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DEVELOPMENT AND APPLICATION OF MOLECULAR ASSAYS FOR MOSQUITO-BORNE ALPHAVIRUSES IN SOUTH AFRICA

Micah Dimaculangan

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HEALTH SCIENCES

UFS

DEVELOPMENT AND APPLICATION OF MOLECULAR ASSAYS FOR MOSQUITO-BORNE ALPHAVIRUSES IN SOUTH AFRICA

by

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Declaration

"I, Micah Dimaculangan, declare that the dissertation hereby submitted to the University of the Free State for the degree Master of Medical Science (Virology) is my own work and has not previously been submitted to any other institution of higher education for a degree."

Signature:  Date: 13 December 2021

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“No one who achieves success does so without acknowledging the help of others.” - Alfred North Whitehead

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Abstract

Surveillance of mosquito-borne alphaviruses is critical for the prevention of diseases and the control of outbreaks caused by these viruses, especially with the absence of approved vaccines and antiviral treatments available. Hence, the continual development of rapid and reliable tools for the surveillance of alphaviruses is important. This will aid in the understanding of which viruses are currently circulating with the potential to cause outbreaks. Molecular nucleic acid amplification tests (NAATs), particularly conventional and real-time reverse transcription (RT)-polymerase chain reaction (PCR), are typically employed in epidemiological surveys. In this study, a conventional nested RT-PCR assay was developed to detect alphaviruses in South Africa. In addition, an isothermal amplification technique, specifically a RT-helicase dependent amplification (HDA) assay, which only requires a simple heating device, for instance a heating block, and lateral flow dipsticks/ cassettes for end point detection, was developed to detect alphaviruses currently circulating in South Africa, as an alternative to the RT-PCR assay for application in low resource settings or for field application. The conventional nested RT-PCR assay was able to detect ≥620 copies of RNA compared to the RT-HDA assay which had a minimum limit of detection of 4.8×10^5 copies of RNA. Both assays were tested for theoretical cross-reactivity with other alphaviruses, which include Sindbis virus (SINV) and chikungunya virus (CHIKV) isolates from other regions and genotypes, and isolates from alphaviruses such as Ross River virus (RRV), Barmah Forest virus (BFV), Mayaro virus (MAYV), eastern equine encephalitis virus (EEEV), Venezuelan equine encephalitis virus (VEEV) and western equine encephalitis virus (WEEV) that are endemic to other parts of world. Alignment of the primers with the sequences of these isolates shows that both assays in theory would be able to detect SINV isolates from northern Europe, taking into account the transcontinental transmission of the virus between South Africa and northern Europe by migratory birds. The conventional nested RT-PCR assay may be able to detect most alphaviruses due to minimal mismatches (0 – 1) detected between the primers and the partial nsP4 sequences of the alphavirus isolates, while the RT-HDA assay may not be well suited to detect other alphaviruses due to the many mismatches (>4) detected between the primers and the partial nsP4 sequences of the alphavirus isolates. Nevertheless, this shows that the RT-HDA is theoretically more specific that the conventional nested RT-PCR assay. The RT-HDA however failed to detect any alphaviruses in the 42 mosquito pools tested, which was not unexpected as the assay could

only detect up to 4.8 x 10^5 copies of RNA. In contrast, the conventional nested RT-PCR assay was able to detect alphaviral RNA in five out of the 42 mosquito pools tested, and the nucleotide sequences were determined to identify the alphavirus species. SINV RNA was detected in three mosquito pools and Middelburg virus (MIDV) was detected in two pools. Phylogenetic analysis was subsequently performed to determine the genetic relationship of these isolates from the Free State with previously published/ reported SINV and MIDV isolates in South Africa, Africa, and around the world.

The conventional nested RT-PCR assay developed in this study has shown to be a useful surveillance tool for the detection of mosquito-borne alphavirus infections. Given the low sensitivity determined for the RT-HDA assay, improvements, or alternative rapid and fieldable NAATs should be considered in the future for alphavirus surveillance applications in low resource settings.

Keywords: Surveillance, detection, mosquito-borne alphaviruses, RNA, RT-PCR, RT-HDA, sensitivity, theoretical specificity, phylogenetic analysis, South Africa

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List of Abbreviations

µg	Micrograms
µl	Microlitres
µM	Micromolar
AuNPs	Gold nanoparticles
Ae.	*Aedes*
amp	Ampicillin
ATP	Adenosine triphosphate (ATP)
BFV	Barmah Forest virus
BHK	Baby hamster kidney
CHIKV	Chikungunya virus
CO_2	Carbon dioxide
CTP	Cytidine triphosphate
Cx.	*Culex*
dH_20	Distilled water
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide triphosphates
ECSA	East/Central/South African
EDTA	Ethylene-diamine-tetra-acetic acid
EEEV	Eastern equine encephalitis virus
ELISA	Enzyme-linked immunosorbent assays
GTP	Guanosine triphosphate
HDA	Helicase dependent amplification
ICTV	International Committee on Taxonomy of Viruses
IFA	Indirect fluorescent antibody
IgG	Immunoglobulin G

IgM	Immunoglobulin M
iiPCR	Insulated isothermal polymerase chain reaction
LAMP	Loop-mediated amplification
LB	Luria Bertani
mAbs	Monoclonal antibodies
MAC-ELISA	IgM antibody capture ELISA
MAYV	Mayaro virus
MDA	Multiple displacement amplification
$MgSO_4$	Magnesium sulphate
MIDV	Middelburg virus
ml	Millilitre
mM	Millimolar
NAATs	Nucleic acid amplification tests
NaCl	Sodium chloride
NASBA	Nucleic acid sequence-based amplification
NC	Nucleocapsid
NDUV	Ndumu virus
ng	Nanogram
nsP	Nonstructural protein
ONNV	O'nyong-nyong virus
pAbs	Polyclonal antibodies
PCR	Polymerase chain reaction
pfu	Plaque forming units
RCA	Rolling circle amplification
RNA	Ribonucleic acid
RPA	Recombinase polymerase amplification
RRV	Ross River virus
RT-HDA	Reverse transcription (RT)-helicase dependent amplification (HDA)
RT-LAMP	Reverse transcription (RT)-loop-mediated amplification (LAMP)

RT-PCR	Reverse transcription (RT)-polymerase chain reaction (PCR)
RT-RPA	Reverse transcription (RT)-recombinase polymerase amplification (RPA)
SAMRS	Self-avoiding molecular recognition system
SDV	Sleeping disease virus
SESV	Southern elephant seal virus
SFV	Semliki Forest virus
SINV	Sindbis virus
SMART	Signal-mediated amplification of RNA technology
SOC	Super optimal broth with catabolite
SPDV	Salmon pancreas disease virus
TAE	Tris-acetate-EDTA
$TCID_{50}$	Median Tissue Culture Infectious Dose
U	Units
UTP	Uridine triphosphate
VEEV	Venezuelan equine encephalitis virus
WEEV	Western equine encephalitis virus
WNV	West Nile virus

CHAPTER 1: LITERATURE REVIEW

1.1 Introduction

Emerging viruses in recent years are most frequently RNA arboviruses or zoonotic viruses which are transmitted between animals and humans. Arboviruses (arthropod-borne viruses) are transmitted to vertebrate hosts by hematophagous (blood-feeding) arthropod vectors, such as mosquitoes and ticks. The term arbovirus is not a taxonomic classification, but rather a term to describe the virus' requirement for an arthropod vector in their transmission cycle (Liang et al., 2015; Velazquez-Salinas et al., 2016). Among vector-borne outbreaks, mosquito-borne outbreaks occur with the highest frequency. Some examples included Zika virus (genus *Flavivirus*) and chikungunya virus (genus *Alphavirus*), both responsible for recent outbreaks.

Global distribution, emergence, re-emergence, and increased prevalence of alphaviruses may be attributed to several factors, which include virus evolution, the wide range of mosquito vectors and host reservoirs, climate change, deforestation, the adaptation of mosquito vectors to new ecological niches, increased urbanization, international travel and trade, and relocation of viraemic vertebrates, such as birds (Morens et al., 2004; Suhrbier et al., 2012; Lwande et al., 2015). Therefore, active surveillance of alphavirus infection is important and will aid in the understanding of which viruses are currently circulating with the potential to cause outbreaks.

1.2 History of alphavirus infections in South Africa

1.2.1 Sindbis virus (SINV)

SINV was initially isolated in Sindbis, a village in Egypt, in 1952 from *Culex pipiens* and *Cx. univittatus* mosquitoes (Taylor et al., 1955). Symptoms of human infection of the virus were not well known until 1961 when the virus was isolated from a patient in Uganda with a febrile disease (Haddow, 1961).

In February 1954, strain AR 86 was isolated from a mosquito pool of *Cx.* spp. mosquitoes collected in Springs near Johannesburg, South Africa. Strains AR 166 and AR 169 were isolated from mosquitoes collected in March 1954 at Isis Estates, with one mosquito pool consisting of 12 *Cx. univittatus* and another mosquito pool consisting of two *Cx. tigripes* and one *Cx. Annulioris* (Weinbren et al., 1956). Investigations were carried out after receiving reports of febrile disease in humans and infection in cattle.

In South Africa, human infections of SINV and West Nile virus (WNV) occur sporadically during the summer across the central plateau region, which includes the Free State, Gauteng, and Northern Cape provinces (Jupp et al., 1986).

SINV was isolated from a patient's skin lesion in 1963 in Johannesburg (Malherbe et al., 1963). The patient presented with febrile illness, malaise, a maculopapular rash, and exhibited joint and tendon pain. Up until 1974, the cases reported were predominantly from the Free State and Gauteng provinces (McIntosh et al., 1976)

The largest epidemics of both SINV and WNV ever recorded in South Africa began in early 1974, with thousands of human infections reported from the Karoo and the Northern Cape Province. From December 1983 until April 1984, hundreds of human SINV infections were reported in the Gauteng. Infection rates for *Cx. univittatus* that were collected during 1983 and 1984 were higher in comparison to those of the previous year, which were attributed to the unusually high temperatures and rainfall recorded in the summer throughout the mosquito season (Jupp et al., 1986).

Surveillance of SINV infection in South Africa between 2006 and 2010 reported the annual occurrence of the virus in humans throughout most of the country but more frequently in the Free State, Gauteng, and Northern Cape provinces. From 2006 – 2009, of the 1606 samples submitted, 21 (1.3%) tested positive for IgM antibodies. In 2010, of the 2025 samples submitted, 208 (10.3%) tested positive for IgM antibody. The increase in reported cases in 2010 accounts for the above-average rainfall providing favourable environments for mosquito breeding (Storm et al., 2013).

In a surveillance study conducted between January 2008 – December 2013, 623 horses presenting with febrile illness and neurological disease were investigated. Of the 623 samples submitted, eight (1.3%) tested positive for SINV, of which three died from neurologic disease. Co-infection of SINV with WNV was reported in two of the three horses that died (van Niekerk et al., 2015).

In a related study, 608 samples collected from animals other than horses that had undiagnosed neurologic, febrile, and respiratory disease or that had died suddenly or unexpectedly during February 2010 – September 2018, were tested for alphavirus infection. Of the 608 animal samples submitted, 32 (5.5%) were identified as alphavirus infections, of which 9 (1.5%) tested positive for SINV. Sudden unexpected deaths were reported in two SINV positive animals, namely a buffalo and a blesbuck (Steyn et al., 2020).

1.2.2 Middelburg virus (MIDV)

MIDV was initially isolated in 1957 in Middelburg in the Eastern Cape in South Africa from *Aedes* mosquitoes during an outbreak of disease among sheep. Two MIDV isolates were reported: isolate AR749 from *Aedes caballus* and isolate AR747 from other *Aedes* mosquitoes (Kokernot et al., 1957). Subsequent positive reactions for MIDV in livestock and humans were obtained from serological tests conducted in KwaZulu-Natal in South Africa (Kokernot et al., 1961; Smithburn et al., 1959). In 1993, MIDV was isolated from a horse in Zimbabwe, a neighbouring country of South Africa, after the horse succumbed to a fatal illness resembling African horse sickness (Attoui et al., 2007).

In a surveillance study (previously mentioned) conducted in South Africa between January 2008 and December 2013, 44/623 horses (7.1%) presenting with unexplained febrile and acute neurologic infections tested positive for MIDV, of which 28 had neurologic disease, 16 had febrile disease, and 12 died (van Niekerk et al., 2015).

In a related a study (previously mentioned) conducted between February 2010 and September 2018, 32/608 samples (5.5%) collected from animals other than horses presenting with unsolved febrile illness and neurological disease, or respiratory signs or unexplained deaths

were identified as alphavirus infections, of which 23 (3.8%) tested positive for MIDV. A sudden unexpected death was reported in a MIDV positive waterbuck (Steyn et al., 2020).

1.2.3 Ndumu virus (NDUV)

NDUV was first isolated in 1959 from *Mansonia uniformis* (Theobald) mosquitoes captured in KwaZulu-Natal, South Africa (Kokernot et al., 1961) and later from *Aedes mcintoshi* and *Aedes ochraceus* mosquitoes in Kenya (Crabtree et al., 2009).
Very little is known about the virus and its vertebrate hosts. Antibodies in humans from eight rural locations were detected, but no known association with disease in humans (Karabatsos, 1985; Kokernot et al., 1961). In South Africa, the mosquitoes from which NDUV was obtained were *Aedes circumluteolus* and *Mansonia uniformis* (Kokernot et al., 1961).

1.2.4 Chikungunya virus (CHIKV)

CHIKV was first described following an outbreak in Tanzania in 1952-1953 (Lumsden, 1955). The name "chikungunya", meaning "that which bends up", was derived from the distorted postures of patients with the viral infection as a result of acute joint pains (Robinson, 1955). The virus has since been implicated in large-scale outbreaks all over the globe. McIntosh stated in a 1975 memoir that CHIKV has tropical distribution in southern Africa, based on antibody surveys in wild primates and humans in Mozambique, Botswana, Namibia, Zimbabwe and South Africa (McIntosh, 1975). While human outbreaks of CHIKV infection have been reported in South Africa in 1957 (Gear & Reid, 1957), 1975/1976 (McIntosh et al., 1977) and 1977 (Fourie & Morrison, 1979; Morrison, 1979), there has been no recent evidence to suggest that the virus circulates in South Africa, although there is potential for re-emergence (Burt et al., 2014).

1.3 Classification of alphaviruses

According to the International Committee on Taxonomy of Viruses (ICTV), 32 species of alphaviruses have been recognised (Chen et al., 2018). Alphaviruses can be classified into antigenic complexes based on their serological cross-reactions (Calisher & Karabatsos, 1988). Originally, seven antigenic complexes were recognised, namely Barmah Forest (BF), Semliki Forest (SF), Middelburg (MID), Ndumu (NDU), eastern equine encephalitis (EEE), western equine encephalitis (WEE), and Venezuelan equine encephalitis (VEE) complexes (Powers et al., 2001). "Trocara" was later recognised as the eighth complex following the discovery of the Trocara virus from mosquitoes in the Amazon jungle (Travassos da Rosa et al., 2001). Today, fish and seal-specific alphaviruses, namely sleeping disease virus (SDV), southern elephant seal virus (SESV), and salmon pancreas disease virus (SPDV), are also recognised as antigenic complexes, forming 11 in total (Chen et al., 2018; Kuhn, 2013).

