The development and validation of a reverse transcription recombinase polymerase amplification assay for detection of flaviviruses

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Submitted in fulfilment of the requirements in respect of the MMedSc Virology degree qualification completed in the Division of Medical Virology in the Faculty of Health Sciences at the University of the Free State

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Division of Medical Virology

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University of the Free State, Bloemfontein, SA

4 February 2019

Declarations

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

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Presentations and publications

Presentations

Bonnet EH, Burt FJ. The development and validation of a reverse transcription recombinase polymerase amplification assay for the detection of flaviviruses. 50th Faculty Research Forum 29th-30th August 2018, Faculty of Health Sciences, University of the Free State. Oral presentation.

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Contents

Abstract

Flaviviruses have been of clinical importance since ancient times. Five flaviviruses are known to occur or have been identified historically in South Africa (SA) namely, West Nile virus (WNV), Usutu virus (USUV), Wesselsbron virus (WSLV), Spondweni virus (SPOV) and Banzi virus (BANV). Medically significant flaviviruses, WNV and WSLV, are known to occur annually in SA. Development of isothermal assays, such as recombinase polymerase amplification (RPA), plays an important role in performing surveillance studies and increasing diagnostic capacity for emerging viral pathogens in limited resource settings. In their native form, WNV and WSLV can only be handled in a biosafety laboratory level 3 and this restricts laboratories that lack such resources, hence transcribed RNA controls were successfully prepared for WNV, USUV and WSLV to develop and validate a RT-RPA for the detection of flaviviruses. A lateral-flow RT-RPA was developed by identifying theoretical cross reactivity between the probe and primer candidates by sequence alignments of the conserved NS5 protein of WNV, USUV and WSLV. It was determined that a few mismatches were present between WNV and USUV in the probe binding region and in the reverse primer, as well as between WNV and WSLV, hence different probe and reverse primer regions were identified for WNV/USUV and WSLV. A limitation of the study was the selection of a reference strain of WNV belonging to lineage 1 as a lineage 2 isolate would have been a more suitable representative of SA lineages. Nonetheless, RNA from SA isolate 93/01 was amplified using the RT-RPA. The sensitivity of the assay was determined by diluting RNA control ten-fold, and was found that the WNV RT-RPA could detect WNV and USUV transcribed RNA diluted 10^9 fold, whereas the WSLV RT-RPA detected WSLV transcribed RNA diluted 10^{10} fold. Testing RNA from other arboviruses suggested that despite the binding tolerability of the assay there was good specificity as no other arboviruses were amplified. However because of similarity in sequence data, USUV transcribed RNA was detected with the WNV RT-RPA and WSLV RT-RPA. Theoretical cross reactivities with other flaviviruses were determined by sequence alignments of the NS5 region and it was proposed that Japanse encephalitis virus (JEV), Zika virus (ZIKV) and dengue virus (DENV) RNA will not be detected by either the WNV RT-RPA or WSLV RT-RPA. Seventeen pools of *Culex* spp mosquitoes were screened for flavivirus RNA, although no flaviviruses were expected within such a small cohort. Lack of amplification inhibitors in the mosquito samples was confirmed by spiking known negative mosquito samples with transcribed flavivirus RNA and performing a RT-RPA using the spiked samples. Detecting WNV by RT-RPA will not only be useful for surveillance studies in SA,

but can also be used as a diagnostic tool for veterinary diagnostics, especially equine. In conclusion, the development and validation of a RT-RPA for the detection of WNV, WSLV and USUV flaviviruses was successful. The RT-RPA proved to be a robust, rapid and sensitive assay that might have potential as a diagnostic tool in the field or resource limited settings.

Keywords: Flaviviruses, West Nile virus, Usutu virus, Wesselsbron virus, limited resource settings, transcribed RNA, RT-RPA, sensitive, robust, rapid, isothermal

List of Figures

Figures 1, 2 and 11 were designed by the author.

Permission was obtained for figure 3, license number: 4566901298447

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List of abbreviations

°C	Degrees celsius
µl	Microliter
µM	Micromolar
amp	Ampicillin
ATP	Adenine triphosphate
BANV	Banzi virus
Biosg	Biotin
bp	Base pair
BSL-3	Biosafety laboratory level 3
C protein	Capsid protein
CCHFV	Crimean-Congo haemorrhagic fever virus
cDNA	Complementary deoxyribonucleic acid
cfu/ug	Colony forming units per microgram
CHIKV	Chikungunya virus
CSF	Cerebrospinal fluid
CTP	Cytosine triphosphate
DENV	Dengue virus
DIG	Dioxygen
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DRC	Democratic Republic of Congo
E protein	Envelope protein
E.coli	*Escherichia coli*
EBOV	Ebola virus
EDTA	Ethylene-diamine-tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay

FAM	Fluorescein amidite
FDA	Food and Drug Administration
fg	Fentogram
FNBG	Free State National Botanical Gardens
GC/rxn	Genome copies per reaction
GE/rxn	Genome equivalents per reaction
GTP	Guanine triphosphate
H_2O_2	Hydrogen peroxide
HAI	Hemagglutination inhibition test
IFA	Immunofluorescent assay
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IVT	*In vitro* transcription
JEV	Japanese encephalitis virus
JEVSAV	Japanese encephalitis virus SA
Kb	Kilobase
kDA	Kilodalton
LAMP	Loop-mediated isothermal amplification
LB	Luria-Bertani broth
LB/amp	Luria-Bertani broth with ampicillin
LFS-RPA	Lateral flow based recombinase polymerase amplification
LGTV	Langat virus
LIV	Louping ill virus
LOD	Limit of detection
MARV	Marburg virus

MgAcO	Magnesium acetate
ml	Milliliter
mM	Millimolar
$MnCl_2$	Manganese chloride
mRNA	Messenger ribosomal nucleic acid
NASBA	Nucleic acid based amplification assay
NC region	Non-coding region
NFW	Nuclease-free water
ng/µl	Nanograms per microliter
NICD	National Institute for Communicable Diseases
Nm	nanometer
nM	Nanomolar
NS1	Non-structural protein 1
NS2A	Non-structural protein 2A
NS2B	Non-structural protein 2B
NS3	Non-structural protein 3
NS4A	Non-structural protein 4A
NS4B	Non-structural protein 4B
NS5	Non-structural protein 5
o/n	overnight
OIE	World Organisation for Animal Health
ORF	Open reading frame
PCR	Polymerase chain reaction
PFU	Plaque forming units
pGEMUSUV	Usutu pGEM® plasmid DNA
pGEMWNV	West Nile pGEM® plasmid DNA
pGEMWSLV	Wesselsbron pGEM® plasmid DNA

pmol/µl	Picomoles per microliter
prM	Premembrane protein
PRNT	Plaque reduction neutralisation test
pUC57USUV	pUC57 Usutu plasmid DNA
pUC57WNV	pUC57 West Nile plasmid DNA
pUC57WSLV	pUC57 Wesselsbron plasmid DNA
qPCR	Quantitative polymerase chain reaction
RFV	Royal farm virus
RNA	Ribonucleic acid
RPA	Recombinase polymerase amplification
RT-PCR	Reverse transcription polymerase chain reaction
RT-RPA	Reverse transcription recombinase polymerase amplification
RVF	Rift Valley fever
SA	South Africa
SINV	Sindbis virus
SOC	Super optimal catabolite repression broth
SPOV	Spondweni virus
SSB	Single-stranded binding molecule
SUDV	Sudan virus
SV	Sigma virus
TBEV	Tick-borne encephalitis virus
THF	Tetrahydrafuran
TMA	Transcription mediated amplification
TMB	Tetramethylbenzidine
U/µl	Units per microliter
Uganda S	Uganda Sigma

uncRNA	Universal control ribonucleic acid
USUV	Usutu virus
UTP	Urasil triphosphate
V	Volt
WHO	World Health Organisation
WNV	West Nile virus
WSLV	Wesselsbron virus
xg	Gravitational force
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
YF	Yellow fever
YFV	Yellow fever virus
ZIKV	Zika virus

Chapter 1: LITERATURE REVIEW

1.1 Introduction and History

Arboviruses are viruses transmitted by arthropod vectors, including mosquitoes. In most instances, humans are incidental hosts as they do not develop high enough levels of viremia to be able to contribute to the transmission cycle. Infected arthropods introduce the virus into the host during a blood meal. However, in some instances laboratory-acquired infections can also occur after handling infected tissues or fluids (Calisher, 1994). Flaviviruses have been of clinical importance since ancient times, especially during the recorded epidemics of yellow fever (YF) in the mid 1600's. The prototype of the *Flavivirus* genus is yellow fever virus (YFV) which probably originated in Africa and spread to countries in Europe and the Americas as a consequence of the slave trade between these continents. Since the isolation of YFV in 1927, many viral species have been included in the genus, and currently it is comprised of over 90 viruses (MacLachen et al., 2017). Not all members of this genus have been associated with human disease; however there are medically significant flaviviruses associated with severe disease, encephalitis and haemorrhagic fever.

1.2 Classification of flaviviruses

The family *Flaviviridae* is comprised of four genera namely, *Flavivirus, Hepacivirus, Pestivirus* and *Pegivirus* (Calisher, 1994; Simmonds et al., 2017). At least 50 members of the *Flavivirus* genus are medically significant vector-borne viruses or have veterinary significance (MacLachen et al., 2017). The classification of the family *Flaviviridae* has recently been updated and with the addition of the Viru Taxonomy: 2018b Release report (Simmonds et al., 2017; https://talk.ictvonline.org/ictv-reports/ictv_online_report/positive-sense-rna-viruses/w/flaviviridae). Currently the family is comprised of four genera, including *Flavivirus, Hepacivrus, Pestivirus* and *Pegivirus* and a total of 89 species. With the genus *Flavivirus* there are 53 species of viruses, most of which are transmitted by arthropods and are grouped accordingly depending on the source of transmission. Within this genus there are species that are significant human pathogens, such as yellow fever virus and Zika virus, as well as species that cause veterinary disease and economic losses. In addition there are a small number that are only known to infect only mammals or only arthropods.

Members of the genus *Flavivirus* have a 50nm in diameter virion comprised of three structural and seven non-structural proteins (NS). The structural proteins include an envelope (E), pre-membrane (prM) and capsid (C), while the non-structural proteins include NS1, NS2A, NS2B,

NS3, NS4A, NS4B and NS5 (Petersen et al., 2001). The genome encased in the virion is a single-stranded, positive sense, infectious RNA molecule, which is approximately 9.2-11.0kb (Simmonds et al., 2017). The genome possesses a cap structure at the 5′ terminus, however unlike most host mRNAs, it lacks 3′ terminal polyadenylation. As an alternative, the 3′ terminal nucleotides form a stable, highly conserved stem-loop structure, which stabilizes the genome and provides signals for initiation of translation for protein synthesis. The structural proteins that form the virions are encoded in the 5′-region of the genome, whereas the NS proteins that form the viral replicase are encoded in the remaining genome (Rice et al., 1985). The genome is positive sense and serves as RNA template for translation and encodes a polyprotein, which is cleaved into ten proteins after post-translational modifications (Lindebach et al., 2003). Figure 1 depicts the genome of flaviviruses.

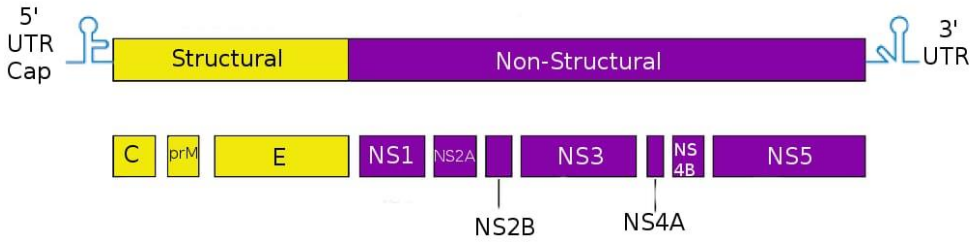

Figure 1. The genome organisation of the polyprotein of flaviviruses depicting structural and non-structural proteins.

The open reading frame (ORF) downstream of the 5′ non-coding (NC) region of the genome encodes for the proteins C, E and prM. Protein C is 11 kilodaltons (kDA) in size and is involved in the formation of the icosahedral nucleocapsid. This hydrophobic protein forms a complex with the genome by binding to the RNA and interacts with the host cellular membranes to allow for assembly of the virus (Markoff et al., 1997). Protein E is the largest structural protein with a size of 50 kDA. This protein is a glycosolated type I membrane protein found on the outer surface of the lipid bi-layered envelope. It functions as a class II viral fusion protein and it has been observed to be the primary target for neutralising antibodies (Sánchez et al., 2005). Protein M is the third structural protein with a size of 8 kDA and is translated into an immature form called prM, which is subsequently cleaved by a cellular enzyme, furin, resulting in the mature protein M and a “pr” segment (Stadler et al., 1997). Protein M, representing the C terminal component of the immature prM protein, is only present in mature virions, whereas the N-terminal “pr” protein segment can be detected in the extracellular growth medium in tissue cell cultures following cleavage (Lindenbach et al., 2007). The prM complex in the immature form protects protein E from degradation as the virion is transported through the secretory pathway during virion assembly (Bray et al., 1991).

The seven NS proteins are vital for replication and, excluding NS1, their primary immunologic roles are as targets for cytotoxic T-cells in the cell-mediated immune response. The NS1 protein is 48 kDA in size and can exist either in cell-associated, cell-surface or extracellular non-virion forms (Macdonald et al., 2005). It is also described to be a co-factor for replication and the soluble extracellular form of this protein is also associated with cell surfaces, correlating with a strong humoral immune response (Chung et al., 2006).

1.3 Epidemiology and transmission of flaviviruses

Most flaviviruses are arthropod-borne viruses transmitted between arthropod vectors and vertebrate hosts. Based on phylogenetic trees, Africa is considered as the ancestral origin of all mosquito and tick-transmitted flaviviruses. Flaviviruses are grouped into clusters namely mosquito-borne, tick-borne and non-vectored/no-known vector viruses. The ancient history of these flaviviruses originated from the Old World; however flaviviruses have recently spread more globally due to the increase in human populations and global movements (Braack et al., 2018).

The mosquito-borne flaviviruses are grouped in six groups based on genetic relationship and vector. There are an additional two groups that are described as probably mosquito-borne (refer to Appendix C) (Simmonds et al., 2017; https://talk.ictvonline.org/ictv-reports/ictv_online_report/positive-sense-rna-viruses/w/flaviviridae). Within the mosquito-borne and probably mosquito-borne groups there are five flaviviruses known to occur or that have been identified historically in South Africa (SA) namely, West Nile virus (WNV), Usutu virus (USUV), Wesselsbron virus (WSLV), Spondweni virus (SPOV) and Banzi virus (BANV). Medically significant flaviviruses, WNV and WSLV, are known to occur annually in SA causing sporadic outbreaks, usually associated with heavy rainfall favouring mosquito breeding (Braack et al., 2018).

1.3.1 West Nile virus

WNV was first isolated in 1937 from the serum of a febrile Ugandan woman (Smithburn et al., 1940). The virus circulates in endemic, and sometimes epidemic, transmission cycles throughout Europe, western Asia, Africa, the Middle East, Australia (Kunjin strain of WNV) and North and Central America. It has long been suspected that migratory birds act as the introductory hosts of WNV into new regions as outbreaks generally occur in temperate regions during late summer or early fall. This coincides with the arrival of large flocks of migratory birds and mosquitoes and occurs in human settlements near wetlands, as a result of high

concentrations of birds coming in contact with ornithophilic mosquitoes (Petersen et al., 2001) (Figure 2).

There are nine genetically distinct lineages of WNV based on the polyprotein nucleotide sequences (Appendix D) (Pachler et al., 2014)). Lineage 1, the most widespread globally, is further divided into lineage 1a, which include the American strains (Lanciotti et al., 1999) and lineage 1b (Kunjin virus) which is mainly found in Australia (Coia et al., 1988). Lineage 1c was identified in a study of 17 isolates from India (Bondre et al., 2007). Lineage 2 isolates have been detected in Africa, including SA (Fall et al., 2014) and parts of Europe such as Greece, Italy and Hungary (Bagnarelli et al., 2011; Bakonyi et al., 2006; Papa et al., 2011).

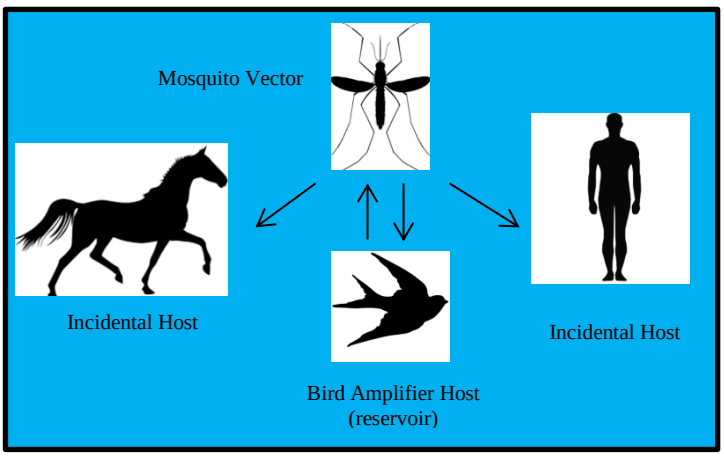

Figure 2. Schematic representation of the transmission of WNV in nature. The virus is maintained between mosquitoes and birds and spill over to incidental hosts is achieved through the bite of an infected mosquito.

Lineage 3 isolates have been isolated in the Czech Republic (Bakonyi et al., 2005) and lineage 4a is found in Russia. Lineage 5 was identified in India based on results from 17 isolates which included an isolate from 1c (see above) and a distinct lineage that was designated lineage 5 (Bondre et al., 2007). Lineage 6 identified in Spain has a 95% identity in nucleotide sequence with sub-lineage 4b, however there is only a partial sequence available for lineage 6 (Pachler et al., 2014; Vazquez et al., 2010). Putative lineages 7 and 8 were found in Senegal (Fall et al, 2014) and it has been proposed that the WNV-Uu-LN-AT-2013 strain from Austria constitute a new lineage (lineage 9) or can be grouped into lineage 4 as a sub-lineage (4c). The complete polyprotein nucleotide sequence of strain WNV-Uu-LN-AT-2013 shares an identity of 83% with lineage 4 WNV strains and 96% identity at the amino acid level (Pachler et al., 2014).

One of the largest WNV outbreaks occurred in 1974 in the Karoo region of SA after exceptionally high summer rains. The outbreak covered 2500 km^2 and occurred concurrently with an outbreak of Sindbis virus (SINV). WNV antibody was present in high levels in wild birds (McIntosh et al., 1976). In a retrospective serological survey after the outbreak, 55% of human sera tested were antibody positive for WNV. Two key factors likely resulted in the occurrence of this epidemic, unusual rains that increased mosquito population density and higher summer temperatures which enhanced viral replication in the vector. Another epidemic outbreak occurred during 1983 and 1984 in the Witwatersrand-Pretoria region (now referred to as Gauteng Province) and resulted in hundreds of human cases that were diagnosed clinically. Twenty-eight cases of SINV infection were confirmed in the laboratory by seroconversion and five cases were confirmed as WNV (Jupp et al., 1986). Cattle sera from 50 herds across SA were tested for antibody against WNV and a seroprevalence of 1.3% for WNV was reported (Burt et al., 1996), while a serological survey of dogs from the Highveld region of SA showed that 37% (138 of 377) had neutralising antibodies against WNV (Blackburn et al., 1989). A recent study done in the Free State region showed a seropositivity rate of 56% from a total of 2 393 serum samples from humans, cattle, sheep and wild animals screened using an in-house enzyme linked immunosorbent assay (ELISA) (Mathengtheng and Burt, 2014).

Prior to 2010, all isolates from SA were identified as belonging to lineage 2 and lineage 2 viruses were presumed to be less virulent than lineage 1 (Burt et al., 2002). Subsequently, pathogenic and neuroinvasive lineage 2 viruses have been identified in SA (Venter et al., 2010; Venter et al., 2017). A surveillance study was conducted during 2008-2015 in South Africa where 1407 animals presenting with neurologic disease were screened for WNV. The study identified a prevalence of lineage 2 WNV in 7.4% of the horses tested, 1.5% of livestock and 0.5% of wildlife (Venter et al., 2017). A WNV isolate belonging to lineage 1 was detected in the brain of a pregnant mare presenting with symptoms of neurological disease and subsequent to onset of illness, the mare aborted her foetus and died (Venter et al., 2011). Further evidence of virulent strains from lineage 2 was reported in five horses with unexplained fever and neurological signs. Four of the horses were located in the Gauteng province and one was in the Western Cape region of SA (Venter et al., 2009). Two human cases caused by WNV lineage 2 were confirmed after a couple visited SA. The 76-year old woman tested positive for WNV IgM by ELISA and WNV RNA was detected in a urine sample by RT-PCR. After two weeks follow-up serum was tested using ELISA and showed seroconversion of WNV IgG. Her 72-year old husband also tested positive for WNV IgM and IgG by ELISA, however WNV RT-PCR on the urine sample was negative (Parkash et al., 2019).

1.3.2 Wesselsbron virus

WSLV is transmitted by *Aedes* mosquitoes and infects sheep and cattle in Africa and can also infect humans with fever and myalgia being the most common symptoms (Weiss, 1956). The agent was first isolated in 1955 from the blood of a febrile man and dead lamb in Wesselsbron in the Free State, SA. WSLV has a wide geographical distribution in Africa including SA, Botswana, Zimbabwe, Uganda, Mozambique, Cameroon, Senegal, Madagascar and Democroatic Republic of Congo (Weyer et al., 2013). WSLV was first identified during an outbreak of Rift Valley Fever virus (RVFV) in the Kroonstad district in 1956 (McIntosh, 1980). WSLV antibodies were detected in lambs, although no virus was isolated. In 1958, WSLV was finally isolated from one lamb within this cluster. Because of the similar clinical presentation of RVFV and WSLV, live partially attenuated vaccines were prepared and sold for dual veterinary use in SA and Zimbabwe. However, the vaccine caused abortion in pregnant sheep and was thus not recommended for use in pregnant animals. To date, no fatal human cases associated with WSLV infection have been reported. A case was reported in 1980, involving a laboratory worker who had exposure to WSLV through a splash of virus suspension into the eye and 29 naturally infected acute human cases have been laboratory confirmed (Heymann et al., 1958; Jupp and Kemp, 1998; Justines and Shope, 1969; McIntosh, 1980; Smithburn et al., 1957; Swanepoel, 1989; Tomori et al., 1981; Weinbren 1959; Weiss et al., 1956). Retrospective serosurveys were conducted prior to 1980 and showed positivity rates of up to 35% in selected populations from Namibia, 30% from southern Mozambique and 22% from northern Botswana. From an outbreak that occurred in SA between 2010 and 2011, WSLV was isolated from two human cases although an initial diagnosis of RVF infection was suspected. These isolates were included in a phylogenetic analysis of collective WSLV isolates based on the sequence of the NS5 gene. Two clades of WSLV were described circulating in southern Africa, with isolates from Zimbabwe and SA clustering in clade 1 and isolates from KwaZulu Natal province in clade 2 (Weyer et al., 2013).

1.3.3 Spondweni virus

SPOV was first isolated in 1954 in Nigeria, however was misidentified as ZIKV (MacNamara, 1954). SPOV was first isolated in SA in 1955 from a pool of *Mansonia uniformis* mosquitoes collected in the Natal region (Kokernot et. al, 1957)). Two cases of SPOV infection occurred in laboratory workers in SA (McIntosh et al., 1961), as well as in the western parts of Africa where Americans resided in Burkina Faso, Cameroon and Gabon (Wolfe et al., 1982). Human clinical disease varies from mild febrile illnesses with headache to fever, chills, nausea, aches and pains,

dizziness, rash and epistaxis. SPOV is pathogenic for suckling and weaning baby mice inoculated intracerebrally, and it was reported that adult white Swiss mice exhibited exclusive tropism with histopathological changes in nervous tissue. An immune response was elicited in experimentally infected vervet monkeys, guinea pigs and rabbits after viral infection; however, no clinical symptoms were observed (Kokernot et al., 1957). SPOV has recently been detected from a pool of *Culex quinquefasciatus* mosquitoes collected in 2016 in Haiti (White et al., 2018).

1.3.4 Banzi virus

BANV was isolated for the first time in 1956 from a febrile boy in Tongaland in the KwaZulu Natal province, SA (Smithburn et al., 1959). The agent was reported to be serologically related to Uganda Sigma (Uganda S) and YF viruses and was classified as belonging to the Uganda S serocomplex. Neutralizing antibodies against BANV have been found in human sera in Angola, Mozambique, SA, Namibia and Botswana (Kokernot et al., 1965; Smithburn et al., 1959). BANV has been isolated from mosquitoes in Kenya and SA and from rodents and hamsters caught in Mozambique (Jupp et al., 1976; McIntosh et al., 1976 a, b; Metselaar et al., 1974).

1.3.5 Usutu virus

USUV virus was first isolated from mosquitoes in 1959 in SA (Williams and Woodall, 1964). USUV was only isolated from two patients in Africa, a patient that presented with a fever and rash in 1981 in the Central African Republic and from a 10-year old patient who had a fever and jaundice in 2004 in Burkina Faso (Nikolay et al., 2011). USUV forms part of the Japanese encephalitis virus (JEV) serocomplex and has been isolated from several mosquito species throughout the African continent, especially in parts of Africa where surveillance programs are implemented, including Senegal, Burkina Faso, Cote d´Ivorie, Nigeria, Uganda (Nikolay et al., 2011) and Kenya (Ochieng et al., 2013). No sequence data is available for USUV in Africa besides SA; however one subtype was isolated from *Culex perfuscus* in the Central African Republic in 1969. In 2001, an outbreak of USUV was observed in Vienna and Austria when a mass of Eurasian blackbirds (*Turdus merula*) died. Within five days of the beginning of the outbreak in August, five Great Gray owls (*Strix nebulosa*) died in the Tiergarten Schönbrunn Vienna Zoo and in addition masses of Bam swallows (*Hirundo rustica*) were observed dead in the Austrian federal state of upper Austria (Weissenböck et al., 2002). In 2009, the first two human cases of USUV infection were reported in Italy. The cases involved two immunocompromised patients that developed meningoencephalitis and USUV was amplified by reverse transcription polymerase chain reaction (RT-PCR) from the cerebrospinal fluid

(CSF) of the patients (Pecorari et al., 2009). The only USUV neuroinvasive infection in humans outside Italy was documented in Croatia in 2013 during a WNV outbreak. Neutralizing antibodies against USUV were detected in all patients and seroconversion was documented by ELISA in two of them (Santini et al., 2015).

1.3.6 Other important flaviviruses

Other medically important mosquito-borne flaviviruses include dengue virus (DENV) and YFV. YFV and DENV are not endemic in SA; however it is important to acknowledge possible risk factors as these viruses can be introduced into SA via air transport, shipping and tourism. An outbreak of DENV was recorded in 1929 but the virus does not appear to established endemnicity. Several imported cases of DENV have been reported in SA, however DENV is not currently circulating in SA and has not been isolated from mosquito populations (Msimang et al., 2018).

YFV endemic areas were mapped in the early 1930's and 1940's by screening large numbers of human serum samples from West African natives (Beeuwkes et al., 1930) before the widespread use of YF vaccination. During the 17^{th}-19^{th} centuries YF epidemics occurred due to the intensity of international trade linking Africa, America and islands in between. YF endemic areas include 43 countries in Africa and Central and South America (Appendix E) (Brent et al., 2018). YF epidemics occurred recently in Angola, Democratic Republic of Congo (DRC) and Brazil. During 2016, yellow fever outbreaks occurred in Angola and DRC where approximately 965 cases and 400 fatal cases were confirmed (Kraemer et al., 2017). An outbreak occurred in Brazil from December 2016 to June 2017 and involved 777 confirmed cases with 261 deaths, however sporadic cases were found after the epidemic (Goldani, 2017). The World Health Organisation (WHO) recognized the ongoing epidemic and recommended vaccination to all residents residing in the State of Rio de Janeiro, Bahia and São Paulo. As of June 2018, 1257 cases were confirmed with 394 deaths (Sakamoto et al., 2018).

Dengue infections are caused by four serotypes (possibly 5), designated DEN-1, DEN-2, DEN-3, and DEN-4 (Normile, 2013). The first known epidemic of dengue occurred in the Philippines in 1953–1954 and spread throughout Southeast Asia, the Pacific Islands, the Americas and sub-Saharan Africa (Gubler, 1998). Two species of mosquitoes, *A. aegypti* and *A. albopictus*, are the primary vectors of DENV responsible for transmission to humans (Christophers, 1960). DENV infections have increased drastically over the past 50 years and it was estimated that 390 million dengue cases occur worldwide per year (Bhatt et al., 2013). Not only are DENV cases increasing, but large outbreaks are occuring. The threat of an outbreak now exists in Europe as

local transmission was reported for the first time in France (La Ruche et al., 2010) and Croatia (Schmidt-Chanasit et al., 2010) in 2010. Approximately 2000 cases were reported in 2012 during an outbreak on the Madeira islands of Portugal and imported cases were recorded in ten other countries in Europe (Alves et al., 2013). The number of cases in the People's Republic of China (Li et al., 2016) increased in 2014 and Delhi in India experienced its worst outbreak in 2015 with over 15000 cases (Ahmed et al., 2015). Large dengue outbreaks occurred during 2016 in the region of the Americas with more than 2.38 million cases and 1032 deaths (Torres et al., 2017), whereas more than 375000 suspected cases were reported in the Western Pacific region. In the African region, a localized outbreak occurred in Burkina Faso with 1327 probable cases (Tarnagda et al., 2018).

Three DENV outbreaks occured in the Kwazulu Natal province of SA in 1897, 1901 and in the summer of 1926/1927. The virus was introduced through infected human travellers and approximately 50000 cases and 60 deaths were reported. Between the period of 2000-2016, 176 dengue cases were laboratory confirmed (Msimang et al., 2018).

