillance increases the likelihood of timely detection of emerging or re-emerging threats minimizing the potentially devastating consequences of the disease spread or epidemics by these viruses (Hoinville et al., 2013). Over the last 40 years, various methods have been developed for the serodiagnosis of arboviruses including techniques such as immunofluorescence assay (IFA), complement fixation test (CFT), hemagglutination inhibition assay, plaque reduction neutralization test (PRNT), microsphere immunoassay (MIA) and enzymelinked immunosorbent assays (ELISAs) for both IgM and IgG (Basile et al., 2013).

Surveillance plays an important role in understanding what viruses are circulating with potential to cause outbreaks and helps in understanding arboviral infections in human and wildlife populations (Ushijima et al., 2021). However, the co-circulation of arboviruses introduces complexities in clinical diagnosis, highlighting the need for reliable laboratory diagnostic tools (Kasbergen et al., 2023). Molecular diagnostic methods are acknowledged for their specificity but are deemed challenging in resource-limited areas. Serology, particularly ELISA and IFA, are vital tools, enabling an extended timeframe diagnosis of arboviruses through the detection of antibodies (Kasbergen et al., 2023).

Previous studies have described the concept of simultaneous detection of viruses through multiplexing. A study of Basile et al. (2013) described multiplexing capability using Luminex platform that allowed for a single small sample to be simultaneously tested against 13 multiple viral antigens. However, Luminex based assays require expensive equipment and are not readily available for low resource countries. In our study, we aimed to implement a cost-effective and time-efficient approach for

serodiagnosis using immunofluorescent polyvalent antigen slides as a multiplexing method. Addressing financial constraints associated with sophisticated technologies like Luminex, our approach aimed to make multiplexing more accessible. Another study by Raulino et al., 2021 also conducted a multiplex study detecting antibodies to arboviruses (Chikungunya, O'nyong-nyong, Zika, Dengue, West Nile and Usutu viruses) in diverse non-human primate species from Cameroon and Congo. Their study used recombinant proteins effectively coupled to Luminex beads for the simultaneous detection of IgG antibodies against these viruses which resonates with the multiplexing theme in our research. In this study our developed IFA were able to target two different arboviruses and the advantage of IFA is that the number of antigens can be increased based on the availability of antigen. Fixation of antigen on the slide is relatively simple and does not require high training and special equipment compared to coupling of antigen to the beads. IFA is a cost-effective alternative compared to Luminex platform, aligning with the imperative for robust yet economically viable diagnostic tools in resource-limited settings.

Infected and/or transfected cells can express target antigen that can be used to screen for antibodies (Dreyer et al., 2010; Gabriel et al., 2018). In-house assay methods allow optimization and adaption of the assay to any choice of antigens (Dobaño et al., 2021). Additionally, it allows the use of different cell lines to express the target antigens. Cells infected with whole virus usually need appropriate biosafety measures, specialized expertise in cell culture techniques and can have significant serological cross reactivity within genera (Peaper & Landry, 2014). Hence recombinant plasmids are used to avoid working with live viruses especially the viruses requiring BSL level 4.

In this study, we started by preparing multiple recombinant constructs expressing arbovirus proteins from selected viruses CCHFV, SINV, and WNV. Genes encoding nucleoprotein (NP), capsid (C), EDIII from CCHFV, SINV, and WNV respectively were codon optimized to express in mammalian cells and cloned into a pcDNA 3.1+ vector using Not1 and BamHI restriction enzymes by GenScript. Expressions of pcDNA 3.1 CCHFV NP, pcDNA 3.1SINV C, and pcDNA 3.1 WNV EDIII in HEK-293 cells were demonstrated using antibodies directed against the HIS tag fused to the protein and anti-viral IgG antibodies. Transfections were optimised with Lipofectamine 3000 and Xtremegene 360. Results showed superior protein expression for pcDNA 3.1 CCHFV NP using Lipofectamine 3000 compared to X-tremeGENE 360. The result extend the

study of Shi et al., 2018 who showed that lipofectamine gave high expression yield in mammalian cells. pcDNA CCHFV NP protein size on both SDS and Western blot was similar to the findings by Shrivastava et al., 2019. Despite different optimization of pcDNA 3.1+ WNV EDIII, the expression remained <20%. The low expression limited our study emphasizing the need for further optimization. Study by Tripathi et al. focused on the expression of the WNV EDIII, and in their study they used the pET28a vector and E. coli BL21(DE3) cells for WNV EDIII expression and achieved high yields protein concentration on the TB and LB growth medium. The disparities in expression systems could be the main reason our results were different. To address the observed limitations in our study, further optimization strategies, including adjustments to growth conditions and potentially exploring alternative expression systems, will be explored in subsequent research endeavours.

The pcDNA 3.1 SINV C showed no expression, despite the different parameters the results remained the same. The low or no expression can be caused by several factors (Li et al., 2022). The protein produced might have been cytotoxic causing cells to destroy them. Protein may have undergone conformational changes or posttranslational modifications altering its recognition by the serum antibodies. These modifications could have masked or altered the antigenic epitopes, rendering them inaccessible to the antibodies present in the positive serum sample. Additionally, variations in the folding and presentation of the antigen could have impacted its binding affinity to the serum antibodies, further contributing to the lack of detectable expression (Reverberi & Reverberi, 2007). However, SINV virus is a BSL-2 pathogen and hence antigens were prepared through infection of cells.

The next part of the study was to prepare polyvalent antigen slides for screening samples for IgG antibodies against arboviruses. A reference assay was needed to compare results with the polyvalent antigen slides. In house ELISA was developed to screen IgG in serum samples from animals and humans. ELISAs were developed by optimal dilutions of key components by checkerboard titrations of the antigens, SINV cell lysate, mock antigen, CCHFV Gc antigen and secondary antibodies. Different time intervals were also used to determine the optimal time for development of colour. Optimization of the ELISAs was conducted with a keen focus on achieving a delicate

balance between sensitivity and specificity. The optimizations were conducted for each ELISA separately.