Alphaviruses have also been described as Old World and the New World alphaviruses (Strauss & Strauss, 1994), based on phylogenetic analysis, geographical distribution and clinical manifestation. Old World alphaviruses are distributed across Europe, Asia, Australia, and Africa. This group includes Barmah Forest virus (BFV), MIDV, NDUV, CHIKV, SINV, Ross River virus (RRV), o'nyong-nyong virus (ONNV) and SFV. These viruses are rarely fatal and are mainly associated with infections typically characterised by malaise, rash, arthritis, and myalgia (Ryman & Klimstra, 2008). New World alphaviruses are distributed across the western hemisphere (the Americas) and are associated with encephalitis (Zacks & Paessler, 2010). This group includes western equine encephalitis virus (WEEV), eastern equine encephalitis virus (EEEV), and Venezuelan equine encephalitis virus (VEEV). The WEE complex contains both Old world and New world alphaviruses, such as SINV and WEEV, respectively (Weaver et al., 1997).

Although Mayaro virus (MAYV) is solely endemic to South America, this virus is suggested to have Old World origin based on symptoms of infection and phylogenetic analysis of the E1 gene (Lavergne et al., 2006).

1.4 Structure and genome of alphaviruses

Alphavirus virions (Figure 1.1 (A)) are small, spherical and icosahedral-shaped, enveloped particles approximately 70 nm in diameter (Fuller, 1987; Mancini et al., 2000; Morgan et al., 1961). The virus particle consists of a nucleocapsid (NC) core surrounding the RNA genome. The NC is enclosed within a lipid bilayer envelope that is embedded with 240 heterodimers assembled into 80 glycoprotein spikes consisting of three E1/E2 heterodimers (Rice & Strauss, 1982; Vogel et al., 1986; Wahlberg et al., 1989). Both the interior protein capsid shell and the exterior glycoproteins have T=4 icosahedral symmetry (Fuller, 1987; Paredes et al., 1993). The E2 glycoprotein contains major epitopes to which neutralization antibodies bind, and also mediates the attachment of the virus to the host cell receptors (Dalrymple et al., 1976; Ubol & Griffin, 1991; Wang et al., 1991), while the E1 glycoprotein includes a fusion domain which facilitates membrane penetration of NC from endosomes into the cytoplasm (Garoff et al., 1980; Rice & Strauss, 1981).

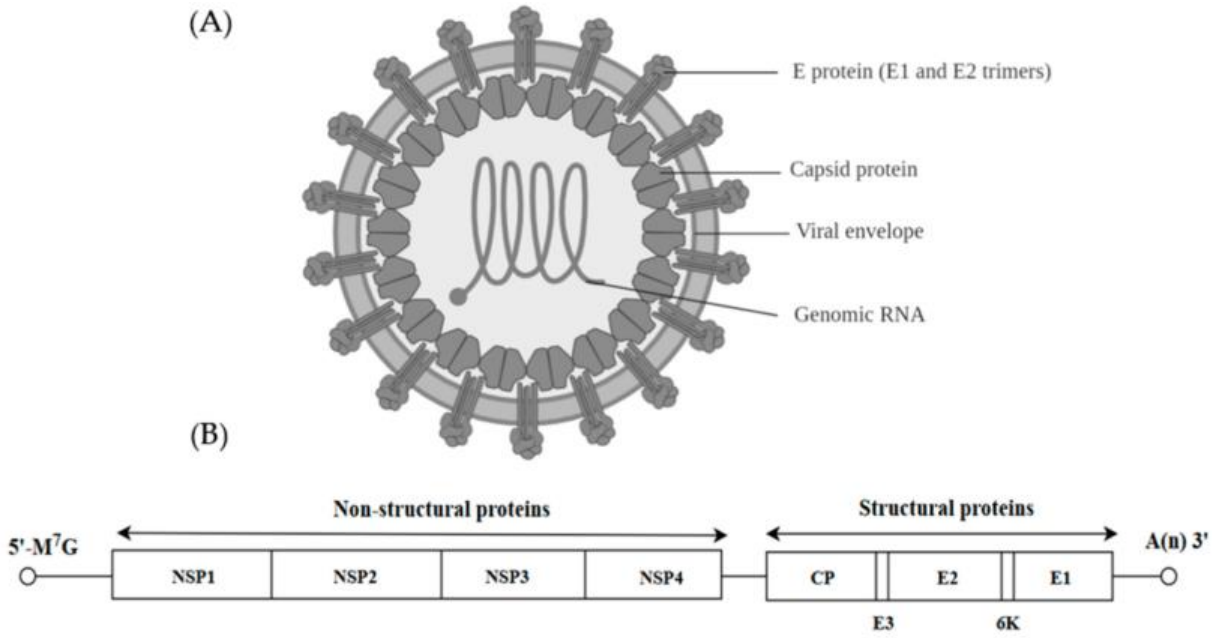

Figure 1.1: The structure and genome of alphaviruses. (A) The alphavirus structure showing the position of the E protein, capsid protein, viral envelope, and genomic RNA. (B) The alphavirus genome showing the 5′ and 3′ untranslated regions, and the nonstructural proteins and structural proteins. | This material is in the public domain – see Appendix C

The alphavirus genome (Figure 1.2 (B)) is a single-stranded, positive-sense RNA genome approximately 11 – 12 kb in length, containing a 5' 7-methyl-G cap and a 3' poly(A)-tail (Cancedda & Shatkin, 1979; Simmons & Strauss, 1972; Strauss et al., 1984).
The genome consists of two open reading frames (ORFs) which encode for nonstructural and structural proteins (Strauss & Strauss, 1994). The first ORF, encompassing two-thirds of the genome from the 5' terminus, encodes the nonstructural proteins, while the second ORF, encompassing one-third of the genome from the 3' terminus, encodes the structural proteins. The nonstructural proteins are translated as one or two polyproteins (P1234, or P123 and P1234) from genomic RNA (gRNA). These polyproteins are cleaved to produce four nonstructural proteins (nsP1 – nsP4) and their cleavage intermediates which are required for replicating the viral genome. The structural domain is translated as a polyprotein from the 26S subgenomic RNA (sgRNA). The structural polyprotein is then cleaved to produce five structural proteins (C, E3, E2, 6K and E1) and their cleavage intermediates which are required for the particle assembly and attachment and penetration into host cells (Strauss & Strauss, 1994).

1.5 Replication of alphaviruses

For alphaviruses to infect host cells, receptor-mediated endocytosis as well as a low pH triggered membrane fusion reaction, which releases the nucleocapsid into the cytoplasm, is required. Viral RNA replication and protein synthesis occurs in the cytoplasm. Envelope proteins are transported from the cytoplasm via the secretory pathway to the plasma membrane where virus budding takes place (Ooi et al., 2015). Alphavirus prototypes SINV and SFV were used to study the replication of alphaviruses (Kääriäinen & Ahola, 2002; Strauss & Strauss, 1994).

Infection of cells begin with the binding of the virus to host receptors (Figure 1.2). This is primarily mediated by the E2 glycoprotein (Salminen et al., 1992; Smith et al., 1995).
Thereafter, clathrin-mediated endocytosis of the virus occurs (Helenius et al., 1980; Marsh et al., 1983). The low pH of the endosome activates a number conformational changes, causing E2/ E1 dimer dissociation and allowing the rearrangement of the E1 homotrimer (Justman et al., 1993; Kielian & Helenius, 1985). This triggers the membrane fusion reaction between the viral and cell membranes (Wahlberg et al., 1992; Wahlberg & Garoff, 1992), depositing the

virus's NC into the cytoplasm, which disassembles, exposing the viral RNA for translation (Helenius, 1984). The gRNA is directly translated to yield one or two nonstructural polyproteins, depending on the virus (Strauss & Strauss, 1994). The polyproteins are cleaved by the virus-encoded protease into the individual nonstructural proteins, constructing a replication complex to replicate the gRNA through a negative-strand RNA intermediate (Kujala et al., 2001; Lemm et al., 1994). When complete cleavage to produce nsP1 – nsP4 is achieved, negative-strand synthesis is inactivated, switching to positive strand gRNA and sgRNA synthesis (Lemm et al., 1994; Shirako & Strauss, 1994).

The sgRNA is translated to yield a polyprotein precursor of the structural proteins (Raju & Huang, 1991). The capsid protein (Cp) is first to be translated and autoproteolytically cleaves itself from the rest of the polyprotein (Aliperti & Schlesinger, 1978; Hahn & Strauss, 1990; Melancon & Garoff, 1987). The Cp packages and assembles the gRNA into NC-like particles (Owen & Kuhn, 1996; Weiss et al., 1989). The remaining polyprotein is subsequently translocated to the endoplasmic reticulum (ER) by transmembrane domains and signal sequences, where it is processed for cleavage and maturation of the structural proteins (Lobigs et al., 1990; Melancon & Garoff, 1987; Sanz et al., 2003). Mature glycoproteins are produced and transported from the ER to the cell surface via the Golgi complex (De Curtis & Simons, 1988; Green et al., 1981; Sariola et al., 1995). Interactions between the NCs and the cytoplasmic domain of the E2 protein drive the budding process, with E1/E2 heterodimers forming an envelope around the NC-like particles (Metsikkö & Garoff, 1990; Owen & Kuhn, 1997; Vaux et al., 1988). Lastly, the virions acquire a membrane lipid bilayer from the host cell upon release from the cell (Acheson & Tamm, 1967; Fuller, 1987; Laine et al., 1973; Vogel et al., 1986).

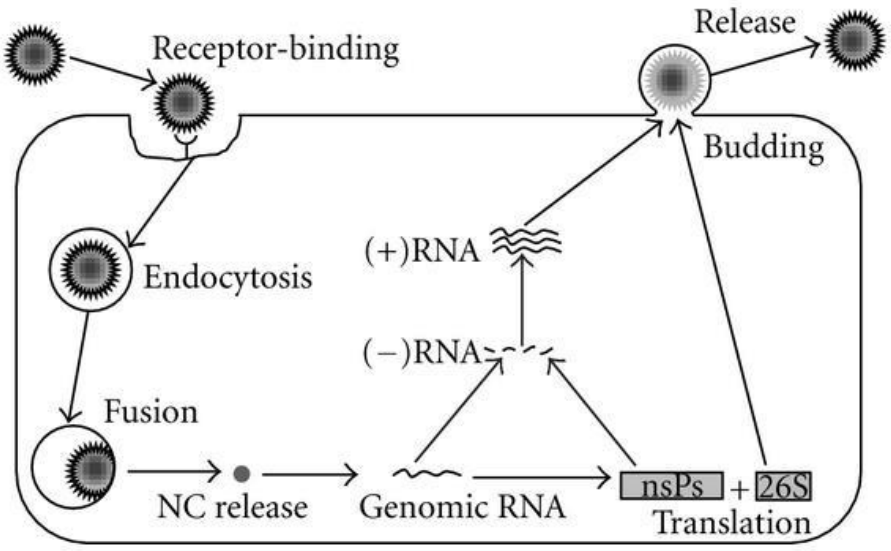

Figure 1.2: The replication of alphaviruses (Leung et al., 2011) | See Appendix C for permission to use this material

1.6 Epidemiology, vectors, and transmission

1.6.1 Widely distributed alphaviruses

1.6.1.1 Chikungunya virus (CHIKV)

CHIKV is associated with explosive epidemics. Outbreaks are linked to viral transmission via *Aedes* mosquitoes that have a wide geographical distribution (Figure 1.3), and since humans serve as amplification hosts, CHIKV has successfully spread via international travel (Suhrbier et al., 2012; Weaver et al., 2012).

Following its discovery in 1952, numerous subsequent outbreaks and epidemics were reported in Africa and South East Asia (Caglioti et al., 2013; Powers et al., 2000). The re-emergence of CHIKV occurred in 2004 when a major outbreak in Kenya (Powers & Logue, 2007; Sergon et al., 2008) initiated a rapid, worldwide dissemination of the virus from Africa to surrounding islands in the Indian Ocean (WHO, 2006), particularly the Réunion Island, to counties in the east which include India (infecting more than 1.5 million people) and Southeast Asia (Charrel et al., 2007; Schwartz & Albert, 2010).

CHIKV was subsequently reported in Europe, affecting Italy (Rezza et al., 2007) and France (Grandadam et al., 2011; Krastinova et al., 2006). The virus was later reported in the Americas, including more than 40 countries with more than 2 million reported cases from 2013 to 2016 (Christian et al., 2017; Lanciotti et al., 2007). The outbreak in Réunion Island between 2005 – 2006 was deemed one of the most significant CHIKV outbreaks, with an estimated one-third of its population (approximately 266 000) infected with the virus (Townson & Nathan, 2008).

Phylogenetic analyses of CHIKV isolates have identified three major genotypes: East/Central/South African (ECSA), West African, and Asian (Powers et al., 2000).