1.4 Laboratory diagnosis of flavivirus infection

Clinical diagnosis of the different flavivirus infections of humans remains difficult owing to the non-specific symptoms. Therefore laboratory diagnosis is mandatory, particularly the diagnosis of isolated cases to confirm the etiology of disease (Gardner and Ryman, 2010). Each case may present with differences in severity and can lead to incorrect recognition of disease. The duration of viremia does vary for different flaviviruses however, in general viremia, can occur for 2-7 days following the onset of disease (Gould and Solomon, 2008). For most flavivirus infections an immune response is usually detectable 5-7 days from onset, with immunoglobulin M antibodies (IgM) peaking after 15 days (Busch, 2008). An IgM antibody response can remain detectable for months to years, depending on the flavivirus (Kapoor et al., 2004). In some flavivirus infections,immunoglobulin G antibodies (IgG) only appear 8-10 days after onset of disease and can be detected throughout the patient's life (Domingo et al., 2011). Viremia in flavivirus infections is usually of short duration and the probability of obtaining a virus isolate from the patient is low. Because of the short lived viremia, diagnosis of flavivirus infections are better achieved by using serological and molecular assays (Calisher, 1994; Kuno, 2003). For frequently identified flaviviruses such as WNV, USUV and YFV, commercial assays are available to compliment in house assays usually performed by reference laboratories. However for lesser known flaviviruses no commercial assays are available and the detection of these is

usually associated with specialised laboratories performing research and specific surveillance using in house assays.

WNV infections in humans is listed by the National Institute for Communicable Diseases (NICD) as a catergory 3 notifiable disease in SA and the World Organisation for Animal Health (OIE) listed animal WNV infections as a notifiable disease. Arbovirus diagnostics for humans are performed at the Arbovirus Reference Laboratory at the NICD, while animal samples are tested at Onderstepoort Veterinary Institute and various private laboratories in SA.

1.4.1 Serological diagnosis

Haemagglutination inhibition assays (HAI) have been widely used for the detection of flaviviruses and the antibodies produced to these viruses. These assays exploit the ability of the E protein to bind and agglutinate red blood cells and subsequently form a lattice of agglutinated cells (Clark and Casals, 1958). The advantages of HAI are that the assay can be performed with minimal training of laboratory workers and non-complex equipment is required (Choi et al., 2013). Nevertheless, multiple different pH buffers are required for each different antigen and there is a high level of cross-reactivity amongst flaviviruses. HAI was used extensively in earlier years to detect flavivirus infections; however, more sensitive and specific assays are used today (Kuno, 2003).

Immunofluorescence assays (IFA) can be used to detect antibodies and differentiate between recent and old infections. The assay involves incubation of patient serum on glass slides, upon which flavivirus infected cells have been fixed. The patient's antibodies specific to the virus can be detected with a fluorophore-conjugated anti-species IgM or IgG immunoglobulin. IFA is very beneficial as a biosafety laboratory level 3 (BSL-3) is not required and results are quickly obtained. A downside to the assay is the cross-reactivity of immune antibodies with closely related flaviviruses which can impair the accuracy of diagnosis (Hobson-Peters, 2012). The first commercial IFA using Euroimmun Biochip technology was evaluated in 2008 for the serodiagnosis of IgG and IgM antibodies against YFV (Niedrig et al., 2008). Euroimmun biochips are available for WNV, USUV, DENV 1-4, ZIKV and JEV; however not for SPOV or BANV (Euroimmun product portfolio; Litzba et al., 2010).

The serological cross reactivity between flaviviruses significantly complicates the interpretation of serological assays. Hence plaque reduction neutralisation test (PRNT) is still considered the gold standard for the serological diagnosis of flavivirus infections although is not generally used routinely for diagnosis and rather for confirmation (Panning, 2017). Neutralisation of the virus

by antibodies in the infected serum is verified by a reduction of PFU relative to the serum dilution. PRNT is highly specific, although the accuracy of interpretation of the results depends upon comparison against other flaviviruses endemic to a given area (Lindsey et al., 1976). These tests are also time-consuming, labour-intensive, require highly skilled personnel and a BSL-3 for handling live virus (Kuno, 2003).

ELISA dramatically changed serologic practices and produced numerous procedural modifications and commercial diagnostic kits. The antibody capture ELISA format has been used for most flavivirus diagnoses, especially WNV infection, and is particularly sensitive in demonstrating IgM responses early in illness (Davis et al., 2001). Only until recently has a commercial USUV IgG ELISA (Euroimmun, Lübeck, Germany) been developed (Saiz and Blazque, 2017). ELISA is a plate based assay technique designed for detecting and quantifying antibodies and antigens. In an ELISA, an antigen needs to be immobilised to a solid surface and subsequently complexed with an antibody attached to an enzyme. Detection of the reaction is accomplished by assessing the conjugated enzyme activity via incubation with a substrate to produce a measurable product. Specificity errors associated with serological flavivirus cross-reactivity have been improved through the use of algorithms (Martin et al., 2004).

The Food and Drug Administration (FDA) approved a lateral flow device for the diagnosis of WNV infection in humans (Sambol et al., 2007). Lateral flow assays consist of antigens or antibodies fixed on nitro-cellulose strips and utilises gold particles as reporter molecules. Briefly, anti-WNV IgM antibodies in patient serum form a tertiary complex with biotinylated anti-human IgM, recombinant WNV E protein and an anti-E monoclonal antibody that is coupled to colloidal gold particles. The complex is captured by the immobilised streptavidin on the nitrocellulose strip and forms a pink line. According to Sambol et al. (2007), the assay displayed 98.8% sensitivity and 95.3% specificity compared to other predicate assays. Lateral flow assays have significant advantages over ELISAs, one being that results are obtained within 15 minutes. However, the lateral flow assay device can only test a small number of samples at a time.

1.4.2 Molecular diagnosis

The development of molecular diagnostic techniques constitutes a major focus in flavivirus research. A lot of work has been invested in the development of reliable molecular methods to detect and differentiate among different species of flaviviruses. All molecular amplification assays involve three basic steps, namely nucleic acid extraction and/or purification from samples, amplification of nucleic acids and the detection of the amplified product. Flavivirus

detection involves extraction and purification of ribonucleic acid (RNA) from a variety of sample types, including human sera or mosquitoes. Traditional extraction methods are gradually being replaced with commercially available kits that use silica to bind nucleic acids after washing. Advantages to using kits include more rapid reproducible results.

In the 1990's, a number of RT-PCR based assays were described for flaviviruses. The most common amplification assay for flavivirus detection used is a basic RT-PCR format. In Austria 2018, 18 of 31 598 blood donors tested positive for USUV by RT-PCR during 28 June and 17 September (Aberle et al., 2018).A standard RT-PCR involves two steps: 1) reverse transcription of viral genomic RNA into complementary deoxyribonucleic acid (cDNA), and 2) amplification of cDNA by Taq polymerase. Either a virus-specific oligonucleotide can be used to prime DNA transcription or random hexamers can be used to initiate transcription. Primer design should include the alignment of as many sequences available to be able to achieve high sensitivity and specificity (Lanciotti, 2003). There are many commercially available thermostable polymerases that can be used for RT-PCR amplification; however they differ in properties such as fidelity, thermal stability and 5′ to 3′ exonuclease activity. Following amplification, a detection method is required to visualise the size of the amplified products. The most widely used detection method is gel electrophoresis where the product can be visualised on an agarose gel by staining the DNA with dye such as ethidium bromide. It is important to note that in some instances non-specific binding of the oligonucleotides can occur, thus generating products with similar/identical mobility to the gene of interest on the agarose gel. SYBR Green is a safer DNA-binding dye compared to ethidium bromide and the fluorescence increases when it binds to DNA. This approach requires a thermocycler that can record fluorescence during temperature cycling. After amplification, the thermocycler can calculate the melting temperature of the DNA fragments so that the predicted amplified product can be distinguished from non-specific amplified DNA fragments (Lanciotti, 2003).

The combination of RT-PCR amplification with fluorescent labelled probes offers numerous advantages over standard RT-PCR. Sequence-specific probe binding assays rely on oligonucleotide probes that hybridise to the complementary sequence in the target PCR product and thus only detect this specific product. Probe formats include hydrolysis probes which fluoresce when the 5′ exonuclease activity of TaqMan DNA polymerase hydrolyses them and hybridisation probes which hybridize to an internal sequence of amplified fragments during the annealing phase of PCR (Holland et al., 1991). This approach requires no post-amplification steps for characterising the amplified DNA and minimizes the possibility of cross-contamination and formation of primer-dimers. A probe based PCR assay is highly specific

since assay fluorescence increases only if the specific target DNA is present in the reaction. Multiplex-PCR protocols can be used to detect and differentiate flaviviral infections in a single reaction as it requires different oligonucleotide probes labelled with different fluorescent dyes. In addition, real-time protocols allow the quantification of the genome copy numbers when a standard curve is included in the assay (Lanciotti, 2003).

Unfortunately, there is a reasonable concern that real-time methodologies are not likely to be adapted for field diagnosis and point-of-care settings as it is more expensive and requires sophisticated equipment as the assays are dependent on use of cyclic temperatures and specialised reagents (Domingo et al., 2011). In an effort to identify new techniques for field purposes, considerable research has been done in developing methods without requiring laboratory equipment. This applies to the isothermal methodologies such as nucleic acid sequence based amplification (NASBA), transcription mediated amplification (TMA), loop-mediated isothermal (LAMP) and recombinase polymerase amplification (RPA) technologies (Domingo et al., 2011).

NASBA and TMA both apply a continuous isothermal process (41°C or 60°C) based on the use of a mixture of reverse transcriptase, RNase H and T7 RNA polymerase. These assays yield approximately 1-billion fold RNA amplification in two hours' time. Products can be visualised using agarose gel electrophoresis with ethidium bromide or by molecular beacons which provides both specificity to the reaction and the opportunity for real-time detection (Compton, 1991). The NASBA assay has been used to detect different flaviviruses, especially for surveillance of WNV (Lanciotti and Kerst, 2001). Although numerous methods are successful in detecting flaviviruses, it was demonstrated that high viral loads, such as USUV, can produce false-positives with TMA in blood donors (Gaibani et al., 2010).

LAMP technology consists of a strand displacement reaction using a DNA polymerase with strand displacement activity at 63-65°C. Results are obtained in less than 30 minutes and can be visualised by spectrophotometric analysis, agarose gel electrophoresis, naked-eye visual turbidity and visual fluorescence by addition of an intercalating dye to the reaction such as SYBR-Green (Mori et al., 2001). Parida and colleagues (2005) designed an RT-LAMP assay targeting the 3′-UTR region for rapid detection of all four DENV serotypes. This RT-LAMP assay demonstrated a detection limit of 0.1–1 pfu, with no cross-reactivity to closely related flaviviruses and showed an increase in sensitivity levels compared to the standard RT-PCR and virus isolation. Performing a RT-LAMP is simple, rapid and accurate.

1.5 Recombinase polymerase amplification assay

RPA is a remarkably well established isothermal molecular technique and compares favourably with other isothermal methods such as LAMP. It exhibits high sensitivity and operates at a constant temperature, allowing for simple processing and rapid amplification of products (Lobato and O' Sullivan, 2018).

RPA technology was developed by Piepenburg and colleagues in 2006 (Lobato and O' Sullivan, 2018) using proteins involved in cellular DNA synthesis, recombination and repair. RPA employs three enzymes namely a recombinase, a single stranded DNA-binding molecule (SSB) and a strand-displacing polymerase. The process starts when a recombinase protein (uvsX) derived from T4-like bacteriophages bind to oligonucleotides in the presence of adenosine triphosphate (ATP) and a crowding agent, such as a polyethylene glycol, resulting in a recombinase-primer complex. The complex binds to double stranded DNA and promotes strand displacement by the primer at the cognate site. The SSB stabilises the displaced DNA strand, preventing ejection of the inserted primer by migration. Lastly, the recombinase disassembles and a DNA polymerase (fragment of *Bacillus subtilis* Pol 1, Bsu) binds to the 3' end of the primer to elongate the strand in the presence of deoxyribonucleotide triphosphates (dNTPs). Exponential amplification is achieved by cyclic repetition of the process (Figure 3). A reverse transcriptase enzyme and a fluorescent probe can be added to the basic RPA to allow detection of RNA template (Wand et al., 2018). RPA is currently commercialised by TwistDX, a company in the United Kingdom (www.twistdx.co.uk).

RPA products can be detected in various ways, depending on the TwistDx RPA kit used for amplification. End point detection is usually recommended as it requires less instrumentation than real-time detection, decreasing the overall cost of the test and also making it more suitable for low resource settings (Lobato and O' Sullivan, 2018).

The majority of published work on lateral flow assay RPA (LFS-RPA) state that the results are obtained extremely rapidly in a visual read-out format. The TwistAmp® nfo kit is compatible with lateral flow strip detection and is achieved by the addition of three different oligonucleotides namely, a labelled probe, forward primer and labelled reverse primer.

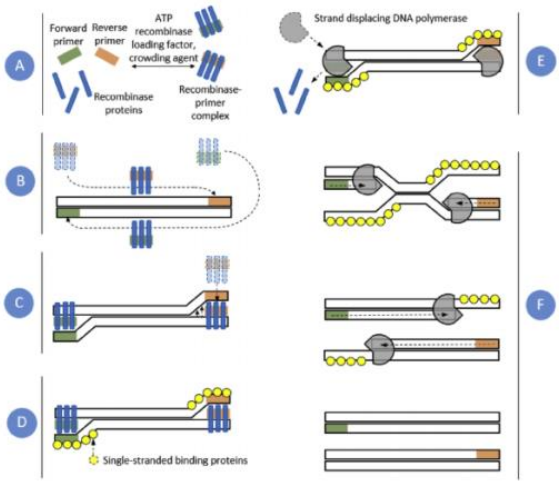

Figure 3. Recombinase polymerase amplification process. Recombinase proteins form complexes with each primer (A) and scans DNA for homologous sequences (B). Each primer is then inserted at the cognate site by the recombinase enzyme (C) and SSB stabilise the displaced DNA strand (D). Afterwards the recombinase disassembles, leaving the 3'-end of the primers accessible to a strand displacing DNA polymerase (E), elongating the primer (F) (Lobato and O' Sullivan, 2018, 4566901298447).

The primers are recommended to be between 30-35 nucleotides long, with the reverse primer labelled at the 5' end (e.g. dioxygen). The probe should be 46-50 nucleotides long and modified at the 5'-end with an antigenic label (e.g. biotin) and a polymerase extension blocking group at the 3'-end (e.g. phosphate). An abasic nucleotide such as tetrahydrofuran (also known as dSpacer) should replace a conventional nucleotide and is placed at least 30 nucleotides from the 5'-end and 15 nucleotides from the 3'-end. The tetrahydrofuran (THF) residue is cleaved by an nfo enzyme and forms a new 3'-hydroxyl group in the probe, transforming the probe into a primer. The amplicons produced in the presence of the probe and two opposing primers will include the two antigenic labels, making detection possible using a lateral flow assay. Refer to Figure 4 for the design of the oligonucleotide probe.

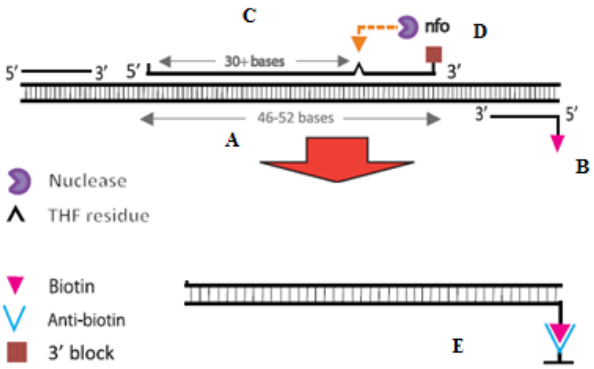

Figure 4. Design of RPA probe. The probe is between 46-50 nucleotides long (A) and modified at the 5'-end with an antigenic label such as biotin (B). A THF residue replaces a conventional nucleotide at least 30 nucleotides from the 5'-end (C) and is cleaved by an nfo enzyme (D), transforming the probe into a primer (E) (Modified from TwistDx manual).

Apart from lateral flow assay, other end point detection strategies can be used to visualise amplicons such as agarose gel electrophoresis. However, it is necessary to purify the products post-amplification to avoid smeared bands due to crowding agent and proteins in the mix. Modified primers or biotin labelled dNTPs can be used in RPA for detection using colorimetric techniques. After amplification, streptavidin-HRP, or 3,3', 5, 5'- Tetramethylbenzidine (TMB) and hydrogen peroxide (H_2O_2) are added to the reaction to produce a change in colour. The intensity of the colour can be correlated to the concentration of the amplicons produced. Bridge flocculation assay is another technique used to visualise RPA products. The assay principle is based on the reversible flocculation of carboxyl-functionalised magnetic beads, which is dependent on the length of DNA, salt concentration and pH of the sample. A magnetic bead solution is added to the RPA products, following a wash with ethanol and re-suspension in a low pH buffer. A positive result is obtained if the beads remain flocculated (Wee et al., 2015; Wee et al., 2015). Fluorescence detection can also be employed as an end-point detection approach. This is achieved by multiplexing using forward primers immobilised onto array spots and fluorophore modified reverse primers. After post-amplification, the amplified products can be spatially resolved and visualised by laser scanner measurements (Kersting et al., 2014). RPA can also be carried out in solution and the amplified products captured on a microtitre plate following denaturation of the duplex RPA amplicons (Santiago-Felipe et al., 2014). In an alternative approach, one of the primers is immobilised on a substrate and solid phase amplification is performed, followed by denaturation, hybridization with an enzyme labelled reporter probe and electrochemical detection (del Rio et al., 2014). An electrochemical biosensor was designed for the detection of plant pathogens using modified primers to produce double labelled amplicons with biotin at one end and an oligonucleotide overhang at the other. The amplicon was purified using biotin and streptavidin magnetic beads and a capture probe was used to bind gold-nanoparticles tagged with a complementary capture probe. After purification, the RPA amplicons were placed on screen printed carbon electrodes and the gold of the gold- nanoparticles were measured using differential pulse voltammetry (Lau et al., 2017).

The robustness of RPA in the presence of inhibitors facilitates amplification from impure samples which is not achievable by PCR. RPA can be performed directly from serum, as well as in the presence of PCR inhibitors, including ethanol and heparin. RPA has successfully been performed directly from urine (Krölov et al., 2014) pleural fluids (Liljander et al., 2015), milk (Choi et al., 2016), stool samples (Wu et al., 2016) and seed powders (Chandu et al., 2016). However, according to a study conducted by Rosser et al. (2015), 1.25% of urine had no impact

on RPA amplification efficacy, but 10% did inhibit amplification when small amounts of target DNA was present in the sample (100fg).

A RT-RPA assay was developed for the detection of YFV (Escadafal et al., 2014). LFS-RPA assay was performed using the TwistAmp™ nfo RT kit from TwistDx (Cambridge, UK) and compared to real-time RT-PCR. Both assays were able to detect 20 YFV strains. The analytical sensitivity was determined by testing RNA extracts from 10-fold dilutions of YFV preparations and it was observed that the real-time RT-PCR could detect 8 genome copies/reaction (GC/rxn), whereas the LFS RT-RPA detected as low as 44 GC/RXN. All 20 different YFV strains used in the study were detected by the LFS RT-RPA and demonstrated no cross-reactions with closely related viruses.

A RT-RPA assay has been described for the molecular detection of Crimean-Congo haemorrhagic fever virus (CCHFV). The aim of the work was to develop a RT-RPA assay as an alternative to an existing RT-PCR and provide a fast and fieldable diagnostic. A serial dilution of a synthetic RNA S-segment of the Europe 1 strain (AY277672) was performed to determine the detection limit of the assay. The detection limit was between 500 and 50 copies of RNA. The CCHFV RPA was tested using a selection of strains representing all seven molecular clades of the virus and detection of viral extracts/synthetic virus RNA of all seven S-segment clades were observed in less than ten minutes. The specificity of the assay was determined by testing RNA derived from viruses representing each genera, *Mammarenavirus*, *Marburgvirus*, *Henipavirus*, *Orthonairovirus* and *Orthohantavirus*. The RPA was unable to detect RNA from any of these viruses including the closely related *Orthonairoviruses* Hazara and Issyk-Kul. The inhibitory effect of the assay was tested by spiking a known quantity of synthetic CCHFV template into crude samples. The assay tolerated the presence of inhibitors in crude preparations of mock field samples, indicating that the assay may be suitable for use in the field. Tajikistan (Central Asia) has experienced seasonal CCHFV outbreaks (Tishkova et al., 2012) since the discovery of CCHFV in the region 40 years ago. The CCHFV RT-RPA was used to screen clinical samples and tick extracts obtained previously during outbreaks in 2013-2015. The CCHFV RPA detected 88% of 8 positive tick samples and 100% of 13 positive sera samples (Bonney et al., 2017).

A Zika virus (ZIKV) RT-RPA assay was developed for the rapid detection of ZIKV nucleic acid using the TwistAmp™ exo RPA kit (Wand et al., 2018). Synthetic RNA fragments from five different ZIKV strains were prepared and tested using the RPA. A ten-fold serial dilution of the synthetic fragment 5 (strain BeH815744, KU365780, from Brazil, 2015), representing

the latest outbreak strain, was prepared and each dilution was tested with the RT-RPA. The assay was able to detect 500 copies of template within 10 minutes and the limit of detection of the RPA was estimated to be between 500 and 5 copies of RNA for all five strains of ZIKV. It was demonstrated that the developed assay was robust and capable of tolerating sequence variability in the primer and probe regions, even if the variability occured in the primer and probe regions of the sequence. The specificity of the assay was determined by testing extracted nucleic acid from a panel of viruses that have clinical relevance, genetic relatedness to ZIKV and co-circulate. The ZIKV RT-RPA did not detect members from Orthobunyavirus, Phlebovirus and Alphavirus genera, which are all mosquito-borne arboviruses. Closely related flaviviruses such as WNV, YFV and DENV 1-4, also tested negative.

1.6 Problem identification, aims and objectives

It would be useful to have sensitive and specific nucleic acid amplification assays for low resource countries and field applications. RPA is a versatile alternative to PCR as fast, portable, nucleic acid detection assays. RPA is ideally suited to field, point-of-care and other settings as it requires no sophisticated laboratory equipment. Unlike PCR, the RPA reaction operates at a constant temperature and results are typically generated within 3-10 minutes.

The aim of this study was to develop and evaluate a reverse transcription recombinase polymerase assay (RT-RPA) for the detection of flaviviruses.

The objectives of this study were as follows:

1. Prepare RNA transcripts for WNV, USUV and WSLV to be used as positive controls
2. Develop and evaluate an isothermal RT-RPA for detection of flaviviral RNA.
3. Compare RT-RPA and conventional RT-PCR with regards to sensitivity and specificity using transcribed RNA.
4. Screen wild caught mosquitoes for flavivirus RNA using RT-RPA.

Chapter 2: THE PREPARATION OF RNA CONTROLS FOR DEVELOPMENT OF IN HOUSE RT-RPA

2.1 Introduction

Many emerging pathogens, including medically significant arboviruses, are classified as biosafety 3 or 4 pathogens which dictate that a biosafety level 3 and 4 facility is required for safe handling and culture. This requirement limits the number of laboratories that can culture the viruses. However performing surveillance is an important aspect of understanding the circulation and prevalence of viruses. Development of molecular assays circumvents the need for high containment facilities for isolation of viruses. However development of in-house assays still requires the use of positive controls for validation of the assays, and determining sensitivity and specificity of assays.

Transcribing RNA from non-infectious partial genes makes it possible for researchers in low resource settings to work with viruses that require high bio-containment laboratories. One advantage of working with transcribed RNA is that one does not require a virus isolate to be able to conduct studies and it is completely safe to work with. Sequence data can easily be retrieved from GenBank and a conserved region of the gene can be identified by multiple sequence alignments. Genes can be synthesised and used to prepare plasmids for various applications including transient expression of proteins or transcription of RNA.

There are several reports describing the application of transcribed RNA as suitable controls for molecular assays. For example, transcribed RNA has been used as a universal reaction-specific internal control in development of a respiratory pathogen detection assay. RNA controls were prepared for the detection of nine clinically important respiratory viruses. High yields of control RNA were transcribed *in vitro* and contaminating plasmid template DNA was not detectable at any of the concentrations tested in the nested RT-PCR. The transcribed RNA proved to be sufficiently stable during storage for routine use (Dingle et al., 2004). Similarly transcribed RNA was described as a control for a ZIKV detection assay. The sensitivity of a published ZIKV real-time RT-PCR and two marker assays were compared using transcribed RNA as controls. Five assay specific quantified *in vitro* transcripts were used as positive controls for the respective genomic target regions. To allow exact analyses of the lower limit of detection (LOD) for all of the assays, all target domains were joined in a quantitative universal control ribonucleic acid (uncRNA) containing all of the assays' target regions on one RNA strand. The uncRNA control ensured high sensitivity and good comparability of qualitative and quantitative results in clinical and diagnostic studies (Corman et al., 2016). Hence the use of transcribed RNA also

allows for flexibility and various modifications and permutations in the preparation of the controls.

The detection of low concentrations of DNA/RNA is of interest for medical diagnostics and surveillance and is a pronounced limitation of some molecular assays. The sensitivity of an assay is described as the smallest amount or concentration of substance in a sample that can accurately be measured by the assay. The lower the detectable concentration, the greater the LOD of the assay (Saah et al., 1997). Performance at a low concentration is often of great importance in molecular infectious disease assays as it defines the ability of the test to diagnose disease and determine treatment endpoints. Knowing the LOD of an assay can also determine the concentration to be used as a low positive control which can be observed to ensure consistency of performance between runs at levels near the cut-off values (Burd, 2010).

The specificity of an assay is the parameter that needs to be determined to validate molecular assays. It refers to the ability of an assay to only detect the target regions of interest and also that the quantification of the regions are not affected by cross-reactivity from related or potentially interfering nucleic acids. To rule out potential cross-reactivity, organisms need to be tested that have similar genetic structure as the pathogen and that cause similar disease symptoms (Burd, 2010).

To develop molecular assays for flaviviruses, a conserved region of the gene needs to be determined to design potential primer candidates. Literature indicates that the NS5 polymerase is the most conserved region throughout all the flaviviruses with 59% nucleotide similarity in mosquito-borne flaviviruses such as WNV, and 78% in tick-borne flavivirus (Danecek et al., 2010; Karothia et al., 2018). In a 2009 study, entropy analysis revealed numerous highly conserved WNV sequences distributed throughout the viral proteins, especially the NS5 protein (Koo et al., 2009).

In their native form, WNV and WSLV can only be handled in BSL-3 and this restricts laboratories that lack such resources. Hence there is a need for preparation of positive controls which are safe to handle in resource limited settings and in instances where the virus is not available but sequence data can be retrieved from a database.

The NS5 region was previously identified as a suitably conserved region for the design of in-house degenerate primers. In previous studies in our laboratory, nested primer pairs were identified targeting an approximately 400bp region of the NS5 gene (Samudzi, 2008; Mathengtheng, 2015). Hence for this study the same region was targeted.

To develop isothermal molecular assays, such as RPA for fieldwork, RNA controls were required to optimise the assays and to investigate specificity and sensitivity. Thus RNA was transcribed from DNA template to prepare positive controls.

2.2 Methods and materials

2.2.1 Synthetic NS5 genes

Synthetic partial genes covering a 370 bp region of WNV (NC_009942.1), 371bp region of USUV (NC_006551.1) and 365bp region of WSLV (JN226796.1) were synthesized and supplied in pUC57 (Appendix F) by GenScript. The plasmid DNA was reconstituted in 20µl of nuclease-free water (NFW) and the DNA concentration and purity of the DNA was measured using a Nanodrop™ 2000 spectrophotometer (ThermoScientific, Massachusetts,USA). The ratio of absorbance at 260nm and 280nm was used to assess the purity of DNA. A ratio of approximately 1.8 is usually accepted as suitable for DNA, however if the ratio is appreciably lower, it may indicate the presence of protein, phenol or other contaminants that absorb at 280nm. pUC57 plasmid containing WNV, WSLV and USUV were designated pUC57WNV, pUC57USUV, pUC57WSLV respectively, and used to transform competent *Escherichia coli* (*E. coli*) cells.

2.2.2 Preparation of plasmid DNA

2.2.2.1 Transformation of E.coli cells

For each transformation, DNA was used to transform JM109 competent cells with a transformation efficiency of $1x10^8$cfu/µg DNA (Promega, Wisconsin, USA). Briefly, 1µl of pUC57WNV, pUC57USUV and pUC57WSLV was added to 25µl of competent cells. The cells were incubated on ice for 20 minutes, followed by heat shock for 50 seconds at 42°C and incubated on ice for two minutes. The transformation mixture was incubated at 37°C for 90 minutes after the addition of 900µl super optimal broth with catabolite repression (SOC) at room temperature. Cells were plated out on Luria-Bertani broth (LB) agar plates containing ampicillin (amp) at a final concentration of 100µg/ml, supplemented with 0.5mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and 80µg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (refer to Appendix P for media composition). The plates were incubated o/n (o/n) at 37ºC.

Blue-white colony selection is a rapid and efficient technique for the identification of possible transformants. It relies on the activity of β-galactosidase which converts the colourless substrate

X-gal to produce blue colonies. The LacZ gene contains the multiple cloning and T/A cloning sites (Appendix F). The gene will be disrupted in positive transformants, thus β-galactosidase will no longer be produced and X-gal can no longer be metabolised to produce blue colonies, resulting in white colonies; however it is possible that the gene may not be disrupted and hence confirmation by PCR or restriction digestion is still required.

A single white colony was inoculated into a volume of 5ml LB containing amp (100µg/ml) and incubated at 37°C o/n. The o/n cultures were used to prepare glycerol stocks for pUC57WNV, pUC57USUV and pUC57WSLV by the addition of 150µl 100% glycerol to 850µl of LB media and cells.

2.2.2.2 Plasmid extraction and purification

Plasmid was extracted and purified from the overnight (o/n) culture using the PureYield™ plasmid miniprep system (Promega, Wisconsin, USA) according to manufacturer's instructions. Briefly, a volume of 3ml of culture was added to 100µl of cell lysis buffer and mixed by inverting the tube four times. An aliquot of 350µl neutralization solution was added and subsequently centrifuged at 14000xg for three minutes. A volume of 200µlendotoxin removal was added to the supernatant and centrifuged at 14000xg for 15 seconds. The columns were washed and DNA was eluted in 35µl NFW by centrifugation. The eluted DNA was stored at -20°C.

The plasmid DNA concentration and purity were measured using absorbance at 260nm and 260:280nm ratio respectively with a Nanodrop™ 2000 spectrophotometer.