With SINV ELISA for human serum samples and SINV ELISA for cattle serum samples, results indicated similar optimal dilution of SINV cell lysate and secondary antibody to be 1:1000 and 1:8000 respectively with optimal time of 20 minutes. Cut-off of the ELISAs were calculated using the mean net OD values of the negative control sera samples plus 3 standard deviations (Lunn et al., 2012). Determining cutoff values for the ELISAs was a crucial step in distinguishing between positive and negative samples. Results of calculated cut-off values for SINV ELISA for human serum samples was 0.25. Same cut-off was used as human ELISA because of similar results when using cattle negative bovine control. Percentage positivity (PP) was calculated to normalise the data by using the net OD of the sample divided by the mean net OD of the positive control multiplied by 100. Calculated PP values for SINV ELISA was 24,38%. Our study show similar results to previous study of Koren et al., 2019 utilising SINV cell lysate to develop ELISA.

The results of CCHFV ELISA for human serum sample showed optimal dilutions of CCHFV Gc and secondary antibodies to be 0.25ug/ml and 1:8000 respectively. The optimal time determined was 30 minutes. Cut-off for CCHFV ELISA for humans were calculated using the mean net OD values of the negative control sera samples plus 3 standard deviations. Cut off was calculated to be 0.21 and PP value was 16,53% providing a standardized measure for identifying positive samples. The results of CCHFV ELISA for cattle serum samples was 0.25ug/ml and 1:4000 for CCHFV Gc and secondary antibody respectively. Cut off used was the same as humans because the anti-bovine controls showed similar results to human negative sera. Results after screening the human and animal samples, prevalence of positive samples in the tested population were 42 out of 386 human serum samples testing positive for SINV IgG antibodies and 3 out of 386 for CCHFV IgG antibodies. Our results extend those of Belij-Rammerstorfer et al., 2022, where they also developed ELISA using the Gc antigen.

After screening the next step was to develop polyvalent and monovalent slides to screen serum samples. The polyvalent slides were optimized to simultaneously screen for SINV and CCHFV antibodies by determining the most effective dilutions and ratios

for SINV and CCHFV NP antigens. Results indicated that the optimal dilution of the polyvalent was 2 CCHFV:1 SINV. Monovalent antigen slides in turn were prepared to detect each IgG antibody separately and were optimised using individual antigen cells:uninfected vero cells. Results obtained showed dilution of CCHFV:uninfected cells to be 2:1 and SINV:uninfected cells to be 1:4. Uninfected cells assist with differentiation of non-specific fluorescence. The utilization of polyvalent and monovalent antigen slides played a pivotal role in the efficient screening for antibodies against SINV and CCHFV in human serum samples. The successful implementation of these slides, particularly in detecting antibodies against both CCHFV and SINV in the tested serum samples, demonstrated a high degree of accuracy and efficacy of our diagnostic approach. Specifically, 39 samples that tested positive for SINV IgG exhibited concordant positive results with the polyvalent slides. Similarly, three samples positive for CCHFV IgG antibodies were correctly identified by the polyvalent slides, while the 8 negative serum samples were accurately classified as negative. CCHFV ELISA borderline samples all below cut off but selected as they were close to the cut off value, accurately tested negative with polyvalent slides. Three borderline samples for SINV ELISA tested positive for SINV. The CCHFV monovalent slide successfully identified the three samples positive and 22 borderline samples negative CCHFV IgG antibodies, effectively confirming the IgG antibody against CCHFV and SINV. The cross reactivity observed for the serum sample known to be anti-CHIKV IgG positive needs to be confirmed using neutralisation assays to make any reliable comment on cross reactivity. The polyvalent slides offered a simultaneous assessment of antibodies, contributing to a robust and reliable screening strategy.

The calculated specificity for the assay using six samples was 83.3%. One sample known to be anti-CHIKV IgG positive reacted against SINV. The sample was tested using the ELISA and reacted positively. However as the immune status of this sample against SINV was unknown and was from a laboratory worker exposed to SINV and living in a region where SINV occurs it was not possible to determine if the result was due to cross reactivity or a positive immune status from previous infection. This sample will require confirmation using neutralisation assays. No cross reactivity was observed using samples positive for IgG against YFV or Mayaro virus.

The accuracy and sensitivity of the multiplex assay were evident through the confirmation of IgG presence by monovalent IFA. The ability to discriminate specific

antibody responses against each virus enhances the diagnostic value of this methodology. The presence of non-specific background signals were minimized through optimization processes. Incorporating controls, such as known negative sera, were used in the assay to identify and address non-specific reactions. Our results show similar results with the study by Lederer et al., 2013 where they successfully simultaneously detected antibodies against old and new world hantaviruses using IFA biochips containing various antigens. However, our study use microscopic IFA slides, mixing cells in a single well to allow for efficient testing of serum samples for multiple pathogens without the added complexity and expense associated with biochip technology reflecting the practical and cost-effective strategy in our study to detect arboviruses.

The use of multiplex assays for serological screening demonstrated significant advantages in this study. Compared to the previous studies by Basile et al. 2013 and Raulino et al.,2021) our assay used an alternative low cost and fast method to simultaneous detect IgG antibodies against multiple viruses on a single platform. This approach allowed for efficient serosurveillance, particularly beneficial in regions with limited resources. The ability to discriminate specific antibody responses against each virus enhanced the diagnostic value of this methodology. The versatility of multiplex assays not only expedites screening processes but also ensures precise identification of virus specific antibodies, contributing to the overall efficacy and reliability of the diagnostic approach.

Previous studies have successfully created multiplex platform using recombinants and infected cells (Beck et al., 2015, Raulino et al., 2021, Surtees et al., 2020). These studies have highlighted the value of simultaneous virus detection, particularly in the surveillance of arboviruses. However, these studies used different approaches to develop multiplex assays compared to this study. The studies use microsphere immunoassays (MIAs) or Luminex assays to create the multiplex. However these techniques requires high cost and specialised equipments. Our study achieved the same goal of multiplex assay focused on using a different approach of IFA, cost-effectiveness and easily accessibility compared to Luminex assays. This study achieved comparable outcomes with the studies by (Beck et al., 2015, Raulino et al., 2021, Surtees et al., 2020) in terms of simultaneously detecting multiple viruses in a

single test, thereby conserving reagents and resources. Moving forward, further research could explore the optimization and refinement of IFA-based multiplex platforms for enhanced virus detection and surveillance.