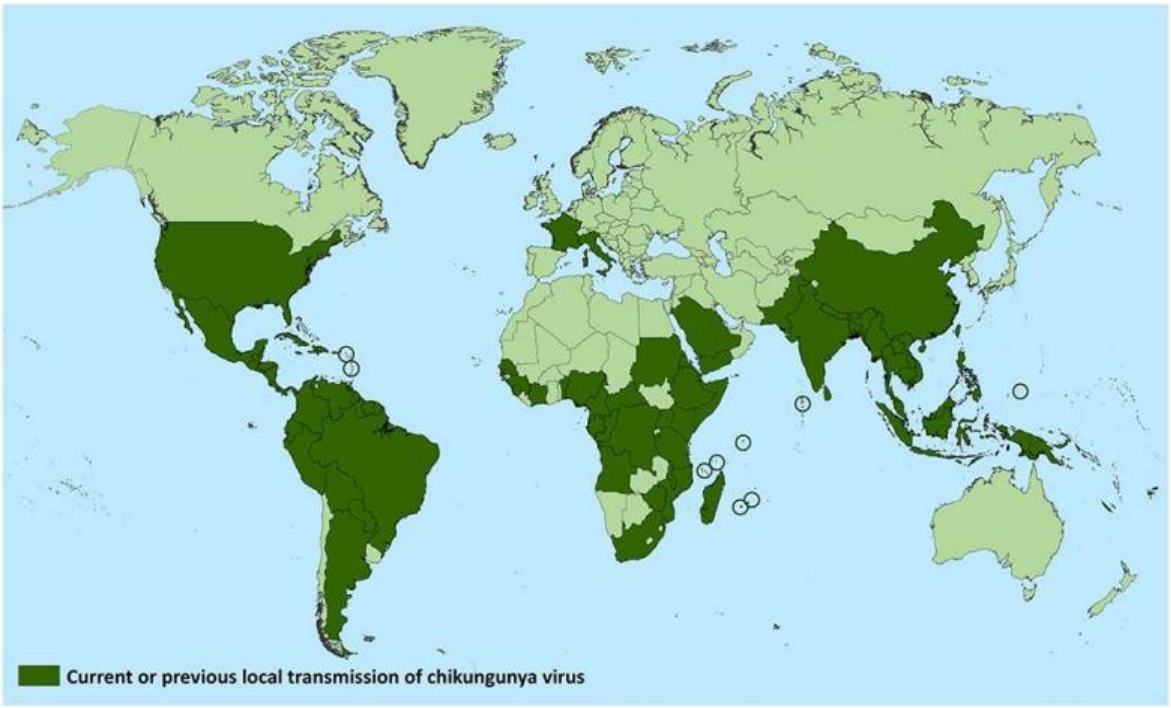

Figure 1.3: Countries and territories where chikungunya cases have been reported (as of September 17, 2019). From the Centers of Disease Control and Prevention (CDC) | This material is in the public domain – see Appendix C

Two distinct transmission cycles for the maintenance of CHIKV have been identified (Figure 1.4). In Africa, CHIKV typically circulates in an enzootic sylvatic cycle between forest dwelling *Aedes* spp. mosquitoes as vectors and wild primates as reservoirs/ amplifying hosts (Vanlandingham et al., 2005). The virus was isolated in *Ae. africanus* in East Africa (McCrae et al., 1971), *Ae. delzieli, Ae. furcifer, Ae. luteocephalus,* and *Ae. taylori* in West Africa (Diallo et al., 1999; Jupp & McIntosh, 1988), and *Ae. codellieri* and *Ae. taylori* in South Africa (Jupp & Kemp, 1996). The enzootic transmission cycle may spill over to infect humans. Human epidemics in Africa tend to be small and occur sporadically, which usually coincides with increased rainfall and consequently, increased sylvatic mosquito populations, predominantly of the *Aedes furcifer-tayleri* group (Diallo et al., 1999; Higgs & Vanlandingham, 2015; Lumsden, 1955). Conversely, CHIKV outbreaks in the coastal regions of Kenya in 2004 followed a period of unusually warm conditions, favouring mosquito breeding, and unusually dry conditions, resulting in improper water storage and enhancing mosquito breeding in close proximity to people (Chretien et al., 2007; Gould & Higgs, 2009).

In Asia, CHIKV mainly circulates in an urban cycle between *Ae. aegypti* or *Ae. albopictus* vectors and human hosts (Jupp & McIntosh, 1988; Weaver, 2006).
Historically, only *Ae. aegypti* mosquitoes were associated with epidemics in Asia (Jupp & McIntosh, 1988), until the introduction of the ECSA strain from Africa to Reunion Island in 2005, a mutation in the E1 protein (A226V E1), which has shown to increase viral fitness in the *Ae. albopictus* vector (Tsetsarkin et al., 2007; Vazeille et al., 2007).

This adaptation played a vital role for the spread of the virus to various geographical regions, especially since *Ae. albopictus* is more widely distributed than *Ae. aegypti*, in addition, *Ae. albopictus* have the ability to survive in regions with temperate climate, seen in Europe and Americas, unlike *Ae. aegypti* which are mainly restricted tropical and subtropical regions (Charrel et al., 2007; Kraemer et al., 2015).

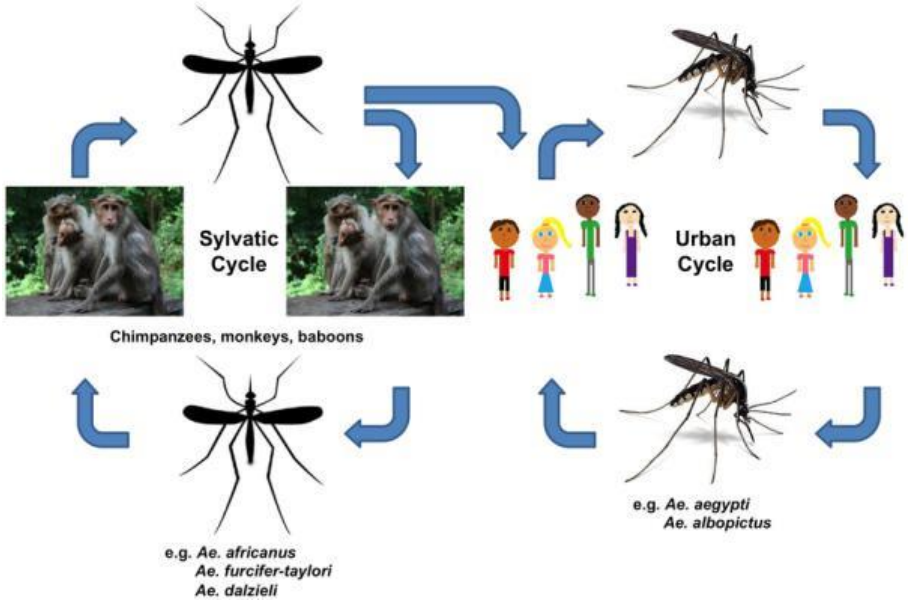

Figure 1.4: Transmission cycles for the maintenance of CHIKV (Thiboutot et al., 2010) | See Appendix C for permission to use this material

1.6.1.2 Sindbis virus (SINV)

SINV is one of the most widely distributed arboviruses in the world, identified in Eurasia, Africa, and Oceania. Six SINV genotypes (SINV-I – SINV-VI) have been identified, each restricted to a specific geographic region (Ling et al., 2019; Lundström & Pfeffer, 2010; Saleh et al., 2003). SINV-I is restricted to Africa, Europe, and the Middle East; SINV-II and SINV-VI are restricted to Australia; SINV-III is restricted to Southeast Asia; SINV-IV is restricted to Asia and the Middle East; and SINV-V (also referred to as Whataroa virus) is restricted to New Zealand.

Although cases of human infection occur in these regions, outbreaks of human infection has only been associated with SINV-I, which were reported in South Africa and northern Europe. In South Africa, significant outbreaks of human infection occurred in 1963, 1974, 1983-1984, and between 2006 and 2010 (Jupp et al., 1986; McIntosh et al., 1976; Storm et al., 2013). In northern Europe, human infections are represented by Ockelbo disease (Sweden), Pogosta disease (Finland), and Karelian fever (the Karelian part of Russia). Significant outbreaks of infection were reported in 1981-1982, 1988, 1995, 2002, and 2013 (Bergqvist et al., 2015; Brummer-Korvenkontio et al., 2002; Kurkela et al., 2005; Lundström, 1999; Niklasson &

Espmark, 1984). There is evidence suggesting that migratory birds are responsible for the transcontinental dissemination of SINV from South Africa to northern Europe (Kurkela et al., 2008). In Finland between 1974 and 2002, outbreaks of Pogosta disease occurred every 7 years, for reasons that may have to do with the behaviour of migratory birds playing a role in dispersal of virus between continents (Bergqvist et al., 2015; Brummer-Korvenkontio et al., 2002).

SINV infections typically occur during late summer or early autumn, particularly after periods of heavy rainfall which favour mosquito breeding (Jupp et al., 1986; Lundström, 1999). The virus is maintained in an enzootic cycle with wild birds as amplifying reservoir hosts and ornithophilic mosquitoes ("bird-loving" mosquitoes) as transmitting vectors (Reusken et al., 2011). Humans are incidental dead-end hosts that do not contribute to the maintenance of the virus in nature since they do not develop sufficient viremia to transmit the virus (Jupp, 2005). For transmission to humans, opportunistic vectors which feed on birds and mammals and serve as bridge vectors are required (Reusken et al., 2011). For example, in Sweden, *Culex torrentium* has been suggested as the main enzootic vector (Figure 1.5), while *Aedes cinereus* has been found to serve as bridge vectors for transmitting the virus from birds to humans (Turell et al., 1990).

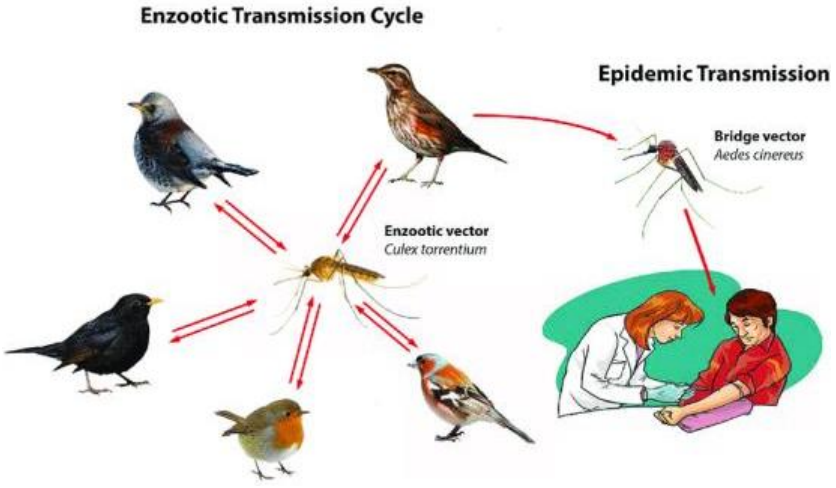

Figure 1.5: Enzootic cycle of SINV transmission in Sweden (Lundström et al., 2019) | See Appendix C for permission to use this material

In South Africa, in the temperate inland plateau regions, *Cx. univittatus* mosquitoes are the primary vectors for transmission, whereas in the coastal lowlands of north-eastern South Africa, *Cx. neavei* mosquitoes are the main vectors (Jupp et al., 1986; McIntosh et al., 1978) While both mosquito vectors have the ability to transmit the virus to humans, *Cx. univittatus* mosquitoes are the more efficient vectors and in addition, liable for the higher incidence rate in human cases in the upper situated inland regions of South Africa (Jupp, 1996).

1.6.2 Alphaviruses endemic to Africa

1.6.2.1 Middelburg virus (MIDV)

MIDV has been identified in humans, cattle, sheep and goats, obtained from serological tests conducted in KwaZulu-Natal in South Africa (Kokernot et al., 1961; Smithburn et al., 1959), horses with neurological disease in South Africa and Zimbabwe (Attoui et al., 2007; van Niekerk et al., 2015), as well as wildlife and nonequine domestic animals showing signs of neurologic disease, febrile illness, or unexplained deaths including white rhinoceros, buffalo, domestic bovids, warthogs lions, birds, lemon dove and blue crane, sable antelopes, waterbucks, and genets (Steyn et al., 2020). Other than South Africa and Zimbabwe, MIDV has also been reported from CAR, Kenya, Senegal and Cameroon (Hubálek et al., 2014; Tricou et al., 2014). Reservoir hosts for MIDV are still unknown, although the widespread distribution of MIDV suggests that birds might contribute to the maintenance of this virus (Steyn et al., 2020). Important mosquito vectors found to host MIDV include *Ae. caballus* and *Mansonia africana* (Hubálek et al., 2014).

1.6.2.2 Ndumu virus (NDUV)

NDUV has not been linked to human or animal morbidity. In South Africa, the virus has been identified from *Mansonia uniformis* and *Aedes circumluteolus* mosquitoes (Kokernot et al., 1961), while in Kenya, the virus has been isolated in several *Ae.* spp. Mosquitoes and *Cx. rubinotus* (Crabtree et al., 2009; Lutomiah et al., 2014; Ochieng et al., 2013). Due to the high number of NDUV isolated from *Cx. rubinotus* mosquitoes (Ochieng et al., 2013), it was suggested that this mosquito may play an important role in the transmission and maintenance of the virus in nature. *Cx. rubinotus* feed on rodents (Jupp et al., 1976), therefore, this vertebrate

may contribute to the natural maintenance of the virus. Other potential hosts for circulation of the virus include domestic pigs (Masembe et al., 2012) and goats (Lutomiah et al., 2014). The virus was also isolated from *Rhipicephalus pulchellus* ticks which were collected from cattle and warthogs (Lwande et al., 2013).

1.6.2.3 O'nyong-nyong virus (ONNV)

A closely related virus to CHIKV, ONNV has also been linked to large epidemics in Africa. The 1959-1962 epidemic in east Africa began in northwestern Uganda (Gulu), spreading to Kenya and southwards to Tanzania, Malawi, and Mozambique, infected more than 2 million people (Lutwama et al., 1999). After an absence of approximately 35 years, the virus re-emerged in 1996-1997 in southern Uganda, causing another major outbreak (Lanciotti et al., 2007; Rwaguma et al., 1997). A more recent epidemic was reported in central Uganda in 2002 (Vanlandingham et al., 2005). Unlike CHIKV and all other alphaviruses that are transmitted by culicine mosquitoes, ONNV is transmitted by anophelines mosquitoes, typically *Anopheles funestus* and *An. Gambiae* (Powers et al., 2000). These vectors live in close associations with humans and contribute to the rapid spread of the virus during epidemics. Humans may be the only natural host for the virus, as no other vertebrate reservoir for the virus has been identified (Powers et al., 2000).

1.6.2.4 Semliki Forest virus (SFV)

SFV was responsible for a large outbreak in Bangui, CAR, in 1987 (Mathiot et al., 1990). During this time, the virus was isolated from *Aedes africanus* and *Ae. aegypti* mosquitoes (Mathiot et al., 1990). SFV antibodies are mainly found in human sera collected from West and East Africa (Weaver & Frolov, 2010). SFV may circulate in a sylvatic cycle between *Aedes* mosquitoes and monkeys (Weaver & Frolov, 2010).

1.6.3 Alphaviruses endemic to Australia

RRV and BFV are endemic and enzootic in Australia (Flexman et al., 1998; Harley et al., 2001), with RRV also endemic and enzootic in Papua New Guinea (Hii et al., 1997; Scrimgeour et al., 1987) and in the Pacific Islands (Aubry et al., 2015; Lau et al., 2017). Most cases occur in northern Australia during the summer (also known as the wet season in the tropical north), particularly between December to February, where high temperatures, rainfall and tides contribute to increased mosquito populations, which increases the dissemination of the viruses (Whelan et al., 2003).

1.6.3.1 Ross River virus (RRV)

RRV is responsible for most mosquito-borne infections in Australia (*Australian Government Department of Health. National Notifiable Diseases Surveillance System*, 2020). Between 1993 and 2017, 120 605 cases of infection (averaging 5000 per year) have been reported (*Australian Government Department of Health. National Notifiable Diseases Surveillance System, 1 April to 30 June 2017*, 2018). RRV has been isolated from over 40 mosquito species, with *Ae. vigilax*, *Ae. camptorhynchus*, and *Cx. annulirostris* recognised as the typical vectors for transmission (Russell, 2002). Macropod marsupials such as kangaroos are generally considered better reservoirs than placental mammals and birds due to their high and long duration of viraemic titers (Koolhof & Carver, 2017).