2.2.2.3 Confirmation of positive transformants

To confirm that the *E.coli* cells were positively transformed, restriction digestions were performed for each plasmid. For pUC57WNV and pUC57USUV, the gene of interest was excised using BamHI and HindIII and for pUC57WSLV the gene was excised using HindIII and SacI (Promega, Wisconsin, USA). The restriction digestion reaction mixture (Table 1) was incubated at 37°C for two hours.

Table 1. Reaction components of restriction digestion using specified restriction enzymes

Reaction components	**Volume (µl)**
BamHI or SacI (10U/µl)	1
HindIII (10U/µl)	1
Plamid DNA*	1
10x restriction enzyme, buffer E	2
NFW	15
Total	**20**

* pUC57WNV (225.1ng/µl), pUC57USUV (240.1ng/µl), pUC57WSLV (347.4ng/µl)

The DNA fragments were separated by electrophoresis at 80V for 60 minutes using a 1% SeaKem® LE agarose gel (Lonza, Maine, USA) and were visualised by addition of 4µl Gel Red™ Nucleic acid gel stain, 2000X solution (Biotium, Hayward, USA) to 20µl digested sample (Refer to Appendix P for media composition). The O'GeneRuler™ DNA Ladder Mix #SM1173 (Fermentas, Illinois, USA) molecular weight marker with a range of DNA fragments from 100-10 000bp was used for estimating the size of the DNA digestion products (Appendix G). The digested products were visualised using a Molecular Imager® Gel Doc™ XR+ System (Bio-Rad, California, USA).

2.2.2.4 Optimisation of GoTaq® DNA polymerase PCR

Primers targeting the partial NS5 gene of WNV, USUV and WSLV were designed in a previous study based on sequence alignments (Appendix H) of the NS5 gene. The primers were designated FlaviF1 and FlaviR1 and target a ~414bp region of the NS5 gene. In another study, two additional reverse primers targeting the the inner region of the ~414bp amplicon were designed and designated FlaviR2 and FlaviR3. The additional reverse primers were necessary due to genetic variability between the flaviviruses.

The forward primer, FlaviF1, and the reverse primer, FlaviR2 (Table 2), were used together with GoTaq® DNA polymerase (Promega, Wisconsin, USA) to amplify the partial NS5 gene. There are some base differences between the primers and aligned viruses such as WSLV but in a previous study these primers had been shown to amplify RNA from the viruses included in this study and hence were used in the current study.The PCR reaction components are listed in Table 3.

Table 2. Consensus primers targeting the NS5 region

Primer name	Genomic positions on NS5*	Oligonucleotide sequence	%GC content	Tm (ºC)	Length of primer (bp)
FlaviF1	1030-1046	5'-ATGGCH˟ATGACW˟GACAC'-3	49	50.1	17
FlaviR2	1380-1360	5'-CY˟XTTY˟CCCATCATGTTXTA-3'	37.5	44	20

*WNV reference isolate (GenBank accession number: NC_009942.1), USUV reference isolate (GenBank accession number: NC_006551.1), WSLV isolate AV259 (Accession number: JN226796.1)
˟ Wobble bases represented as follows H: A or C or T; W: A or T; Y: C or T

Table 3. Reaction components for plasmid DNA PCR

PCR components	Volume (µl)	Final concentration
$MgCl_2$ solution, 25mM	4	2mM
5x Green GoTaq® flexi buffer	10	1x
PCR nucleotide mix, 10mM each	1	0.2mM each dNTP
Flavi F1 forward primer (20pmol/µl)	1	2µM
Flavi R2 reverse primer (20pmol/µl)	1	2µM
GoTaq®G2 Hot start polymerase (5U/µl)	0,25	1.25U
WNV plasmid DNA	1	-
NFW	31,75	-
Total	**50**	

The PCR was optimised by testing different annealing temperatures. The annealing temperatures that were tested were 42ºC, 43ºC, 44ºC, 46ºC, 48ºC and 50ºC. The following PCR cycling conditions were used with each annealing temperature: a single initial denaturation hold for two minutes at 95°C for one cycle, 95°C for 30 seconds for 30 cycles, specific annealing temperature for 30 seconds and an extension phase at 72°C for one minute using a ProFlex PCR system (Applied Biosystems, London, England). A final extension step was performed at 72°C for five minutes and the PCR products were left at 4°C indefinitely.

The PCR amplicons were separated by electrophoresis at 80V for 60 minutes using a 1% SeaKem® LE agarose gel. A volume of 1µl 2000x Gel Red™ Nucleic acid gel stain solution was added to 4µl PCR amplicons and visualised using a Molecular Imager® Gel Doc™ XR+ System.

2.2.2.5 Amplification of NS5 genes

After optimisation of the PCR, amplification of pUC57WNV, pUC57USUV and pUC57WSLV was performed using GoTaq® DNA polymerase as described in section 2.2.2.4 and using an annealing temperature of 44ºC (Appendix J).

The positive amplicons were purified from excised gel slices using the Wizard® SV Gel and PCR Clean-Up System (Promega, Wisconsin, USA). Gel purification was performed by the addition of 10µl/mg membrane binding solution to the excised gel slices and incubated at 65°C until the gel was completely dissolved. The dissolved gel mixture was transferred to a Wizard® SV Minicolumn inserted into a collection tube and centrifuged at 14000xg for one minute. DNA was bound to a silica membrane on the minicolumn and washes were performed with 700µl and 500µl of membrane wash solution, with centrifugation at 14000xg between each subsequent wash. DNA was eluted in 35µl NFW. The DNA concentration of the PCR amplicon was not determined and rather the volume of DNA to add was estimated from visualisation of an aliquot on an agarose gel.

2.2.3 Preparation of RNA transcripts

2.2.3.1 Cloning of amplicons into pGEM®-T easy bacterial vector and transformation of cells

To prepare DNA template for transcribing RNA, purified PCR amplicons were ligated into pGEM®-T easy vector (Promega, Madison, USA) by T/A cloning using T4 DNA ligase. The pGEM®-T easy vector is a linearized vector with a single 3'-terminal thymidine at both ends. The vector has a high copy number and contains T7 and SP6 RNA polymerase promoters flanking a multiple cloning region within the alpha peptide coding region of the enzyme β-galactosidase. Refer to Appendix K for illustration of the vector map and multiple cloning sites of the pGEM®-T easy vector. The ligation components listed in Table 4 were incubated o/n at 4°C.

Table 4. Ligation reaction components

Reaction components	**Insert (µl)**	**Positive (µl)**
2x Rapid ligation buffer, T4 DNA ligase	5	5
pGEM T easy vector (50ng)	1	1
PCR amplicon	3	-
Control DNA	-	2
T4 DNA ligase (3 Weiss units/µl)	1	1
NFW	-	1
Total	**10**	**10**

Ligation mixtures were used to transform JM109 competent cells and afterwards plasmid was extracted and purified as explained in section 2.2.2.2. Positive transformants were selected using blue/white colony selection and confirmed by digestion of plasmids using restriction

endonuclease NotI (Promega, Wisconsin, USA) which cleaves at sites flanking the inserted gene. Section 2.2.2.3 describes the components and reaction setups required. pGEM® plasmid DNA constructs were designated pGEMWNV, pGEMUSUV and pGEMWSLV.

2.2.3.2 Preparation of DNA template for RNA transcription

pGEM®-T easy vector contains a SP6 promoter site with a sequence of 5'ATTTAGGTGACACTATAG3', located downstream of the inserted partial gene (Appendix K). SP6 primer and FlaviF1 were used to amplify the partial NS5 gene of pGEMWNV, pGEMUSUV and pGEMWSLV to confirm the orientation of the ligated genes 0and to obtain DNA template for transcription. The genes were amplified using GoTaq® DNA polymerase as previously described in section 2.2.2.4. The positive PCR amplicons were excised and purified as explained in section 2.2.2.2. The DNA concentration of the PCR amplicon was not determined and rather the volume of DNA to add was estimated from visualisation of an aliquot on an agarose gel.

2.2.3.3 Sequencing

Determination of the nucleotide sequence of each amplicon was performed using the ABI PRISM Big Dye Terminator v3.1 sequencing ready reaction kit (Applied Biosystems, Foster City, USA) according to manufacturer's instructions. Sequencing reaction components are shown in Tables 5 and 6 respectively. For each amplicon bidirectional sequencing was performed.

Table 5. Sequencing reaction components

Components	**Volume (µl)**
Ready reaction	1
Sequencing primer (0.8pmol/µl)	4
Dilution buffer	2
Template DNA	3
Total	**10**

Table 6. Control sequencing reaction

Components	**Volume (µl)**
Ready reaction	1
Control sequencing primer (0.8pmol/µl)(M13)	4
Dilution buffer	2
Control sequencing plasmid (pGEM-3z(f)t)	1
NFW	2
Total	**10**

Reactions were cycled using the following cycling conditions: initial denaturation at 96ºC for one minute for one cycle, followed by 25 cycles of denaturation at 96ºC for 10 seconds, an annealing temperature of 44°C for five seconds and an extension stage at 60°C for four minutes. Lastly samples were held at 4°C indefinitely.

Post-reaction cleanup of sequencing amplicons was performed by using the Ethylene-Diamine-Tetra-Acetic acid (EDTA)/ethanol precipitation method. Briefly, 0.5M EDTA was diluted with NFW to 125mM at a pH level of 8.0. A volume of 5µl 125mM EDTA and 60µl absolute ethanol was added to a 1.5ml microcentrifuge tube. The sequencing reaction volume was adjusted to 20µl by the addition of 10µl NFW and afterwards added to the EDTA/ethanol solution. The reaction was vortexed for five seconds and incubated at room temperature for 15 minutes to allow for precipitation. After incubation the reaction was centrifuged at 14000xg for 20 minutes at 4ºC. The supernatant was completely aspirated and the pellet was re-suspended in 500µl of 70% ethanol and centrifuged at 14000xg for another 10 minutes. After centrifugation the supernatant was removed and the remaining pellet was incubated at 37ºC until dry. The samples were stored in the dark at 4ºC and submitted for electrophoresis to the Department of Microbial, Biochemical and Food Biotechnology of the University of the Free State. The 3130x/ Genetic Analyzer (Applied Biosystems, California, USA) was used for the sequencing.

Sequence data was edited using Chromas Pro version 1.6. The flaviviruses were determined by comparison with nucleotide sequence data originally retrieved from GenBank for synthesis of the genes and by Blast analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.2.3.4 Transcribing RNA from DNA template

Transcription relies on the complementary pairing of bases. The double stranded DNA separate and one strand acts as the template, onto which free nucleotides align to their complementary bases. The free nucleotide adenine (A) aligns with thymine (T) on the strand, guanine (G) with cytosine (C), C with G and urasil (U) with adenine. The reaction is catalysed by the enzyme RNA polymerase which adds ribonucleotides to the DNA strand (Poveda et al., 2010).

RNA was transcribed from DNA template using the MEGAscript™ SP6 Kit (Invitrogen, Massachusetts, USA) according to manufacturer's instructions. Briefly, equal volumes of the four nucleotides solutions (ATP, GTP, CTP and UTP) were mixed together and added to a standard 20µl reaction containing NFW, 10X reaction buffer, linear template DNA and enzyme mix. The tube was gently flicked and briefly centrifuged to collect the reaction mixture at the

bottom of the tube. The reaction mixture was incubated at 37 °C for 16 hours. Refer to Table 7 below for the reaction components.

The RNA transcript was purified using SV Total RNA Isolation System (Promega, Madison, USA) according to manufacturer's conditions. Briefly, 175µl of RNA lysis buffer and 20µl of reaction mixture were added to 350µl RNA dilution buffer and incubated at 70°C for three minutes. After centrifugation at 14 000xg for 10 minutes, 200µl of 95% ethanol was added to the lysate reaction mixture and subsequently transferred to the spin basket assembly followed by centrifugation for one minute. For each solution, a 50µl DNase incubation mix was prepared by chronologically adding 40µl yellow core buffer, 5µl 0.09M manganese (II) chloride ($MnCl_2$) and 5µl of DNase enzyme 1. This mixture was added directly to the column assembly and incubated at 20-25°C for 25 minutes. The enzyme activity was stopped by the addition of 200µl DNase stop solution. Afterwards, the columns were washed with 600µl and 250µl RNA wash solution, respectively. RNA was eluted in 100µl NFW and stored at -80°C.

Table 7. Reaction components for RNA transcription

Components	**Volume (µl)**
ATP (5mM)	2
GTP (5mM)	2
CTP (5mM)	2
UTP (5mM)	2
10 x Reaction buffer	2
SP6 enzyme mix (20U/µl)	2
DNA template*	1
NFW	7
Total	**20**

*WNV (66.5ng/µl), USUV (25.8ng/µl), WSLV (42.2ng/µl)

The RNA transcript was purified using SV Total RNA Isolation System (Promega, Madison, USA) according to manufacturer's conditions. Briefly, 175µl of RNA lysis buffer and 20µl of reaction mixture were added to 350µl RNA dilution buffer and incubated at 70°C for three minutes. After centrifugation at 14 000xg for 10 minutes, 200µl of 95% ethanol was added to the lysate reaction mixture and subsequently transferred to the spin basket assembly followed by centrifugation for one minute. For each solution, a 50µl DNase incubation mix was prepared by chronologically adding 40µl yellow core buffer, 5µl 0.09M manganese (II) chloride ($MnCl_2$) and 5µl of DNase enzyme 1. This mixture was added directly to the column assembly and incubated at 20-25°C for 25 minutes. The enzyme activity was stopped by the addition of 200µl DNase stop solution. Afterwards, the columns were washed with 600µl and 250µl RNA wash solution, respectively. RNA was eluted in 100µl NFW and stored at -80°C.

Transcribed RNA was treated with RQ1 RNase-free DNase (Promega, Madison, USA) to remove any remaining DNA from the RNA transcript. Briefly, 6µl of RNA was added to a mixture of 1µl RQ1 RNase-free DNase 10X reaction buffer, 1µl of RQ1 RNase-free DNase and 2µl of NFW. The 10µl reaction mixture was incubated at 37ºC for 30 minutes. After incubation, 1µl of RQ1 DNase stop solution was added and incubated at 65ºC for 10 minutes to terminate the reaction.

PCR was performed using the DNase treated transcribed RNA as template to detect whether DNA was still present after being treated with DNase 1 enzyme. PCR was performed with GoTaq® Hot Start DNA polymerase and FlaviF1 and FlaviR2 were used as the opposing primers (Section 2.2.2.4).

2.2.3.5 Optimisation of one-step reverse transcription PCR

Purified RNA was used to optimise a one-step RT-PCR using OneTaq One-step RT-PCR kit (New England Biolabs, Massachusetts, USA). FlaviF1 was used as the forward primer and FlaviR2 as the reverse primer. The parameters that were tested included the annealing temperature and primer concentration. According to the manufacturers', optimal final primer concentration is between 100nM-800nM, thus 100nM, 200nM, 400nM, 600nM and 800nM were tested. The annealing temperature was tested at 44ºC, 46ºC, 48ºC, 50ºC and 52ºC. Refer to Table 8 for RT-PCR reaction components.

Table 8. RT-PCR reaction components per tube

Components	**Volume (µl)**	**Final concentration**
OneTaq One-Step Reaction mix, 2X	12.5	1x
OneTaq One-Step Enzyme mix, 25X	1	1x
Flavi F1 (10µM/µl)	X	Y
Flavi R2 (10µM/µl)	X	Y
NFW	Z	-
WNV template RNA*	1	-
Total	**25**	-

X Amount of 10µM primer added (0.25µl, 0.5µl, 1µl, 1.5µl, 2µl)
Y Final primer concentration (range from 100nM to 800nM)
Z Amount of NFW added (10µl, 9.5µl, 8.5µl, 7.5µl, 6.5µl)
* Concentration approximately 24.6ng/µl

The reaction was cycled as follows: reverse transcription step at 48°C for ten minutes followed by 94°C for one minute. The tubes were cycled 40 times at 94°C for 15 seconds, annealing temperature for 30 seconds and an extension phase for 30 seconds at 68°C. A final extension

step was programmed for five minutes at 68°C and held at 4°C indefinitely. The PCR products were visualised on a 1% agarose gel as previously described in section 2.2.2.3.

It was observed that the optimal PCR conditions were at 44ºC for the annealing temperature and 400nM for final primer concentration. Refer to Appendix L for the optimisation results. After optimisation, RT-PCR was performed using transcribed RNA of WNV, USUV and WSLV.

2.2.3.6 Determination of the lower detection limits of one-step RT-PCR

The RNA copy number could not be calculated accurately because of the low concentration of RNA yield and the introduction of carrier RNA during the purification step of transcribed RNA which can influence the RNA concentration, especially if the yield of RNA was low. RNA concentrations were not standardised and hence could not be used to compare assays for different viruses and were only used to compare different assays detecting the same virus i.e. RT-RPA and RT-PCR. For the comparision ten-fold dilutions of the transcribed RNA controls were prepared and five standards with the lowest dilutions were used to give an indication of the LOD of the assay. An aliquot of 5µl of RNA template was added to the RT-PCR reaction described in section 2.2.3.5.

The amplified products were separated by electrophoresis at 80V for 60 minutes using a 1% SeaKem® LE agarose gel. A volume of 4µl 2000x Gel Red™ Nucleic acid gel stain solution was added to 20µl PCR amplicon and visualised using a Molecular Imager® Gel Doc™ XR+ System.

2.3. Results

2.3.1 Synthetic NS5 genes

Synthetic genes representing the conserved partial NS5 region of WNV, USUV and WSLV were synthesized and supplied in pUC57 by GenScript. Referring to Table 9, high concentrations of reconstituted DNA were measured for each plasmid and the purity values were acceptable. A ratio of approximately 1.8 is usually accepted as suitable for DNA, however if the ratio is appreciably lower, it may indicate the presence of protein, phenol or other contaminants that absorb at 280nm.

Table 9. Concentrations and purities of reconstituted DNA

Plasmid	Concentration (ng/µl)	Purity (260/280nm)
pUC57WNV	405.4	1.9
pUC57USUV	385.6	1.9
pUC57WSLV	403.3	1.89

2.3.2 Preparation of plasmid DNA

2.3.2.1 Transformation of cells and confirmation of positive transformants

Glycerol stocks of plasmid DNA were made by transforming JM109 competent cells and storing an aliquot of the o/n culture. Plasmid was extracted from the remaining volume of o/n culture and purified using the PureYield^{TM} plasmid miniprep system. The concentration and purity of purified plasmid DNA were measured using the Nanodrop 2000 (Table10).

Table 10. Concentrations and purities of plasmid DNA

Plasmid	Concentration (ng/µl)	Purity (260/280nm)
pUC57WNV	225.1	1.84
pUC57USUV	240.1	1.86
pUC57WSLV	347.4	1.85

Restriction endonuclease digestion was performed on plasmid DNA to confirm positive transformation of competent JM109 cells. pUC57 vector was linearized by restriction digestion using BamHI and HindIII enzymes for pUC57WNV and pUC57USUV, and SacI and HindIII for pUC57WSLV. The 20µl reaction volume was separated by electrophoresis and visualised under a UV transilluminator. Figure 5 A-C shows the expected size for the digested partial NS5 gene of pUC57WNV at 371bp, 370bp for pUC57USUV and 365bp for pUC57WSLV. The linearized plasmid was approximately 2340bp for WNV and USUV, and 2345bp for WSLV.

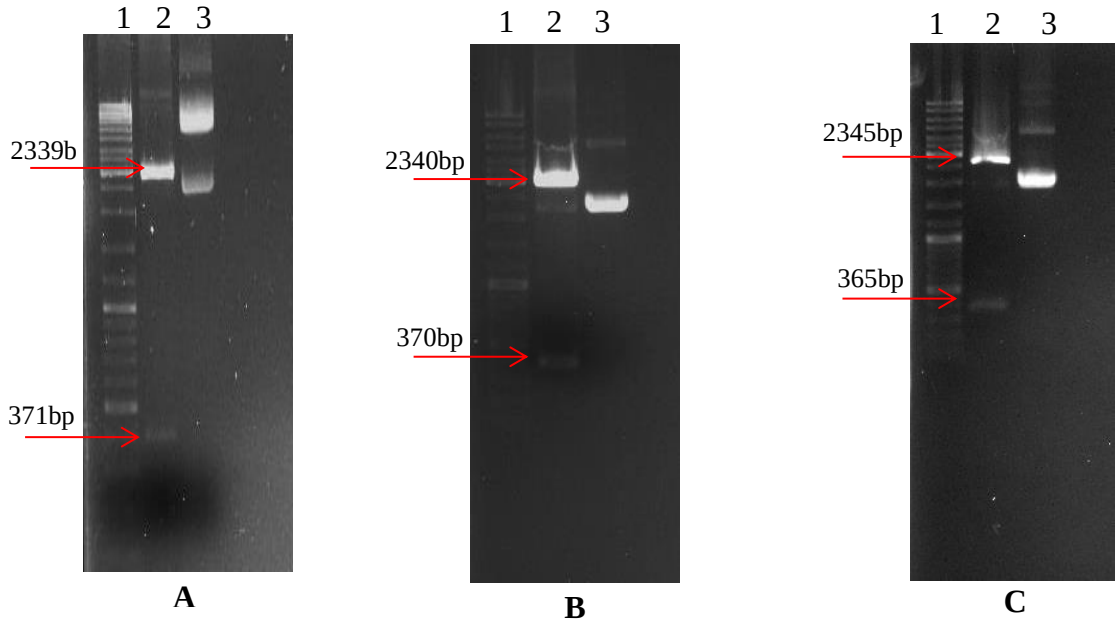

Figure 5. A 1% agarose gel electrophoresis analysis of restriction digestion. Figure 5A Lane 1: O'GeneRuler™ DNA ladder Plus, SM 1173; Lane 2: Digested pUC57WNV; Lane 3: Undigested pUC57WNV. Figure 5B Lane 1: O'GeneRuler™ DNA ladder Plus, SM 1173; Lane 2: Digested pUC57USUV; Lane 3: Uncut pUC57USUV. Figure 5C Lane 1: O'GeneRuler™ DNA ladder Plus, SM 1173; Lane 2: Digested pUC57WSLV; Lane 3: Undigested pUC57WSLV.

2.3.3 Preparation of RNA transcripts

2.3.3.1 Cloning of partial NS5 gene into pGEM®-T easy bacterial vector

To prepare DNA for insertion of partial genes into pGEM®-T easy vector, PCR was performed on plasmid DNA using FlaviF1 as forward primer and FlaviR2 as reverse primer. A 5µl aliquot of each PCR amplicon was separated by electrophoresis on a 1% agarose gel and visualised using a transilluminator. Figures 6A-C depicts the expected amplicon sizes: 371bp for pUC57WNV, 370bp for pUC57USUV and 365bp for pUC57WSLV. The bands present at 2339bp for pUC57WNV, 2340bp for pUC57USUV and 2345bp for pUC57WSLV is likely DNA plasmid template.

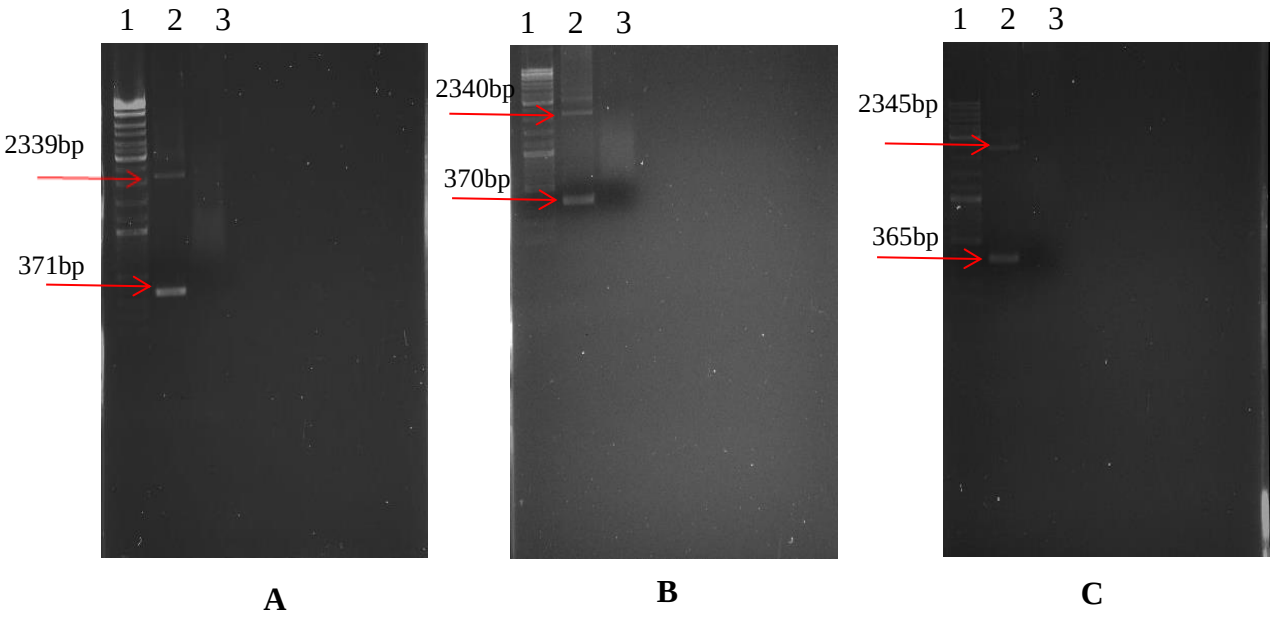

Figure 6. A 1% agarose gel electrophoresis showing results of plasmid DNA PCR. Figure 6A Lane 1: O'GeneRuler™ DNA ladder Plus, SM 1173; Lane 2: pUC57WNV; Lane 3: Negative Control. Figure 6B Lane 1: O'GeneRuler™ DNA ladder Plus, SM 1173; Lane 2: pUC57USUV; Lane 3: Negative control. Figure 6C Lane 1: O'GeneRuler™ DNA ladder Plus, SM 1173; Lane 2: pUC57WSLV; Lane 3: Negative control.

The remaining amplicons were separated and excised for purification using Wizard® SV Gel and PCR Clean-Up System according to the manufacturer's instructions. The purified PCR amplicons were ligated into pGEM®-T easy vector.

2.3.3.2 Confirmation of positive transformants

One white colony from each ligation reaction was selected and cultured o/n in LB/amp broth. Plasmid preparations were purified using PureYield™ Plasmid Miniprep system and DNA concentrations were measured using the Nanodrop 2000. Refer to Table 11 below for concentration and purity values of the pGEM® constructs. Confirmation of positive transformants was done by restriction endonuclease digestion using NotI endonucleases. The expected band size was 412bp for pGEMWNV (Figure 7A), 411bp for pGEMUSUV (Figure 7B) and 406bp for pGEM®WSLV (Figure 7C). The linearized plasmid was approximately 2603bp for pGEM®WNV and pGEMUSUV, and 2609bp for pGEMWSLV.

Table 11. DNA concentrations and purities of pGEM®-T easy plasmid DNA

Plasmid	Concentration (ng/µl)	Purity (260/280nm)
pGEMWNV	602.2	1.83
pGEMUSUV	444.2	1.88
pGEMWSLV	515.5	1.82

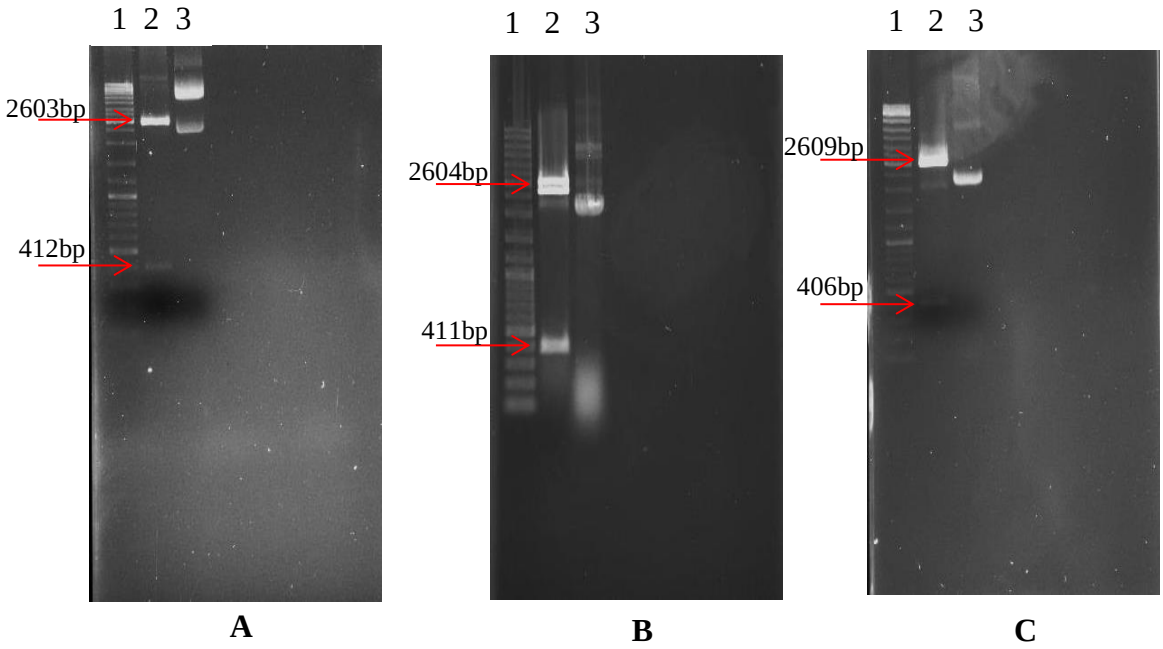

Figure 7. A 1% agarose gel electrophoresis analysis depicting the correct orientation of inserted genes in pGEM-T easy vector. Figure 7A Lane 1: O'GeneRuler™ DNA ladder Plus, SM 1173; Lane 2: pGEMWNV ; Lane 3: Negative control. Figure 7B Lane 1: O'GeneRuler™ DNA ladder Plus, SM 1173; Lane 2: pGEMUSUV; Lane 3: Negative control. Figure 7C Lane 1: O'GeneRuler™ DNA ladder Plus, SM 1173; Lane 2: pGEMWSLV; Lane 3: Negative control.