Limitations were encountered throughout the study, which warrant acknowledgment. Firstly, errors in the designing of the synthetic genes obtained from GeneScript may have compromised their functionality, potentially impacting the intended outcomes of the study. Despite efforts to rectify these issues, alterations made to the genes during the optimization process could have affected their original integrity. Additionally, the inability to successfully express WNV plasmid limited the scope of the study, restricting the examination of multiple antigens to only a subset. Furthermore, the delayed receipt of the Bunyamwera viral isolate prevented its timely inclusion in the multiplex assay, thereby constraining the breadth of viruses investigated. Moreover, the absence of both positive and negative IgG-tested cattle sera samples for SINV and Crimean-Congo hemorrhagic fever virus (CCHFV) in the laboratory hindered the establishment of accurate cutoff values for the ELISAs, potentially affecting the interpretation of results. Furthermore, the preparation of a limited number of cells expressing the antigens of interest restricted the amount of multiplex slides produced, and the inability to repeat testing on serum samples.

This study enhanced our understanding of diagnostic methodologies but also laid the groundwork for future advancements in research. This study contributes to the broader goal of combatting arboviral diseases. The chapter concludes with insights from the development process, including the successful expression of certain antigens and the challenges faced in expressing others, emphasizing the need for further optimization and alternative strategies.

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Appendix A: Ethics approval of application and amendment

Environmental and Biosafety Research Ethics Committee



Environment & Biosafety Research Ethics Committee

07-Jun-2022

Dear Nyiko Maswanganyi

Project Title: **Expression of arbovirus antigens for preparing multiplex immunofluorescent platform for serology**

Department: **School of Pathology Department (Bloemfontein Campus)**

APPLICATION APPROVED

This letter confirms that this research proposal was given ethical clearance by the Environment & Biosafety Research Ethics Committee of the University of the Free State.

Your ethical clearance number, to be used in all correspondence is: **UFS-ESD2022/0119/22**

Please note the following:

1. This ethical clearance is valid for two years from the issuance of this letter.
2. If the research takes longer than two years to complete, please submit a **Continuation Report to the Ethics Committee before ethical clearance expires.**
3. If any changes are made during the research process (including a change in investigators), please inform the Ethics Committee by submitting an **Amendment.**
4. When the research is concluded, please submit a **Final Report to the Ethics Committee.**

Thank you for your application and we wish you well in all of your research endeavours.

Yours Sincerely

Prof. RR (Robert) Bragg
Chairperson: Environment & Biosafety Research Ethics Committee
University of the Free State

Directorate: Research Development
T: +27 (0)51 401 9398 | +27 (0)51 401 2075 | E: smitham@ufs.ac.za
Johannes Brill Building, Room 106D, First Floor
205 Nelson Mandela Drive | Park West, Bloemfontein 9301 | South Africa





Environment & Biosafety Research Ethics Committee

10-Jul-2023

Dear Nyiko Maswanganyi

Project Title: **Expression of arbovirus antigens for preparing multiplex immunofluorescent platform for serology**

Department: **School of Pathology Department (UFS Main Campus)**

AMENDMENT APPROVED

This letter confirms that the amendment to your research proposal and henceforth your ethical clearance has been approved by the Biosafety & Environmental Research Ethics Committee of the University of the Free State.

Your ethical clearance number, to be used in all correspondence is: **UFS-ESD2022/0119/22/3**

Please note the following:

- 1. Ethical clearance is valid for one year from the issuance of the initial ethical clearance letter.**
- 2. If the research takes longer than one year to complete, please submit a Continuation Report to the Ethics Committee before ethical clearance expires.**
- 3. If any further changes are made during the research process (including a change in investigators), please inform the Ethics Committee by submitting another Amendment.**
- 4. When the research is concluded, please submit a Final Report to the Ethics Committee.**

Thank you for your application and we wish you well in all of your research endeavours.

Yours Sincerely

Prof. RR (Robert) Bragg
Chairperson: Environment & Biosafety Research Ethics Committee
University of the Free State

Directorate: Research Development
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P.O. Box 339 | Bloemfontein 9300 | South Africa | www.ufs.ac.za



Health Science Research Ethics Committee

Dear Nyiko Maswanganyi

Ethics Clearance: **Expression of arbovirus antigens for preparing multiplex immunofluorescent platform for serology**

Principal Investigator: Nyiko Maswanganyi

Department: **School of Pathology Department (Bloemfontein Campus)**

[Submission Page](#)

APPLICATION APPROVED

Please ensure that you read the whole document

With reference to your application for ethical clearance with the Faculty of Health Sciences, I am pleased to inform you on behalf of the Health Sciences Research Ethics Committee that you have been granted ethical clearance for your project.

Your ethical clearance number, to be used in all correspondence is: **UFS-HSD2022/1278/2510**

The ethical clearance number is valid for research conducted for one year from issuance. Should you require more time to complete this research, please apply for an extension.

We request that any changes that may take place during the course of your research project be submitted to the HSREC for approval to ensure we are kept up to date with your progress and any ethical implications that may arise. This includes any serious adverse events and/or termination of the study.

A progress report should be submitted within one year of approval, and annually for long term studies. A final report should be submitted at the completion of the study.

Research conducted in any Department of Health facility: Researchers are required to sign and return the HSREC approval letters to the provincial Department of Health where they applied. It is also a requirement for researchers to submit electronic copies of their final research findings, and/or make a presentation of their findings and recommendations at departmental research days when and where indicated.

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(2020); 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; International Council for Harmonisation (ICH) Harmonised Guideline, Integrated Addendum to ICH E6(R1), Guideline for Good Clinical Practice (GCP) E6(R2), 2016, SAHPRA Guidelines as well as Laws and Regulations with regard to the Control of Medicines, Constitution of the HSREC of the Faculty of Health Sciences.

The Principal Investigator (PI) bears final responsibility for the RIMS application. In the event of any misconduct or improper activities perpetrated by a third party, the PI will be held vicariously liable. The HSREC will bear no responsibility or liability for any actions of a PI and/or third party or breach of confidentiality caused by the PI and/or third party.

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 proposal for ethical clearance and we wish you every success with your research.