1.6.3.2 Barmah Forest virus (BFV)

BFV is the second most common mosquito-borne virus in Australia (*Australian Government Department of Health. National Notifiable Diseases Surveillance System*, 2020), with approximately 1000 to 2000 cases per year. BFV has a wide range of mosquito vectors, with *Cx. annulirostris* and *Ae. vigilax* identified as the main vectors (Jacups et al., 2008; Ryan & Kay, 1999). Although a vertebrate reservoir of BFV has yet to be identified, serosurveys have implicated quokkas (Johansen et al., 2005), brush tail possums (Kay et al., 2007), and horses (Gummow et al., 2018) as potential natural hosts.

1.6.4 Alphaviruses endemic to the Americas

1.6.4.1 Mayaro virus (MAYV)

MAYV is endemic to South America. The virus circulates in an enzootic sylvatic cycle between the tree-canopy dwelling *Haemagogus* spp. mosquitoes and wild vertebrates, mainly monkeys, birds and reptiles (Valencia-Marín et al., 2020). Since its discovery in 1954 in Trinidad and Tobago (Anderson et al., 1957), three genotypes have been identified – genotype D (widely dispersed), genotype L (limited) and genotype N (new). Genotype D is widely distributed and has been identified in Trinidad, Tobago, Brazil, Bolivia, Peru, French Guiana, Argentina, Surinam, Colombia, and Venezuela (Auguste et al., 2015; Powers et al., 2006), genotype L has a limited distribution and has been identified in Brazil and Haiti (Lednicky et al., 2016; Long et al., 2011; Powers et al., 2006), and genotype N has been identified in Peru (Auguste et al., 2015). *Aedes aegypti* mosquitoes have also proven to be competent vectors of the virus in laboratory studies (Long et al., 2011), suggesting urban vectors have the potential to spread the virus over a wider scale than sylvatic regions.

1.6.4.2 Eastern equine encephalitis virus (EEEV)

EEEV is naturally maintained between birds and *Culiseta melanura* mosquitoes. Principal bridging vectors for transmission of the virus to horses and humans include *Aedes*, *Coquillettidia*, and *Culex* species. EEEV vectors mostly inhabit freshwater swamps in the Atlantic and Gulf Coast states, as well as the Great Lakes area. Human infections were mostly reported in Florida, Georgia, Massachusetts, and New Jersey (Zacks & Paessler, 2010).

1.6.4.3 Western equine encephalitis virus (WEEV)

WEEV is maintained in an enzootic sylvatic cycle between passerine birds and *Culex tarsalis* mosquitoes. Transmission of the virus to horses and humans is facilitated by bridging vectors including *Ae. campestris* (New Mexico), *Ae. dorsalis* (Utah and New Mexico) and *Ochlerotatus melanimon* (California) (Zacks & Paessler, 2010).

1.6.4.4 Venezuelan equine encephalitis virus (VEEV)

VEEV is transmitted between *Culex (Melanoconion*) species mosquitoes and rodents in enzootic cycles, and between *Ochlerotatus taeniorhynchus* mosquitoes and horses/ humans in epizootic/ epidemic cycles. Equids develop high viremic titers, serving as sources of infection for feeding mosquitoes (Zacks & Paessler, 2010). Outbreaks of the virus in humans and equids have been reported in South America (Venezuala, Colombia, Ecuador, and Peru), in Central America (Costa Rica, Honduras, Guatemala, Mexico, El Salvador, Panama, and Nicaragua) and in the United States (Zacks & Paessler, 2010).

1.7 Detection and diagnosis of alphaviruses

1.7.1 Virus isolation

Traditionally, alphavirus diagnostics is achieved by virus isolation, which has been useful in defining viral agents in serum, cerebrospinal fluid, and mosquito vectors (Powers & Roehrig, 2010). The conventional procedure for isolating and identifying an alphavirus begins with inoculating the virus in neonatal mice or a cell culture system in which cytopathic effects or plaques can develop (Powers & Roehrig, 2010). Cell culture systems include mammalian cells such as African green monkey kidney (Vero) cells and baby hamster kidney (BHK) cells, and mosquito cells such as AP-61 (cloned from *Ae. pseudoscutellaris*) and C6/36 (cloned from *Ae. albopictus*) (Powers & Roehrig, 2010). Virus identification has been significantly simplified with the application of direct or indirect fluorescent microscopy in conjunction with polyclonal (pAbs) or monoclonal antibodies (mAbs). MAbs with high functional affinities to allow for specific binding to viral antigens in complex protein mixtures have enhanced the rapid identification of viral agents in situ (Powers & Roehrig, 2010). In contrast to other arboviruses, alphaviruses are rapid growing viruses which can induce cytopathic effects within 1 – 3 days post-infection if kept under favourable conditions (Lwande et al., 2015). However, virus isolation is expensive and time-consuming, which requires subsequent diagnostic techniques such as reverse transcription polymerase chain reaction (RT-PCR) and immunofluorescence assays to characterise successful isolates (Soto-Garita et al., 2018). Isolation of the virus from acute-phase sera is impractical for managing patients since several days are required to obtain final results and the patients are frequently not viremic when presented with clinical signs

(Najioullah et al., 2014). Furthermore, a biosafety level 3 laboratory is required for most alphaviruses that may be transmitted by aerosols at high concentrations (Soto-Garita et al., 2018)

1.7.2 Serology

Serological diagnosis of alphavirus infections has changed significantly over the past number of years. Historically, the diagnosis of these infections relied on four tests to detect immunoglobulin M (IgM) and IgG antibodies in serum samples, which include plaque reduction neutralization tests, complement fixation, hemagglutination inhibition, and indirect fluorescent antibody (IFA). Positive identification using these antibody assays require a fourfold titer increase in samples collected between the acute and convalescent stages of infection (Powers & Roehrig, 2010). Plaque reduction assay is the gold standard for antibody detection, particularly towards viruses that belong to the same genus, in this case alphaviruses (Lwande et al., 2015). The more recent development of solid-phase antibody-binding assays, such as enzyme-linked immunosorbent assays (ELISAs) has provided a tool for more rapid serodiagnostic testing, especially with the adaptation and application of IgM antibody capture ELISA (MAC-ELISA) utilised in early infection (Powers & Roehrig, 2010). Serological diagnosis of recent alphavirus infections is demonstrated by an increase in specific IgM or IgG antibodies, when testing acute and convalescent samples in parallel (Lloyd, 2004). IgM assays are useful in diagnosing acute infection for a particular alphavirus, however cross-reactions may occur between other members belonging to the same alphavirus antigenic complex, and thus caution should be taken in the interpretation of results (Calisher et al., 1986; Lloyd, 2004). Also, IgM antibodies are usually not detected until 4 to 5 days post-infection, delaying diagnosis (Wang et al., 2006).

1.7.3 Molecular techniques

Molecular assays or nucleic acid amplification tests (NAATs) provide a rapid and more sensitive means to detect alphaviruses infections (Hodneland & Endresen, 2006; Kang et al., 2010; Lambert et al., 2003; Linssen et al., 2000). These methods include conventional and real-time RT-PCR assays which are widely used for the detection of alphavirus RNA in clinical samples and for epidemiological surveillance (Lwande et al., 2015). Table 1.1 below provides

a summary of genus-specific and RT-PCR assays that have been developed for the detection of alphaviruses.

Table 1.1 Genus-specific RT-PCR assays developed for the detection of alphaviruses

RT-PCR method	Target region(s)	Alphaviruses detected	Sensitivity	References
Conventional semi-nested RT-PCR	nsP1	27 alphaviruses	1 200 plaque forming units (pfu)	(Pfeffer et al., 1997)
Conventional nested RT-PCR	nsP4	EEEV, VEEV, WEEV, RRV, SINV, SFV and CHIKV	25 pfu	(Sánchez-Seco et al., 2001)
Conventional RT-PCR followed by multiplex nested PCR	nsP1	14 Brazilian alphaviruses including MAYV, Aura virus, EEEV, WEEV and VEEV	$10^{0.5}$ $TCID_{50}$/ml	(Bronzoni et al., 2004)
Conventional nested RT-PCR	nsP4	BFV, CHIKV, MAYV, ONNV, RRV, SFV, SINV, EEEV, WEEV, VEEV	5 to 100 RNA copies	(Grywna et al., 2010)
Real-time Taqman RT-PCR	nsP4	19 alphaviruses	40 RNA copies	(Giry et al., 2017)

RT-PCR diagnosis of alphavirus infection in serum is only useful if clinical samples are taken in the acute stage of infection when viremia levels are high (Suhrbier et al., 2012). For CHIKV infection for instance, the viremic period generally lasts 5 – 7 days after symptom onset (Gibney et al., 2011; Jaffar-Bandjee et al., 2010), with viral loads usually ranging from 1×10^5 to 1×10^9 copies of RNA per ml of blood (Laurent et al., 2007). However, the virus may be cultured from viraemic samples in cell culture systems such as C6/36 (mosquito cell line) or Vero (monkey kidney cell line) cells for later applications (Suhrbier et al., 2012).

Contrarily, in mosquito vectors, arboviral infection is persistent and may endure the insect's whole life (Reyes-Ruiz et al., 2019), hence mosquitoes are convenient for epidemiological surveys to determine if there are alphaviruses circulating in the area.

Modern molecular techniques, which include isothermal amplification assays such as RT-LAMP and RT-RPA have been described for point-of-care testing and rapid diagnosis of CHIKV infections (Hayashida et al., 2019; Parida et al., 2007; Prüger et al., 2016). Unlike PCR-based techniques, isothermal amplification techniques do not require a thermocycler and is therefore useful in low-resource or field settings. Isothermal amplification methods include "recombinase polymerase amplification (RPA), loop-mediated amplification (LAMP), helicase-dependent amplification (HDA), nucleic acid sequence-based amplification (NASBA), multiple displacement amplification (MDA), signal-mediated amplification of RNA technology (SMART) and rolling circle amplification (RCA)".
A simple isothermal amplification method such as RT-HDA, see Figure 1.6 for schematic diagram, where only a heating block is needed, combined with lateral flow devices such as PCRD cassettes and dipsticks (Abingdon Health, UK) for end-point detection (Figure 1.7) may be useful for alphavirus detection in low resource settings.

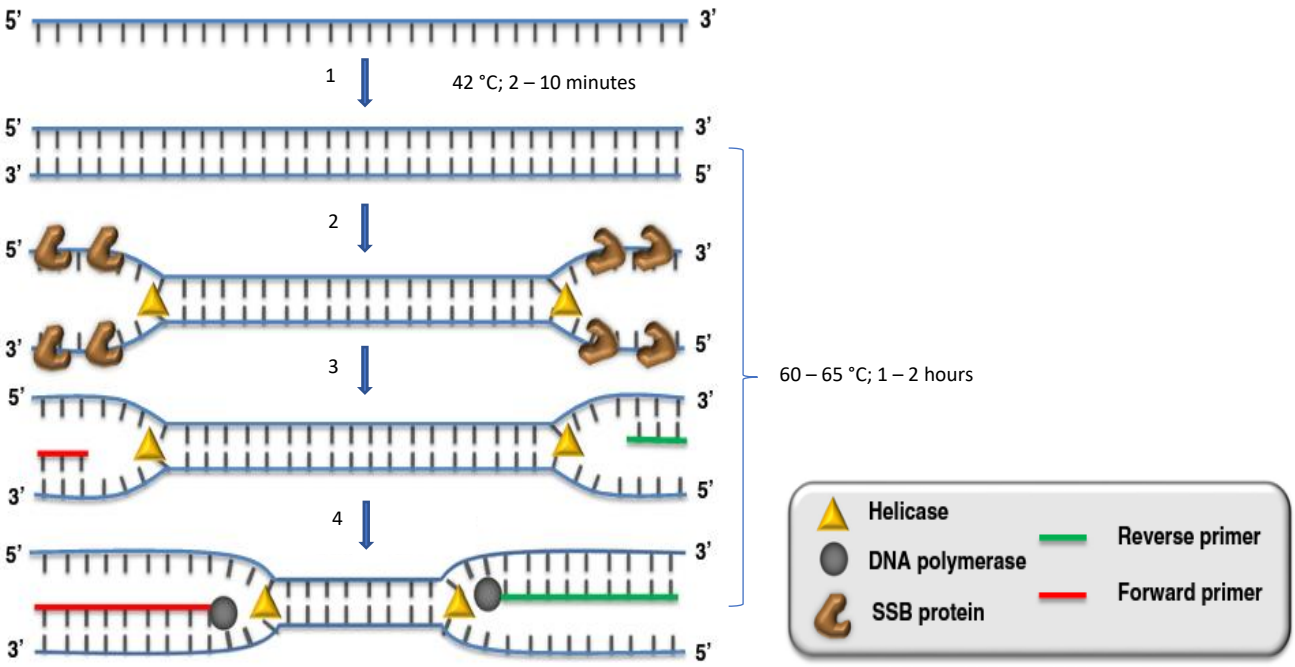

Figure 1.6: Schematic diagram of the RT-HDA system, adapted from Barreda-garcía & Miranda-castro, 2018. 1. Synthesis of DNA from RNA template, 2. Unwinding of dsDNA by helicase enzyme and stabilisation of ssDNA by SSB protein, 3. Annealing of forward and reverse primers to ssDNA strands, 4. DNA replication using DNA polymerase

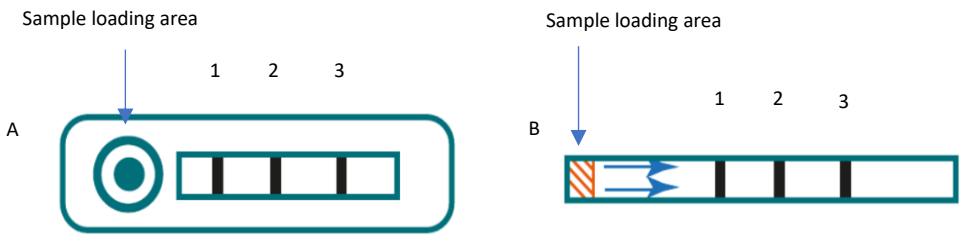

Figure 1.7: PCRD lateral flow devices. A. PCRD cassette, B. PCRD FLEX dipsticks. A1 and B1: Test line 1 (T1) – detects DIG/Biotin labelled amplicons, A2 and B2: Test line 2 (T2) - Detects FAM/Biotin or FICT/Biotin labelled amplicons, A3 and B3: Control line (C)

Although molecular techniques surpass virus isolation and antibody detection methods in terms of turnaround time and sensitivity (Hodneland & Endresen, 2006; Kang et al., 2010; Lambert et al., 2003; Linssen et al., 2000), nucleic acid determination is often required to identify the species, isolate or strain of alphaviral RNA detected.