2.3.3.3 Transcribing RNA from DNA template

PCR was performed on each pGEM® construct using FlaviF1 as forward primer and Sp6 as reverse primer. A 5µl aliquot of each PCR amplicon was separated by electrophoresis on a 1% agarose gel and visualised using a transilluminator. The expected amplicon size for pGEMWNV was 470bp, 469bp for pGEMUSUV and 464bp for pGEMWSLV (Figure 8A-C). The bands present at approximately 2545bp for pGEMWNV, 2546bp for pGEMUSUV and 2551bp for pGEMWSLV was circular plasmid.

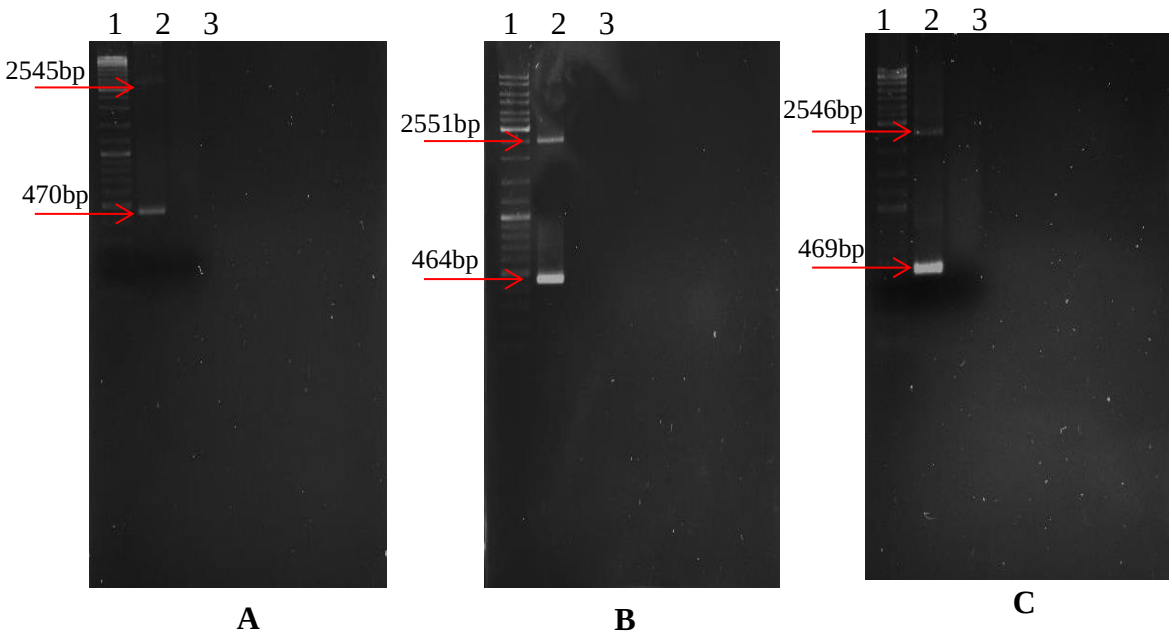

Figure 8. A 1% agarose gel electrophoresis analysis depicting the correct orientation of inserted genes in pGEM-T easy vector. Figure 8A Lane 1: O'GeneRuler™ DNA ladder Plus, SM 1173; Lane 2: pGEMWNV ; Lane 3: Negative control. Figure 8B Lane 1: O'GeneRuler™ DNA ladder Plus, SM 1173; Lane 2: pGEMWSLV; Lane 3: Negative control. Figure 8C Lane 1: O'GeneRuler™ DNA ladder Plus, SM 1173; Lane 2: pGEMUSUV; Lane 3: Negative control.

The remaining PCR products were excised from a 1% gel and purified using Wizard® SV Gel and PCR Clean-Up System according to the manufacturer's instructions.

The purified PCR amplicons were sequenced to confirm the presence of the genes of interest prior to transcription and then used as DNA template for the preparation of RNA transcripts. Raw sequence data from positive PCR amplicons were edited using Chromas Pro version 1.6 and the sequence of the synthetic genes were determined by BLAST analysis. WNV had a 100% identity to WNV isolate AVA1506113, USUV had a 100% identity to isolate V24 and WSLV had a 100% identity to strain AV259. Refer to Appendix L for the sequence data.

Transcribed RNA was isolated and purified using SV Total RNA Isolation System kit according to manufacturers' instructions and RNA was DNase treated prior to RT-PCR. A PCR was performed using RNA as template to confirm that there was no contaminating DNA present. The PCR reaction was performed without a positive control to avoid false positives and in retrospect it would have been more appropriate to include a positive. The PCR primers, enzymes and cycling conditions have been used previously and are optimal; however it is recognised that the actual reaction was not suitably controlled. As can be seen in Figure 9, transcribed RNA of WNV (Figure 9A), USUV (Figure 9B, lane 3) and WSLV (Figure 9B, lane 2) were free from any remaining DNA template.

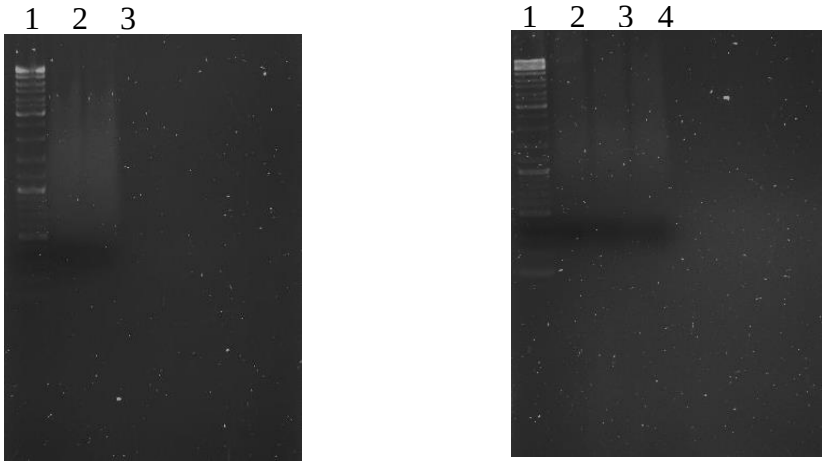

Figure 9. A 1% agarose gel electrophoresis analysis depicting the presence of DNA. Figure 9A Lane 1: O'GeneRuler™ DNA ladder Plus, SM 1173; Lane 2: WNV transcribed RNA; Lane 3: Negative control. Figure 9B Lane 1: O'GeneRuler™ DNA ladder Plus, SM 1173; Lane 2: WSLV transcribed RNA; Lane 3: USUV transcribed RNA; Lane 4: Negative control.

2.3.3.4 Amplification by RT-PCR

After optimisation of the RT-PCR (Appendix K), each RNA control was amplified using the OneTaq One-Step RT-PCR kit according to manufacturers' conditions. Flavivirus type specific forward and reverse primers, FlaviF1 and FlaviR2, were used. PCR products were separated and visualised using a 1% agarose gel electrophoresis. The expected size of transcribed RNA can be viewed in Figure 10 at 371bp for WNV, 370bp for USUV and 365bp for WSLV.

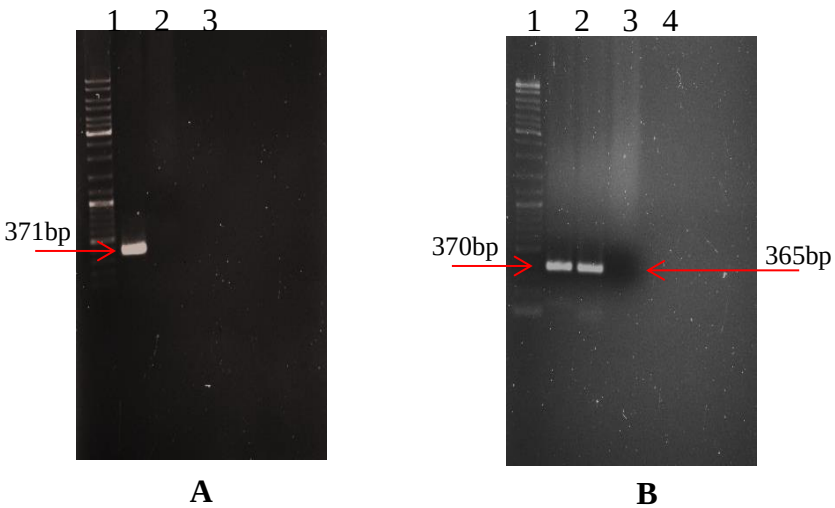

Figure 10. A 1% agarose gel electrophoresis analysis depicting transcribed RNA RT-PCR. Figure 10A Lane 1: O'GeneRuler™ DNA ladder Plus, SM 1173; Lane 2: WNV RNA ; Lane 3: Negative control. Figure 10B Lane 1: O'GeneRuler™ DNA ladder Plus, SM 1173; Lane 2: USUV RNA; Lane 3: WSLV RNA; Lane 4: Negative control.

2.3.3.5 Determination of the lowest detection limits of RT-PCR

The approximate RNA concentrations were measured; however it was decided to use ten fold dilutions for the LOD because the RNA concentrations were not considered accurate. Thus sensitivity of the RT-PCR (for comparison with RT-RPA for each virus in Chapter3) was determined by diluting the transcribed RNA ten-fold and performing RT-PCR on the five or 6 highest dilutions using a one-step RT-PCR. A volume of 5µl template was added to each RT-PCR reaction. The RT-PCR detected a minimum of 10^6 for WNV and USUV RNA and 10^5 for WSLV RNA (Table 12). Refer to Appendix M for the LOD gel electrophoresis analysis.

Table 12. Sensitivity determination of one-step RT-PCR

Flavivirus RNA	Volume (µl)	Dilution	RT-PCR
West Nile	5	10^0	Positive
	5	10^6	Positive
	5	10^7	Negative
	5	10^8	Negative
	5	10^9	Negative
	5	10^{10}	Negative
Usutu	5	10^0	Positive
	5	10^6	Positive
	5	10^7	Negative
	5	10^8	Negative
	5	10^9	Negative
	5	10^{10}	Negative
Wesselsbron	5	10^0	Positive
	5	10^5	Positive
	5	10^6	Negative
	5	10^7	Negative
	5	10^8	Negative
	5	10^9	Negative
	5	10^{10}	Negative

2.4 Discussion

Many countries have limited laboratory facilities and are not able to handle BSL-3 pathogens, such as WNV and WSLV, safely. Recently, there has been a proliferation of molecular assays that can be easily adapted for low resource and field settings (Bonney et al., 2017), hence transcribed RNA was prepared as controls for developing molecular assays.

Synthetic NS5 genes of WNV, USUV and WSLV were supplied in pUC57. *E.coli* competent cells were transformed and conventional PCR was performed for the production of plasmid

DNA amplicons. Primers used for amplification were designed in previous studies based on the conserved region of the partial NS5 gene (Mathengtheng, 2015; Samudzi, 2008).

Transcribed RNA was prepared for WNV, USUV and WSLV by the ligation of PCR amplicons into pGEM®-T easy bacterial cloning vector. DNA template for transcription was prepared by amplification of the target genes. The SP6 promoter site is situated downstream of the pGEM®-T easy vector and was used to confirm the orientation of the ligated genes. SP6 positive PCR amplicons were sequenced and the DNA template was used to transcribe RNA. It is of utmost importance to ensure that transcribed RNA is DNA free, especially for downstream applications such as RT-PCR (Sirakov, 2016). The RNA transcripts were free from any remaining DNA and positive controls were confirmed by RT-PCR.

The number of RNA copies could not be accurately calculated to represent only flaviviral RNA due to the introduction of carrier RNA in the transcribed RNA during the purification step using the SV Total RNA Isolation System. Certain purification steps, such as precipitation, are inefficient at low concentrations of RNA. To form a precipitate a number of molecules need to aggregate into a single particle. This is achieved by the addition of a carrier molecule, such as RNA, to increase the concentration and proximity required to form aggregates (Shaw et al., 2009). RNA concentrations were not standardised and thus could not be used to compare assays for different viruses and were only used to compare different assays detecting the same virus i.e. RT-RPA and RT-PCR. Thus, ten-fold dilutions of the transcribed RNA controls were prepared and five standards with the lowest dilutions were used to give an indication of the LOD of the assay. Therefore, the LOD of the RT-PCR was determined by diluting transcribed RNA ten-fold instead. However this was a limitation to the study as RNA copy number to determine LOD was not possible. It was found that the RT-PCR could detect WNV RNA diluted up to 10^6 fold, USUV RNA diluted up to 10^6 fold and WSLV RNA up to 10^5 fold.

In conclusion, the primers, FlaviF1 and FlaviR2, had the tolerability to detect different species of flaviviruses, including WNV, USUV and WSLV. A RT-PCR is not considered a quantitative assay; however determining the LOD by dilutions can give an indication as to how sensitive a RT-PCR is. The positively transcribed RNA transcripts were used to develop and validate a RT-RPA for the detection of flaviviruses.

Chapter 3: DEVELOPMENT AND EVALUATION OF RT-RPA FOR DETECTION OF FLAVIVIRAL RNA

3.1 Introduction

A conventional PCR requires sophisticated laboratory equipment and reagents. This makes it difficult to perform surveillance studies or screening of clinical samples in low resource settings with limited access to conventional laboratory facilities. There has been a recent demand for research into next-generation molecular diagnostics with improvements in performance relative to conventional PCR.

RPA is an isothermal nucleic acid amplification technology used for molecular detection for a diversity of pathogens (Daher et al., 2016). The RPA adopts a single incubation period with a rapid turnaround time. It employs a recombinase enzyme to separate the DNA duplex and single stranded DNA-binding molecules to stabilise the open complex, allowing amplification and detection with probe technology. TwistAmp® DNA amplification kits provide the reagents needed to amplify nucleic acid template from trace levels to detectable amounts of amplicon (Lobato and O' Sullivan, 2018).

RPA products can be visualised in various ways, however end point detection is usually recommended as it requires less instrumentation than real-time detection, decreasing the overall cost of the test and also making it more fit for low resource settings (Escadafel et al., 2014). End-point detection of RPA products rely mostly on lateral flow assays, as it is rapid and presented in a visual read-out format. This is achieved by using the TwistAmp® nfo kit together with three different oligonucleotides (two primers and a probe) that are modified according to TwistAmp® guidelines (Lobato and O' Sullivan, 2018). The TwistAmp® nfo kit includes the basic amplification reagents and a nuclease (nfo) that generates new polymerase extension substrates with suitable probes (TwistDx Manual).

According to RPA guidelines, primers designed for a given PCR assay may work in an RPA, however it may not be optimal for TwistAmp® reactions. The sequences of PCR primers may promote primer-primer interactions, secondary structures or hairpins (Lobato and O' Sullivan, 2018). TwistAmp® primers are ideally 30 to 35 nucleotides long, and in contrast to PCR, the melting temperature of an oligonucleotide is not the critical factor for its performance as a primer. RPA utilises proprietary probe systems designed for providing specific and sensitive detection of amplicons. The probes are designed to have homology to regions within an amplicon between the main amplification primers. It should typically be 46-52 nucleotides long, at least 30 of which are placed 5' to the THF site and at least a further 15 are located 3' to the

THF site. The THF residue replaces a nucleotide that would normally base pair to the complementary sequence. PCRD Nucleic Acid Detector (Abingdon Health, Birmingham, UK) is a single use *in vitro* immunochromatographic test for the qualitative visual detection of labelled RPA products (Li et al., 2019). The lateral flow test strip is generic and can detect any amplification product in which appropriately labelled primers are included (Jauset-Rubio et al., 2016).

The low RPA incubation temperature and rapid processing time makes assay systems based on RPA technology ideally suited for fieldable diagnostics. A RT-RPA was designed for the rapid and sensitive detection of ZIKV. The assay did not require any sophisticated equipment and results were available in less than 15 minutes (Chan et al., 2016). Two RT-RPA assays were developed for the detection of DENV 1-4. Two RNA molecular standards were used and the analytical sensitivity was found to range from 14 (denv 4) to 241 (DENV 1-3) RNA molecules detected (Abd El Wahed et al., 2015). To investigate the feasibility of developing a RT-RPA for flaviviruses, RT-RPA assays were investigated for the detection of two flaviviruses, WNV and WSLV, which are known to occur in SA, and for USUV, that was previously identified in the country and is likely to be present. Sequence data for 11 mosquito and tick borne flaviviruses were aligned in a previous study. The flaviviruses included WNV, WSLV, DENV-2, YFV, ZIKV, tick-borne encephalitis virus (TBEV), LGTV, Louping ill virus (LIV), Royal farm (RFV), Kadam virus (KADV) and Japanese encephalitis virus strain SA (JEVSAV). Based on this alignment a 414bp region of the partial NS5 gene was identified as one of the most conserved regions of the genome and a pair of degenerate consensus primers were identified that targeted this region (Mathengtheng, 2015). These primers are currently being used for flavivirus surveillance studies in our laboratory and hence the region was selected as a target for the RT-RPA.

3.2 Methods and materials

3.2.1 Alignment of sequence data for design of primers and probes

As the aim was to develop assays for detection of flaviviruses known or likely to occur in SA, alignments of sequence data retrieved from GenBank for each of the three viruses were initially performed to determine if it would be possible to identify one set of consensus primers and a probe, or if multiple sets would be required to accommodate sequence variability.

Representative sequences for each virus were aligned using Clustal Omega version 1.2.1 to determine the possibility of identifying consensus primers and probes for the three different

viruses. Multiple sequence alignments were performed using sequence data retrieved from GenBank for each lineage of WNV where available, which included lineages 1, 2, 3, 4 and 9. In addition sequence data for 7 SAn isolates from lineage 2 were included. Alignments were also constructed using sequence data retrieved from GenBank for four isolates of WSLV and four isolates of USUV. Table 13 depicts the data retrieved from GenBank for the alignment of sequences.

Table 13. Virus isolates/strains retrieved from GenBank

Virus	Strain/Isolate	Lineage	Origin	GenBank accession number
WNV	Reference isolate	1	USA	NC_009942.1
	H442	2	SA	EF429200.1
	SA381/00	2	SA	EF429199.1
	SA93/01	2	SA	EF429198.1
	SPU116/89	2	SA	EF429197.1
	N/A	2	SA	HM147822.1
	HS101_08	2	SA	JN393308.1
	349/77	2	SA	KM052152.1
	97/-103	3	Rabensburg	AY765264.1
	LEIV-Krnd88-190	4	Russia	AY277251.1
	Uu-LN-AT-2013	9	Austria	KJ831223.1
WSLV	AV259	N/A	SA	JN226796.1
	IP259570/SEN/2013	N/A	Senegal	KY056258.1
	SAH177	N/A	SA	EU707555.1
	IP248525/SEN/2013	N/A	Senegal	IP248525/SEN/2013
USUV	Vienna 2001	N/A	Austria	NC_006551.1
	SAAR-1776	N/A	SA	AY453412.1
	HautRhin7316/France/2015	N/A	France	KX601691.1
	V44	N/A	Germany	KJ438780.1

N/A: Not applicable, USA: United States of America

3.2.2 Design of RPA primers and probes

The primer pairs and probes were designed based on gene sequence alignments of the conserved partial NS5 gene of WNV, USUV and WSLV. FlaviF1, designed in a previous study, was used as the forward primer for the RPA reactions. According to PCRD Nucleic Acid Detector assay guidelines, the PCRD cassette requires 5' modifications of the primer pair. The PCRD Nucleic Acid Detector has three reaction lines including line 1 that is closest to the sample application port and detects dioxygen (DIG) or biotin (Biosg) labelled amplicons, line 2 which detects fluorescein amidite (FAM) or Biosg labelled amplicons and line C which is the control line. Suppliers do not provide information regarding the composition of the control line but they do describe it as a flow check control line to confirm that the samples have migrated. It is possible

that it is a substance that reacts with the carbon coated particles in the buffer. When the sample is applied to the application the neutravidin coated carbon particles are rehydrated and react with the tag/biotin labelled products in the sample. The mixture travels along the membrane by a capillary mechanism. As the sample flows through the test membrane the coated particles migrate and in the presence of FAM/Biogs and DIG/Biosg labelled products, are captured by the antibodies immobilised at the test lines to form a coloured complex (visible line). Refer to Figure 11 for the illustration of the PCRD Nucleic Acid Detector assay.

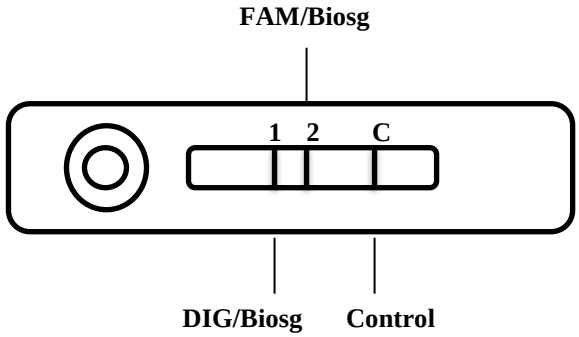

Figure 11. PCRD Nucleic Acid Detector assay.

A reverse primer with a dioxygen label at the 5' end was designed according to PCRD Nucleic Acid Detector guidelines as described in the previous paragraph. The probe was designed according to TwistAmp® nfo recommendations with a 5′ DIG label, an internal spacer (idSp) replacing a base and a 3′ carbon or phosphate blocker. The primer and probe sequences are listed in Table 14 as follows**:**

Table 14. Nucleotide sequences and properties used in the development of RPA assays

Primer name	**Virus**	**Genomic positions on partial NS5***	**Oligonucleotide sequence**	**%GC content**	**Tm (ºC)**	**Size of primer or probe (nt)**
FlaviF1	WNV,USUV, WSLV	1030-1046	5'-ATGGCHATGACWGACAC'-3	49	50.1	17
WNV RPA R	WNV, USUV	1379-1356	5'/DigN*/CTCTTTCCCATCATGTTGTAAATGCAAGT -3'	37.9	57.8	24
WNV RPA Probe	WNV, USUV	1276-1227	5'/5Biosg×/GCTTTGGGTGCCATGTTTGAAGAGCAGAATC/idSpº/ATGGAGGAGCGCCAG/3PhosA/ -3'	54.3	70.5	50

WESS RPA R	WSLV	1379-1350	5'/DigN*/CGTTTTCCCATCATGTTGTACACACATGTC-3'	59.8	43.3	30
WESS RPA Probe	WSLV	1263-1309	5'/5Biosg×/GAAGAGTGCAAGTGAGGCTGTTCAGGATCC/idSpº/CAGTTCTGGAAACTGG/3Phos$^{\Delta}$/-3'	52.2	68.8	47

* Dioxygen label, × Biotin label, º Internal spacer, $^{\Delta}$ Phosphate blocker
××WNV reference isolate (GenBank accession number: NC_009942.1), USUV reference isolate (GenBank accession number: NC_006551.1), WSLV isolate AV259 (Accession number: JN226796.1)

3.2.3 Optimisation of RT-RPA

According to TwistAmp® guidelines, the standard reaction conditions allow both fast and sensitive amplification for most RPA assays. However, the performance can be improved by optimisation of the reaction conditions. Parameters that can easily be changed include reaction temperature and primer concentrations. The TwistAmp® nfo kit operates optimally in the temperature range of 37ºC - 42ºC and the concentration of each primer can be varied between approximately 150nM and 600nM. The temperature was tested in the range of 39ºC-41ºC and primer concentration of 340nM, 380nM and 420nM.

RT-RPA was performed using WNV transcribed RNA produced in the previous chapter and the TwistAmp® nfo kit according to manufacturer's conditions (TwistAmp, Cambridge, UK). Transcribed RNA was used as template and FlaviF1 as the forward primer, together with the gene specific reverse primer (WNV RPA R) and probe (WNV RPA probe). The RT-RPA components are listed in Table 15:

Table 15. RT-RPA components per tube

RPA components	**Volume (µl)**	**Final concentration**
Flavi F1 (10µM)	X	Y
Reverse primer (10µM)	X	Y
RPA Probe (10µM)	0.6	0.12pmol/µl
Rehydration buffer	29.5	-
M-MLV reverse transcriptase (20U/µl)	1	0.4U/µl
RNase inhibitor(20U/µl)	1	0.4U/µl
RNase-free water	Z	-
Template	1	-
MgAcO (280mM)	2.5	14mM
Total:	**50**	

X Volume of 10µM primer added to reaction (2.1µl, 1.9µl, 1.7µl)
Y Final concentration of primer in reaction (420µM, 380µM, 340µM)
Z Volume of RNase-free water added (10.2µl, 10.6µl, 10.8µl)
MgAcO Magnesium acetate

The reaction mixture was added to the freeze-dried pellet, mixed thoroughly and incubated at a range of temperatures from 39ºC-41ºC for 20 minutes using ProFlex PCR system. PCRD Nucleic acid detection strips were used to visualise the products (Abingdon Health, York, UK). In summary, a 1:14 dilution of the reaction mix was performed by mixing 6µl reaction sample with 84µl of PCRD extraction buffer in a 1.5ml microcentrifuge tube. A volume of 75µl of diluted reaction mixture was added to the sample well of the PCRD test cassette and left in a horizontal position for 10 minutes. Results were read visually after 10 minutes. To confirm the results, amplicons were purified using the Wizard® SV Gel and PCR Clean-Up System and visualised using a 1% agarose gel as described in chapter 2, sections 2.2.2.2 and 2.2.2.3.

3.2.4 Amplification of partial NS5 genes by RT-RPA

After optimisation of the RT-RPA, it was observed that the ideal temperature was at 40ºC and the primers at a final concentration of 420nM. RT-RPA was performed using transcribed WNV, USUV and WSLV RNA template and the gene specific reverse primers and probes (Table 16) with FlaviF1 as the forward primer. Refer to section 3.2.3 for the reaction components and set up. To determine the reproducibility of the assay, RT-RPA was repeated in triplicate for each control, two reactions in the same day and one reaction the following day.

3.2.5 Sequencing

The nucleotide sequences of positive RPA amplicons were determined by sequencing reactions as previously described in section 2.2.3.3. Raw sequence data was edited using Chromas Pro version 1.6. The flaviviruses were determined by comparison with nucleotide sequence data retrieved from GenBank and by Blast analysis.

3.2.6 Sensitivity of RT-RPA

The sensitivity of each RT-RPA was evaluated by testing ten-fold dilutions of transcribed RNA controls produced in chapter 2. RT-RPA was performed as previously described using a 5µl aliquot of template per reaction and diluting the reaction mix 1:14 prior to detection of RPA products using the PCRD lateral flow assay. The results were compared to a conventional RT-PCR as described in section 2.3.3.5.

3.2.7 Specificity of RT-RPA

The specificity of each RT-RPA was tested using transcribed RNA for WNV, WSLV and USUV. In addition RNA from a flavivirus (YFV) was tested and two unrelated arboviruses,

SINV and CCHFV. Stored SINV (SAAR86) and YFV (vaccine strain 17D) RNA isolated from infected cell cultures and frozen at -80°C was tested. CCHF RNA was donated by Professor J Paweska from the NICD, Johannesburg, SA. Each of the RNA species included for specificity was verified in related studies using virus specific primers. In addition, alignments of sequence data constructed as described in section 3.2.1 were compared with other flaviviruses including ZIKV, DENV-2 and JEV to determine theoretical potential cross reactivities. Refer to Table 16 for the GenBank data.

Table 16. GenBank data for other flaviviruses

Virus	Strain/Isolate	Country	GenBank accession number
Zika	PF13/251013-18	French Polynesia	KX369547.2
Dengue-2	TSV01	Australia	AY037116.1
Yellow fever	Uganda 2010	Uganda	JN620362.1
Japanese encephalitis	Vellore P20778	India	AF080251.1
West Nile	Reference	USA	NC_009942.1
Usutu	Vienna 2001	Austria	NC_006551.1
Wesselsbron	AV259	SA	JN226796.1

3.2.8 Screening of wild-caught mosquitoes

For a related study, pools of wild-caught mosquitoes were collected from three sites in the Bloemfontein region during 2016 and 2017. The sampling sites were identified based on locality, presence of a permanent water body/retainer and an active bird population. The sites included the Bloemfontein Zoo, Free State National Botanical Gardens (FNBG) and Krugersdrift Dam (Terblanche, 2019, unpublished MMedSc).

The Bloemfontein Zoo is situated 29º19’44”S; 26º05’56”E and presented as an urban environment because it is surrounded by human settlement and the Loch Logan Spring. The site also had several different artificial water containers that could act as breeding sites for mosquitoes. The NFBG is situated 29º05’26” S; 26º21’28”E in Bloemfontein. This sampling site was selected as a peri-urban environment. This means that the environment forms the boundary between an urban and rural area. The area has large expanses of grassland and has a fast-growing human population. This area has an active bird population. The Krugersdrift Dam is situated 28º88’36”S; 25º95’83”E and was selected as a rural environment. It is situated 50km from Bloemfontein and is surrounded by farmland. There is very little human disturbances as the dam is located near a tar road, with very few human activities. The dam acts as an active water body and has a bird population.

Mosquitoes were collected using Shannon traps and CDC light traps baited with dry ice. The mosquito collections were performed at intervals between December 2016 and April 2017,

especially during the periods of February 2017 to April 2017 as those are the peak periods for arbovirus circulation in SA. After morphological identification of mosquito species the bodies of mosquitoes were pooled according to species, collection site and date. The pools ranged from 1-10 mosquitoes per pool depending on the amount of mosquitoes collected.

RNA was extracted from mosquito tissue using Qiazol® lysis reagent (Ambion Inc., Texas, USA) and the Qiazol® quick-start protocol according to manufacturers' instructions.

In this study, RNA from selected pools was tested using the RT-RPA. A total of 17 pools containing *Culex* species mosquitoes were tested (Table 17). The RT-RPA reaction was performed as described previously but without a positive control to avoid false positives.