Yours Sincerely



Prof. A. Sherriff

Chairperson: Health Sciences Research Ethics Committee

Health Sciences Research Ethics Committee

Office of the Dean: Health Sciences

T: +27 (0)51 401 7795/7794 | E: ethicsfhs@ufs.ac.za

IRB 00011992; REC 230408-011; IORG 0010096; FWA 00027947

Block D, Dean's Division, Room D104 | P.O. Box/Postbus 339 (Internal Post Box G40) | Bloemfontein 9300 | South Africa
www.ufs.ac.za





Health Sciences Research Ethics Committee

31-Jul-2023

Dear Nyiko Maswanganyi

Ethics Number: UFS-HSD2022/1278/2510-0002

Ethics Clearance: **Expression of arbovirus antigens for preparing multiplex immunofluorescent platform for serology**

Principal Investigator: **Nyiko Maswanganyi**

Department: **School of Pathology Department (UFS Main Campus)**

[Submission Page](#)

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:

- The title of the MMedSc has been revised to "Preparation of multiplex immunofluorescent platform for serology" as the platform will include infected as well as transfected cells.
- Objective 4 which was to "select and expand stable clones expressing protein of interest" has been replaced as we have been unable to prepare a stable cell line and instead the multiplex assay has been expanded to include Bunyamwera infected cells.
- Additionally, we will use Sindbis infected cells as we were unable to express recombinant Sindbis protein antigen (new objective 4).
- Bunyamwera virus in particular has been included in the study as we have detected Bunyamwera in mosquitoes collected in Bloemfontein in an unrelated study. Hence the multiplex antigen slides will be prepared using recombinant CCHF antigen, Sindbis virus infected cells and Bunyamwera virus infected cells.
- The assay will be used to screen serum samples from human and cattle sera collected for unrelated studies (new objective 5).
- Initially 15 to 20 serum samples from patients that would have potentially been exposed to the virus because of where they live or work collected in our laboratory for unrelated studies were going to be used to screen the antigen slides prepared for IgG detection. But now 136 serum samples from healthy volunteers, collected between 2016 and 2017 for seroepidemiology studies on Crimean-Congo haemorrhagic fever virus and other vector borne diseases (HSREC 34/2016) will be screened for CCHF, Bunyamwera and Sindbis IgG. Copies of informed consent documents indicating permission to store samples and use for related studies are attached in the appendices of the protocol.
- Stored serum samples from 30 cattle bled on at least 5 occasions for an unrelated study and stored will be screened using the multiplex antigen slides for antibody against CCHFV, Sindbis virus and Bunyamwera virus. AREC will be obtained for this animal study. A section 20 permit and a letter from Dr van dalen for using the samples is available.

AREC approval is also required.

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(2020); 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; International Council for Harmonisation (ICH) Harmonised Guideline, Integrated Addendum to ICH E6(R1), Guideline for Good Clinical Practice (GCP) E6(R2), 2016. SAHPRA Guidelines as well as Laws and Regulations with regard to the Control of Medicines, Constitution of the HSREC of the Faculty of Health Sciences.

The Principal Investigator (PI) bears final responsibility for the RIMS application. In the event of any misconduct or improper activities perpetrated by a third party, the PI will be held vicariously liable. The HSREC will bear no responsibility or liability for any actions of a PI and/or third party or breach of confidentiality caused by the PI and/or third party.

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

Prof. A. Sherriff
Chairperson : Health Sciences Research Ethics Committee

Health Sciences Research Ethics Committee
Office of the Deans: Health Sciences
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Animal Research Ethics

06-Sep-2023

Dear Nyiko Maswanganyi

Student Project Number: UFS-AED2023/0040

Project Title: Preparation of multiplex immunofluorescent platform for serology

Department: School of Pathology Department (UFS Main Campus)

You are hereby kindly informed that, the UFS Animal Research Ethics Committee approved the above project.

Kindly take note of the following:

1.
A progress report with regard to the above study has to be submitted Annually and on completion of the project. Reports are submitted by logging in to RIMS and completing the report as described in SOP AEC007: Submission of Protocols, Modifications, Amendments, Reports and Reporting of Adverse Events which is available on the UFS intranet.
2.
Researchers that plan to make use of the Animal Experimentation Unit must ensure to request and receive a quotation from the Head, Mr. Seb Lamprecht.
3.
Fifty (50%) of the quoted amount is payable when you receive the letter of approval.

Yours Sincerely



Dr Walter Janse van Rensburg
Chair: Animal Research Ethics Committee

Appendix B: Sequence data West_Nile_Virus_pcDNA3.1(+)

BamHI- GGATCC

Kozak sequence- GCCACC

Unwanted bases (removed after PCR)- ATGG

NotI- GCGGCCGC

His tag- CATCACCATCACCATCAC

ATG start- codon

TGA/TAA Stop- codon

GACGGATCGGGAGATCTCCCGATCCCCTATGGTGC ACTCTCAGTACAATCTGC
TCTGATGCCGCATAGTTAAGCCAGTATCTGCTCCCTGCTTGTGTGTTGGAGGTC
GCTGAGTAGTGC GCGAGCAAATTTAAGCTACAACAAGGCAAGGCTTGACCGA
CAATTGCATGAAGAATCTGCTTAGGGTTAGGCGTTTTGCGCTGCTTCGCGATGT
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AGCTAGAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTAT
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CTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAAACGAAA
ACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGAT
CCTTTTAAATTA AAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTT

GGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTC
TATTCGTTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATAC
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GTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAACAAATAGGGGT
TCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTC

Appendix C: Sequence data Sindbis_virus_pcDNA3.1(+)

BamHI- GGATCC

Kozak sequence- GCCACC

Unwanted bases (removed after PCR)