1.8 Clinical manifestations of alphavirus infections

Human infections vary from rashes and fever, to transient or incapacitating arthritis, or encephalitis (Tesh, 1982; Whitley, 1990). CHIKV infections are often underreported, possibly because the symptoms of disease overlap extensively with malaria, dengue fever, and several other communicable tropical ailments (Sunil, 2021). CHIKV infection has been linked to fatalities with outbreaks in India and Réunion Island (Mavalankar et al., 2008), and several cases included neurological disease (Casolari et al., 2008; Ganesan et al., 2008). Cases of SINV affecting humans have been reported primarily in South Africa (Jupp et al., 1986; McIntosh et al., 1976; Storm et al., 2013) and northern Europe (Bergqvist et al., 2015; Brummer-Korvenkontio et al., 2002; Kurkela et al., 2005; Lundström, 1999; Niklasson & Espmark, 1984). Like CHIKV, infection with SINV usually results in acute arthralgia, rash, febrile illness, and malaise. SINV was identified as the causative agent of Ockelbo disease in Sweden, Pogosta disease in Finland, and Karelian fever in Russia, in the mid-1980s (Adouchief et al., 2016; L'vov et al., 1985; Niklasson & Espmark, 1984).

Central nervous system disorders are mainly associated with New world alphaviruses, which include severe encephalitis (Zacks & Paessler, 2010).

1.9 Treatment of alphavirus infections

Despite decades of research and several fundamental discoveries in virology, there are no vaccines or antivirals approved for infections caused by alphaviruses, although numerous proposed treatments have been described (Nagata et al., 2013; Phelps et al., 2021; Suchowiecki et al., 2021; Torres-Ruesta et al., 2021; Zaid et al., 2021).

For arthritogenic alphavirus infections, antihistamines can be used to relieve itching associated with rash, and analgesics and non-steroidal anti-inflammatory drugs (NSAIDs) can be used to relieve pain and reduce inflammation associated with joint symptoms (Adouchief et al., 2016; Mylonas et al., 2002, 2004). However, since dengue fever can be clinically indistinguishable from alphavirus infections, NSAIDs or aspirin should not be used until alphavirus infection is confirmed to avoid bleeding complications coupled with dengue fever (Vairo et al., 2019). Hence acetaminophen use for 14 days is appropriate for pain relief in the acute stage of infection (Vairo et al., 2019). Pentosan polysulfate sodium is currently the only drug formulation to advance to clinical trial for the treatment of RRV disease (Herrero et al., 2015; Krishnan et al., 2021).

Antibody therapies have been tested in mouse models for potential efficacy against CHIKV, RRV and MAYV (Powell et al., 2020). In addition to potential therapeutics for arthritogenic alphaviral infections, vaccine candidates have been described for CHIKV (Chen et al., 2020; Ramsauer et al., 2019; Schrauf et al., 2020), RRV (Wressnigg et al., 2015; Yu & Aaskov, 1994) and MAYV (Robinson et al., 1976; Weise et al., 2014).

For encephalitic alphavirus infections, supportive care remains to be the main approach, which frequently includes admission to intensive care and ventilatory support (Morens et al., 2019). Routine monitoring of intracranial pressure have been described in patients with worsening encephalitis (Silverman et al., 2013). Vaccine candidates have been described for EEEV, VEEV and WEEV (Nagata et al., 2013; Phelps et al., 2021; Torres-Ruesta et al., 2021).

1.10 Prevention, control, and surveillance

Since no vaccines or antivirals are currently available, prevention remains the key approach to control the transmission of alphaviruses. Prevention includes efforts to reduce potential sites for vector breeding, implementation of insecticides and protective measures such as protective clothing, mosquito repellents, and mosquito bed nets. Hence mosquito-based surveillance programs can allow society enough time to enact efficient strategies for disease prevention and outbreak control.

As previously described in section 1.7.3, molecular techniques, most commonly RT-PCR based techniques are widely used to detect alphavirus infections for epidemiological surveys due to reduced processing time in comparison to virus isolation and serological detection methods, allowing for high throughput screening. Newer, more rapid isothermal amplification methods have also been developed to detect alphaviral RNA. Table 1.1 below provides a summary of molecular assays used for mosquito-based arbovirus surveillance:

Table 1.2 Summary of molecular assays used for mosquito-based alphavirus surveillance

Description of molecular assay	**Alphaviruses detected in mosquito samples**	**References**
Real-time RT-PCR assays were described for the detection of CHIKV and ONNV	The CHIKV assay was tested in a field setting in Thailand, and no positive isolates identified	(Smith et al., 2009)
A real-time RT-PCR assay was described for the detection of arboviruses in Taiwan	Alphavirus detection in field-collected mosquitoes was not successful	(Yang et al., 2010)
A two-step conventional RT-PCR assay was described for the detection alphavirus detection in Kenya	NDUV and SINV	(Ochieng et al., 2013)

Description of molecular assay	Alphaviruses detected in mosquito samples	References
A conventional nested RT-PCR assay was described for the detection of arboviruses in Colombia	VEEV	(Hoyos-lópez et al., 2016)
A RT-LAMP assay was described for the detection of Zika virus, dengue virus and CHIKV	CHIKV	(Yaren et al., 2017)

1.11 Problem identification, aim and objectives

There is a lack of vector surveillance and monitoring programs for most arboviral diseases in South Africa (Mensah & El Zowalaty, 2018). PCR-based methods such as conventional and real-time RT-PCR are typically used in epidemiological surveys. Although RT-PCR remains the method of choice in high resource settings and well-funded institutions, RT-PCR techniques may not be adequate in low resource settings where sophisticated laboratories and electricity are limited. Thus, the development of rapid, fieldable assays should be considered as an alternative to RT-PCR techniques for application in low resource settings.

The aim of this study was to develop molecular assays with application for mosquito-borne alphaviruses in South Africa.

The objectives of the study were as follows:

1. To develop a conventional nested RT-PCR assay and a RT-HDA assay to detect alphaviruses in South Africa.
2. To determine and compare the sensitivity and specificity of the molecular assays.
3. To use the molecular assays to screen for potential alphaviruses in wild mosquitoes caught in the Free State.
4. To assess the phylogeny of the positive alphavirus isolates from wild, caught mosquitoes in the Free State.

CHAPTER 2: DEVELOPMENT OF MOLECULAR ASSAYS FOR DETECTION OF ALPHAVIRUSES IN MOSQUITO VECTORS IN SOUTH AFRICA

2.1 Introduction

Alphaviruses are widely distributed across the globe and are found on all continents (Kim et al., 2016; Lwande et al., 2015). As most alphaviruses are transmitted by mosquito vectors, the global dissemination of these viruses may be attributed to the presence of diverse mosquito vectors (Lwande et al., 2015), and with a changing global climate, the rise in temperatures and high levels of precipitation may contribute to increased mosquito breeding (Jupp et al., 1986; Lundström, 1999). Hence, continual development of rapid and reliable tools for the detection of alphaviruses in mosquito vectors is important and will aid in the understanding of which viruses are currently circulating with the potential to cause outbreaks.

Alphaviruses can be detected using various assays including virus isolation, antigen detection, and molecular techniques targeting the viral genome. Among these methods, molecular techniques such as RT- PCR are routinely used for diagnosis in acutely infected patients and for surveillance of mosquito vectors. RT-PCR allows for detection of RNA viruses, semi-nested and nested PCR/ RT-PCR increase assay sensitivity and specificity, and real-time PCR/ RT-PCR allow quantitative analysis. RT-PCR assays that have been used for alphavirus detection in mosquito vectors include a real-time RT-PCR assay for the detection of chikungunya and o'nyong-nyong viruses (Smith et al., 2009), a SYBR Green I-based real-time RT-PCR for alphavirus detection in Taiwan (Yang et al., 2010), a two-step conventional RT-PCR for alphavirus detection in Kenya (Ochieng et al., 2013), and a conventional nested RT-PCR to detect alphaviruses in Colombia (Hoyos-lópez et al., 2016).

Although benefits of real-time RT-PCR over conventional RT-PCR include speed, decreased risk of contamination, and quantitative ability, conventional RT-PCR is still one of the most commonly used methods for virus detection due to its accessibility and ease (Ma et al., 2018). Conventional assays are also more amenable to accommodating diversity for targeting genera

using consensus primers as they do not require additional conserved regions for designing probes.

While RT-PCR remains the method of choice for epidemiological surveys in developed areas, RT-PCR requires the use of a thermocycler and reliable power supply, which may pose a challenge in low resource areas where equipped laboratories are limited. These limitations have spurred the development of isothermal amplification methods, such as RPA, LAMP, HDA, NASBA, MDA, SMART and RCA. These methods are broadly described in previously published reviews (Li & Macdonald, 2015; Zanoli & Spoto, 2013). Isothermal amplification methods are generally easier to operate and do not require any thermocycling, saving time, energy, and reducing system complexity, thus expanding their use to low resource settings. Isothermal amplification assays have been described for arbovirus surveillance in mosquito samples, including a RT-LAMP assay for the detection of chikungunya virus and two flaviviruses (Zika virus and dengue virus), and a RT-RPA assay for the detection of Zika virus.

HDA technology, developed by Vincent et al. in 2004, mimics an *in vivo* process of DNA replication using a thermostable helicase enzyme instead of heat to separate nucleic acids and amplify the target DNA under the action of the polymerase (Vincent et al., 2004).
Modifications to the HDA system, such as RT-HDA development, has allowed for the detection of RNA by incorporating a thermostable reverse transcriptase into the reaction mixture (Goldmeyer et al., 2007).

In this study, a conventional nested RT-PCR assay was developed to detect alphaviruses known to circulate in South Africa, such as MIDV and SINV, alphaviruses historically reported in South Africa such as CHIKV and NDUV, and alphaviruses reported in other regions of Africa such as ONNV and SFV to consider the possibility of resurgence or cross-border infections, with application for the surveillance of these viruses in mosquito vectors.
Many of these viruses required a biosafety level 3 laboratory that was not available at that time for the preparation of controls, hence non-infectious RNA was prepared for assay development and validation.

In addition, we describe a rapid fieldable isothermal assay for alphavirus surveillance purposes and without the need for sophisticated equipment in this study. Due to its accessibility and ease

in design, to simultaneously detect alphaviruses currently circulating in South Africa such as SINV and MIDV, an RT-helicase dependent amplification (HDA) assay was developed in this study as an alternative to the conventional nested RT-PCR assay. This assay would have application in low resource settings, and to potentially screen for MIDV and SINV in mosquitoes. The development and proof of concept of the assay was focused on alphaviruses known to occur in South Africa.

2.2 Materials and methods

2.2.1 Primer design

For the conventional nested RT-PCR assay, degenerate consensus primers (Table 2.1), designated Alphavirus nsP4 (F1), Alphavirus nsP4 (R1), Alphavirus nsP4 (F2) and Alphavirus nsP4 (R2), were designed using conserved regions identified in the nsP4 gene following the alignment of sequence data retrieved for South African and other African alphavirus isolates from NCBI GenBank (https://www.ncbi.nlm.nih.gov/genbank/). nsP4 sequence data was available for a total of 40 alphavirus isolates (table 2.3), and the sequences were aligned using Clustal Omega version 1.2.4 (https://www.ebi.ac.uk/Tools/msa/clustalo/). The alignment and conserved regions identified for primer design are shown in Appendix D.

Alphavirus nsP4 (F1) and Alphavirus nsP4 (R1) primers, identified as outer primers for the first round of nested amplification, were designed to target a 570 bp region, while Alphavirus nsP4 (F2) and Alphavirus nsP4 (R2), identified as inner primers for the nested amplification, were designed to target a 200 bp region. Position of the primers on the gene relative to the isolates used as positive controls are also provided in Appendix D.

For the RT-HDA assay, Degenerate consensus primers (Table 2.2), designated HDA Forward (5' biotin) and HDA Reverse (5' 6-FAM), were designed using conserved regions identified in the nsP4 gene following the multiple alignment of sequence data retrieved for South African and other African SINV and MIDV isolates which contain the nsP4 region in NCBI GenBank. A total of five SINV isolates and five MIDV isolates were previously identified (Table 2.3), and the sequences were aligned using Clustal Omega version 1.2.4. The multiple alignment and conserved regions identified for primer design are shown in Appendix E. The primers were

designed to target a 116 bp region using the recommended specifications for the isothermal technique (Barreda-garcía & Miranda-castro, 2018). Position of the primers on the gene relative to the isolates used as positive controls are provided in Appendix E.

Table 2.1 Conventional nested RT-PCR primers

Primer	Sequence (5' – 3')	No. of bases	No. of degenerate bases	Tm (℃)	GC%	Amplicon size
Alphavirus nsP4 (F1)	AARTTYGGVGCNATGATGAA	20	4	60.7	45	570 bp
Alphavirus nsP4 (R1)	CWATTTAGGWCCRCCGTASA	20	4	60	47.5	
Alphavirus nsP4 (F2)	GCNATGATGAARTCNGGHATG	21	4	60.9	49.2	200 bp
Alphavirus nsP4 (R2)	TTMACYTCCATGTTSAKCCA	20	4	60.4	42.5	

Degenerate bases are highlighted in yellow

Tm and %GC content calculated using the OligoAnalyzer 3.1Tool

https://eu.idtdna.com/calc/analyzer

Table 2.2 RT-HDA primers

Primers	Sequence	No. of bases	No. of degenerate bases	Tm (°C)	GC %	Amplicon size
HDA Forward (5' biotin)	/5Biosg/ATGAAATCYGGMATGTTCCTSACGCT	26	3	69.3	46.2	116 bp
HDA Reverse (5' 6-FAM)	/56-FAM/TCGCCGATRAAKGCKGCACATTTRGA	26	4	71.1	50	

Degenerate bases are highlighted in yellow

Tm and %GC content calculated using the OligoAnalyzer 3.1 Tool

https://eu.idtdna.com/calc/analyzer

Table 2.3 South African and other African alphavirus isolates with nsP4 sequences available in GenBank used in multiple alignment for primer design

Species	Isolate	Country of isolation	Year of isolation	Source of isolation	Accession number
SINV	AR18132	South Africa	1974	*Culex univittatus*	MK045247
	SAAR_18141	South Africa	1976	*Culex univittatus*	MK045246
	Girdwood S.A.	South Africa	1962	*Homo sapiens*	U38304
	SAAR_6071	South Africa	1964	*Culex univittatus*	MK045250
	S.A.AR86	South Africa	1954	*Culex spp.*	U38305
MIDV	SaAr 749	South Africa	1957	*Aedes caballus*	AF339486
	ArB-8422	Central African Republic	1977	*Aedes vittatus*	KM115530
	ArTB-5290	Central African Republic	1984	*Amblyomma variegatum*	KM115531
	SAE25_11	South Africa	2011	Horse	KF680222
	MIDV857	Zimbabwe	1993	Horse	EF536323