Table 17. Wild-caught mosquitoes from three locations in and around Bloemfontein, Free State Province, SA

Pool number	Number of mosquitoes	Location of capture	Mosquito species
4	6	Bloemfontein Zoo	*Culex theileri*
5	1	NFBG	*Culex univittatus*
6	7	NFBG	*Culex theileri*
7	1	NFBG	*Culex pipiens*
8	1	Bloemfontein Zoo	*Culex theileri*
10	1	NFBG	*Culex theileri*
12	6	Bloemfontein Zoo	*Culex theileri*
13	1	Bloemfontein Zoo	*Culex tigripes*
14	1	Bloemfontein Zoo	*Culex terzii*
22	4	Bloemfontein Zoo	*Culex theileri*
25	3	Bloemfontein Zoo	*Culex univittatus*
30	4	NFBG	*Culex theileri*
43	2	Krugersdrift Dam	*Culex theileri*
44	3	Krugerdrift Dam	*Culex theileri*
47	2	Bloemfontein Zoo	*Culex theileri*
54	1	NFBG	*Culex pipiens*
55	6	NFBG	*Culex theileri*
Total number of mosquitoes	**50**		

NFBG : National Free State Botanical Gardens

3.2.9 Effect of possible inhibitors in mosquito extracts

Isothermal methodologies such as RPA are usually tolerant to crude samples and allow detection in arthropod preparations with minimal processing (Bonney et al, 2017). However, the potential inhibitory effect of interfering agents within a crude mosquito sample was tested by spiking two mosquito pools that were negative for WNV, USUV and WSLV RNA as

confirmed by real-time qRT-PCR in a previous study. Each pool was spiked with 1µl of 10^0 transcribed RNA and RT-RPA was performed on each pool

3.3 Results

3.3.1 Alignment of flavivirus sequence data and design of primers and probes

Sequence data of the conserved partial NS5 region was retrieved from GenBank for WNV (Reference strain: NC_009942.1), USUV (Reference strain, GenBank accession number: NC_006551.1) and WSLV (GenBank accession number: JN226796.1).

Sequence data retrieved from GenBank was used to identify possible primer and probe candidates by sequence alignments of the partial NS5 region. According to Figure 12, a probe was designed for WNV in which there were four mismatches between the probe and USUV and three mismatches between the reverse primer and USUV. Within the same region there were 16 mismatches between the probe and WSLV and six mismatches between the reverse primer and WSLV, hence an additional primer was designed specifically for WSLV and an additional probe based on WSLV sequence.

```
WSLV      actcttctggccatgactgacacgactccgtttgggcagcaaagggtgtttaaagagaaa      1072
WNV       accaccatggccatgactgacactactcccttcgggcagcagcgagtgttcaaagagaag      1080
USUV      accaccatggcgatgactgacaccactccgtttgggcagcagagggttttcaaagaaaag      1080
          **     **** *********** ***** ** ********  * ** ** ***** **

WSLV      gtggacacaaaagccccgcctccacctccaggaacacgtgcaatcatgcgcgtcgttaat      1132
WNV       gtggacacgaaagctcctgaaccgccagaaggagtgaagtacgtgctcaacgagaccacc      1140
USUV      gttgacaccaaggccccggaaccccccttctggagttagagaggtgatggatgagaccacc      1140
          ** ***** ** ** **    ** **    ***          *  *    *     *

WSLV      gcctggttgtttcaacacttggctcgcaagaagaagccccgcatttgcacacgcgaagag      1192
WNV       aactggttgtgggcgtttttggccagagaaaaacgtcccagaatgtgctctcgagaggaa      1200
USUV      aattggctgtgggcttttctcgcacgagaaaagaagccaaggttgtgcaccagggaagag      1200
             *** ***         * **  *  * **    **  *  * *** *  * ** **

WSLV      ttcgtggctaaggttcgaagccatgccgccctcggagcgtatctggaagagcaggacaaa      1252
WNV       ttcataagaaaggtcaacagcaatgcagctttgggtgccatgtttgaagagcagaatcaa      1260
USUV      tttaagaggaaggtcaacagcaacgctgctttgggagccatgtttgaagagcagaaccaa      1260
          **       *****    *** * ** **  * ** **     * ********* *  **

WSLV      tggaagagtgcaagtgaggctgttcaggatccacagttctggaaactggttgatgatgag      1312
WNV       tggaggagcgccagagaagcagttgaagatccaaaattttgggagatggtggatgaggag      1320
USUV      tggagcagtgccagggaggctgtagaggaccctcggttctgggaaatggtggacgaagaa      1320
          ****  ** ** ** ** ** **  * ** **    ** *** *  **** ** ** **

WSLV      aggaaactgcatctgcaaggccaatgccgcacatctgtgtcacaacatgatgggaaaacgt      1372
WNV       cgcgaggcacatctgcgggggaatgtcacacttgcatttacaacatgatgggaaagaga       1380
USUV      agggagaaccatctgaaaggagagtgccacacatgcatttacaacatgatggggaagcgt      1380
           *  *    ******   **  * ** *  ** **  * ************** **  *
```

Forward primer

WNV RPA probe

WSLV RPA probe

Reverse WSLV primer

Reverse WNV primer

Figure 12. Sequence alignments of WNV, USUV and WSLV for the design of primers and probes for RT-RPA.

The reference strain of WNV isolated in the USA was used as the NS5 gene in this study. In retrospect this was unfortunately an incorrect isolate to select for screening SA populations and hence alignments of sequence data from seven SAn WNV isolates were retrieved from GenBank and aligned with the reference strain (Figure 13). There were multiple mismatches between the probe and the nucleotide sequence for SA isolates, with six mismatches in the region 5' upstream of the THF site on the probe. There were two mismatches in the SA isolates compared to the reverse primer. To investigate the potential for the probe to detect a SA lineage 2 isolate, RNA from SA isolate SA93/01 was tested using the RT-RPA (Figure 14). The line is not visible in the photograh but was faintly present and observed in the laboratory.

```
NC_009942.1       gttaccaccatggccatgactgacactactccttcgggcagcagcgagtgttcaaagag
         1077

HM147822.1        gttaccacgatggctatgactgacaccactcccttcggtcaacaacgagtgttcaaggaa
         1080

HS101_08          gtgaccacgatggccatgacagacactactcctttcggtcaacaacgagtattcaaggaa
         1077

SPU116/89         gtgaccacgatggccatgacagacactactcctttcggtcaacaacgagtgttcaaggaa
         1077

SA93/01           gtgaccacgatggccatgacagacactactcctttcggtcaacaacgagtgttcaaggaa
         1077

SA381/00          gtgaccacgatggccatgacagacactactcctttcggtcaacaacgagtgttcaaggaa
         1077

H442              gtgaccacaatggccatgacagacaccactcctttcggtcaacaacgagtgttcaaggaa
         1077

349/77            gtgaccacaatggccatgacagacaccactcctttcggtcaacaacgagtgttcaaggaa
         1080

** ***** ***** ***** ***** ***** ***** ** ** ***** ***** **


NC_009942.1       aaggtggacacgaaagctcctgaaccgccagaaggagtgaagtacgtgctcaacgagacc
         1137
```

```
HM147822.1      aaggtggatacgaaggctccagagccttcagaaggggtcaaatatgtccttaatgagacc
        1140

HS101_08        aaggtggacacaaaggctccagagcctccggaaggggtcaaatacgtcctcaatgagacc
        1137

SPU116/89       aaggtggacacaaaggctccagagcctccagaaggggtcaaacacgtcctcaatgagaca
        1137

SA93/01         aaggtggacacaaaggctccagagcccccagaaggggtcaaatacgtcctcaatgaaacc
        1137

SA381/00        aaggtggacacaaaggctccagagcctccagaaggagtcaaatacgtcctcaatgaaacc
        1137

H442            aaggtggacacaaaggctccagagcctccagaaggagtcaaatacgtcctcaatgagacc
        1137

349/77          aaggtggacacaaaggctccagagcctccagaaggagtcaaatacgtcctcaatgagacc
        1140

******** ** ** ***** ** **  * ***** ** **  * ** ** ** ** **


NC_009942.1     accaactggttgtgggcgtttttggccagagaaaaacgtcccagaatgtgctctcgagag
        1197

HM147822.1      acaaattggctgtgggccttcttggcccgtgagaagaagcccaggatgtgctcccgggag
        1200

HS101_08        acgaactggctgtgggccttttagcccgcgacaagaaaccaaggatgtgctcccgggag
        1197

SPU116/89       acgaactggctgtgggccttttagcccgcgataagaaacccaggatgtgttcccgggag
        1197

SA93/01         acgaactggctgtgggccttcttagcccgcgataagaaacccaggatgtgttcccgggag
        1197

SA381/00        acgaactggctgtgggccttcttagcccgcgataagaaacccaggatgtgttcccgggag
        1197

H442            acgaactggctgtgggccttttagcccgcgataagaaacccaggatgtgttctcgggag
        1197

349/77          acgaactggctgtgggccttttagcccgcgacaagaaacccaggatgtgttccagggag
        1200

** ** *** ******* ** ** *** * ** **    ** ** ***** **  * ***


NC_009942.1     gaattcataagaaaggtcaacagcaatgcagctttgggtgccatgtttgaagagcagaat
        1257

HM147822.1      gagttcattaataaagtcaacagcaacgctgctctcggagcaatgtttgaagaacagaac
        1260

HS101_08        gaattcattggaaaagtcaacagtaatgccgctctaggagcgatgtttgaagaacagaac
        1257

SPU116/89       gaattcattggaaaagtcaacagtaatgccgccctaggagcgatgtttgaagaacagaac
        1257

SA93/01         gaattcattggaaaagtcaacagtaatgccgccctaggagcgatgtttgaagaacagaac
        1257
```

```
SA381/00          gaattcattggaaaagtcaacagtaatgccgccctaggagcgatgtttgaagaacagaac
        1257

H442              gaattcattggaaaagtcaacagtaatgccgccctaggagcgatgtttgaagaacagaac
        1257

349/77            gaattcattggaaaagtcaacagtaatgccgccctaggagcgatgtttgaagaacagaac
        1260

** *****    ** ******** ** ** **  * ** ** *********** *****


NC_009942.1       caatggaggagcgccagagaagcagttgaagatccaaaattttgggagatggtggatgag
        1317

HM147822.1        caatggaagaacgccagagaagctgtagaggatccaaagttctgggaaatggttgatgag
        1320

HS101_08          caatggaagaacgcccgggaagctgtagaggatccaaagttttgggagatggtggatgaa
        1317

SPU116/89         caatggaagaacgcccgggaagctgtagaggatccaaagttttgggagatggtggatgaa
        1317

SA93/01           caatggaagaacgcccgggaagctgtagaggatccaaagttttgggagatggtggatgaa
        1317

SA381/00          caatggaagaacgcccgggaagctgtagaggatccaaagttttgggagatggtggatgaa
        1317

H442              cagtggaagaacgcccgggaagctgtagaggatccaaagttttgggagatggtggatgag
        1317

349/77            caatggaagaacgcccgggaagctgtagaggatccaaagttttgggagatggtggatgag
        1320

 ** **** ** **** * ***** ** ** ******** ** ***** ***** *****


NC_009942.1       gagcgcgaggcacatctgcgggggggaatgtcacacttgcatttacaacatgatgggaaag
        1377

HM147822.1        gaacgtgaggcacatctccgtggagaatgcaacacctgcatttacaacatgatgggcaag
        1380

HS101_08          gagcgtgaggcgcatctccgtggagaatgcaacacctgcatctacaacatgatgggaaag
        1377

SPU116/89         gagcgtgaggcgcatctccgtggagaatgcaacacctgcatctacaacatgatgggaaag
        1377

SA93/01           gagcgtgaggcgcatctccgtggagaatgcaacacctgcatctacaacatgatgggaaag
        1377

SA381/00          gagcgtgaggcgcatctccgtggagaatgcaacacctgcatctacaacatgatgggaaag
        1377

H442              gagcgtgaagcgcatctccgtggagaatgcaacacctgcatctacaacatgatgggaaag
        1377

349/77            gagcgtgaagcgcatctccgtggagaatgcaacacctgcatctacaacatgatgggaaag
        1380

** ** ** ** ***** ** ** *****  **** ***** ************** ***
```

```
NC_009942.1       agagagaaaaaacccggagagttcggaaaggccaagggaagcagagccatttggttcatg
         1437

HM147822.1        agagagaagaaacctggagagtttggcaaagccaaaggcagcagagccatttggtttatg
         1440

HS101_08          agagagaagaagcctggagagttcggcaaagctaaaggcagcagagccatctggttcatg
         1437

SPU116/89         agagagaagaagcctggagagttcggcaaagctaaaggcagcagagccatttggttcatg
         1437

SA93/01           agagagaaaaagcctggagagttcggcaaagctaaaggcagcagagccatctggttcatg
         1437

SA381/00          agagagaaaaagcctggagagttcggcaaagctaaaggcagcagagccatctggttcatg
         1437

H442              agagagaagaagcctggagagttcggcaaggctaaaggcagcagagccatctggttcatg
         1437

349/77            agagagaagaagcctggagagttcggtaaggctaaaggcagcagagccatctggttcatg
         1440

******** ** ** ******** ** ** ** ** ** *********** ***** ***
```

Forward primer

RPA WNV Probe

RPA WNV reverse

Figure 13. Sequence alignments of SA WNV isolates.

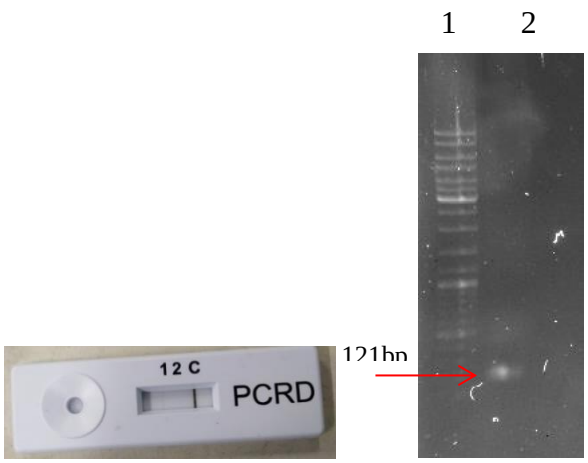

Figure 14. Gel electrophoresis analysis of isolate SA93/01 RT-RPA. Cassette: Positive WNV RPA product; Lane 1: O'GeneRuler™ DNA ladder Plus, SM 1173; Lane 2: WNV SA93/01 RNA ; Lane 3: Negative control.

To determine the similarity between the WNV lineages 1 and 2 which circulate more commonly globally and the more recently identified lineages, sequence data from GenBank for WNV lineages 1, 2, 3, 4 and 9 were aligned. Sequence data for the partial NS5 regions of lineages 5, 6, 7 and 8 were not available. According to the sequence alignment shown in Figure 15, there were 11 mismatches between the WNV probe and lineage 9, 12 mismatches between lineage 3 and the WNV probe and 10 mismatches between lineage 4 and the probe. Two to four mismatches were identified in the reverse primer and the less common lineages.

```
L4      accaccatggccatgactgataccacccctttcgggcagcagcgtgtgttcaaagaaaaa      1080
L9      accacaatggccatgaccgacaccacccccttgggcagcagcgcgtgttcaaggaaaag      1080
L3      accaccatggctatgacggataccacacccttggacagcagcgcgtgtttaaggagaag      1072
L1      accaccatggccatgactgacactactcccttcgggcagcagcgagtgttcaaagagaag      1080
L2      accacgatggccatgacagacactactcctttcggtcaacaacgagtgttcaaggaaaag      1080
        ***** ***** ***** ** ** ** ** ** ** ** ** ** ***** ** ** **

L4      gtcgacaccaaggcacccgaaccagctgaaggtgtgaaactggttttgaacgaaaccaca      1140
L9      gttgacacgaaggcccctgaaccaacggaaggcgttaagatagtattaaatgaaaccacc      1140
L3      gttgacaccaaggctccagaaccacctgaggggggtaaagtgcgtgcttaatgagaccacc      1132
L1      gtggacacgaaagctcctgaaccgccagaaggagtgaagtacgtgctcaacgagaccacc      1140
L2      gtggacacaaaggctccagagcccccagaaggggtcaaatacgtcctcaatgaaaccacg      1140
        ** ***** ** ** ** ** **  * ** ** ** **    **  * ** ** *****

L4      aactggctgtggaccttcttggcaagaaacaagcgaccacgcatgtgcaccagagaggaa      1200
L9      aactggctgtgggcttttctggcgcgagagaagcgaccacgcatgtgcacgagagaagag      1200
L3      aactggttgtggtcgcacctcgagcgtgaaaagcgtcccaggatgtgctcccgagaggag      1192
L1      aactggttgtgggcgtttttggccagagaaaaacgtcccagaatgtgctctcgagaggaa      1200
L2      aactggctgtgggccttcttagcccgcgataagaaacccaggatgtgttcccgggaggaa      1200
        ****** ***** *     * *   *  * **    **  * *****  *  * ** **

L4      ttcattagcaaggttaacagcaacgcagctctcggagccatgttcgaagaacaaaaccaa      1260
L9      ttcataagcaaggtcaacagcaacgcggcattgggagctatgtttgaggaacagaaccag      1260
L3      tttattcgcaaagtgaacagcaatgcagcgctaggagctatgtttgaggaacagaaccag      1252
L1      ttcataagaaaggtcaacagcaatgcagctttgggtgccatgtttgaagagcagaatcaa      1260
L2      ttcattggaaaagtcaacagtaatgccgccctaggagcgatgtttgaagaacagaaccaa      1260
```

```
        ** **  * ** ** ***** ** ** **  * ** ** ***** ** ** ** ** **

L4      tggtcgaacgctcgcgaggcggtggaagaccccaagttctgggaaatggttgacgatgag        1320
L9      tggtcaaacgccagagacgctgtagaagatcctaagttctgggagatggtggatgaagag        1320
L3      tggaaaagcgctagagaagctgtcgaggactccacgttttgggagatggttgatgaagaa        1312
L1      tggaggagcgccagagaagcagttgaagatccaaaattttgggagatggtggatgaggag        1320
L2      tggaagaacgcccgggaagctgtagaggatccaaagttttgggagatggtggatgaagag        1320
        ***   * ***  * ** ** ** ** **  * *  ** ***** ***** ** ** **


L4      cgtgaggcacatctccgtggcgaatgctacacctgcatttacaacatgatgggcaagaga        1380
L9      cgcgaggcccatctccgaggtgaatgccacacgtgcatatataacatgatgggcaagaga        1380
L3      cgcgaagttcacctgcgtggagaatgcaacacctgcatctacaacatgatggggaaaaga        1372
L1      cgcgaggcacatctgcgggggggaatgtcacacttgcatttacaacatgatgggaaagaga        1380
L2      cgtgaggcgcatctccgtggagaatgcaacacctgcatctacaacatgatgggaaagaga        1380
        ** ** *  ** ** ** ** *****  **** ***** ** ********** ** ***
```

Forward primer

WNV RPA Probe

WNV reverse primer

Figure 15. Sequence alignments for identification of primer pair and probe to detect WNV lineages 1, 2, 3, 4 and 9.

NS5 sequence data for four USUV isolates from SA and Europe were aligned. There was reasonable similarity among all the isolates with three mismatches in the probe upstream of the THF site and two mismatches in the reverse primer. The forward primer was well conserved for all the flaviviral species with one or two mismatches.

```
SAAR-1776          accactatggcgatgactgacaccactccgtttgggcagcagagggttttcaaagaaaag
        1080

NC_006551.1        accaccatggcgatgactgacaccactccgtttgggcagcagagggttttcaaagaaaag
        1080

HautRhin7316       accaccatggcgatgactgacaccactccgtttgggcagcaaagggttttcaaagaaaag
        900
```

```
V44             accaccatggcgatgactgacaccactccgtttgggcagcaaagggttttcaaagaaaag
        1080

***** ********************************** ******************


SAAR-1776       gttgacaccaaggctccagaaccccccttctggagttagagaagtgatggatgagaccacc
        1140

NC_006551.1     gttgacaccaaggccccggaaccccccttctggagttagagaggtgatggatgagaccacc
        1140

HautRhin7316    gttgacaccaaggccccggaaccccccttctggagttagagaggtgatggatgagaccacc
        960

V44             gttgacaccaaggccccggaaccccccttctggagttagagaggtgatggatgagaccacc
        1140

************** ** ********************** ******************


SAAR-1776       aattggctgtgggcttttctcgcacgagaaaagaagccaaggttgtgcaccagggaagag
        1200

NC_006551.1     aattggctgtgggcttttctcgcacgagaaaagaagccaaggttgtgcaccagggaagag
        1200

HautRhin7316    aattggctgtgggcttttctcgcacgagaaaagaagccaaggctgtgcaccagggaagag
        1020

V44             aattggctgtgggcttttctcgcacgagaaaagaagccaaggctgtgcaccagggaagag
        1200

****************************************** *****************


SAAR-1776       tttaagaggaaggtcaacagcaacgctgctttgggagccatgtttgaggagcagaaccaa
        1260

NC_006551.1     tttaagaggaaggtcaacagcaacgctgctttgggagccatgtttgaagagcagaaccaa
        1260

HautRhin7316    tttaagaggaaggtcaacagcaacgctgctttgggagccatgtttgaagagcagaaccaa
        1080

V44             tttaagaggaaggtcaacagcaacgctgctttgggagccatgtttgaagagcagaaccaa
        1260

********************************************** ************


SAAR-1776       tggagcagtgctagggaggctgtagaggaccctcggttctgggaaatggtggacgaagaa
        1320

NC_006551.1     tggagcagtgccagggaggctgtagaggaccctcggttctgggaaatggtggacgaagaa
        1320

HautRhin7316    tggagcagtgccagggaggctgtagaggaccctcggttctgggaaatggtggacgaagaa
        1140

V44             tggagcagtgccagggaggctgtagaggaccctcggttctgggaaatggtggacgaataa
        1320
```

```
********** ********************************************** **


SAAR-1776          agggagaaccatctgaaaggagagtgccacacatgcatttacaacatgatggggaagcgt
         1380

NC_006551.1        agggagaaccatctgaaaggagagtgccacacatgcatttacaacatgatggggaagcgt
         1380

HautRhin7316       agggagaaccatctgaaaggagagtgccacacatgcatttacaacatgatggggaagcgt
         1200

V44                agggagaaccatctgaaaggagagtgccacacatgcatttacaacatgatggggaagcgt
         1380

************************************************************


SAAR-1776          gagaagaagctcggagagtttggcaaagcgaaaggcagtcgagccatatggttcatgtgg
         1440

NC_006551.1        gagaagaagctcggagagtttggtaaagcgaaaggtagtcgagccatatggttcatgtgg
         1440

HautRhin7316       gagaagaagctcggagagtttggcaaagcgaaaggtagtcgagccatatggttcatgtgg
         1260

V44                gagaagaagctcggagagtttggcaaagcgaaaggtagtcgagccatatggttcatgtgg
         1440

********************** *********** ************************
```

Forward primer

WNV RPA Probe

WNV reverse primer

Figure 16. Sequence alignments for the identification of primers and probe that will detect all isolates of USUV.

Sequence data for four isolates of WSLV were aligned. There was significant similarity among the isolates in the targeted regions with one mismatch upstream of the THF site and one mismatch in the reverse primer.

```
SAH177             caatggcattgtcaaaatgctgtcgatgccatgggacaaattcgaatctgtgactcttct
         1020

JN226796.1         taatggcattgtcaaaatgttgtctatgccatgggacaaattcgagtctgtgactcttct
         1020

SEN/2013           taatggcattgtcaaaatgttgtctatgccatgggacaaattcgagtctgtgactctttt
         964

IP248525           taatggcattgtcaaaatgttgtctatgccatgggacaaattcgagtctgtgactctttt
         974
```

```
****************** **** ******************** ************ *


SAH177          ggccatgactgacacaaccccgtttgggcagcaaagggtgtttaaagagaaagtggacac
         1080

JN226796.1      ggccatgactgacacgactccgtttgggcagcaaagggtgtttaaagagaaagtggacac
         1080

SEN/2013        ggccatgactgacacgactccgtttgggcagcaaagggtgttcaaggagaaagtggacac
         1024

IP248525        ggccatgactgacacgactccgtttgggcagcaaagggtgttcaaggagaaagtggacac
         1034

*************** ** ********************** ** **************


SAH177          aaaagccccgcctccacctccaggaacacgtgcgatcatgcgtgttgtcaatgcttggtt
         1140

JN226796.1      aaaagccccgcctccacctccaggaacacgtgcaatcatgcgcgtcgttaatgcctggtt
         1140

SEN/2013        aaaagccccgcctccacctccaggaacacgcgcaatcatgcgcgtcgtcaatgcctggtt
         1084

IP248525        aaaagccccgcctccacctccaggaacacgcgcaatcatgcgcgtcgtcaatgcctggtt
         1094

****************************** ** ******** ** ** ***** *****


SAH177          gttccaacacctcgctcgtaagaagaagccccgcatttgcacgcgtgaagagtttgtggc
         1200

JN226796.1      gtttcaacacttggctcgcaagaagaagccccgcatttgcacacgcgaagagttcgtggc
         1200

SEN/2013        gttccaacacttggctcgcaagaagaagccccgcatttgcacacgcgaagagttcgtggc
         1144

IP248525        gttccaacacttggctcgcaagaagaagccccgcatttgcacacgcgaagagttcgtggc
         1154

*** ****** * ***** ********************** ** ******** *****


SAH177          caaagttcgtagccatgccgcccttggagcgtatctcgaagagcaggacaaatggaagag
         1260

JN226796.1      taaggttcgaagccatgccgccctcggagcgtatctggaagagcaggacaaatggaagag
         1260

SEN/2013        taaggttcgaagccatgccgccctcggagcgtatctggaagagcaggataaatggaagag
         1204

IP248525        taaggttcgaagccatgccgccctcggagcgtatctggaagagcaggataaatggaagag
         1214

** ***** ************** *********** *********** ***********
```

```
SAH177          tgcaagtgaggctgtccaggatccacagttctggaaactggttgatgatgagaggaagct
        1320

JN226796.1      tgcaagtgaggctgttcaggatccacagttctggaaactggttgatgatgagaggaaact
        1320

SEN/2013        tgcaagtgaggctgttcaggatccacagttctggaaactggttgatgatgagaggaaact
        1264

IP248525        tgcaagtgaggctgttcaggatccacagttctggaaactggttgatgatgagaggaaact
        1274

*************** ****************************************** **


SAH177          gcatctgcaaggccaatgccggacatgtgtgtacaacatgatggggaaacgtgaaaagaa
        1380

JN226796.1      gcatctgcaaggccaatgccggacatgtgtgtacaacatgatgggaaaacgtgaaaagaa
        1380

SEN/2013        gcatctgcaaggccaatgccggacatgtgtgtacaacatgatggggaaacgtgaaaagaa
        1324

IP248525        gcatctgcaaggccaatgccggacatgtgtgtacaacatgatggggaaacgtgaaaagaa
        1334

****************************************** **************
```

Forward primer

WESS Probe

WESS Reverse primer

Figure 17. Sequence alignments for the identification of primers and probe that will detect all isolates of WSLV.

3.3.2 Optimisation of RT-RPA using transcribed WNV RNA

Transcribed RNA prepared as described in chapter 2 was used as template for the optimisation of RT-RPA. Parameters that were tested included the reaction temperature and primer concentrations. The temperature was tested in the range of 39ºC-41ºC and primer concentrations of 340nM, 380nM and 420nM. The RT-RPA was able to detect transcribed WNV RNA at 39ºC, 40ºC and 41ºC, however the brightest band was visualised at 40ºC (Figure 18). As can be seen in Figure 19, the RNA was amplified using a primer concentration of 420nM, 380nM and 340nM. However amplification decreased at 340nM, therefore the recommended primer concentration of 420nM was used for the RPA reactions.

RT-RPA products were visualised on PCRD cassettes and the remaining amplicon was purified and visualised on an agarose gel to confirm results. For each reaction two bands were identified as approximately 121bp and approximately 380bp. In the absence of contaminating plasmid DNA which was confirmed in section 2.3.3.3, the larger band of DNA was likely the result of an amplification product from the forward and reverse primers. The principle of the reaction is based on cleavage of the probe which subsequently acts as a primer. If the probe is not completely cleaved due to high concentrations of probe and insufficient enzyme for cleavage then a longer amplification product will be produced. Hence on an agarose gel two products were visible, the smaller product the amplification product from the forward primer and "cleaved probe" and the larger product from the forward and reverse primer.

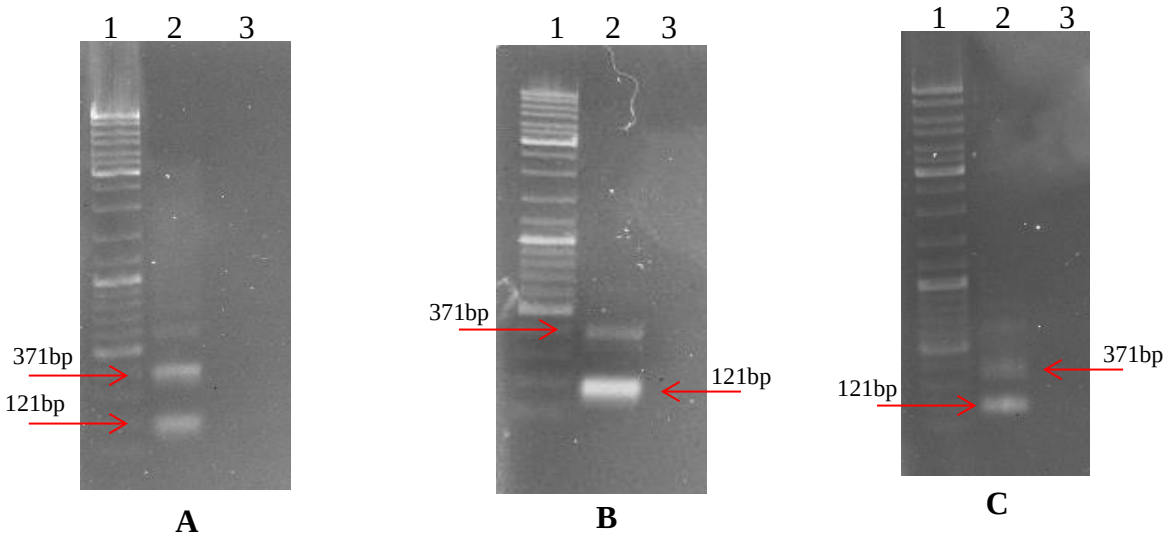

Figure 18. Temperature optimisation of RT-RPA. Figure 18A Lane 1: O'GeneRuler™ DNA ladder Plus, SM 1173; Lane 2: 39ºC; Lane 3: Negative control. Figure 18B Lane 1: O'GeneRuler™ DNA ladder Plus, SM 1173; Lane 2: 40ºC; ; Lane 3: Negative control. Figure 18C Lane 1: O'GeneRuler™ DNA ladder Plus, SM 1173; Lane 2: 41ºC; Lane 3: Negative control.