NotI- GCGGCCGC

His tag- CATCACCATCACCATCAC

ATG start- codon

GACGGATCGGGAGATCTCCCGATCCCCTATGGTGC ACTCTCAGTACAATCTGC
TCTGATGCCGCATAGTTAAGCCAGTATCTGCTCCCTGCTTGTGTGTTGGAGGTC
GCTGAGTAGTGCGCGAGCAAATTTAAGCTACAACAAGGCAAGGCTTGACCGA
CAATTGCATGAAGAATCTGCTTAGGGTTAGGCGTTTTGCGCTGCTTCGCGATGT
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AGCCCTGTCTGTGGTGACATGGAATAGCAAGGGCAAGACAATCAAAACAACAC
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GTCTAGAGGGCCCGTTTAAACCCGCTGATCAGCCTCGACTGTGCCTTCTAGTT
GCCAGCCATCTGTTGTTTGCCCCCTCCCCCGTGCCTTCCTTGACCCTGGAAGGT
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AGTAGGTGTCATTCTATTCTGGGGGGTGGGGTGGGGCAGGACAGCAAGGGGG
AGGATTGGGAAGACAATAGCAGGCATGCTGGGGATGCGGTGGGCTCTATGGC
TTCTGAGGCGGAAAGAACCAGCTGGGGCTCTAGGGGGTATCCCCACGCGCCC
TGTAGCGGCGCATTAAAGCGCGGGGTGTGGTGGTTACGCGCAGCGTGACCG
CTACACTTGCCAGCGCCCTAGCGCCCGCTCCTTTTCGCTTTTCTTCCCTTCTTTC
TCGCCACGTTGCGCGGCTTTCCCCGTCAAGCTCTAAATCGGGGGCTCCCTTTA
GGGTTCCGATTTAGTGCTTTACGGCACCTCGACCCCAAAAACTTGATTAGGGT
GATGGTTCACGTAGTGGGCCATCGCCCTGATAGACGGTTTTTCGCCCTTTGAC
GTTGGAGTCCACGTTCTTTAATAGTGGACTCTTGTTCCAAACTGGAACAACACT
CAACCCTATCTCGGTCTATTCTTTTGATTTATAAGGGATTTTGCCGATTTCCGGCC
TATTGGTTAAAAAATGAGCTGATTTAACAAAAATTAACGCGAATTAATTCTGTG
GAATGTGTGTCAGTTAGGGTGTGGAAAGTCCCCAGGCTCCCCAGCAGGCAGAA
GTATGCAAAGCATGCATCTCAATTAGTCAGCAACCAGGTGTGGAAAGTCCCCAG
GCTCCCCAGCAGGCAGAAAGTATGCAAAGCATGCATCTCAATTAGTCAGCAACC
ATAGTCCCGCCCCTAACTCCGCCCATCCCGCCCCTAACTCCGCCCAGTTCCGC
CCATTCTCCGCCCATGGCTGACTAATTTTTTTTATTTATGCAGAGGCCGAGGC
CGCCTCTGCCTCTGAGCTATTCCAGAAGTAGTGAGGAGGCTTTTTTTGGAGGCC
TAGGCTTTTGCAAAAAGCTCCCGGGAGCTTGTATATCCATTTTCGGATCTGATC
AAGAGACAGGATGAGGATCGTTTCGCATGATTGAACAAGATGGATTGCACGCA
GGTTCTCCGGCCGCTTGGGTGGAGAGGCTATTCGGCTATGACTGGGCACAACA

GACAATCGGCTGCTCTGATGCCGCCGTGTTCCGGCTGTCAGCGCAGGGGGCGC
CCGGTTCTTTTTGTCAAGACCGACCTGTCCGGTGCCCTGAATGAACTGCAGGA
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GTGCTCGACGTTGTCACTGAAGCGGGAAGGGACTGGCTGCTATTGGGCGAAGT
GCCGGGGCAGGATCTCCTGTCATCTCACCTTGCTCCTGCCGAGAAAGTATCCA
TCATGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACCTGCCCA
TTCGACCACCAAGCGAAACATCGCATCGAGCGAGCACGTA CTGGATGGAAGC
CGGTCTTGTCGATCAGGATGATCTGGACGAAGAGCATCAGGGGCTCGCGCCA
GCCGAACTGTTCCGCCAGGCTCAAGGCGCGCATGCCCGACGGCGAGGATCTCG
TCGTGACCCATGGCGATGCCTGCTTGCCGAATATCATGGTGGAAAATGGCCGC
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CATAGCGTTGGCTACCCGTGATATTGCTGAAGAGCTTGGCGGCGAATGGGCTG
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TTCTATCGCCTTCTTGACGAGTTCTTCTGAGCGGGACTCTGGGGTTCGAAATGA
CCGACCAAGCGACGCCCAACCTGCCATCACGAGATTTTCGATTCCACCGCCGCC
TTCTATGAAAGGTTGGGCTTCGGAATCGTTTTCCGGGACGCCGGCTGGATGAT
CCTCCAGCGCGGGGATCTCATGCTGGAGTTCTTCGCCACCCCAACTTGTTTAT
TGCAGCTTATAATGGTTACAAATAAAGCAATAGCATCACAAATTTACAAATAAA
GCATTTTTTTCACTGCATTCTAGTTGTGGTTTGTCCAAACTCATCAATGTATCTTA
TCATGTCTGTATACCGTTCGACCTCTAGCTAGAGCTTGGCGTAATCATGGTCATA
GCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGC
CGGAAGCATAAAGTGTAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATT
AATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGC
TGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCG
CTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTTCGTTCCGGCTGCGGC
GAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGG
GATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCG
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CCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGC
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AGCTGGGCTGTGTGCACGAACCCCGTTTCAGCCCGACCGCTGCGCCTTATCC
GGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCA

GCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGA
GTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGAACAGTATTTGGTAT
CTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATC
CGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTTGTTTGGCAAGCAGCAGAT
TACGCGCAGAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTC
TGACGCTCAGTGGAACGAAAACCTCACGTTAAGGGATTTTGGTCATGAGATTATC
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GTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAAT
GATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGC
CAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATC
CAGTCTATTAATTGTTGCCGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGT
TTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTACGCTCGTCTGTTT
GGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCC
CCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGA
AGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCT
CTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACC
AAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTC
AATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGG
AAAACGTTCTTCGGGGCGAAAACCTCTCAAGGATCTTACCGCTGTTGAGATCCAG
TTCGATGTAACCCACTCGTGCACCCAACCTGATCTTCAGCATCTTTTACTTTACC
AGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAAT
AAGGGCGACACGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGA
AGCATTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGA
AAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACG
TC