Species	Isolate	Country of isolation	Year of isolation	Source of isolation	Accession number
CHIKV	Lamu33	Kenya	2004	*Homo sapiens*	HQ456255
	KPA15	Kenya	2004	*Homo sapiens*	HQ456254
	CAR256	Central African Republic	Unknown	Unknown	HM045793
	HB78	Central African Republic	1978	*Homo sapiens*	HM045822
	M2022	Angola	1962	Unknown	HM045823
	UgAg4155	Uganda	1982	*Homo sapiens*	HM045812
	DakAr B 16878	Central African Republic	1984	*Anopheles funestus*	HM045784
	LSFS	Democratic Republic of the Congo	1960	*Homo sapiens*	HM045809
	AR 18211	South Africa	1976	*Aedes furcifer*	HM045805
	SAH2123	South Africa	1976	*Homo sapiens*	HM045795
	A301	Senegal	1963	Chiroptera	HM045821
	Vereeniging	South Africa	1956	*Homo sapiens*	HM045792
	Ross low-psg	Tanzania	1953	*Homo sapiens*	HM045811
NDUV	GSA_S5_4278	Kenya	Unknown	Mosquito	JX644171
	SaAr 2204	South Africa	1959	Unknown	AF339487
	UGPV	Uganda	2010	*Sus scrofa*	JN989958
	BSA_S4_2268	Kenya	Unknown	Mosquito	JX644169
	BAR_S2_3527	Kenya	Unknown	Mosquito	JX644167
	GSA_S1_936	Kenya	Unknown	Mosquito	JX644170
	BSA_S4_2265	Kenya	Unknown	Mosquito	JX644168
	BAR_S2_3526	Kenya	Unknown	Mosquito	JX644166
SFV	ATH00510	Kenya	2010	Goat	KF283988
	Tanzania53	Tanzania	1953	*Homo sapiens*	MK280688

Species	Isolate	Country of isolation	Year of isolation	Source of isolation	Accession number
	A7	Unknown	Unknown	Unknown	Z48163
	A7(74)	Unknown	Unknown	Unknown	Y14761
ONNV	Gulu strain	Uganda	1959	*Homo sapiens*	M20303
	Ahero	Kenya	2001	*Anopheles funestus*	KX771232
	SG650	Uganda	1996-1997 epidemic	*Homo sapiens*	AF079456
	HB67652	Central African Republic	1966	*Homo sapiens*	MF409176
	IBH10964	Nigeria	1966	*Homo sapiens*	AF079457

2.2.2 Preparation of transcribed RNA positive controls

The positive controls used in this study included total SINV RNA extracted from SINV infected cells and four transcribed RNA controls (CHIKV, MIDV, NDUV and ONNV). SINV total RNA, extracted from isolate S.A.A.R86 cultured in the laboratory in a previous study (Litabe, 2020), was used as a positive control to represent SINV circulating in South Africa.

One isolate for CHIKV, MIDV, NDUV, and ONNV was selected to prepare a positive control to represent each member. Many of these viruses require biocontainment and hence non-infectious RNA was prepared for development and optimization of the assays. Transcribed RNA was prepared for each member using synthetic genes obtained from GenScript (Hong Kong) Ltd. Briefly, a representative isolate was selected for each member - CHIKV (isolate AR 18211), MIDV (isolate SAE25_11), NDUV (isolate SaAr 2204) and ONNV (isolate SG650). The target region, sized 570 bp, was identified within the nsP4 gene following the multiple alignment of alphavirus isolates as described previously. The 570 bp sequences for each isolate was modified by adding T7 and SP6 promoter regions to the 5’ and 3’ ends of the genes respectively (Appendix F), for downstream transcription. The genes were synthesised and supplied in pUC57 vectors by GenScript (Appendix F).

PCR was performed using the GoTaq Hot Start Polymerase Kit (Promega, USA) and Alphavirus nsP4 (F1) primer (Table 2.1) and SP6 primer (5'-ATTTAGGTGACACTATAG-3') to amplify the target region (570 bp) and downstream SP6 promotor region (20 bp) required for RNA transcription. For each control, PCR was performed in duplicate to increase template yield for transcription. The reaction mixtures were prepared to a total of 50 µl using the components listed in Table 2.4:

Table 2.4 Reaction components for plasmid DNA PCR prior to transcription

Components	Volume (µl)	Final concentration
5X Green or Colorless GoTaq® Flexi Buffer	10	1X
$MgCl_2$ Solution, 25 mM	4	2 mM
PCR Nucleotide Mix, 10 mM each	1	0.2 mM each dNTP
Alphavirus nsP4 (F1) forward primer (20 µM)	1	0.4 µM
SP6 reverse primer (20 µM)	1	0.4 µM
GoTaq® Hot Start Polymerase (5 U/µl)	0.25	1.25 U
Template DNA	1	-
Nuclease Free Water	31.75	-
Total	**50**	**-**

The PCR reactions were performed in a ProFlex™ PCR system thermocycler (ThermoScientific, California, USA) using the following cycling conditions: initial denaturation at 95 °C for 2 minutes, followed by 30 cycles of denaturation at 95 °C for 30 seconds, annealing at 45 °C for 30 second and an elongation at 72 °C for 35 seconds, ending with one final elongation cycle at 72 °C for 5 minutes. The reactions were subsequently held at 4 °C indefinitely.

To confirm amplification of the target genes, a 5 µl aliquot of each PCR product was used and the DNA fragments were separated by electrophoresis on a 1% Seakem®LE agarose gel (Lonza, Maine, USA) prepared in 1X Tris-acetate-EDTA (TAE) buffer (pH 8.0). See Table G1 in Appendix G regarding the % agarose gel used. A 3 µl aliquot of the molecular marker,

O'GeneRuler™ DNA Ladder Mix, ready-to-use, SM1173 (Thermo Scientific, USA) was used to obtain an estimated fragment size of the amplified product. Electrophoresis was powered by PowerPac™ Basic (Bio-rad, USA) at 90 Volts for 45 minutes, followed by post-staining for 45 minutes using GelRed™ Nucleic Acid Gel Stain 10000X solution (Biotium, Hayward, USA). Refer to Appendix G for composition of the post-stain. The DNA fragments were visualised using Image Lab™ Software (Bio-Rad, USA) using a Molecular Imager® Gel Doc™ XR System (BioRad, USA).

The remaining PCR products were subsequently separated by electrophoresis and excised and purified from a 1% agarose gel using the Wizard® SV Gel and PCR Clean-Up System (Promega, USA) according to manufacturer's instructions. Briefly, 10 µl Membrane Binding Solution was added per 10 mg gel slice which was subsequently vortexed and incubated at 65 °C until the gel slice was completely dissolved. For each control, the duplicate gel mixtures were transferred to one Wizard® SV minicolumn (inserted into a collection tube) consisting of a silica membrane to which DNA binds. The column assembly was incubated at room temperature for 1 minute, followed by centrifugation at 14,000 × g for 1 minute. The membrane was washed with 700 µl Membrane Wash Solution, followed by centrifugation at 14,000 × g for 1 minute. The membrane was washed for a second time with 500 µl Membrane Wash Solution, followed by centrifugation at 14,000 × g for 5 minutes. To allow evaporation of any residual ethanol, the column assembly was recentrifuged for 1 minute without the microcentrifuge lid. The minicolumn was subsequently transferred to a clean 1.5 ml microcentrifuge tube. To elute the DNA, 30µl of nuclease-free water was added to the column assembly and incubated at room temperature for 1 minute, followed by centrifugation at 14,000 × g for another minute. The purified products were visualised by gel electrophoresis as previously described, and the concentration was measured using a NanoDrop™ 2000 Spectrophotometer (Thermo Scientific, USA).

The purified DNA products were transcribed to RNA using the MEGAscript™ SP6 Transcription Kit (Thermo Scientific, USA) according to the manufacturer's instructions. The transcription reaction mixtures were prepared to a total of 20 µl each using the components listed in Table 2.5:

Table 2.5 Reaction components for transcription

Components	Volume (µl)
ATP solution	2
CTP solution	2
GTP solution	2
UTP solution	2
10X Reaction Buffer	2
Purified DNA template (~ 0.1 – 0.2 µg)	1
Enzyme mix	2
Nuclease Free Water	7
Total	**20**

The reaction mixtures were incubated at 37 °C for 4 hours.

The transcribed RNA products were subsequently purified using the MEGAclear™ Kit (Thermo Scientific, USA) according to the manufacturer's instructions. Briefly, for each transcribed product, 80 µl of Elution Solution was added to bring the sample to 100 µl. Following gentle mixing, 350 µl of Binding Solution Concentrate was added to the sample, and after gentle mixture, 250 µl of 100% ethanol was added to the sample, followed by gentle mixture. The mixture was added to a filter cartridge - collection tube assembly and centrifuged at 14,000 × g for 1 minute. After the flow-through was discarded, 500 µl of Wash Solution was added to the filter cartridge - collection tube assembly and centrifuged at 14,000 × g for 1 minute. This wash step was repeated with a second 500 µl aliquot of Wash Solution, and after the flow-through was discarded, the assembly was centrifuged for an additional 30 seconds to remove the last traces of Wash Solution. A 50 µl aliquot of Elution Solution was added to the center of the filter cartridge. The closed tube was incubated at 65 °C for 5–10 minutes in a heat block, and the eluted RNA was recovered by centrifuging at 14,000 × g for 1 minute at room temperature. To maximise RNA recovery, the elution step was repeated with a second 50 µl aliquot of Elution Solution. The purified RNA product was stored at -80 °C.

Prior to RT-PCR and RT-HDA applications, RNA working solutions were prepared by treating the transcribed RNA with RQ1 RNase-Free DNase (Promega, USA) to eliminate traces of genomic DNA. Briefly, the DNase digestion reaction was prepared to a total volume of 10 µl using the components listed in Table 2.6:

Table 2.6 Reaction components for DNase treatment

Components	Volume (µl)
Transcribed RNA	1
RQ1 RNase-Free DNase 10X Reaction Buffer	1
RQ1 RNase-Free DNase	1
Nuclease Free Water	7
Total	**10**

The reaction mixtures were incubated at 37 °C for 1 hour. Subsequently, 1 µl of RQ1 DNase Stop Solution was added to the reaction mixtures which were incubated at 65 °C for 10 minutes to inactivate the DNase. Subsequent to performing the PCR, amplicons were visualised using agarose gel electrophoresis as described in sections 2.2.3 and 2.2.4 to confirm the absence of amplifiable genomic DNA in the RNA working solutions. The working solutions were stored at -80 °C.

2.2.3 Development of the conventional nested RT-PCR assay

RT-PCR was performed using the Transcriptor One-Step RT-PCR Kit (Roche, USA) according to the manufacturer's instructions. The transcribed RNA controls prepared in the previous sections and total SINV RNA available in the laboratory were used as template, and Alphavirus nsP4 (F1) and Alphavirus nsP4 (R1) primers (Table 2.1) were used to amplify the target region of 570 bp. The reaction mixtures were prepared to a total of 50 µl using the components listed in Table 2.7:

Table 2.7 Reaction components for conventional RT-PCR

Components	Volume (µl)	Final concentration
5X Reaction Buffer	10	1X
Alphavirus nsP4 (F1) forward primer (20 µM)	1	0.4 µM
Alphavirus nsP4 (R1) reverse primer (20 µM)	1	0.4 µM
Transcriptor Enzyme Mix	1	-
Template RNA	5	-
Nuclease Free Water	32	-
Total	**50**	**-**

The reactions were cycled as follows: reverse transcription at 50 °C for 30 minutes followed by initial denaturation at 94 °C for 7 minutes. Subsequently, a standard PCR profile was followed consisting of 10 cycles of denaturation at 94 °C for 10 minutes, annealing at 56 °C for 30 minutes, elongation at 68 °C for 34 seconds, and 25 cycles of denaturation at 94 °C for 10 minutes, annealing at 56 °C for 30 minutes, elongation at 68 °C for 34 seconds with 3 seconds added for each successive cycle, ending with one cycle for final elongation at 68 °C for 7 minutes. The reactions were held at 4 °C indefinitely.
The RT-PCR products were visualised by gel electrophoresis, as previously described.

Subsequently, nested PCR was performed using the GoTaq Hot Start Polymerase Kit (Promega, USA), using the PCR products produced in the previous round as template and Alphavirus nsP4 (F2) and Alphavirus nsP4 (R2) primers to amplify the target region of 200 bp. The reaction mixtures were prepared to a total of 50 µl using the components listed in Table 2.8:

Table 2.8 Reaction components for nested PCR

Components	Volume (µl)	Final concentration
5X Green or Colorless GoTaq® Flexi Buffer	10	1X
$MgCl_2$ Solution, 25 mM	4	2 mM
PCR Nucleotide Mix, 10 mM each	1	0.2 mM each dNTP
Alphavirus nsP4 (F2) forward primer (20 µM)	1	0.4 µM
Alphavirus nsP4 (R2) reverse primer (20 µM)	1	0.4 µM
GoTaq® Hot Start Polymerase (5 U/µl)	0.25	1.25 U
Template DNA	1	-
Nuclease Free Water	31.75	-
Total	**50**	**-**

The reactions were cycled as follows: initial denaturation at 95 °C for 2 minutes, followed by 30 cycles of denaturation at 95 °C for 30 seconds, annealing at 45 °C for 30 second and an elongation at 72 °C for 12 seconds, ending with one final elongation cycle at 72 °C for 5 minutes. The reactions were held at 4 °C indefinitely. The nested PCR products were subsequently visualised by gel electrophoresis, as previously described.

2.2.4 Development of the RT-HDA assay

The IsoAmp® II Universal tHDA Kit (New England BioLabs, USA) used for RT-HDA development in this study provided two possible protocols that are compatible with lateral flow end-point detection. Hence two methods for RT-HDA development were compared.

2.2.4.1 Development and optimization of the One-Step RT-HDA method

A One-Step RT-HDA was performed using the IsoAmp® II Universal tHDA Kit (New England BioLabs, USA) according to the manufacturer's instructions. Total SINV RNA and transcribed MIDV RNA were used as template RNA. HDA Forward (5' biotin) and HDA Reverse (5' 6-FAM) primers (Table 2.2) were used to amplify the 116 bp partial nsP4 gene. The reaction mixtures were prepared to a total of 50 µl using the components listed in Table 2.9:

Table 2.9 Reaction components for One-Step RT-HDA

Components	Volume (µl)	Final concentration
10X Annealing buffer II	5	-
MgSO4 (100 mM)	1.75	3.5 mM
NaCl (500 mM)	4	40 mM
IsoAmp® dNTP Solution	3.5	-
RNA template	5	-
Forward Primer (5 µM)	0.75	75 nM
Reverse Primer (5 µM)	0.75	75 nM
IsoAmp® Enzyme Mix	3.5	-
ProtoScript II RT (2 U/µl)	1.0	2 U
Nuclease Free Water	24.75	-
Total	**50**	**-**

Initially, RNA was reverse transcribed by incubating the reaction tubes in a heating block at 42 °C for 2 minutes, followed 65 °C for 2 hours for amplification. A 5 µl aliquot of each RT-HDA amplicon was added to a clean microcentrifuge tube containing 70 µl of PCRD Extraction Buffer, and the 75 µl diluted reaction mixture was added to the sample well of a PCRD test cassette. The results were visualised within 10 minutes.

In addition, to verify that the primers amplified the RNA using the conditions described, the RT-HDA amplicons were separated and visualised by electrophoresis using a 2% agarose gel. See Table G1 in Appendix G regarding the % agarose gel used.