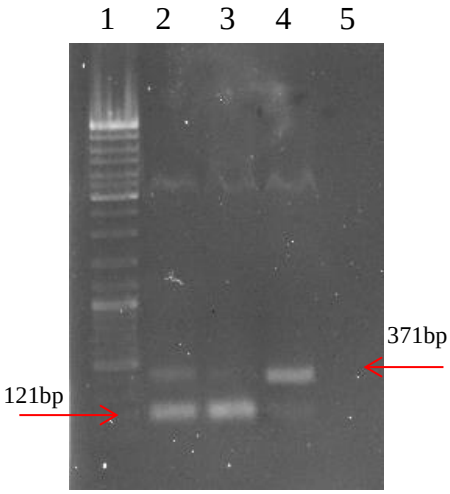

Figure 19. Primer concentration optimisation of RT-RPA. Lane 1: O'GeneRuler™ DNA ladder Plus, SM 1173; Lane 2: 420nM, Lane 3: 380nM, Lane 4: 340nM; Lane 5: Negative control.

3.3.3 Detection of transcribed RNA using RT-RPA

Transcribed RNA was used as template to amplify the partial NS5 gene of WNV, USUV and WNV. The RPA products were visualised by addition of 75µl diluted RPA amplicon to the sample pad of the PCRD Nucleic Acid Detector cassettes. To confirm the results, an aliquot of 5µl purified RT-RPA product was visualised after separation by electrophoresis. The reproducibility of the assay was determined by performing reactions in triplicate. Two assays were performed on the same day and the third reaction on the following day.

Figures 20A and B refer to the two reactions that were performed on the same day and Figure 20C was performed the following day. All three of the PCRD lateral flow assays were positive as a coloured complex formed at line 1. The results were confirmed by purifying the RPA amplicons using the Wizard® SV Gel and PCR Clean-Up System. As can be viewed from the gel electrophoresis pictures, a RT-RPA product of 121bp was present and a larger product visible at approximately 380bp, likely the result of uncleaved probe.

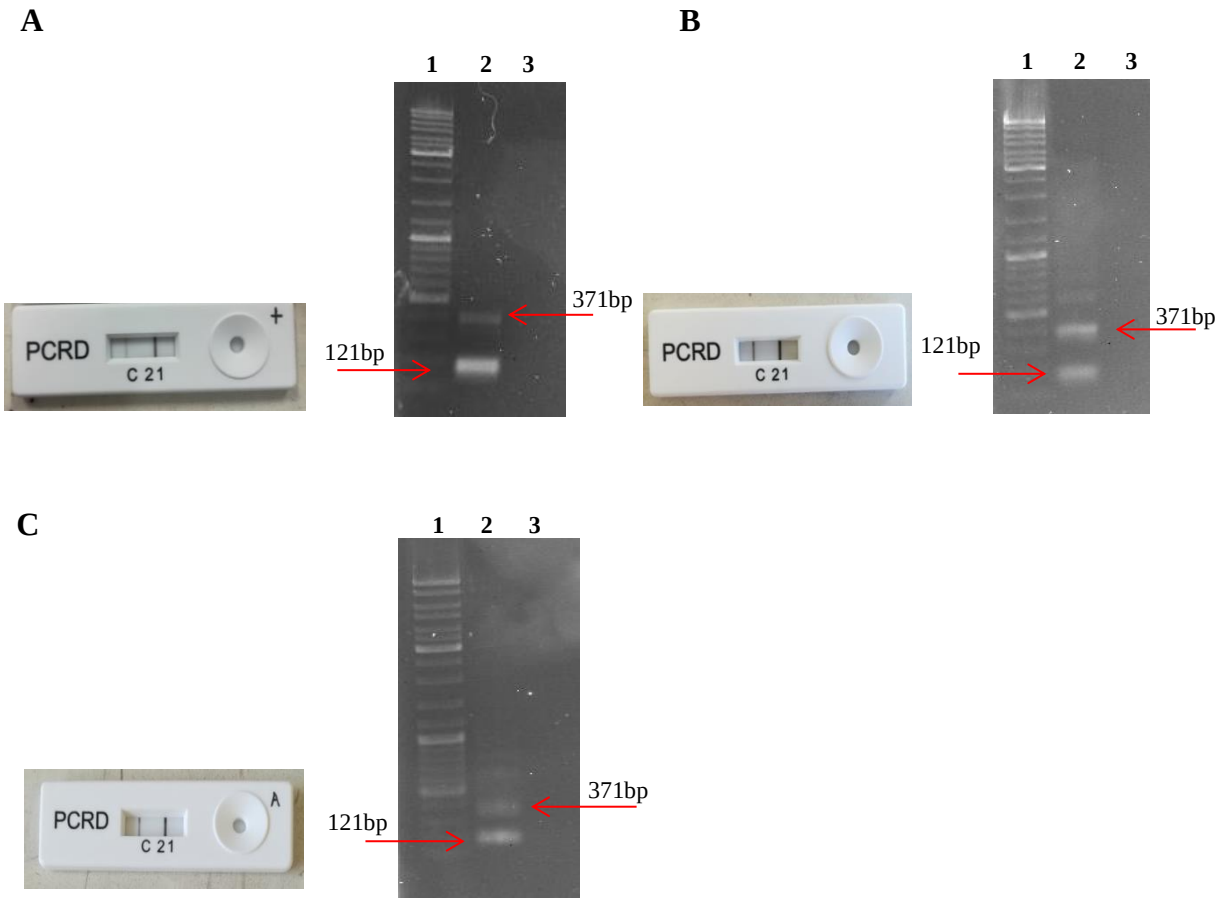

Figure 20. Detection of WNV RNA using RT-RPA. Figure 20A: Cassette: WNV RNA; Lane 1: O'GeneRuler™ DNA ladder Plus, SM 1173; Lane 2: WNV DNA; Lane 3: Negative control. Figure 20B: Cassette: WNV DNA Lane 1: O'GeneRuler™ DNA ladder Plus, SM 1173; Lane 2: WNV DNA; Lane 3: Negative control. Figure 20C: Cassette: WNV DNA; Lane 1: O'GeneRuler™ DNA ladder Plus, SM 1173; Lane 2: WNV RNA; Lane 3: Negative control.

Figure 21 depicts the results obtained for USUV and WSLV RT-RPA. Referring to Figure 21A lane 2, a visible band of 115bp and 365bp was obtained for WSLV and a product of 120bp was visualised for USUV (Figure 21A, lane 3). Two more reactions for USUV and WSLV were performed the following day and the exact same results were visualised on a 1% gel (Figures 21B-D). All the PCRD cassettes showed positive results as indicated by the coloured complex at line 1.

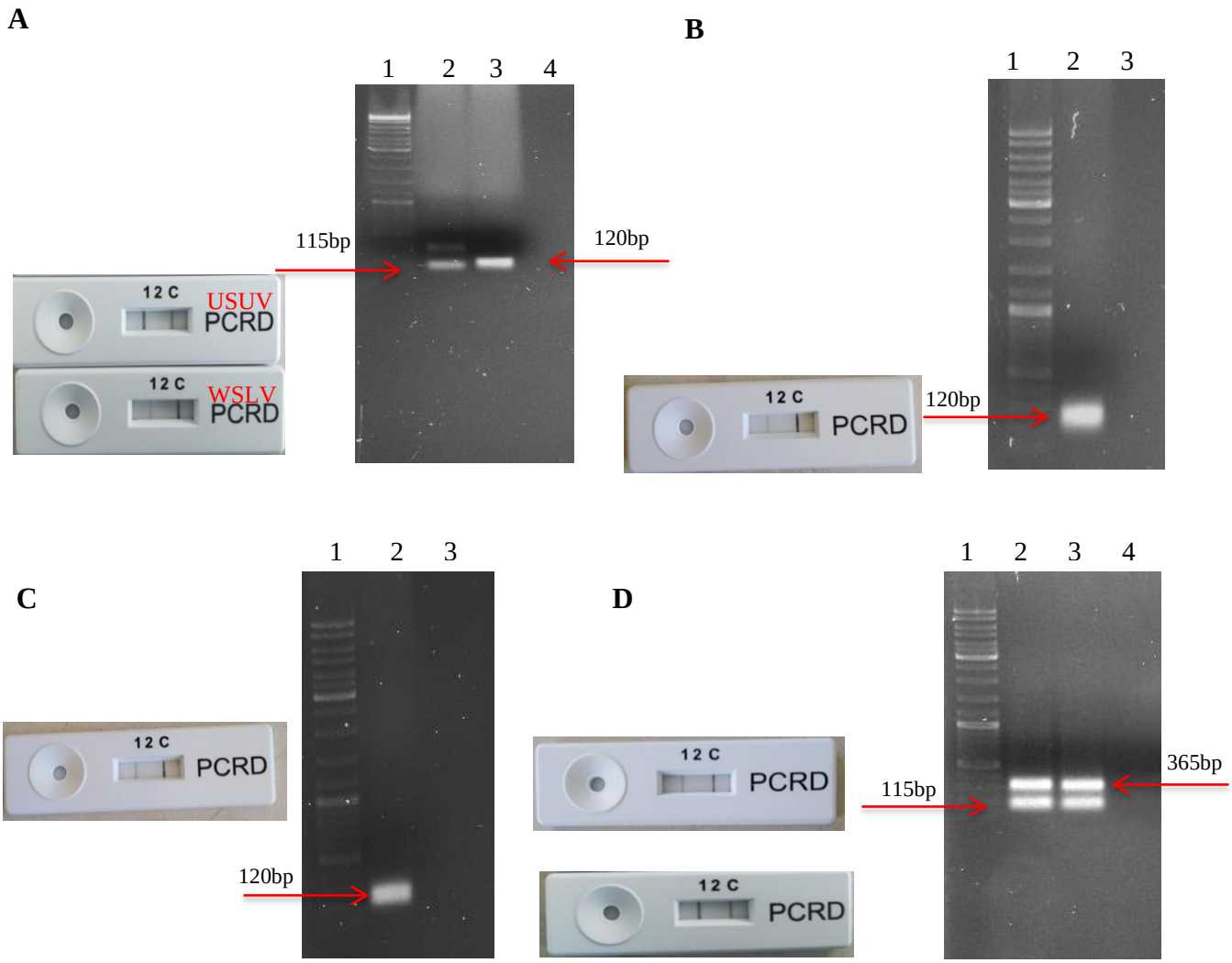

Figure 21. Detection of USUV and WSLV RNA using RT-RPA. Figures 21A Lane 1: O'GeneRuler™ DNA ladder Plus, SM 1173; Lane 2: WSLV RPA product; Lane 3: USUV RPA product; Lane 4: Negative control. Figure 21B Lane 1: O'GeneRuler™ DNA ladder Plus, SM 1173; Lane 2: USUV RPA product; Lane 3: Negative control. Figure 21C Lane 1: O'GeneRuler™ DNA ladder Plus, SM 1173; Lane 2: USUV RPA product; Lane 3: Negative control. Figure 21D Lane 1: O'GeneRuler™ DNA ladder Plus, SM 1173; Lane 2: WSLV RPA product; Lane 3: WSLV RPA product.

3.3.4 Sequencing

The nucleotide sequences of positive RPA amplicons were determined by sequencing as previously described in section 2.2.3.3. The obtained raw sequence data was edited using Chromas Pro version 1.6 (Appendix N) and the flaviviruses were determined by comparison with nucleotide sequence data retrieved from GenBank and by Blast analysis. WNV shared a similarity of 100% with WNV isolate AVA1506113, USUV a 100% similarity with USUV isolate V24 and WSLV a 100% sequence identity with WSLV isolate AV259.

3.3.5 Sensitivity of RT-RPA

RT-RPA was performed on 10-fold dilutions of transcribed RNA to determine the minimum detection levels of the assay (Table 18). The RT-RPA detected WNV and USUV RNA diluted 10^9 fold and WSLV 10^{10} fold . The sensitivity levels of the RT-RPA were compared to LOD results of the RT-PCR described in Chapter 2. Table 19 shows that the RT-RPA was able to detect RNA at higher dilutions than RT-PCR.

Table 18. Sensitivity testing of RT-RPA

Flavivirus	**Volume (µl)**	**Dilution**	**RT-RPA**
West Nile	5	10^0	Positive
	5	10^6	Positive
	5	10^7	Positive
	5	10^8	Positive
	5	10^9	Positive
	5	10^{10}	Negative
Negative control	5		Negative
Usutu	5	10^0	Positive
	5	10^6	Positive
	5	10^7	Positive
	5	10^8	Positive
	5	10^9	Positive
	5	10^{10}	Negative
Negative control			Negative
Wesselsbron	5	10^0	Positive
	5	10^5	Positive
	5	10^6	Positive
	5	10^7	Positive
	5	10^8	Positive
	5	10^9	Positive
	5	10^{10}	Positive
	5	10^{11}	Negative
Negative control			Negative

Table 19. The comparison of RT-RPA sensitivity levels with one-step RT-PCR

Flavivirus	Volume (µl)	Dilution	RT-RPA	RT-PCR
West Nile	5	10^0	Positive	Positive
	5	10^6	Positive	Positive
	5	10^7	Positive	Negative
	5	10^8	Positive	Negative
	5	10^9	Positive	Negative
	5	10^{10}	Negative	Negative
Negative control	5		Negative	Negative
Usutu	5	10^0	Positive	Positive
	5	10^6	Positive	Positive
	5	10^7	Positive	Negative
	5	10^8	Positive	Negative
	5	10^9	Positive	Negative
	5	10^{10}	Negative	Negative
Negative control			Negative	Negative
Wesselsbron	5	10^0	Positive	Positive
	5	10^5	Positive	Positive
	5	10^6	Positive	Negative
	5	10^7	Positive	Negative
	5	10^8	Positive	Negative
	5	10^9	Positive	Negative
	5	10^{10}	Positive	Negative
	5	10^{11}	Negative	Negative
Negative control			Negative	Negative

3.3.6 Specificity

RT-RPA was performed using RNA from five arboviruses including CCHFV, SINV, YFV, WSLV, WNV and USUV. Referring to Tables 20-22, no cross-reactivity was detected for CCHF, SINV, YFV or WNV RNA, however USUV RNA was detected with the WNV RT-RPA and WSLV RT-RPA. This is due to the mismatches present in USUV and the probes and reverse primers of WNV and WSLV. There were four mismatches between the WNV probe and USUV and three mismatches between the reverse primer and USUV. Ten mismatches were present between the WSLV probe and USUV and six mismatches between the reverse primer and USUV.

Table 20. Specificity determination of WNV RT-RP

Virus	Positive	Negative
Crimean-Congo Haemorrhagic fever		X
Sindbis		X
Yellow fever		X
Wesselsbron		X
Usutu	X	
Negative control		X

Table 21. Specificity determination of USUV RT-RPA

Virus	Positive	Negative
Crimean-Congo Haemorrhagic fever		X
Sindbis		X
Yellow fever		X
Wesselsbron		X
West Nile	X	
Negative control		X

Table 22. Specificity determination of WSLV RT-RPA

Virus	Positive	Negative
Crimean-Congo Haemorrhagic fever		X
Sindbis		X
Yellow fever		X
West Nile		X
Usutu	X	
Negative control		X

Theoretical cross reactivities with other flaviviruses including ZIKV, DENV and JEV were determined by sequence alignments of the partial NS5 region (Figure 22). As observed in the sequence alignments below, WNV and YFV had 14 base mismatches in the probe and 22 in the reverse primer, WNV and DENV had 18 mismatches in the probe and 17 mismatches in the reverse primer, WNV and ZIKV had 16 mismatches in the probe and 5 in the reverse primer and lastly WNV and JEV had 13 mismatches in the probe sequence and 4 in the reverse primer. The WSLV probe also showcased many mismatches with the other flaviviruses. JEV had 14 base pair mismatches with WSLV probe and 5 in the reverse primer, WSLV and ZIKV had 19 mismatches in the probe sequence and 3 in the reverse primer, WSLV and DENV shared 17

mismatches in the probe and 3 in the reverse primer and lastly WSLV and YFV had 12 mismatches in the probe and 6 in the reverse primer.

```
YFV       gtcaccaggatggcaatgactgacactacgccttttggccaacaaagagttttcaaggaa      1074
WSLV      gtgactcttctggccatgactgacacgactccgtttgggcagcaaagggtgtttaaagag      1069
DENV      gtgacacagatggcaatgacagacacgactccatttgggcaacagcgcgttttcaaagag      1071
ZIKV      gtcacaggaatagccatgaccgacaccacaccgtatggtcagcaaagagttttcaaggaa      321
WNV       gttaccaccatggccatgactgacactactcccttcgggcagcagcgagtgttcaaagag      1077
USUV      gtgaccaccatggcgatgactgacaccactccgtttgggcagcagagggttttcaaagaa      1077

JEV       gtcaccaccatggccatgactgacaccacccccttcggacagcaaagagttttcaaggag      1077
          ** **     * ** ***** ***** ** ** *  ** ** **  * ** ** ** **

YFV       aaggtggacacgagagccaaagatcctcctgctggaactaggaaaatcatgaaggtggta      1134
WSLV      aaagtggacacaaaagccccgcctccacctccaggaacacgtgcaatcatgcgcgtcgtt      1129
DENV      aaagtggacacgagaacccaagaaccgaaagaaggcacgaaaaaactaatgaaaatcacg      1131
ZIKV      aaagtggacactagggtgccagacccccaagaaggcactcgtcaggttatgagcatggtc      381
WNV       aaggtggacacgaaagctcctgaaccgccagaaggagtgaagtacgtgctcaacgagacc      1137
USUV      aaggttgacaccaaggccccggaaccccccttctggagttagagaggtgatggatgagacc      1137
JEV       aaagttgacacgaaggctcctgagccaccagctggagtcagggaagtgctcaatgagacc      1137
          ** ** ***** *           **       **           *  *

YFV       aaccgttggctgttccgtcatctggcccgggagaagaaccctaggctgtgcacaaaggag      1194
WSLV      aatgcctggttgtttcaacacttggctcgcaagaagaagccccgcatttgcacacgcgaa      1189
DENV      gcagaatggctctggaaagaactaggaaagaaaaagacacctaggatgtgcaccagagaa      1191
ZIKV      tcttcctggttgtggaaagagctaggcaaacacaaacggccacgagtctgtaccaaagaa      441
WNV       accaactggttgtgggcgtttttggccagagaaaaacgtcccagaatgtgctctcgagag      1197
USUV      accaattggctgtgggcttttctcgcacgagaaaagaagccaaggttgtgcaccagggaa      1197
JEV       accaattggctgtgggcccacttgtcacgggaaaaaagacctcgcttgtgcaccaaggaa      1197
                *** * *         *        * **    **  *  * **  *    **

YFV       gaattcatagccaaggtgcgcagtcatgctgcggtaggagcctttctagaagagcaggag      1254
```

```
WSLV      gagttcgtggctaaggttcgaagccatgccgccctcggagcgtatctggaagagcaggac      1249
DENV      gaattcacaagaaaggtgagaagcaatgcagccttaggtgccatattcactgatgagaac      1251
ZIKV      gagttcatcaacaaggttcgtagcaatgcagcattaggggcaatatttgaagaggaaaaa      501
WNV       gaattcataagaaaggtcaacagcaatgcagctttgggtgccatgtttgaagagcagaat      1257
USUV      gagtttaagaggaaggtcaacagcaacgctgctttgggagccatgtttgaagagcagaac      1257
JEV       gaattcataaagaaagttaatagcaacgcggctcttggagcagtgtttgctgaacagaat      1257
          ** **       ** **    **  * ** **  * ** **     *    **  *  *

YFV       caatggaagacagccaatgaagcagttcaagaccctaagttttgggagatggttgatgct      1314
WSLV      aaatggaagagtgcaagtgaggctgttcaggatccacagttctggaaactggttgatgat      1309
DENV      aagtggaagtcggcacgtgaggctgttgaagatagtggattttgggaattggttgacaag      1311

ZIKV      gagtggaagactgcagtggaagctgtgaacgatccaaggttctgggctctagtggacaag      561
WNV       caatggaggagcgccagagaagcagttgaagatccaaaattttgggagatggtggatgag      1317
USUV      caatggagcagtgccagggaggctgtagaggaccctcggttctgggaaatggtggacgaa      1317
JEV       caatggagcacagcacgtgaggctgtgaatgacccacggttttgggagatggtcgatgaa      1317
           * ****     **    ** ** **  * **       ** ***    * ** **

YFV       gagcgcaaactacatcaacaggggcgttgccagtcttgtgtctataacatgatgggaaag      1374
WSLV      gagaggaaactgcatctgcaaggccaatgccggacatgtgtgtacaacatgatgggaaaa      1369
DENV      gaaaggaatctccatcttgaaggaaagtgtgagacatgtgtgtataacatgatgggaaag      1371
ZIKV      gaaagagagcaccacctgagaggagagtgccagagttgtgtgtacaacatgatgggaaaa      621
WNV       gagcgcgaggcacatctgcgggggggaatgtcacacctgcatttacaacatgatgggaaag      1377
USUV      gaaagggagaaccatctgaaaggagagtgccacacatgcatttacaacatgatggggaag      1377
JEV       gagagggaaaaccacctgcgaggagagtgtcacacatgtatctacaacatgatgggaaaa      1377
          **  *  *    ** *     **    **       **  * ** ********** **

YFV       agagagaaaaaactgtctgagtttgggaaagcaaaaggaagtcgtgcgatctggtacatg      1434
WSLV      cgtgaaaagaaaccttctgaatttggcaaagcaaagggaagtagagcaatttggtacatg      1429
DENV      agagagaagaagctaggggagttcggcaaagcaaaaggcagcagagccatatggtacatg      1431
ZIKV      agagaaaagaaacaaggggaatttggaaaggccaagggcagccgcgccatctggtatatg      681

WNV       agagagaaaaaacccggagagttcggaaaggccaagggaagcagagccatttggttcatg      1437
```

```
USUV       cgtgagaagaagctcggagagtttggtaaagcgaaaggtagtcgagccatatggttcatg      1437

JEV        agagagaagaaacctggagagtttggaaaagctaaaggaagcagggccatttggttcatg      1437

            * ** ** ** *     ** ** ** ** ** ** ** **  * ** ** ****  ***
```

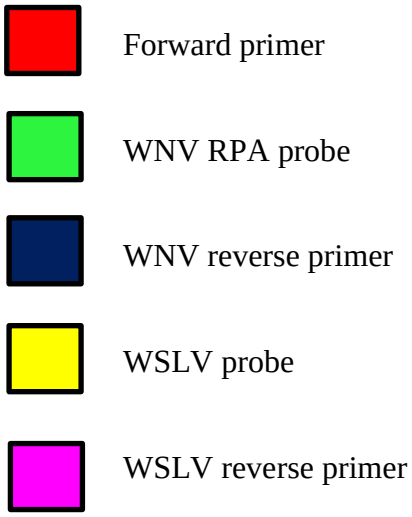

Figure 22. Sequence alignments for potential cross-reactivities with other flaviviruses.

3.4 RT-RPA application in a field setting

3.4.1 Screening of wild-caught mosquitoes

Mosquitoes were collected and identified using morphological features and pooled according to species, date of collection and site captured. Extracted RNA was available for 60 pools of mosquitoes, and 17 were selected. RT-RPA was performed using each of the primers and probes sets for WNV/USUV and WSLV. As shown in Table 23, no WNV, USUV or WSLV RNA was detected in any of the 17 selected pools.

Table 23. Screening of wild-caught mosquitoes for WNV, USUV and WSLV RNA using RT-RPA

Pool number	Number of mosquitoes	Location of capture	Mosquito species	West Nile virus	Usutu virus	Wesselsbron virus
4	6	Bloemfontein Zoo	*Culex theileri*	-	-	-
5	1	NFBG	*Culex univittatus*	-	-	-
6	7	NFBG	*Culex theileri*	-	-	-
7	1	NFBG	*Culex pipiens*	-	-	-
8	1	Bloemfontein Zoo	*Culex theileri*	-	-	-
10	1	NFBG	*Culex theileri*	-	-	-
12	6	Bloemfontein Zoo	*Culex theileri*	-	-	-
13	1	Bloemfontein Zoo	*Culex tigripes*	-	-	-
14	1	Bloemfontein Zoo	*Culex terzii*	-	-	-
22	4	Bloemfontein Zoo	*Culex theileri*	-	-	-
25	3	Bloemfontein Zoo	*Culex univittatus*	-	-	-
30	4	NFBG	*Culex theileri*	-	-	-
43	2	Krugersdrift Dam	*Culex theileri*	-	-	-
44	3	Krugersdrift Dam	*Culex theileri*	-	-	-
47	2	Bloemfontein Zoo	*Culex theileri*	-	-	-
54	1	NFBG	*Culex pipiens*	-	-	-
55	6	NFBG	*Culex theileri*	-	-	-
Negative control				-	-	-
Total number of mosquitoes	**50**					

NFBG: National Free State Botanical Gardens

3.4.2 Effect of possible inhibitors in mosquito extracts

The inhibitory effect of interfering agents within a crude mosquito sample was tested by spiking two mosquito pools that were negative for WNV, USUV and WSLV RNA with a known amount of transcribed RNA (neat WNV, WSLV and USUV RNA). RT-RPA was performed on the spiked mosquito pools and as can be seen in Table 24, no inhibition of the reaction was detected.

Table 24. Effect of possible inhibitors in crude mosquito pools

Virus	Pool number	Mosquito species	Positive	Negative
West Nile	25	*Culex univittatus*	X	
	47	*Culex theileri*	X	
Negative control				X
Usutu	5	*Culex univittatus*	X	
	30	*Culex theileri*	X	
Negative control				X
Wesselsbron	4	*Culex theileri*	X	
	12	*Culex theileri*	X	
Negative control				X

3.5 Discussion

Molecular assays, such as RT-PCR, are used to detect flaviviruses; however it is time consuming and requires sophisticated equipment and reagents (Escadafal et al., 2014). It is difficult to adapt RT-PCR to be used in low resource or field settings for the detection of flaviviruses. RPA is becoming a molecular assay of choice for the rapid, specific and sensitive identification of pathogens, even as a fieldable diagnostic (Daher et al., 2016). Thus, the aim of this chapter was to develop and validate a RT-RPA using transcribed RNA prepared in chapter 2.

The RT-RPA assay was designed based on sequence alignments of the partial NS5 gene of WNV, USUV and WSLV. The aim was to identify a region which would allow uniform detection of all three viruses; however the similarity between WSLV and WNV sequences was not enough. Therefore, two sets of primers and probes were designed to detect the transcribed RNA.

The RT-RPA nfo® assay was performed using the transcribed RNA as template. After optimisation of the assay, it was observed that the incubation temperature was at 40ºC and a final primer concentration of 420nM. According to another study, 420nM of final primer concentration and incubation at 40ºC is optimal for a RPA reaction to take place (Bonney et al., 2017). All three RNA controls tested positive with RT-RPA.

The LOD of the RT-RPA was determined by diluting transcribed RNA ten-fold. It was found that the RT-RPA could detect WNV RNA diluted up to 10^9 fold, USUV RNA diluted up to 10^9 fold and WSLV RNA up to 10^{10} fold. It was found that the RT-RPA was more sensitive than the RT-PCR. The sensitivity of the RT-PCR could likely have been improved using a nested reaction. As mentioned in Chapter 1 section 1.6, a THF residue replaces a conventional nucleotide at least 30 nucleotides from the 5'end. The THF residue gets cleaved by an nfo enzyme and transforms the probe into a primer, resulting in a smaller amplification product. The sensitivity of the RT-RPA was also enhanced as a result of the reverse primers that were labelled with biotin. Biotinylation is the process of attaching a biotin label to molecules, such as nucleic acid. As the RPA product increases, the sensitivity of the assay also increase (Vina-Rodriguez et al., 2017).

The specificity of the RT-RPA was determined by screening available RNA from six viruses including, CCHFV, SINV, WSLV, USUV, WNV and YFV. The developed RT-RPA only detected RNA for USUV, WNV and WSLV. The highest mismatch tolerability for RPA reported so far is nine nucleotide base pairs across the primer and probe binding sites (Abd El Wahed et al., 2013; Boyle et al., 2013; Daher et al., 2015; Kissenkötter et al., 2018; Lillis et al., 2016; Moore et al., 2017; Patel et al., 2016), however ten mismatches were present between WNV isolate SA93/01 and the reference isolate; nonetheless transcribed RNA from isolate SA93/01 was detected by RT-RPA.

Seventeen pools of collected mosquitoes were screened as a side project using the RT-RPA, however all 17 pools tested negative for USUV, WNV and WSLV RNA. No *Aedes* mosquitoes, which are the main vector of WSLV, were present in the mosquito pools. Screening of mosquitoes will be done on a much larger scale as ongoing surveillance studies. The inhibitory effect of interfering agents within a crude sample on the RT-RPA was tested by spiking real-time qRT-PCR confirmed negative samples with a known amount of transcribed RNA. There was no evidence of inhibition of the RT-RPA, indicating that the RT-RPA could be used for mosquito screening in a field setting.

In conclusion, the RT-RPA proved to be a robust, sensitive and specific assay that is not time consuming and can be used as a possible fieldable diagnostic or in surveillance studies.

Chapter 4: CONCLUSION

The genus *Flavivirus* includes arthropod-borne viruses that are either transmitted by mosquitoes or ticks to vertebrate hosts (Gould et al., 2008). Some viral species in the genus are associated with major public health impact worldwide causing disease, encephalitis and haemorrhagic fever (Schweitzer et al., 2009). There are five known, or historically found, flaviviruses in SA namely WNV, USUV, WSLV, SPOV and BANV. WNV and WSLV occur annually in SA, causing sporadic outbreaks associated with heavy rainfall leading to an increase in mosquito population (McIntosh et al., 1961; McIntosh et al., 1976; Smithburn et al., 1959; Weyer et al., 2013; Williams and Woodall, 1964).

Molecular detection of flaviviruses relies on assays such as RT-PCR; however more developments for molecular diagnosis using isothermal assays are being implemented such as NASBA or LAMP techniques. These new approaches may offer a real chance to implement flavivirus molecular diagnosis in point-of-care settings (Domingo et al., 2011). The best option for developing detection tools is to use native infectious virus for optimisation and validation, however WSLV and WNV are classified as BSL-3 pathogens, thus laboratories with low-resource settings are not able to handle the pathogens safely. This study was conducted to overcome the safety limitation by preparing *in vitro* transcription (IVT) RNA transcripts to use in the development of a RT-RPA for the detection of flaviviruses.