Appendix D: Raw ELISA plates data

SINV ELISA for human serum samples

Positive control= **Red**

Negative control= **Blue**

Plate 1 Cell lysate plates

	1	2	3	4	5	6	7	8	9	10	11	12
A	0,111	0,136	0,102	0,113	0,113	0,105	0,101	0,103	0,113	0,198	0,105	0,105
B	0,64	1,024	0,204	0,173	0,264	0,105	0,104	0,107	0,098	0,127	0,487	0,131
C	1,579	0,985	0,108	0,146	0,111	1,106	0,107	0,123	0,111	0,129	0,105	0,105
D	0,126	0,11	0,175	0,155	0,12	0,11	0,118	0,103	0,123	0,131	0,115	0,129
E	0,619	0,127	0,126	0,122	0,138	0,111	0,569	0,18	0,163	0,123	0,117	0,099
F	0,12	0,888	1,743	0,585	0,125	1,068	0,714	0,309	0,146	0,167	0,11	0,109
G	0,141	0,13	0,171	0,18	1,978	0,12	0,086	0,195	0,135	0,119	0,129	0,128
H	0,133	0,155	0,177	0,203	0,163	0,195	0,165	0,08	1,763	1,718	0,291	0,224

Mock plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	0,104	0,13	0,099	0,108	0,12	0,106	0,092	0,109	0,122	0,108	0,103	0,102
B	0,113	0,098	0,096	0,116	0,096	0,108	0,107	0,113	0,106	0,129	0,116	0,12
C	0,114	0,122	0,099	0,117	0,109	0,101	0,105	0,115	0,112	0,127	0,109	0,107
D	0,109	0,108	0,105	0,123	0,102	0,104	0,101	0,091	0,11	0,115	0,101	0,122
E	0,118	0,111	0,107	0,111	0,119	0,101	0,109	0,138	0,128	0,111	0,11	0,101
F	0,109	0,103	0,107	0,11	0,108	0,097	0,114	0,108	0,115	0,122	0,105	0,105

G	0,117	0,109	0,139	0,126	0,107	0,097	0,082	0,147	0,118	0,106	0,119	0,111
H	0,108	0,129	0,137	0,14	0,134	0,179	0,136	0,081	0,238	0,231	0,156	0,138

Plate 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	0,024	0,043	2,038	0,016	0,003	0,01	0,291	0,053	0,007	0,005	0,008	0,004
B	0,019	0,008	0,051	0,08	0,012	0,02	0,016	0,005	0,009	0,005	0,009	0,002
C	0,051	0,039	0,022	0,333	0,016	0,006	0,005	0,347	0,005	0,011	0,014	0,07
D	0,018	0,022	0,028	0,025	0,014	0,038	0,007	0,01	0,016	0,017	0,02	0,014
E	0,203	0,028	0,023	0,049	0,007	0,018	0,011	0,019	0,025	0,028	0,029	0,015
F	0,022	0,032	0,014	0,011	0,072	0,002	0,009	0,02	0,075	0,106	0,011	0,115
G	0,001	0,007	0,014	0,02	0,09	0,011	0,016	0,008	0,018	0,009	0,009	0,007
H	0,001	0,004	0,006	0,008	0,019	0,006	0,005	0,013	1,213	1,187	0,221	0,234

	1	2	3	4	5	6	7	8	9	10	11	12
A	0,103	0,111	0,104	0,104	0,108	0,097	0,095	0,113	0,092	0,089	0,1	0,095
B	0,094	0,09	0,117	0,103	0,088	0,116	0,087	0,103	0,094	0,088	0,092	0,094
C	0,112	0,108	0,112	0,093	0,114	0,091	0,098	0,095	0,091	0,092	0,093	0,162
D	0,101	0,102	0,093	0,115	0,096	0,101	0,096	0,116	0,095	0,103	0,099	0,098
E	0,102	0,097	0,095	0,114	0,094	0,105	0,094	0,103	0,098	0,106	0,101	0,098
F	0,13	0,106	0,09	0,089	0,095	0,098	0,093	0,103	0,109	0,095	0,099	0,095
G	0,111	0,096	0,101	0,124	0,117	0,11	0,114	0,102	0,104	0,102	0,102	0,132
H	0,144	0,098	0,103	0,186	0,142	0,102	0,127	0,11	0,226	0,214	0,141	0,138

Plate 3

	1	2	3	4	5	6	7	8	9	10	11	12
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A	0,003	0,005	0,01	0,013	0,017	0,004	0,034	0,02	0,009	0,011	0,025	0,028
B	0,045	0,001	1,562	0,008	0,582	0,918	0,009	0,451	0,025	0,019	0,013	0,014
C	0,81	0,006	0,001	0,003	0,009	0,075	0,014	0,021	0,021	0,05	0,013	0,023
D	0,015	0,182	0,015	0,029	0,002	1,724	0,015	0,028	0,017	0,065	0,031	0,018
E	0,074	1,992	0,886	0,02	0,016	0,016	0,02	0,272	0,031	0,052	0,046	0,039
F	0,049	0,085	1,743	0,02	0,865	0,025	0,072	0,047	0,038	0,024	0,02	0,012
G	0,028	0,161	0,843	0,044	0,005	0,002	0,025	0,079	0,096	0,029	0,053	0,59
H	0,074	0,013	0,022	0,001	0,033	0,013	1,983	0,028	1,632	1,571	0,315	0,301

Mock

	1	2	3	4	5	6	7	8	9	10	11	12
A	0,107	0,103	0,098	0,136	0,122	0,11	0,113	0,112	0,121	0,117	0,11	0,116
B	0,152	0,112	0,137	0,128	0,099	0,112	0,088	0,094	0,107	0,1	0,093	0,09
C	0,141	0,116	0,096	0,11	0,106	0,11	0,105	0,104	0,096	0,105	0,092	0,102
D	0,114	0,13	0,114	0,137	0,101	0,103	0,106	0,107	0,123	0,116	0,11	0,098
E	0,134	0,123	0,109	0,111	0,122	0,099	0,112	0,13	0,108	0,12	0,123	0,109
F	0,146	0,167	0,158	0,106	0,13	0,117	0,144	0,154	0,116	0,113	0,113	0,106
G	0,114	0,181	0,096	0,113	0,117	0,13	0,117	0,133	0,104	0,147	0,13	0,131
H	0,16	0,169	0,128	0,129	0,149	0,108	0,145	0,107	0,263	0,262	0,156	0,172