The method was optimised to counter false positive results and improve the outcome of true positive results by experimenting with parameters such as duration of amplification (2 hours, 1.5 hours and 1 hour), primer concentration (50 nM, 75 nM and 100 nM), duration of reverse transcription (2 minutes and 10 minutes) and units of reverse transcriptase (1 unit, 2 units, 4 units and 8 units). Transcribed MIDV RNA was used as RNA template during all optimization experiments.

Using the optimal parameters, the One-Step RT-HDA method was performed using the IsoAmp® II Universal tHDA Kit, SINV and MIDV positive controls as RNA template, a negative control using nuclease free water as template, and HDA Forward (5' biotin) and HDA Reverse (5' 6-FAM) primers to amplify the 116 bp partial nsP4 gene. The reaction mixtures were prepared to a total of 50 µl using the components listed in Table 2.10:

Table 2.10 Reaction components for optimized One-Step RT-HDA

Components	Volume (µl)	Final concentration
10X Annealing buffer II	5	-
MgSO4 (100 mM)	1.75	3.5 mM
NaCl (500 mM)	4	40 mM
IsoAmp® dNTP Solution	3.5	-
RNA template	5	-
Forward Primer (5 µM)	0.75	75 nM
Reverse Primer (5 µM)	0.75	75 nM
IsoAmp® Enzyme Mix	3.5	-
ProtoScript II RT (2 U/µl)	4.0	8 U
Nuclease Free Water	21.75	-
Total	**50**	**-**

The reaction tubes were incubated in a heating block at 42 °C for 10 minutes for reverse transcription, followed 65 °C for 1 hours for amplification. The RT-HDA products were visualised on PCRD test cassettes as previously described.

2.2.4.2 Development and optimization of the Two-Step RT-HDA method

A Two-Step RT-HDA was performed using the IsoAmp® II Universal tHDA Kit (New England BioLabs, USA) according to the manufacturer's instructions. Total SINV RNA and transcribed MIDV RNA were used as template RNA, and HDA Forward (5' biotin) and HDA Reverse (5' 6-FAM) primers (Table 2.2) were used to amplify the 116 bp partial nsP4 gene.

Two reaction mixtures, designated Mix A and Mix B, were prepared to a total of 25 µl each using the components listed in Table 2.11:

Table 2.11 Reaction components for Two-Step RT-HDA

Mix A		
Components	**Volume (µl)**	**Final concentration**
10X Annealing buffer II	2.5	-
RNA template	5	-
Forward Primer (5 µM)	0.75	75 nM
Reverse Primer (5 µM)	0.75	75 nM
Nuclease Free Water	16	-
Total	**25**	**-**
Mix B		
Components	**Volume (µl)**	**Final concentration**
10X Annealing buffer II	2.5	-
MgSO4 (100 mM)	1.75	3.5 mM
NaCl (500 mM)	4	40 mM
IsoAmp® dNTP Solution	3.5	-
IsoAmp® Enzyme Mix	3.5	-
ProtoScript II RT (2.0 U/µl)	0.5	1 U
Nuclease Free Water	9.25	-
Total	**25**	**-**

The tubes were subsequently placed on ice.

Mix A reaction tubes were incubated in a heating block at 95 °C for 2 minutes for denaturation, then placed immediately on ice. The contents of Mix B were added to Mix A, and the Mix A tubes were returned to the heating block and incubated at 42 °C for 10 minutes for reverse transcription, followed 65 °C for 2 hours for amplification. The RT-HDA products were visualised on PCRD test cassettes as previously described in section 2.2.4.1. Although certain parameters were tested to see if the method could be optimised further, such as primer

concentration (75 nM, 100 nM, 200 nM and 400 nM), units of reverse transcriptase (1 unit, 2 units, 4 units and 8 units) and duration of reverse transcription (increased to 30 minutes), the original parameters proved to be optimal.

2.2.5 Minimum detection limit

Freshly transcribed MIDV RNA was prepared, and RNA working solutions treated with RQ1 RQ1 RNase-Free DNase were prepared as described in section 2.2.2. The RNA working solutions were measured using a Qubit™ 4 Fluorometer (Thermo Scientific, USA). RNA copy number was determined using the following equation:

$$Number\ of\ RNA\ copies = \frac{amount\ (ng) \times 6.022x10^{23}}{length\ (bp) \times molecular\ weight\ of\ ssRNA}$$

The RNA controls were serially diluted 10-fold and amplified using the conventional nested RT-PCR assay described in section 2.2.3, and the One-Step and Two-step RT-HDA methods described in section 2.2.4.1 and 2.2.4.2, to determine the minimum limit of detection of the assays.

For the RT-PCR assay, the amplicons were visualised by gel electrophoresis as previously described. For the One-Step and Two-Step RT-HDA methods, and the second round nested PCR assay were visualised by gel electrophoresis, as previously described in section 2.2.4. A 10 µl aliquot of each RT-HDA amplicon was added to a clean microcentrifuge tube containing 140 µl of PCRD Extraction Buffer, and a PCRD FLEX dipstick was inserted into the tube. The results were visualised within 10 minutes. The PCRD FLEX dipsticks were used in place of PCRD test cassettes used in prior RT-HDA experiments due to the lower price of the dipsticks.

2.2.6 Theoretical specificity

Theoretical cross-reactivity with other alphaviruses was assessed by multiple alignment of the primer sequences with the partial nsP4 sequences of SINV and CHIKV isolates from other regions and genotypes, and isolates from alphaviruses endemic to other parts of world, such as RRV, BFV, MAYV, EEEV, VEEV and WEEV (Table 2.12). The sequences were aligned

using Clustal Omega version 1.2.4 (https://www.ebi.ac.uk/Tools/msa/clustalo/), and the alignment is shown in Appendix H.

Table 2.12 Alphavirus isolates used to test theoretical specificity of the assays

Alphavirus	Isolate	Country of isolation	Year of isolation	Source of isolation	Accession number
SINV	95M116 (European isolate from SINV-I genotype)	Sweden	1995	*Aedes cinereus*	MK045231
	XJ-160 (SINV-IV genotype)	China	1970	*Culicidae* spp.	AF103728
	M78 (SINV-V genotype)	New Zealand	1962	*Culex pervigilans*	AF339479
	SW6562 (SINV-VI genotype)	Australia	1990	*Culex annulirostris*	AF429428
CHIKV	SVUKDP-09 (Asian genotype)	India	2009	*Homo sapiens*	JN558836
RRV	PW14	Australia	2009	*Homo sapiens*	MK028843
BFV	SW94093	Australia	2012	*Ochlerotatus camptorhynchus*	MW835350
MAYV	TRVL 4675	Trinidad and Tobago	1954	*Homo sapiens*	MK070492
EEEV	VT125787	USA	2012	*Culiseta melanura*	KT153581

Alphavirus	Isolate	Country of isolation	Year of isolation	Source of isolation	Accession number
VEEV	PE21-0029	Peru	1999	*Culex gnomatos*	MH086249
WEEV	Lake43	USA	1994	*Culex tarsalis*	KJ554985

2.3 Results

2.3.1 Preparation of transcribed RNA positive controls

Transcribed RNA was prepared for CHIKV, MIDV, NDUV and ONNV using synthetic genes that were supplied in pUC57 vectors by GenScript, to be used as positive controls for this study. A 570 bp target region was identified within the nsP4 gene following the multiple alignment of 40 alphavirus isolates from South Africa and Africa, and the sequences of the representative isolates selected for the four alphavirus members were modified by adding T7 and SP6 promoter regions to the 5' and 3' ends of the genes respectively. To amplify the partial nsp4 gene (570 bp) of interest and a promoter region (SP6 promoter region: 20 bp) for downstream transcription, PCR was performed using the GoTaq Hot Start Polymerase Kit and the Alphavirus nsP4 (F1) forward primer and SP6 reverse primer. Amplification of the predicted 590 bp products was confirmed on a 1% agarose gel (Figure 2.1).

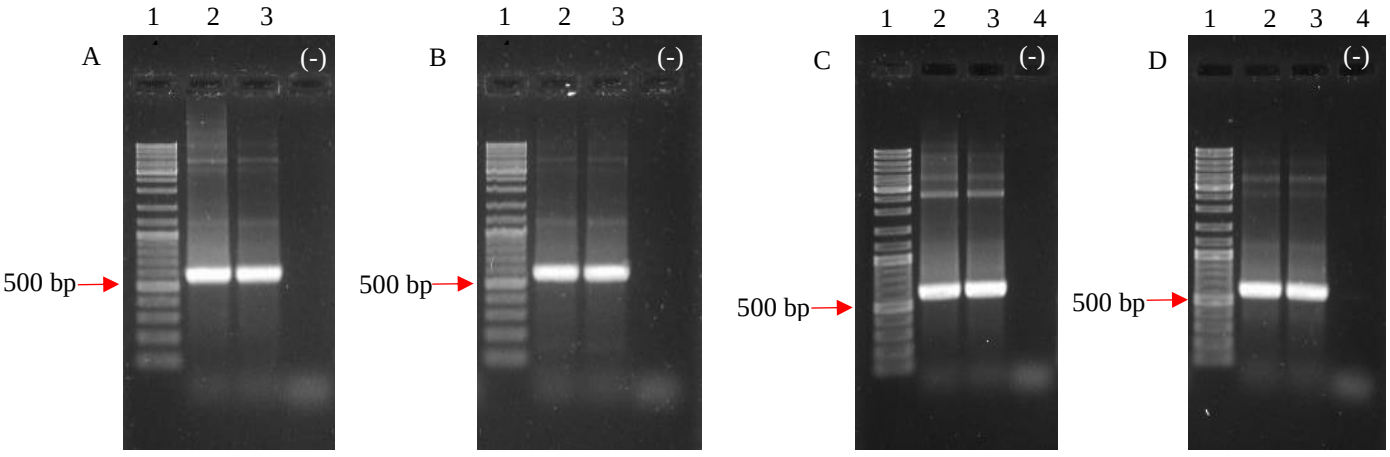

Figure 2.1: PCR amplification of synthetic genes. Lanes 1: O'GeneRuler DNA Ladder Mix (Thermo Scientific, USA), lanes 2 and 3: PCR amplification of the partial nsP4 gene + SP6 promotor region (590 bp), lanes 4: negative control. A. CHIKV, B. MIDV, C. NDUV, D. ONNV

For each alphavirus, the PCR products were excised from 1% agarose gels and combined during purification using the Wizard® SV Gel and PCR Clean-Up System. The purified 590 bp products were confirmed on a 1% agarose gel (Figure 2.2), and DNA concentrations were measured using a NanoDrop spectrophotometer (Table 2.13)

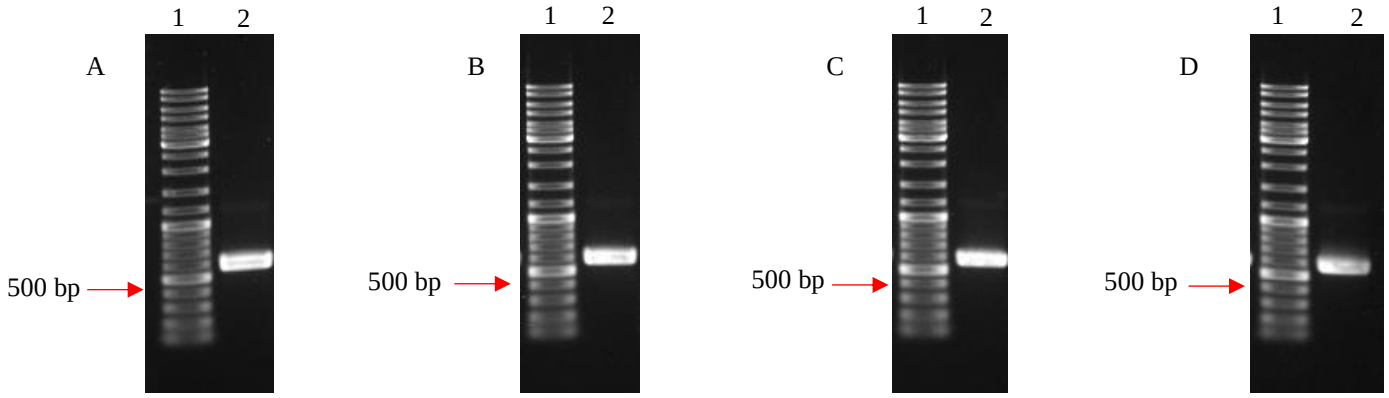

Figure 2.2: Purified alphavirus PCR products. Lanes 1: O'GeneRuler DNA Ladder Mix (Thermo Scientific, USA), lanes 2: purified PCR products (590 bp). A. CHIKV, B. MIDV, C. NDUV, D. ONNV

Table 2.13 DNA concentration of purified PCR products used as template for transcription

Alphavirus control	**DNA concentration (ng/µl)**
CHIKV	211.3
MIDV	146.7
NDUV	272.3
ONNV	197.6

Approximately 0.1 – 0.2 µg of the purified DNA products were transcribed to RNA using the MEGAscript™ SP6 Transcription Kit.

RNA working solutions were prepared by treating the transcribed RNA with RQ1 RNase-Free DNase to eliminate traces of genomic DNA. PCR followed by agarose gel electrophoresis was performed to confirm the absence of amplifiable genomic DNA in the RNA working solutions (Figure 2.3).

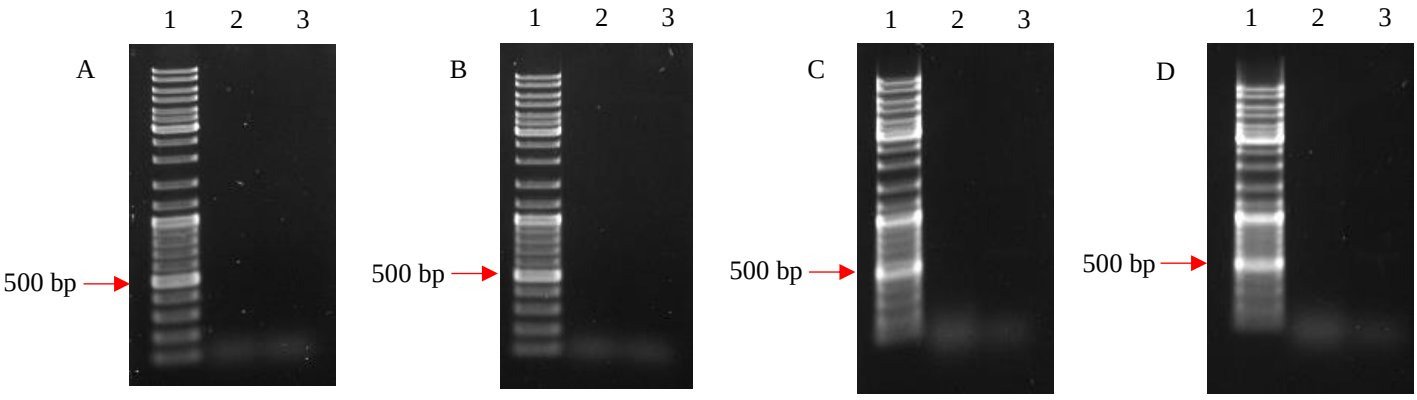

Figure 2.3: Results from PCR amplification of DNase treated transcribed RNA. Lanes 1: O'GeneRuler DNA Ladder Mix (Thermo Scientific, USA), lanes 2 and 3: DNase treated transcribed RNA, lanes 4: negative control. A. CHIKV, B. MIDV, C. NDUV, D. ONNV

2.3.2 Development of conventional nested RT-PCR assay

The transcribed RNA controls and the SINV total RNA control were used to develop and validate the assay. The controls were used as template for amplification using the Transcriptor One-Step RT-PCR Kit. Alphavirus nsP4 (F1) and Alphavirus nsP4 (R1) primers were used to amplify the 570 bp target region. The RT-PCR products were visualised by gel electrophoresis, which showed amplicons of the expected size (approximately 570 bp) for each RNA control (Figure 2.4), therefore, the degenerate primers worked and were capable of amplifying each virus.