Working with transcribed RNA has a few advantages compared to working with live virus. Transcribed RNA is completely safe to work with and does not require any specific biocontainment. Transcribed RNA made from synthesised genes can mimic any target region of a virus, it can be modified and more RNA can be synthesised in a short time. IVT also offers a rapid and cost effective method for the making of RNA. However, the LOD of an assay can be determined more accurately by using native infectious virus because the assay will react according to a natural sample.

IVT has been used to prepare RNA transcripts for a variety of molecular assays, for example PCR (Walker et al., 2003). RNA transcripts were prepared *in vitro* for the simultaneous detection of ZIKV, Chikungunya (CHIKV) and DENV using a multiplex real-time RT-PCR assay. The triplex assay was able to detect 15 copies/reaction of transcribed DENV-1 RNA and less than 10 copies/reaction of transcribed ZIKV and CHIKV RNA (Pabbaraju et al., 2016). In another study, RNA transcripts were successfully prepared for the detection of transcribed WNV RNA and the real-time RT-PCR was able to detect approximately 40 copies of transcribed RNA (Shi et al., 2001).

RNA can be synthesized conveniently by IVT using T7 or Sp6 RNA polymerases which have a high level of activity for IVT as they are composed of a single polypeptide chain and hence do not employ a dissociating initiation factor. There are advantages to using phage RNA polymerase. RNA polymerases, such as SP6, are very specific for their own promoter sequence, thus there is no detectable transcription from other prokaryotic or eukaryotic promoters. The activity of the RNA polymerase remains extremely strand specific when used *in vitro* for example, the RNA synthesis by SP6 polymerase is efficaciously limited to the strand specified by the SP6 promoter (Beckert et al., 2011).

The target gene to be transcribed is firstly inserted into a plasmid vector downstream from the promoter. The plasmid DNA is then digested with a restriction endonuclease downstream of the inserted DNA sequence. When DNA template is added to a mixture of a simple salt buffer, ribonucleotide triphosphates and RNA polymerase, transcription commences at the specific bacteriophage promoter. The process proceeds through the cloned DNA sequence and finally terminates the reaction when the polymerase reaches the end of the linear template. The RNA polymerase reinitiates transcription at the promoter site many times so that a number of RNA copies are made from every molecule of DNA. After reaction completion, the template DNA is removed by digestion using a DNase enzyme to ensure pure synthesised RNA (Huang et al., 2013).

RPA is a novel isothermal alternative to PCR. RPA employs three enzymes including a SSB, a polymerase and a recombinase (Escadafel et al., 2014). RPA is performed at a single temperature using the recombinase enzyme that separates the DNA template to which SSB molecules attach to stabilise the open complex (Yan et al., 2014).

RPA can be advantageous compared to PCR in numerous ways. The primers and probes are slightly longer compared to PCR; however this can be an advantage and disadvantage. The longer probe increases the sensitivity of the assay; however the specificity decreases because the RPA is able to tolerate multiple base mismatches in the primer and probe regions which may result in non-specific binding. Lyophilized reagents are supplied in the kit, thus you only need to add primers, probe and template. RNA viruses can also be amplified using a suitable reverse transcriptase enzyme, and lyophilized reverse transcriptase enzymes are available to make RT-RPA more suitable for low resource settings. The reagents have a long shelf-life and can tolerate high temperatures without losing activity. It has been presented in a study that reagents stored at 45°C for up to three weeks were able to detect 10 HIV-1 copies of DNA and only after another three weeks was a reduction in assay sensitivity observed (Lillis et al., 2016). The process begins

operating immediately as melting of double-stranded DNA is not necessary. As a result, the amount of energy necessary and the cost of equipment are minimal. Furthermore, RPA products can be amplified in a short period of time and products can be visualised in numerous ways, presenting a significant advance in the development of portable nucleic acid based tests (Yang et al., 2017; Zaghloul et al., 2014). In addition, the target gene can either be DNA or RNA, thus making RPA suitable for the detection of RNA viruses as well (Zaghloul et al., 2014), such as WNV. This is achieved by the addition of a suitable reverse transcription enzyme to the RPA reaction. RPA has also shown high tolerance to inhibitors present in crude samples of patients and arthropod vectors including serum, urine and tick homogenate (Bonney et al., 2017). This advantage means that crude samples can be used without extensive extraction of viral nucleic acid and therefore making the assays more readily adaptable to field situations. The use of a recombinase to separate DNA strands and therefore not requiring a denaturing step also enhance adaptation to field work as denaturing temperatures and cycling are not required. Another advantage is that RPA is a very sensitive assay that can detect as little as a few copies of analyte (Li et al., 2019).

Like all molecular assays, RPA has some concerns. Deviations from the manufacturer's protocol and/or storage conditions could influence performance of RPA in limited resource settings. Using lateral flow assays as detection method for RPA products is very cost effective and simple; however there are a few limitations to using it: An inaccurate sample volume can reduce the sensitivity of the assay, there is no possibility of enhancing the response by enzyme activity and obstruction of pores can occur due to matrix components (Koczula et al., 2016). A LFS-RPA designed for the detection of YFV experienced specificity problems as a faint nonspecific band appeared in the negative controls. It was concluded that false-positive results are not necessarily due to contamination, but from the clotting of proteins or primers which do not bind to any template (Escadafel et al., 2014).

In this study, a RT-RPA was developed and validated for the detection of flaviviruses. An approximately 414bp region of the conserved partial NS5 gene was identified in a previous study and within that region a pair of degenerate consensus primers, FlaviF1 and FlaviR2 targeting roughly 380bp were identified (Mathengtheng, 2015). These primers are currently being used for flavivirus surveillance studies in our laboratory and hence the region was selected as a target for the RT-RPA. According to TwistAmp® guidelines, primers designed specifically for a PCR may work in an RPA, thus FlaviF1 was used as the forward primer for the RPA; however reverse primers were designed and modified according to TwistAmp® and PCRD Nucleic Acid Detector guidelines. To design primers and probes, sequence data of the partial

NS5 region was retrieved from GenBank and used to identify theoretical cross reactivity between the probe and reverse primer of USUV, WNV and WSLV. It was determined that four mismatches were present between WNV and USUV in the probe binding region and three in the reverse primer, and 16 between WNV and WSLV in the probe and four in the reverse primer regions, hence different probe and reverse primer regions were identified for WNV/USUV and WSLV. According to other studies, the highest mismatch tolerability for RPA reported so far is nine nucleotide base pairs across the primer and probe binding sites (Abd El Wahed et al., 2013; Boyle et al., 2013; Daher et al., 2015; Kissenkötter et al., 2018; Lillis et al., 2016; Moore et al., 2017; Patel et al., 2016). An RPA was designed for the detection of all of the major HIV-1 global subtypes. The one variant that amplified had three mismatches in the reverse primer, at the 3' end, and five mismatches in the probe sequence (Boyle et al., 2013). The length of the primer and probe compensates for mismatches in the target sequence, which usually causes PCR to fail or lose sensitivity (Whiley et al., 2006). RPA also operates at lower temperatures compared to PCR and this may increase tolerance to mismatches in the primer/probe binding sites (Boyle et al., 2013). However the validation of an assay required specificity testing due to this high degree of mismatch tolerance.

A limitation of the study was the selection of a reference strain of WNV belonging to lineage 1. Lineage 1 is the most widespread globally and circulates in the Americas, Australia, India and more recently, has been identified in SA (Bondre et al., 2007; Coia et al., 1988; Lanciotti et al., 1999; Venter et al., 2010). However a lineage 2 isolate would have been a more suitable representative of SAn lineages. Despite multiple mismatches between lineage 2 isolates from SA and the reference strain, RNA from SA isolate 93/01 was amplified using the RT-RPA. The RT-RPA was able to detect the transcribed RNA of isolate SA93/01, which suggests that the RPA might also detect isolates 349/77, SA381/00 and SPU116/89 based on sequence data available from Genbank. Although the assay needs to be modified for SA isolates, the proof of concept was confirmed that transcribed RNA provides useful controls for viruses requiring biocontainment and that the assay is sensitive for detection of low concentrations of viral nucleic acid. Before the assay can be deemed suitable for surveillance studies in SA, a probe and reverse primer will be designed based on SA lineage 2 isolates and validated.

The theoretical determination of the ability of the RT-RPA to detect all nine lineages of WNV was not possible as sequence data was only available on GenBank for lineages 3 and 9 which circulate in Austria, and lineage 4 which circulates in Russia. Nevertheless, sequence alignments were performed for WNV lineages 3, 4 and 9. Based on the sequence alignments, the possibility of the RPA detecting WNV lineages 3 and 9 are very slim as approximately 16

mismatches were present in the primer/probe regions. However, lineage 4 presented with ten mismatches in the probe region and two in the reverse primer region, suggesting that the RT-RPA may be able to detect WNV lineage 4. Confirmation would require testing using RNA extracted from whole virus or transcribed RNA.

Sequence alignments of the partial NS5 region were also performed for the uniform detection of all the USUV and WSLV isolates available on GenBank. The RT-RPA should theoretically be able to detect USUV isolates V44, SAAR, HautRhin7361 as there were no, or only one, mismatch in the probe and reverse primer regions. Similarly the WSLV RPA probe and reverse primer showed high similarity.

The RT-RPA was optimised using transcribed WNV RNA and after optimisation, transcribed WSLV and USUV RNA were tested using RT-RPA. The sensitivity of the assay was determined by performing ten-fold dilutions on transcribed RNA and the specificity of the assay was determined by screening CCHF, SINV and YFV RNA that was available in our facility. Multiple sequence alignments of ZIKV, DENV-2 and JEV were performed to identify possible cross-reactivities with other flaviviruses. The inhibition of the assay was tested by spiking two pools of mosquitoes that were confirmed negative for WNV, USUV and WSLV RNA by real-time qRT-PCR with 1µl of neat RNA. Lastly, 17 pools of mosquitoes that were collected in a previous study were screened using the RT-RPA.

Considering the sensitivity of the assay, the WNV RT-RPA could detect WNV and USUV transcribed RNA diluted 10^9 fold, whereas the WSLV RT-RPA detected WSLV transcribed RNA diluted 10^{10} fold. Using dilutions rather than RNA copy numbers was a limitation of the study; however the RT-RPA and RT-PCR could be compared for each virus.

More importantly, testing RNA from other arboviruses suggested that despite the binding tolerability of the assay there was good specificity. No other arboviruses were amplified, including YFV. However because of similarity in sequence data, USUV transcribed RNA was detected with the WNV RT-RPA and WSLV RT-RPA. Biothreat RPAs developed for the detection of pathogens like EBOV, SUDV, *Yersinia pestis* and *Bacillus anthrax* showed similar specificity (Euler et al., 2013).

Possible cross reactivities with other flaviviruses were theoretically determined by aligning sequences of the partial NS5 region. Sequence alignments showed that WNV and DENV had 18 mismatches in the probe and 17 mismatches in the reverse primer, WNV and ZIKV had 16 mismatches in the probe and five in the reverse primer and lastly WNV and JEV had 13

mismatches in the probe sequence and four in the reverse primer. For the WSLV RT-RPA, JEV and WSLV had 14 base pair mismatches in the probe and five in the reverse primer, WSLV and ZIKV had 19 mismatches in the probe sequence and three in the reverse primer, and lastly WSLV and DENV shared 17 mismatches in the probe and three in the reverse primer. From the data obtained from the sequence alignments, it can be proposed that JEV, ZIKV and DENV RNA will likely not be detected by either the WNV RT-RPA or WSLV RT-RPA, although this limits the application of the assay for detecting other flaviviruses that may emerge or be circulating in SA. Identifying consensus primers can be limited by a lack of conserved regions between different species and for RPA assays requiring longer probes and primers it can be even more difficult to find consensus primers and probes.

The robustness of a RPA assay in the presence of inhibitors facilitates amplification from a variety of crude extracts including whole blood, anticoagulants, tissues, soil and background DNA (Francois et al., 2011; Rohrman et al., 2015; Schrader et al., 2012). No inhibition of the RT-RPA was shown to occur in the mosquito pools that were spiked with a known amount of WNV, WSLV and USUV transcribed RNA.

As of yet, RPA has not been approved by the FDA and may only be used for research applications. RPA is used in laboratory-based settings and its exploitation will increase in the near future including a range of commercial devices for molecular diagnostics, medical diagnostics, food quality control, environmental analysis and detection of biowarfare agents (Lobato and O' Sullivan, 2018). A small cohort of mosquito samples was tested. Seventeen pools of *Culex* spp were screened for flavivirus RNA, although no flaviviruses were expected within such a small cohort it provided the opportunity to confirm absence of non-specific amplification from mosquito samples and provided RNA extracts from mosquito pools that could be spiked to test for inhibition of amplification. To adapt this RT-RPA for field settings, a suitable RNA isolation method needs to be identified to extract RNA from mosquito samples. Micro-chip solid-phase RNA extraction includes silica-coated paramagnetic beads to absorb RNA in a high-ionic-strength chaotropic solution (Gimenez et al., 2017). This methodology is cost-effective, rapid, does not require sophisticated reagents and equipment, and can be used in point-of-care settings. Detecting WNV by RT-RPA will not only be useful for surveillance studies in SA, but can also be used as a diagnostic tool for veterinary diagnostics, especially equine. Human cases are seldom diagnosed using molecular assays due to the short duration of viremia however WNV is a significant disease in horses causing severe neurological complications and can present with clinical signs similar to equine encephalitis. Rapid assays for differentiation would have application as a veterinary diagnostic tool. Testing clinical

samples would be essential for validation of the assay and comparison of crudely prepared samples with samples in which RNA is isolated using isolation kits would indicate the usefulness of the test for crude preparations. Final considerations for testing crude samples would include inactivation of samples, usually achievable by heat inactivation at 56°C for a specified time period, and simple methods to disrupt the viral envelop and expose viral RNA.

In conclusion, the development and validation of a RT-RPA for the detection of WNV, WSLV and USUV flaviviruses was successful. The RT-RPA proved to be a robust, rapid and sensitive assay that might have potential as a diagnostic tool in the field or resource limited settings.

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Appendixes

Appedix A: Ethics Approval

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

UFS·UV
HEALTH SCIENCES
GESONDHEIDSWETENSKAPPE

IRB nr 00006240
REC Reference nr 230408-011
IORG0005187
FWA00012784

01 November 2017

MS ELISABETH H BONNET
DEPT OF MEDICAL MICROBIOLOGY
FACULTY OF HEALTH SCIENCES
UFS

Dear Ms Elisabeth H Bonnet

HSREC 95/2016E (UFS-HSD2017/1248)
PRINCIPAL INVESTIGATOR: MS ELISABETH H BONNET
PROJECT TITLE: THE DEVELOPMENT AND VALIDATION OF A REVERSE TRANSCRIPTION RECOMBINASE POLYMERASE AMPLIFICATION ASSAY FOR DETECTION OF FLAVIVIRUSES

APPROVED

1. You are hereby kindly informed that the Health Sciences Research Ethics Committee (HSREC) approved this project at the meeting held on 31 October 2017 after all conditions were met.
2. The Committee must be informed of any serious adverse event and/or termination of the study.
3. Any amendment, extension or other modifications to the protocol must be submitted to the HSREC for approval.
4. A progress report should be submitted within one year of approval and annually for long term studies.
5. A final report should be submitted at the completion of the study.
6. Kindly use the **HSREC NR** as reference in correspondence to the HSREC Secretariat.
7. The HSREC functions in compliance with, but not limited to, the following documents and guidelines: The SA National Health Act. No. 61 of 2003; Ethics in Health Research: Principles, Structures and Processes (2015); SA GCP(2006); Declaration of Helsinki; The Belmont Report; The US Office of Human Research Protections 45 CFR 461 (for non-exempt research with human participants conducted or supported by the US Department of Health and Human Services- (HHS), 21 CFR 50, 21 CFR 56; CIOMS; ICH-GCP-E6 Sections 1-4; The International Conference on Harmonization and Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH Tripartite), Guidelines of the SA Medicines Control Council as well as Laws and Regulations with regard to the Control of Medicines, Constitution of the HSREC of the Faculty of Health Sciences.

Yours faithfully

DR SM LE GRANGE
CHAIR: HEALTH SCIENCES RESEARCH ETHICS COMMITTEE

Health Sciences Research Ethics Committee
Office of the Dean: Health Sciences
T: +27 (0)51 401 7795/7794 | E: ethicsfhs@ufs.ac.za
Block D, Dean's Division, Room D104 | P.O. Box/Posbus 339 (Internal Post Box G40) | Bloemfontein 9300 | South Africa
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Appendix B: Section 20 Permit

agriculture,
forestry &
fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries Private Bag X138, Pretoria 0001

Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerrvG@daff.gov.za
Reference: 12/11/1/4

FJ Burt

Department of Medical Microbiology and Virology

Faculty of Health Sciences, Francois Retief

Building Bloemfontein

Email: burtfj@ufs.ac.za

RE: PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO. 35 OF 1984)

Dear FJ Burt

Your application dated 30 May 2017 requesting permission under Section 20 of the Animal

Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers.

I am pleased to inform you tha! permission is hereby granted to perform the following study,

with the following conditions :

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. All potentially infectious material utilised or collected during the study is to be destroyed at the completion of the study. Records must be kept for five years for audit purposes. A dispensation application may be made to the Director Animal Health in the event that any of the above is to be stored or distributed;
3. This approval is granted for research involving non-infectious Arboviruses virus only, as stipulated in the application;
4. Approval may be required in terms of the Genetically Modified Organisms Act, 1997 (Act No 15 of 1997) prior to the start of the study;

5. A veterinary import permit may be required prior to the importation of synthesised genes from China;
6. All imported samples must be packaged and transported in accordance with International Air Transport Association (IATA) requirements and/or the National Road Traffic Act, 1996 (Act No. 93 of 1996);
7. Only a registered waste disposal company may be utilised for the removal of waste generated during the study.

Title of research/study: Arboviruses and zoonotic diseases: molecular assays
Researcher: FJ Burt
Your Ref./ Project Number: UFS 95/2016
Institution: University of the Free State
Our ref Number: 12/11/1/4
Expiry: 31 December 2022

Kind regards,

DR. MPHO MfJA
DIRECTOR O ANIMAL HEALTH

Date: 2017 ..07.. 2 0

SUBJECT: PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO. 35 OF 1984)

agriculture, forestry
& fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerrvG@daff.gov.za
Reference: 12/11/1/4

FJ Burt
Department of Medical Microbiology and Virology
Faculty of Health Sciences, Francois Retief Building
Bloemfontein

Email: burtfj@ufs.ac.za

RE: DISPENSATION ON SECTION 20 APPROVAL IN TERMS OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984) FOR: "ARBOVIRUSES AND ZOONOTIC DISEASES: MOLECULAR ASSAYS"

A dispensation is hereby granted on Point 2 of the Section 20 approval that was issued for the above mentioned study (attached):

i) Viral nucleic acid, RNA, transcribed RNA and plasmids constructed for transcription of RNA may be stored in the Department of Medical Microbiology and Virology, Faculty Health Sciences, University of the Free State, for future studies;

ii) Stored samples may not be outsourced or used for further research without prior written approval from DAFF.

Kind regards,

Maja.

DR. MPHO MAJA
DIRECTOR: ANIMAL HEALTH
Date: 2017-07-20

Appendix C: Overview of mosquito-borne flaviviruses

(Simmonds et al., 2017; https://talk.ictvonline.org/ictv-reports/ictv_online_report/positive-sense-rna-viruses/w/flaviviridae)

Virus species	Virus name	Accession number	Abbreviation
Mosquito-borne, Aroa virus group			
Aroa virus	Aroa virus Bussuquara virus Iguape virus Naranjal virus	AY632536 AF013366 AF013375 AF013390	AROAV BSQV IGUV NJLV
Mosquito-borne, Dengue virus group			
Dengue virus	Dengue virus 1 Dengue virus 2 Dengue virus 3 Dengue virus 4	U88536 U87411 M93130 AF326573	DENV-1 DENV-2 DENV-3 DENV-4
Mosquito-borne, Japanese encephalitis virus group			
Cacipacore virus	Cacipacoré virus	KF917526	CPCV
Japanese encephalitis virus	Japanese encephalitis virus	M18370	JEV
Koutango virus	Koutango virus	AF013384	KOUV
Murray Valley encephalitis virus	Alfuy virus Murray Valley encephalitis virus	AF013360 AF161266	ALFV MVEV
St Louis encephalitis virus	St. Louis encephalitis virus	DQ525916	SLEV
Usutu virus	Usutu virus	AY453411	USUV
West Nile virus	Kunjin virus West Nile virus	D00246 M12294	KUNV WNV
Yaounde virus	Yaounde virus	AF013413	YAOV
Mosquito-borne, Kokobera virus group			
Kokobera virus	Kokobera virus	AY632541	KOKV
	Stratford virus	AF013407	STRV
Mosquito-borne, Ntaya virus group			
Bagaza virus	Bagaza virus	AY632545	BAGV
Ilheus virus	Ilhéus virus Rocio virus	AY632539 AF013397	ILHV ROCV
Israel turkey meningoencephalitis virus	Israel turkey meningoencephalitis virus	AF013377	ITV
Ntaya virus	Ntaya virus	JX236040	NTAV

Tembusu virus	Tembusu virus	JF895923	TMUV
Zika virus	Zika virus	AY632535	ZIKV
Mosquito-borne, yellow fever virus group			
Sepik virus	Sepik virus	DQ837642	SEPV
Wesselsbron virus	Wesselsbron virus	EU707555	WESSV
Yellow fever virus	yellow fever virus	X03700	YFV
Probably mosquito-borne, Kedougou virus group			
Kedougou virus	Kédougou virus	AY632540	KEDV
Probably mosquito-borne, Edge Hill virus group			
Banzi virus	Banzi virus	DQ859056	BANV
Bouboui virus	Bouboui virus	DQ859057	BOUV
Edge Hill virus	Edge Hill virus	DQ859060	EHV
Jugra virus	Jugra virus	DQ859066	JUGV
Saboya virus	Potiskum virus Saboya virus	DQ859067 DQ859062	POTV SABV
Uganda S virus	Uganda S virus	DQ859065	UGSV

Appendix D: Overview of West Nile virus lineages

(Pachler et al., 2014)

Lineage	Representative strain	Reference
1a	NY99-flamingo382-99, New York, 1999	Lanciotti et al., 1999
1b	Kunjin MRM61C, Australia, 1960	Coia et al., 1988
1c/5∞	804994, India, 1980	Bondre et al., 2007
2	B956, Uganda 1937	Smithburn et al., 1940
3	Rabensburg virus 97-103, Czech Republic	Bakonyi et al., 2005
4a	LEIV-Krnd88-190, Russia 1998	Lvov et al., 2004
6/4b*	HU2925/06, Spain	Vazquez et al., 2010
7	Dak-Ar-D-5443, Senegal	Fall et al., 2014
8	ArD94343, Senegal	Pachler et al., 2014
9/4c▪	WNV-Uu-LN-AT-2013, Austria 2013	Pachler et al., 2014

∞ Lineage 5 was identified in India based on results from 17 isolates which included an isolate from 1c

***** Lineage 6 identified in Spain has a 95% identity in nucleotide sequence with sub-lineage 4b

▪ Lineage 9 constitute a new lineage or can be grouped into lineage 4 as a sub-lineage 4c

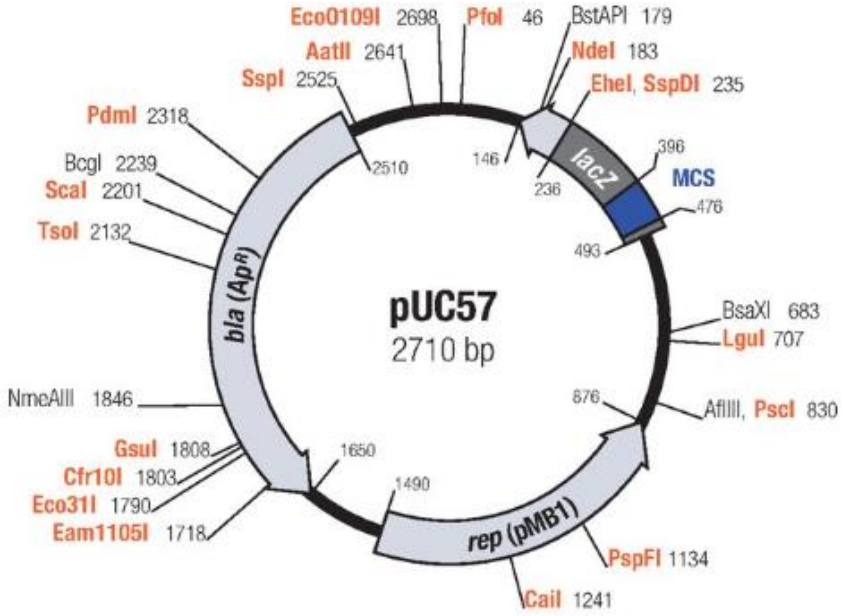
Appendix E: Yellow fever endemic countries in Africa and South America

(Brent et al., 2018)

Africa	**South America**
Mauritania	Brazil
Mali	Columbia
Niger	Ecuador
Chad	Peru
Sudan	Venezuela
Senegal	Guyana
Burkina Faso	Suriname
Nigeria	Paraguay
Ethiopia	Bolivia
South Sudan	Trinidad
Central African Republic	French Guyana
Cameroon	Panama
Benin	
Togo	
Ghana	
Côte D' Ivoire	
Liberia	
Sierra Leone	
Guinea	
Guinea-Bissou	
Gambia	
Equatorial Guinea	
Gabon	
Republic of Congo	
Democratic Republic of Congo	
Burundi	
Uganda	
Kenya	
Angola	
Rwanda	

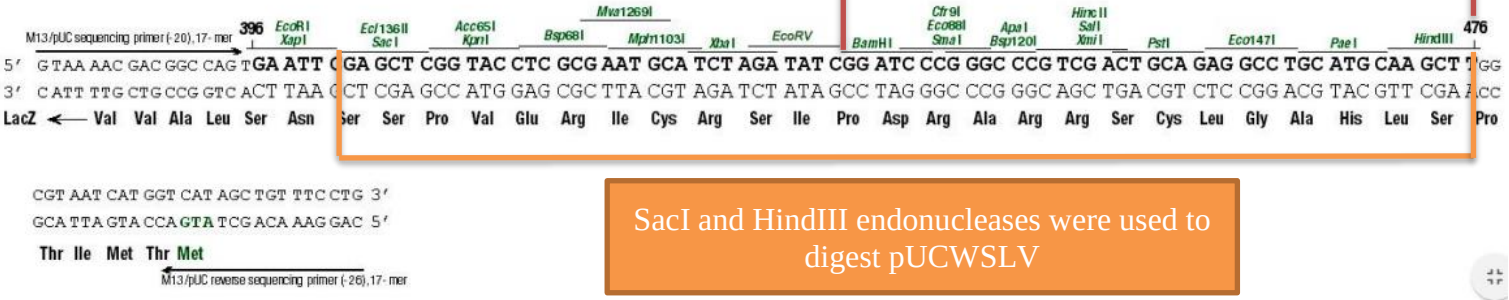
Appendix F: Vector map with sequence reference points of pUC57 plasmid and partial NS5 genes of WNV, USUV and WSLV.