Plate 4

	1	2	3	4	5	6	7	8	9	10	11	12
A	0,021	0,017	0,013	0,042	0,005	0,01	0,009	0,006	1,32	0,001	0,007	0,011
B	0,017	0,484	0,017	0,007	0,012	0,009	0,012	0,236	0,007	0,003	0,002	0,009
C	0,164	0,032	0,029	0,007	0,023	0,022	0,029	0,022	0,006	0,003	0,012	0,006
D	0,003	0,015	0,008	0,004	0,289	0,104	0,032	0,039	1,204	1,097	0,191	0,198

Mock

	1	2	3	4	5	6	7	8	9	10	11	12
A	0,095	0,089	0,085	0,138	0,09	0,088	0,093	0,095	0,089	0,091	0,098	0,1
B	0,114	0,109	0,094	0,095	0,09	0,095	0,093	0,099	0,089	0,105	0,093	0,087
C	0,128	0,115	0,114	0,124	0,114	0,107	0,098	0,104	0,09	0,099	0,093	0,101
D	0,125	0,099	0,096	0,131	0,11	0,091	0,113	0,108	0,183	0,172	0,142	0,143

Plate 5

	1	2	3	4	5	6	7	8	9	10	11	12
A	0,012	0,017	0,027	0,035	0,028	0,012	0,01	0,006	0,006	0,006	0,187	0,067
B	0,009	0,082	0,006	0,002	0,002	0,017	0,001	0	0	0,877	0,013	0,001
C	0,015	0,006	0,006	0,007	0,001	0,001	0,005	0,001	0,005	0,012	0,025	0,021
D	0,016	0,013	0,169	0,005	0,009	0,003	0,003	0,011	0,008	0,005	0,017	0,021
E	0,151	0,001	0,002	0,002	0,004	0,002	0,002	0,004	1,467	0,001	0,002	0,013
F	0,006	0	0,002	0,001	0,025	1,306	1,292	0,224	0,234			

SINV ELISA for cattle serum samples

Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	0,068	0,074	0,065	0,066	0,063	0,065	0,064	0,062	0,062	0,063	0,064	0,064
B	0,068	0,068	0,122	0,087	0,097	0,075	0,098	0,067	0,071	0,082	0,07	0,091
C	0,069	0,088	0,071	0,117	0,07	0,073	0,073	0,067	0,104	0,073	0,065	0,065
D	0,065	0,068	0,062	0,063	0,077	0,062	0,08	0,066	0,068	0,064	0,063	0,067
E	0,281	0,067	0,07	0,072	0,08	0,064	0,071	0,075	0,067	0,069	0,083	0,083

F	0,072	0,067	0,072	0,062	0,066	0,081	0,066	0,066	0,074	0,077	0,081	0,066
G	0,092	0,063	0,068	0,065	0,066	0,063	0,077	0,068	0,068	0,077	0,072	0,091
H	0,097	0,062	0,086	0,066	0,069	0,076	0,068	0,072	1,417	1,34	0,252	0,229

Mock

	1	2	3	4	5	6	7	8	9	10	11	12
A	0,063	0,072	0,064	0,064	0,061	0,063	0,062	0,061	0,061	0,063	0,063	0,061
B	0,065	0,062	0,075	0,067	0,067	0,066	0,072	0,062	0,065	0,067	0,065	0,071
C	0,066	0,071	0,066	0,076	0,062	0,064	0,064	0,064	0,081	0,066	0,064	0,063
D	0,065	0,065	0,063	0,063	0,069	0,062	0,078	0,064	0,065	0,063	0,062	0,065
E	0,14	0,068	0,07	0,068	0,068	0,061	0,065	0,065	0,064	0,065	0,068	0,071
F	0,065	0,067	0,069	0,062	0,064	0,066	0,065	0,062	0,065	0,077	0,067	0,063
G	0,071	0,062	0,064	0,065	0,064	0,062	0,065	0,066	0,065	0,066	0,064	0,071
H	0,078	0,117	0,069	0,063	0,066	0,071	0,065	0,064	0,216	0,211	0,14	0,134

Plate 2

	1	2	3	4	5	6	7	8	9
A	0,067	0,066	0,061	0,061	0,235	1,47	1,455		

CCHFV ELISA for human serum samples

Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	0,131	0,308	0,112	0,122	0,109	0,149	0,127	0,14	0,202	0,176	0,183	0,2
B	0,104	0,111	0,118	0,166	0,105	0,131	0,158	0,132	0,131	0,142	0,165	0,123

C	0,126	0,154	0,167	0,125	0,132	0,153	0,201	0,152	0,2	0,178	0,163	0,146
D	0,101	0,12	0,144	0,153	0,131	0,133	1,066	0,116	0,198	0,195	0,188	0,171
E	0,113	0,137	1,12	0,106	0,135	0,15	0,174	0,191	0,269	0,177	0,188	0,173
F	0,128	0,128	0,12	0,141	0,139	0,142	0,113	0,153	0,22	0,191	0,149	0,188
G	0,172	0,122	0,128	0,129	0,108	0,173	0,091	0,287	0,185	0,168	0,201	0,19
H	0,149	0,125	0,146	0,099	0,11	0,128	0,122	0,095	1,719	1,571	0,027	0,017

Plate 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	0,182	0,112	0,085	0,125	0,044	0,122	0,043	0,078	0	0,012	0,042	0
B	0,137	0,06	0,043	0,142	0,086	0,084	0,07	0,131	0,005	0,005	0,01	0,025
C	0,196	0,169	0,15	0,099	0,14	0,118	0,05	0,134	0,002	0	0	0,11
D	0,101	0,152	0,139	0,101	0,094	0,129	0,115	0,213	0,007	0,199	0	0,012
E	0,108	0,126	0,126	0,054	0,142	0,139	0,049	0,131	0	0	0,002	0
F	0,205	0,16	0,091	0,046	0,051	0,062	0,025	0,069	0,013	0,002	0,021	0,016
G	0,107	0,109	0,106	0,062	0,11	0,11	0,088	0,064	0,045	0	0,02	0,042
H	0,189	0,156	0,182	0,135	0,136	0,135	0,077	0,182	1,247	1,151	0	0

Plate 3

	1	2	3	4	5	6	7	8	9	10	11	12
A	0,034	0,068	0,063	0,008	0,072	0,085	0,042	0,09	0,138	0,174	0,155	0,128
B	0,183	0,051	0,059	0,092	0,024	0,073	0,01	0,072	0,125	0,126	0,086	0,04
C	0,022	0,09	0,144	0,113	0,136	0,159	0,097	0,085	0,072	0,135	0,006	0,14
D	0,054	0,08	0,062	0,04	0,173	0,071	0,157	0,162	0,206	0,058	0,174	0,074
E	0,21	0,158	0,083	0,005	0,061	0,042	0,044	0,144	0,098	0,158	0	0,043

F	0,144	0,143	0,103	0,048	0,104	0,116	0,219	0,154	0,165	0,136	0,074	0,042
G	0,144	0	0,031	0,056	0,065	0,099	0,1	0,185	0,124	0,166	0,192	0,164
H	0,144	0,102	0,197	0,08	0,178	0,076	0,145	0,092	1,389	1,422	0	0

Plate 4

	1	2	3	4	5	6	7	8	9	10	11	12
A	0,001	0,003	0,01	0,018	0,014	0,167	0,07	0,088	0,057	0,019	0,112	0,105
B	0,011	0,009	0,018	0,013	0,018	0,061	0,075	0,08	0,052	0,099	0,058	0,054
C	0,033	0,036	0,046	0,107	0,07	0,123	0,084	0,12	0,044	0,095	0,056	0,052
D	0,001	0,007	0,009	0,157	0,013	0,047	0,044	0,158	1,134	1,213	0,031	0,04

Plate 5

	1	2	3	4	5	6	7	8	9	10	11	12
A	0,016	0,198	0,1	0,031	0,043	0,016	0,787	0,006	0,008	0,001	0,008	0,017
B	0,112	0,026	0,051	0,111	0,029	0,018	0	0,001	0,001	0,028	0,016	0,007
C	0,046	0,053	0,033	0,057	0,017	0,007	0,016	0,006	0,042	0,029	0,034	0,012
D	0,052	0,038	0,038	0,021	0,046	0,008	0,044	0,011	0,019	0,019	0,03	0,035
E	0,025	0,029	0,052	0,015	0,059	0	0,055	0,021	0,021	0,016	0,034	0,005
F	0,094	0,042	0,076	0,027	0,039	1,345	1,364	0,014	0,024			

CCHFV ELISA for cattle serum samples

Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0,004	0,001	0,001	0,003	0,007	0	0,004	0,002	0,001	0	0,001
B	0,003	0,003	0,016	0	0,042	0,022	0,086	0,096	0,02	0,261	0,291	0,077

C	0,1	0,002	0,002	0,003	0,022	0,004	0,049	0,03	0,208	0,043	0,002	0,113
D	0,019	0,043	0,004	0,003	0,011	0,014	0,014	0,008	0,343	0,36	0,01	0,004
E	0	0,041	0,001	0,007	0,017	0,005	0,001	0,012	0,007	0,001	0,002	0,003
F	0	0,04	0	0	0,006	0,004	0,012	0,004	0,006	0,003	0,002	0,003
G	0,004	0,062	0,084	0,054	0,028	0,009	0,003	0,002	0,011	0,001	0,036	0,002
H	0,047	0,027	0,052	0,2	0,121	0,037	0,03	0,002	0,344	0,422	0,006	0,004

*

Plate 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	0,001	0,002	0,015	0,002	0	0,001	0,002	0,001	0,007	0,001	0	0
B	0,178	0,227										

Appendix E: Media composition, buffers and solutions

SOC medium

20g Bacto-tryptone

5g Bacto-yeast extract 2ml

5M NaCl 2. 5ml

1M KCl 10ml

1M MgSO₄ 20ml

1M Glucose

(Dissolved in 900ml distilled water and autoclaved)

Stored at 4°C.

1xTBS

Dissolve 6,05 g Tris and 8,76 g NaCl in 800 ml deionised water. Adjust pH to 7,5 using 1 M HCl, adjust volume to 1 L using deionised water and store at 4°C.

1% Agarose gel

1g Agarose 100ml

1X TAE buffer

6µl 10mg/ml Ethidium bromide

50x TAE buffer

242ml Tris base in 500ml distilled water 57.1ml Glacial acetic acid 100ml 0.5M EDTA (pH 8) Adjust with water to 1L 1xTAE prepared by diluting 1:50

IFA Blocking solution

50L 1X PBS

5g saccharose

250µl Triton x-100

IFA wash solution

1ml TWEEN® 20 detergent (Cal Biochem, Darmstadt, Germany)

100ml 1xPBS

0.1% PBS solution

2µl TWEEN® 20 detergent (Cal Biochem, Darmstadt, Germany)

2 L 1x PBS

LB (amp or kan) broth

5g Yeast extract

10g Tryptone

10g NaCl

Dissolved in 1L distilled water 100ug/ml Ampicillin or 10µg/ml

Kanamycin (filter sterilized)

LB/amp or kan plates

2g Yeast extract

5g Tryptone

5g NaCl

7.5g Agar

Dissolved in 500ml distilled water

100ug/ml Ampicillin or 10µg/ml Kanamycin (filter sterilized)

0,2% Coomassie Brilliant Blue stain (1 L)

Dissolve 1 g Coomassie Brilliant Blue in 450 ml methanol and 100 ml glacial acetic acid. Adjust volume to 1 L using deionised water and store at room temperature.

Destaining solution (2 L)

Mix 600 ml methanol, 200 ml glacial acetic acid and 1,2 L deionised water and store at room temperature.

Transfer buffer (1 L)

Dissolve 2,9 g glycine, 5,8 g Tris and 0,37 g SDS in 200 ml methanol. Adjust volume to 1 L using deionised water and store at 4°C.

BSA buffer

Dissolve 4,3g NaCl (MW=58,44g/mol), 1,04g Na₂EDTA:2H₂O (MW=372,24g/mol) and 3,02g tris (hydroxymethyl) aminomethane (MW121.14g/mol) in 500 ml d H₂O. Add 2,5ml Nonidet P40 and mix thoroughly. Store at room temperature. Supplement an aliquot of lysis buffer with 0,1% bovine serum albumin before use.