A: Vector map



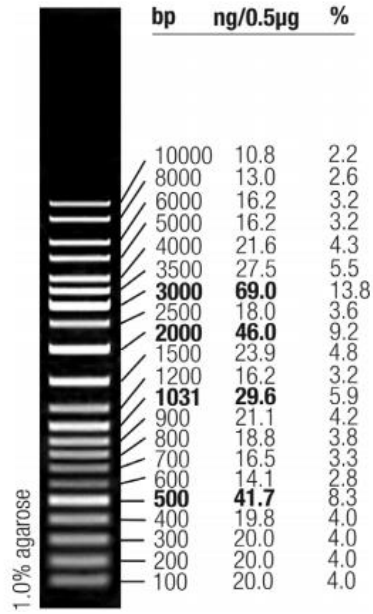
B: Multiple cloning sites

BamHI and HindIII endonucleases were used to digest pUC57WNV and pUCUSUV



SacI and HindIII endonucleases were used to digest pUCWSLV

Appendix G: O'GeneRuler™ DNA ladder Plus, SM #1173



Appendix H: Alignment of flavivirus NS5 sequence data

```
LIV             ATGGCCATGACTGACACGACAGCCTTTGGGCAGCAGAGAGTGTTTAAAGAGAAAGTTGAC 60
TBEV            ATGGCTATGACTGACACAACGGCTTTCGGACAGCAGAGAGTGTTCAAAGATAAAGTTGAC 60
Langat          ATGGCAATGACTGACACAACTGCATTCGGGCAGCAGAGGGTCTTTAAGGACAAAGTCGAC 60
Royal           ATGGCCATGACTGATACAACGGCATTTGGGCAGCAGAGGGTCTTCAAGGACAAGGTGGAT 60
Kadam           ATGGCTATGACAGACACCACGGCTTTTGGACAACAGAGAGTGTTCAAGGAGAAAGTCGAC 60
Yellow          ATGGCAATGACTGACACAACCCCCTTTGGACAGCAGAGGGTGTTCAAAGAAAAAGTTGAC 60
Wesselsbron     CTGGCCATGACTGACACAACCCCGTTTGGGCAGCAAAGGGTGTTTAAAGAGAAAGTGGAC 60
Zika            ATAGCCATGACTGACACCACACCATACGGCCAACAAAGAGTCTTCAAAGAAAAAGTGGAC 60
Dengue2         ATGGCAATGACAGACACGACTCCATTTGGACAACAGCGCGTTTTCAAAGAGAAAGTGGAC 60
West            ATGGCCATGACTGACACCACTCCTTTTGGCCAGCAGCGAGTGTTTAAAGAGAAGGTGGAC 60
JEVSAV          ATGGCCATGACTGACACCACCCCTTTTGGACAGCAAAGAGTTTTCAAGGAGAAAGTTGAC 60
                 * ** ***** ** ** **  * *  ** ** **  * ** ** ** ** ** ** **

LIV             ACAAAAGCACAGGAGCCCCAACCGGGGACAAGAGTGATCACGAGAGCAGTGAATGATTGG 120
TBEV            ACAAAGGCACAGGAGCCTCAGCCCGGTACAAGAGTCATCATGAGAGCTGTAAATGATTGG 120
Langat          ACCAAGGCACAGGAGCCGCAACCCGGGACCAAAATCATCATGAGAGCGGTGAATGACTGG 120
Royal           ACAAAAGCTCAGGAGCCACAACCGGGAACCCGTGTCATCATGCGCTGCGTCAATGATTGG 120
Kadam           ACAAAGGCTCATGAACCCAGGCCAGGCACACAGGTTATAATGCGAGCAACCAACGATTGG 120
Yellow          ACCAGAGCAAAGGATCCACCCGCGGGAACCAGAAAAATCATGAAAGTTGTCAACAGATGG 120
Wesselsbron     ACAAAAGCCCCGCCTCCACCTCCAGGAACACGTGCGATCATGCGTGTTGTCAATGCTTGG 120
Zika            ACCAGGGTGCCAGATCCCCAAGAAGGCACTCGCCAGGTAATGAACATAGTCTCTTCCTGG 120
Dengue2         ACGAGAACCCAAGAACCGAAAGAAGGCACGAAAAAACTAATGAAAATCACGGCAGAATGG 120
West            ACGAAAGCTCCAGAGCCTCCAGAGGGAGTCAAGTACGTCCTCAATGAGACGACAAACTGG 120
JEVSAV          ACGAAGGCTCCTGAGCCACCAGCTGGAGCCAAGGAAGTGCTCAACGAGACCACCAACTGG 120
                ** *           **       **          *                  ***

LIV             ATTCTAGAGCGTCTGGCCCAGAAAAGCAAACCACGCATGTGCAGCAGAGAGGAATTCATA 180
TBEV            ATTTTGGAACGACTGGCGCAGAAAAGCAAACCGCGCATGTGCAGCAGGGAAGAATTCATA 180
Langat          CTGCTTGAGCGACTGGTCAAGAAGAGTCGGCCACGCATGTGCTCCAGGGAGGAGTTCATA 180
Royal           ATGATGGATAGACTGGCAAAAAGGAGCAAGCCGCGCCTGTGCACAAAAGAAGAGTTTTGC 180
Kadam           CTACTTGAGAGACTAGTAAAGAAAAGGAAGCCCAGGATGTGCTCCCGGGAGGAATTCAGA 180
Yellow          CTCTTCCGTCACTTGGCCAGGGAGAAGAATCCTAGACTGTGCACAAAAGAAGAATTCATT 180
Wesselsbron     TTGTTCCAACACCTCGCTCGTAAGAAGAAGCCCCGCATTTGCACGCGTGAAGAGTTTGTG 180
Zika            CTGTGGAAGGAGCTGGGGAAACGCAAGCGGCCACGCGTCTGCACCAAAGAAGAGTTTATC 180
Dengue2         CTCTGGAAAGAATTAGGAAAGAAAAAGACACCTAGGATGTGCACCAGAGAAGAATTCACA 180
West            TTGTGGGCTTACTTAGCTCGAGAGAAAAAACCGAGGATGTGCTCCCGAGAAGAGTTTATC 180
JEVSAV          CTGTGGGCCCACTTGTCACGGGAAAAAAGACCCCGCTTGTGCACCAAGGAAGAATTCATA 180
                 *           *          *     **  *  * ***      ** ** **

LIV             GCAAAAGTGAGATCAAATGCAGCCCTGGGAGCTTGGTCGGATGAGCAGAACAGATGGGCA 240
TBEV            GCAAAAGTGAAATCAAATGCAGCCTTGGGAGCTTGGTCAGATGAGCAAAACAGATGGGCA 240
Langat          GCGAAAGTCCGTTCGAATGCGGCCCTTGGCGCTTGGTCAGATGAGCAAAACAAATGGAAG 240
Royal           GCAAAGGTGAGATCAAATGCAGCGTTGGGAGCATGGACAGATGAGCAGAATATATGGAAG 240
Kadam           GAGAAGGTGCGGTCAAATGCCGCTCTGGGTGCCTGGCTTGATGAGCAGAACCAGTGGAAA 240
Yellow          GCAAAGGTCCGCAGCCACGCAGCCATTGGAGCCTACCTGGAAGAACAAGAACAGTGGAAG 240
Wesselsbron     GCCAAAGTTCGTAGCCATGCCGCCCTTGGAGCGTATCTCGAAGAGCAGGACAAATGGAAG 240
Zika            AACAAGGTGCGCAGCAATGCAGCACTGGGAGCAATATTTGAAGAGGAAAAAGAATGGAAG 240
Dengue2         AGAAAGGTGAGAAGCAATGCAGCCTTAGGTGCCATATTCACTGATGAGAACAAGTGGAAG 240
West            AGTAAAGTCAACAGCAACGCGGCTCTGGGAGCAATGTTTGAGGAACAGAACCAATGGAAA 240
JEVSAV          AAGAAAGTCAACAGCAACGCGGCTCTTGGAGCAGTGTTCGCTGAACAGAATCAATGGAGC 240
                   ** **        * ** **  * ** **          **  *  *    ***

LIV             AGTGCGAGAGAGGCTGTGGTGGTCCCTGCATTCTGGGCCCTCGTGGATGAGGTGAGAGAG 300
TBEV            AGTGCAAGAGAGGCTGTAGAGGATCCTGCATTCTGGCGCCTCGTGGATGAAGAGAGAGAA 300
Langat          AGTGCGAGAGAAGCAGTGGAAGATCCTGAGTTCTGGAGTCTTGTTGAGGCTGAGAGAGAG 300
Royal           AACGCCAGGGAAGCTGTGGAAGACCCAGGATTTTGGAACCTTGTTGATGCTGAGCGTGAG 300
Kadam           ACCGCCCGTGAGGCTGTTGAGGACCCAGCTTTCTGGAATCTTGTTGAGAAAGAACGTGAG 300
Yellow          ACTGCCAACGAGGCTGTCCAGGACCCGAAGTTTTGGGAATTGGTGGATGAAGAAAGGAGG 300
Wesselsbron     AGTGCAAGTGAGGCTGTCCAGGATCCACAGTTCTGGAAACTGGTTGATGATGAGAGGAAG 300
Zika            ACGGCTGTGGAAGCTGTGAATGATCCAAGGTTTTGGGCCCTAGTGGATAGGGAGAGAGAA 300
Dengue2         TCGGCACGTGAGGCTGTTGAAGATAGTGGATTTTGGGAACTGGTTGACAAGGAAAGGAAT 300
West            AATGCCAGAGAGGCTGTAGAAGACCCGAAGTTTTGGGAGATGGTGGACGAGGAGCGCGAG 300
JEVSAV          ACGGCGCGTGAGGCTGTGGATGACCCGCGGTTTTGGGAGATGGTTGATGAAGAGAGGGAA 300
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                    **    ** ** **    *        ** ***    * ** **    *   *

LIV                 AGGCATCTCGTGGGGTGGTGCGCACACTGCGTGTACATCATGATGGGCATGAGAGAAAAG 360
TBEV                AGGCACCTCATGGGGAGATGTGCGCACTGCGTGTACAACATGATGGGCAAGAGAGAAAAG 360
Langat              CGCCACTTGCAGGGGAGATGCGCCCATTGTGTGTACAACATGATGGGGAAAAGAGAGAAA 360
Royal               CTACACAGGCAGGGAAGATGTGCTCAATGCGTATACAACATGATGGGGAAGCGTGAGAAG 360
Kadam               TTGCACCTCACGGGACGTTGCCAGCAGTGCGTGTATAACATGATGGGGAAAAGAGAGAAA 360
Yellow              CTGCACCAACAAGGCAGGTGCCGGACTTGCGTGTACAACATGATGGGGAAAAGAGAGAAG 360
Wesselsbron         CTGCATCTGCAAGGCCAATGCCGGACATGTGTGTACAACATGATGGGGAAACGTGAAAAG 360
Zika                CACCACCTGAGAGGAGAGTGTCACAGCTGTGTGTACAACATGATGGGAAAAAGAGAAAAG 360
Dengue2             CTTCATCTTGAAGGAAAGTGTGAGACATGTGTGTACAACATGATGGGAAAGAGAGAGAAG 360
West                GCTCACCTTCGCGGCGAATGTAACACCTGCATTTACAACATGATGGGCAAGCGTGAGAAG 360
JEVSAV              AACCATCTGCGAGGAGAGTGTCACACATGTATCTACAACATGATGGGAAAAAGAGAGAAG 360
                       **       **    **       **  * ** * ********* *   * ** **

LIV                 AAACTGGGAGAGTTCGGAGTGGCGAAGGGCAGCAGGGCCATCTGGTACATGTGG 414
TBEV                AAACTGGGAGAGTTCGGAGTGGCGAAAGGAAGTCGGGCCATTTGGTACATGTGG 414
Langat              AAGCTTGGTGAATTTGGAGTAGCCAAGGGCAGCAGGGCCATCTGGTACATGTGG 414
Royal               AAGCTGGGAGAATTTGGCTCGGCCAAAGGAAGCAGAGCCATATGGTACATGTGG 414
Kadam               AAACGTGGGGAGTTTGGCGTCGCGAAGGGAAGCCGAGCCATCTGGTACATGTGG 414
Yellow              AAGTTGTCAGAGTTTGGGAAAGCAAAAGGAAGCCGTGCCATCTGGTACATGTGG 414
Wesselsbron         AAACCCTCTGAATTTGGCAAAGCAAAGGGAAGCAGGGCGATCTGGTACATGTGG 414
Zika                AAGCAAGGAGAGTTCGGGAAAGCAAAAGGTAGCCGCGCCATCTGGTACATGTGG 414
Dengue2             AAGCTAGGGGAGTTCGGCAAAGCAAAAGGCAGCAGAGCCATATGGTACATGTGG 414
West                AAACCAGGAGAGTTTGGCAAGGCCAAGGGTAGTAGAGCCATTTGGTTTATGTGG 414
JEVSAV              AAGCCTGGAGAGTTTGGAAAAGCTAAAGGAAGCAGGGCCATTTGGTTCATGTGG 414
                    **       ** ** **    ** ** ** **  * ** ** ****  ******
```
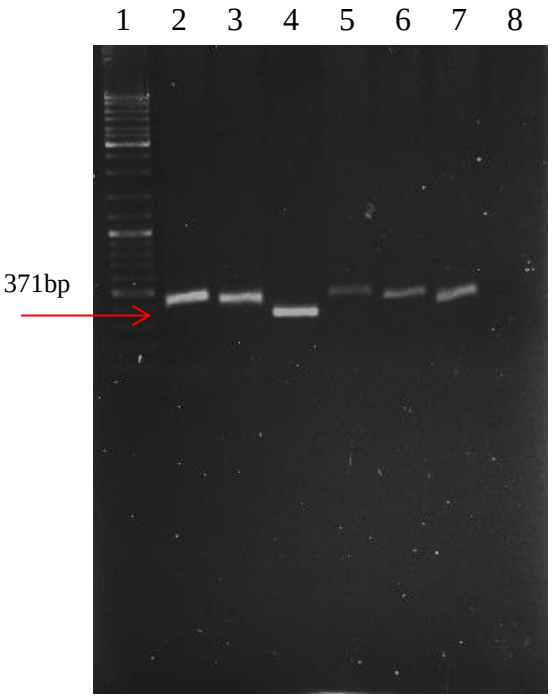

Forward primer is highlighted in yellow ATGGCCATGACTGACAC
Reverse primers are highlighted in blue GCCATCTGGTACATGTGG; TGCGTGTACATCATGATGGGCA; TACAACATGATGGGGAAACG

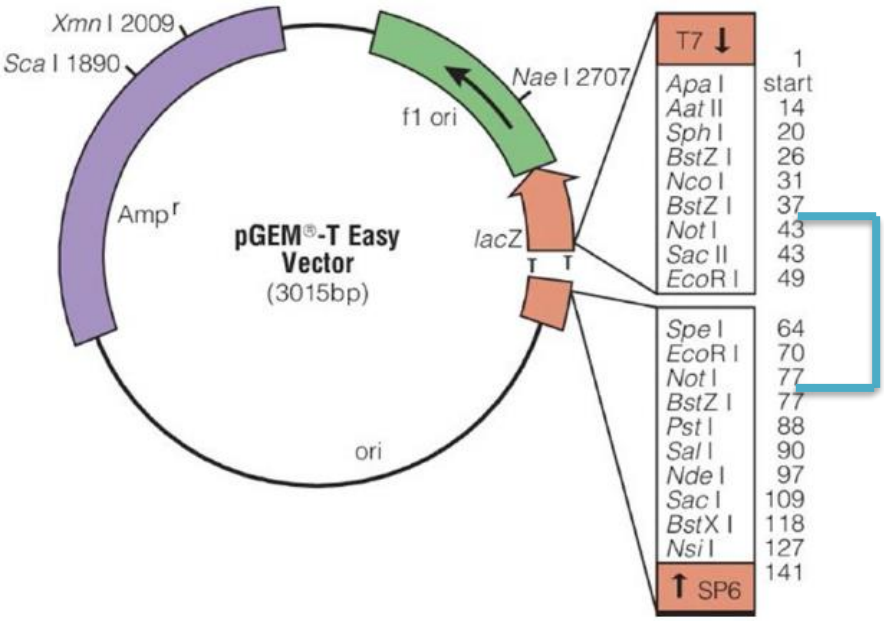
Appendix I: Annealing temperature optimisation of WNV GoTaq® DNA polymerase PCR



A 1% agarose gel electrophoresis showing results for PCR optimisation regarding annealing temperature. Lane 1: O'GeneRuler™ DNA ladder Plus, SM 1173; Lane 2: 42ºC; Lane 3: 43ºC; Lane 4: 44ºC; Lane 5: 46ºC; Lane 6: 48ºC; Lane 7: 50ºC; Lane 8: Negative control.

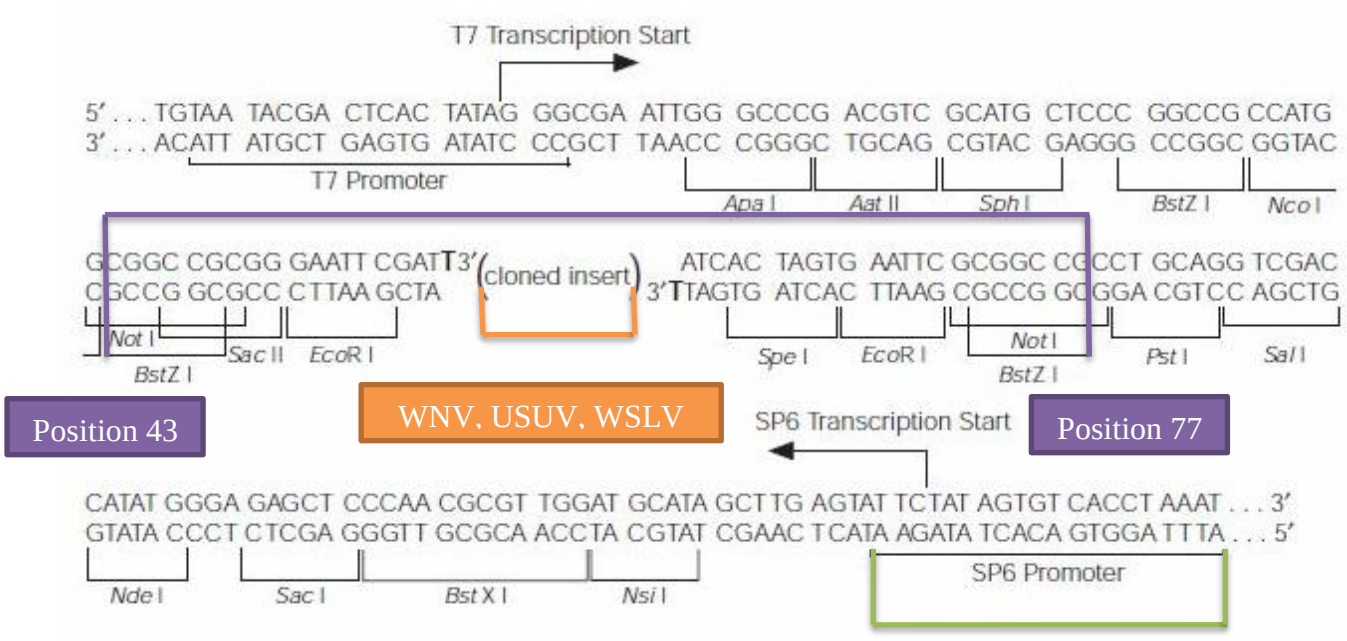
Appendix J: The vector map and multiple cloning sites of pGEM®-T easy vector.

A: Vector map

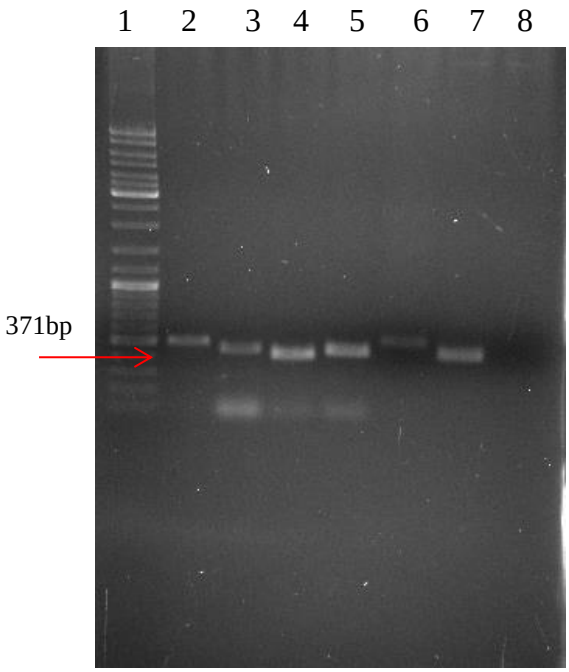


NotI endonuclease was used to digest pGEMWNV, pGEMUSUV and pGEMWSLV

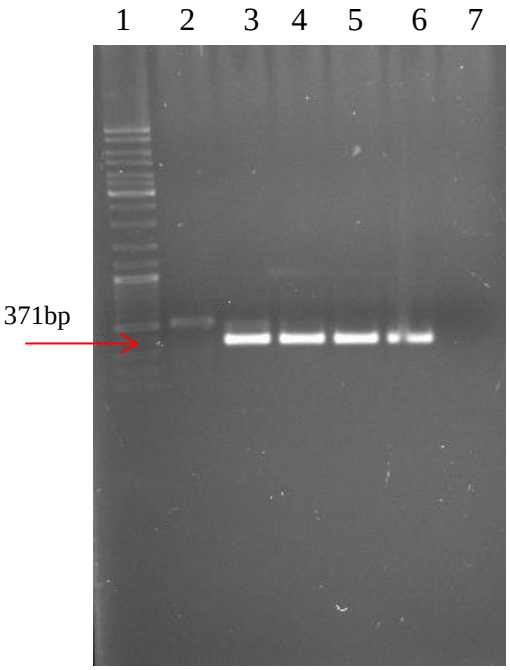
B: Multiple cloning sites



Appendix K: Optimisation of annealing temperature and final primer concentration for RT-PCR



A 1% agarose gel electrophoresis analysis of the optimisation of RT-PCR regarding annealing temperature. Lane 1: O'GeneRuler™ DNA ladder Plus, SM 1173; Lane 2: 42ºC; Lane 3: 44ºC; Lane 4: 46ºC; Lane 5: 48ºC; Lane 6: 50ºC; Lane 7: 52ºC; Lane 8: Negative control.



A 1% agarose gel electrophoresis analysis of the optimisation of RT-PCR regarding final primer concentration. Lane 1: O'GeneRuler™ DNA ladder Plus, SM 1173; Lane 2: 100nM; Lane 3: 200nM; Lane 4: 400nM; Lane 5: 600nM; Lane 6: 800nM; Lane 7: Negative control.

Appendix L: Nucleotide sequences for PCR products of WNV, USUV and WSLV

The Basic Local Alignment Search Tool (BLAST) is an algorithm that was optimized to compare sequences and search sequence databases for optimal local alignments to a query (Altschul et al., 1990). The Except value, also known as the E-value, is a parameter that describes the amount of hits one can observe when searching a database of a particular size. The lower the E-value, the more significant the score and the alignment. The score (S-value) is the extent to which nucleotide sequences are related and is expressed as percentage sequence identity (Jones et al., 2002).

WNV DNA template

5'AGATGTGCCTCGCGCTCCTCATCCACCATCTCCCAAAATTTTGGATCTTCAACT
GCTTCTCTGGCGCTCCTCCATTGATTCTGCTCTTCAAACATGGCACCCAAAGCTGC
ATTGCTGTTGACCTTTCTTATGAATTCCTCTCGAGAGCACATTCTGGGACGTTTTT
CTCTGGCCAAAAACGCCCACAACCAGTTGGTGGTCTCGTTGAGCACGTACTTCAC
TCCTTCTGGCGGTTCAGGAGCTTTCGTGTCCACCTTCTCTTTGAACACTCGCTGCT
GCCCGAAGGGAGTAGTGTCAG'3

The sequence data retrieved for WNV had a 100% identity to isolate AVA1506113 (Genbank accession number: MF175875.1), an E-value of $5e^{-152}$ and a query cover of 100%.

USUV DNA template

5'TTCAGATGGTTCTCCCTTTCTTCGTCCACCATTTCCCAGAACCGAGGGTCCTCTA
CAGCCTCCCTGGCACTGCTCCATTGGTTCTGCTCTTCAAACATGGCTCCCAAAGC
AGCGTTGCTGTTGACCTTCCTCTTAAACTCTTCCCTGGTGCACAACCTTGGCTTCT
TTTCTCGTGCGAGAAAAGCCCACAGCCAATTGGTGGTCTCATCCATCACCTCTCT
AACTCCAGAAGGGGGTTCCGGGGCCTTGGTGTCAACCTTTTCTTTGAAAACCCTC
TGCTGCCCAAACGGAGTGGTGTCAGTCATCGCCAT'3

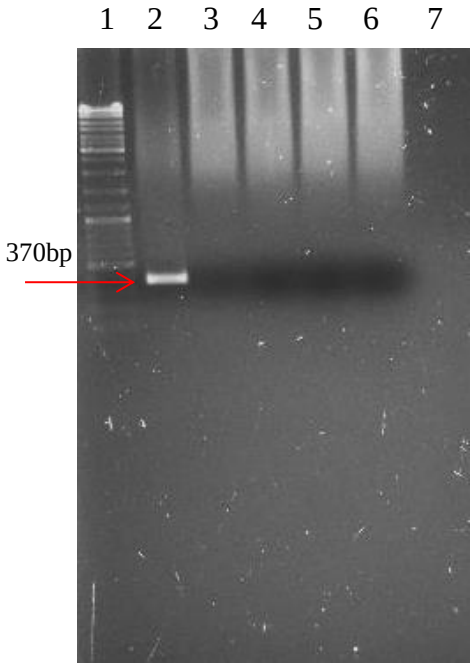
The sequence data retrieved for USUV had a 100% identity to isolate V24 (Genbank accession number: KJ438752.1), an E-value of $3e^{-160}$ and a query cover of 100%.

WSLV DNA template

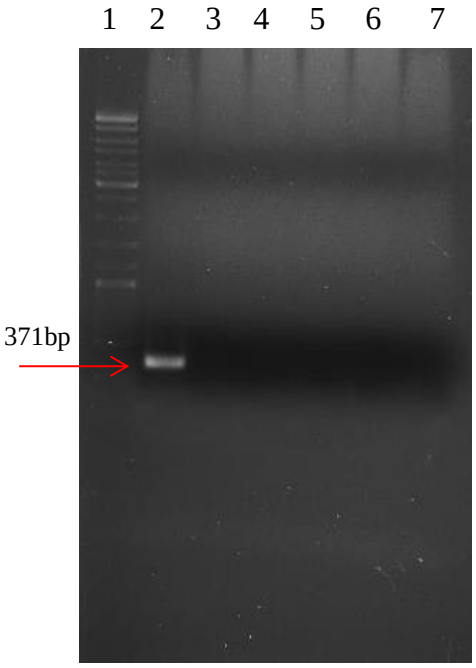
5’GCATTGGCCTTGCAGATGCAGTTTCCTCTCATCATCAACCAGTTTCCAGAACTG
TGGATCCTGAACAGCCTCACTTGCACTCTTCCATTTGTCCTGCTCTTCCAGATACG
CTCCGAGGGCGGCATGGCTTCGAACCTTAGCCACGAACTCTTCGCGTGTGCAAAT
GCGGGGCTTCTTCTTGCGAGCCAAGTGTTGAAACAACCAGGCATTAACGACGCG
CATGATTGCACGTGTTCCTGGAGGTGGAGGCGGGGCTTTTGTGTCCACTTTCTCTT
TAAACACCCTTTGCTGCCCAAACGGAGTCGTGTCAGTCATGGCCA’3

The sequence data retrieved for WSLV had a 100% identity to strain AV259 (Genbank accession number: JN226796.1), an E-value of $3e^{-165}$ and a query cover of 100%.

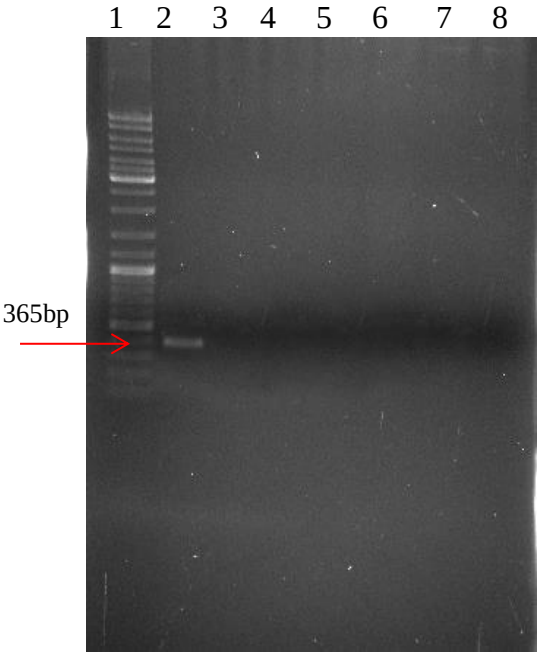
Appendix M: Gel electrophoresis analysis of the sensitivity levels of RT-PCR



Gel electrophoresis analysis of USUV RT-PCR sensitivity results. Lane 1: O'GeneRuler™ DNA ladder Plus, SM 1173; Lane 2: 10^5 copies of RNA; Lane 3: 10^4 copies of RNA; Lane 4: 10^3 copies of RNA; Lane 5: 10^2 copies of RNA; Lane 6: 10^1 copies of RNA; Lane 7: Negative control.



Gel electrophoresis analysis of WNV RT-PCR sensitivity results. Lane 1: O'GeneRuler™ DNA ladder Plus, SM 1173; Lane 2: 10^5 copies of RNA; Lane 3: 10^4 copies of RNA; Lane 4: 10^3 copies of RNA; Lane 5: 10^2 copies of RNA; Lane 6: 10^1 copies of RNA; Lane 7: Negative control.



Gel electrophoresis analysis of WSLV RT-PCR sensitivity results. Lane 1: O'GeneRuler™ DNA ladder Plus, SM 1173; Lane 2: 10^6 copies of RNA; Lane 3: 10^5 copies of RNA; Lane 4: 10^4 copies of RNA; Lane 5: 10^3 copies of RNA; Lane 6: 10^2 copies of RNA; Lane 7: 10^1 copies of RNA; Lane 8: Negative control.

Appendix N: Nucleotide sequences for RPA products of WNV, USUV and WSLV

WNV RPA product

5’TGTGCCTCGCGCTCCTCATCCACCATCTCCCAAAATTTTGGATCTTCAACTGCTT
CTCTGGCGCTCCTCCATTGATTCTGCTCTTCAAACATGGCACCCAAAGC’3

The sequence data retrieved for WNV had a 100% identity to isolate AVA1506113 (Genbank accession number: MF175875.1), an E-value of $1e^{-45}$ and a query cover of 100%.

USUV RPA product

5’CTTTCTTCGTCCACCATTTCCCAGAACCGAGGGTCCTCTACAGCCTCCCTGGCA
CTGCT CCATTGGTTCTGCTCTTCAAACATGGCTCCCAAAGC’3

The sequence data retrieved for USUV had a 100% identity to isolate V24 (Genbank accession number: KJ438752.1), an E-value of $9e^{-45}$ and a query cover of 100%.

WSLV RPA product

5’TGCAGATGCAGTTTCCTCTCATCATCAACCAGTTTCCAGAACTGTGGATCCTGA
ACAGCC TCACTTGCACTCTTC’3

The sequence data retrieved for WSLV had a 100% identity to isolate AV259 (Genbank accession number: JN226796.1), an E-value of $8e^{-30}$ and a query cover of 100%.

Appendix O: Composition of media, buffers and solutions used

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

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

2. **Luria Bertani broth/agar plates (1L) with ampicillin**

Dissolve 10g Bacto-Tryptone, 5g Bacto-Yeast extract and 10g NaCI in 900ml H_2O. Adjust the pH to 7 .0 with 10 M NaOH (app. 200 µL) and adjust the volume to 1 literwith deionized H_2O. Sterilize by autoclaving and allow cooling down. Add 100mg/ml of ampicillin and store at 4°C.

Agar plates were prepared by the addition of 15g of Bacteriological-agar per 1L liquid media. After sterilization by autoclaving, media was allowed to cool and the ampicillin was added, sterile plates were poured and stored in an inverted position at 4°C after solidification.

3. **50 X *Tris-acetate-EDTA* (TAE) stock**
   - Tris-base: 242g
   - Acetate (100% acetic acid): 57.1ml
   - EDTA: 100ml (0.5M sodium EDTA)
   - Add dH_2O to one liter.

4. **1X TAE (pH 8.0)**
   - Dilute 20ml of 50X stock into 980ml dH_2O

5. **1% Agarose gel**

Weigh one gram of Seakem® LE agarose powder and mix with 100ml of 1XTAE buffer pH 8. Heat mixture in a microwave oven until dissolved. Wait until cool.

6. **1 M IPTG (Isopropyl β –D -1 thiogalactopyranoside)**
   - Dissolve 2.38g of IPTG in 8ml distilled H_2O
   - Top-up to 10ml with distilled H_2O
   - Filter sterilize with a 0.22µ syringe filter
   - Store 1ml aliquots at -20°C