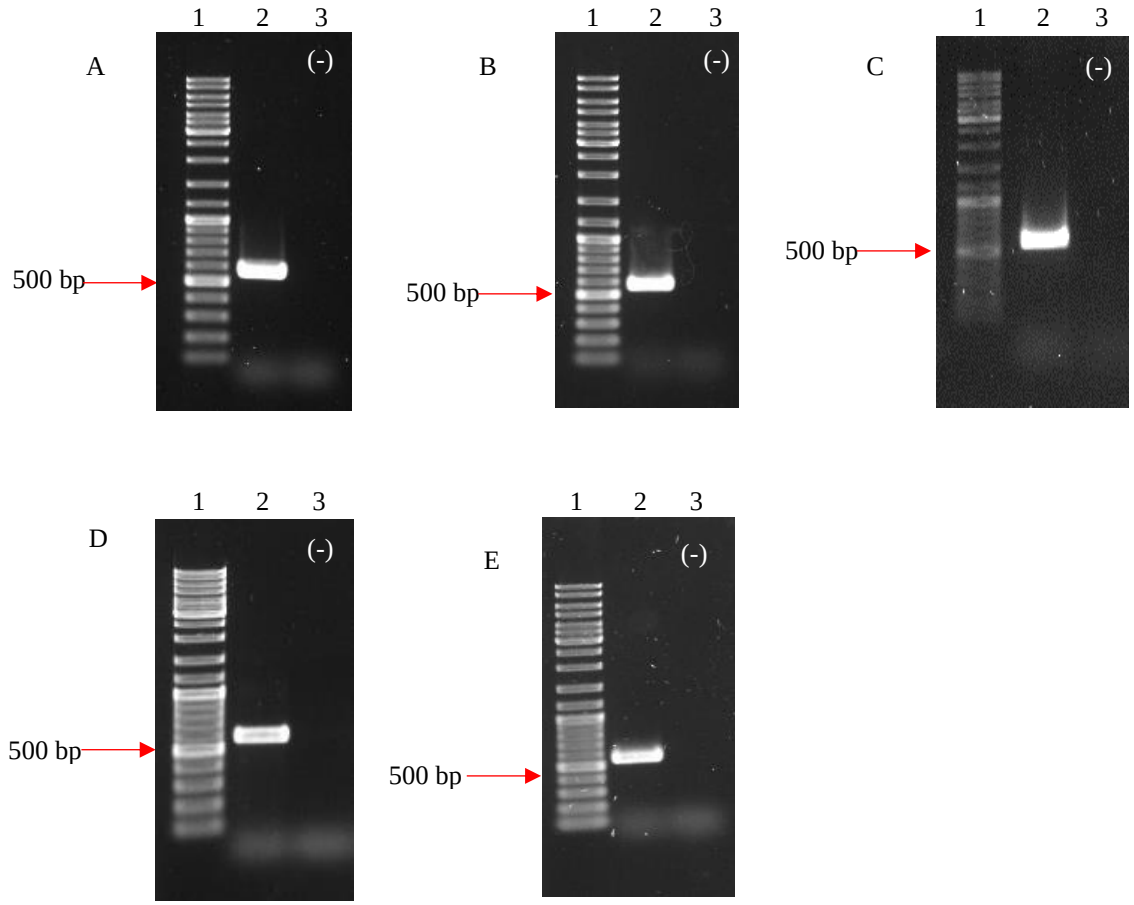

Figure 2.4: First round PCR products from conventional RT-PCR. Lanes 1: O'GeneRuler DNA Ladder Mix (Thermo Scientific, USA), lanes 2 and 3: RT-PCR products (570 bp), lanes 4: negative control. A. CHIKV, B. MIDV, C. NDUV, D. ONNV, E. SINV

Nested PCR using the GoTaq Hot Start Polymerase Kit was subsequently performed using the PCR products produced in the previous round as template. Alphavirus nsP4 (F2) and Alphavirus nsP4 (R2) primers to amplify the target region of 200 bp. The RT-PCR products were visualised by gel electrophoresis, which displayed amplification bands of the expected size (approximately 200 bp) for each RNA control (Figure 2.5), therefore, the degenerate primers worked and were capable of amplifying each virus.

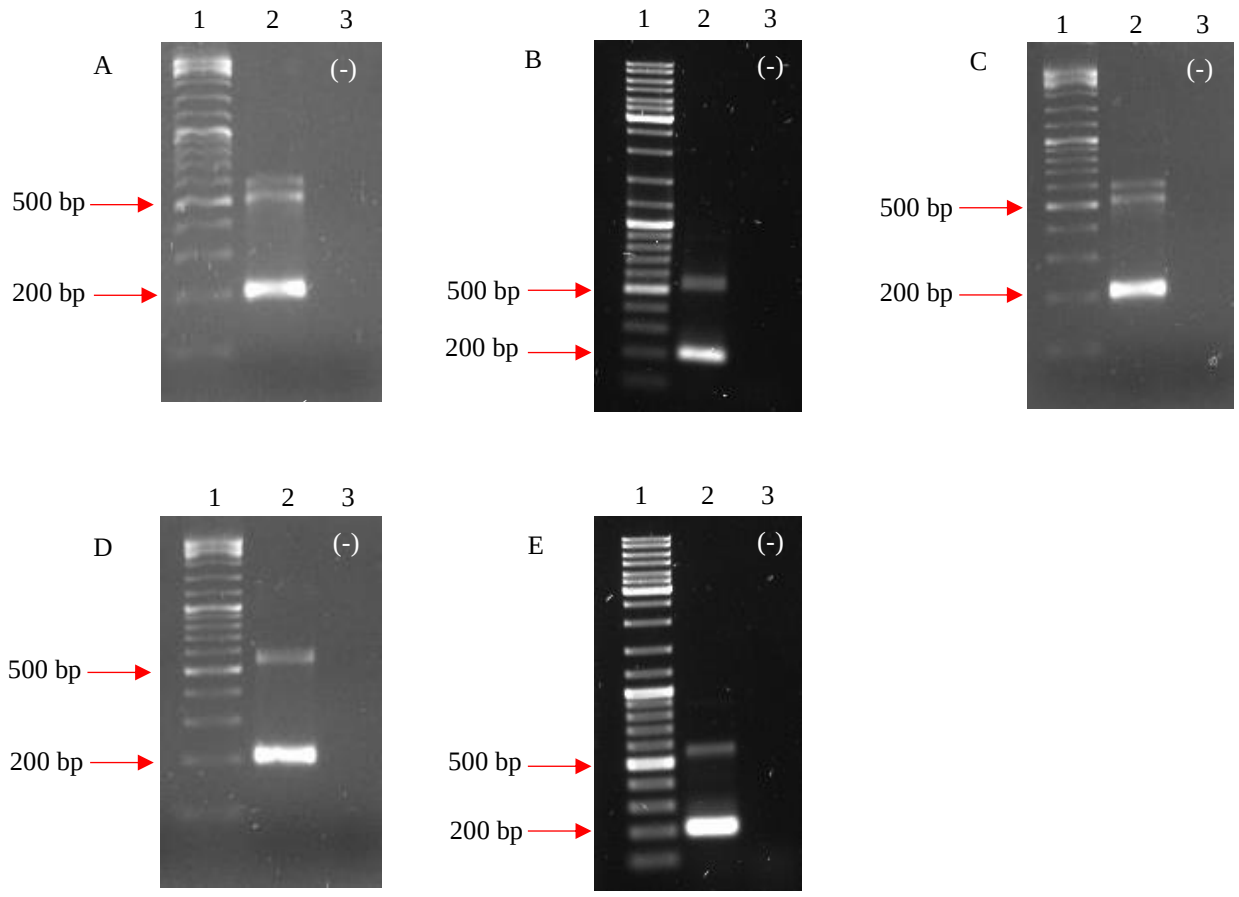

Figure 2.5: Second round (nested) PCR products. Lanes 1: O'GeneRuler DNA Ladder Mix (Thermo Scientific, USA), lanes 2 and 3: nested PCR products (200 bp), lanes 4: negative control. A. CHIKV, B. MIDV, C. NDUV, D. ONNV, E. SINV

2.3.3 Development of the RT-HDA assay

The IsoAmp® II Universal tHDA Kit (New England BioLabs, USA) used for RT-HDA development in this study provided two possible protocols that are compatible with lateral flow end-point detection. Hence two methods for RT-HDA development were compared. Total SINV RNA and transcribed MIDV RNA were used as controls to develop, optimise, and validate the assay. SINV and MIDV controls were selected as these alphaviruses are known to occur in South Africa.

2.3.3.1 One-Step RT-HDA and optimization

The One-Step RT-HDA method was performed using the IsoAmp® II Universal tHDA Kit, and HDA Forward (5' biotin) and HDA Reverse (5' 6-FAM) primers to amplify the 116 bp partial nsP4 gene. Total SINV RNA and transcribed MIDV RNA positive controls and a negative control were used. The RT-HDA products were visualised on PCRD test cassettes (Figure 2.6). The PCRD test cassettes contain three reaction lines. Line 1 detects DIG-Biotin labelled amplicons, line 2 detects FAM-Biotin or FITC-Biotin labelled amplicons, and line C is a flow-check control line. Since FAM-Biotin labels were used, lines 2 and C will indicate a positive result. As shown in Figure 2.6, a line was present for the negative control. However, when the products were separated by electrophoresis and visualised there was no indication of a positive amplicon for the negative control (Figure 2.7). The PCRD is highly sensitive and may detect the presence of non-specific amplification products and primer dimers as "positive".

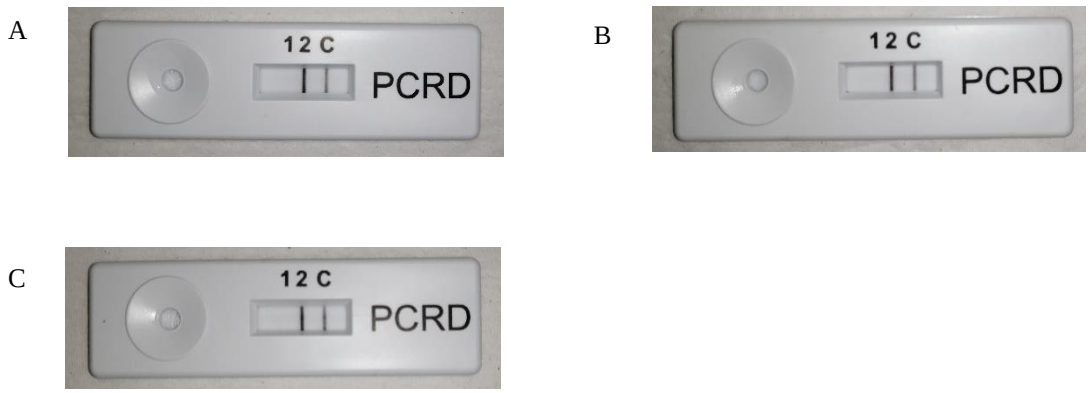

Figure 2.6: One-Step RT-HDA (using original parameters) products visualised on PCRD test cassettes. A. Transcribed MIDV RNA positive control, B. Total SINV RNA positive control, C. Negative control

In addition, to confirm that the primers amplified the target region, the amplicons were visualised on a 2% agarose gel. Amplicons of the expected size (116 bp) were confirmed (Figure 2.7), therefore, the degenerate primers worked and were capable of amplifying each virus.

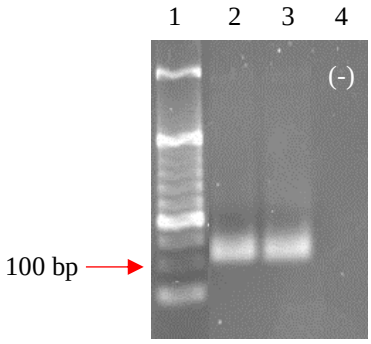

Figure 2.7: One-Step RT-HDA (using original parameters) products visualised on a 2% agarose gel. Lane 1: O'RangeRuler 50 bp DNA Ladder (ThermoScientific, USA), lane 2: MIDV RT-HDA amplicon, lane 3: SINV RT-HDA, lane 4: negative control

Therefore, to overcome false positive results in the PCRD device, the method was repeated by experimenting with the duration of amplification (2 hours, 1.5 hours and 1 hour), using the lowest recommended primer concentration (50 nM), and nuclease free water as template for negative control. The absence of the false positive test line was achieved by decreasing the amplification time to 1 hour (Figure 2.8).

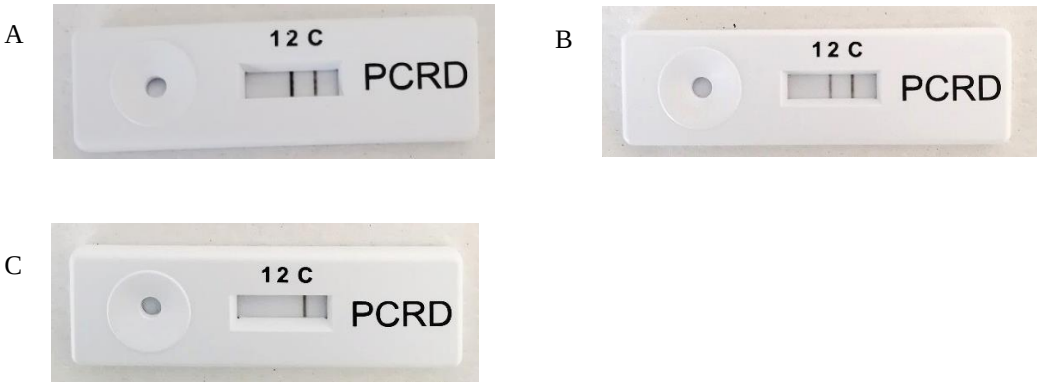

Figure 2.8: Results from One-Step RT-HDA using varying times for amplification. A. 2 hours, B. 1.5 hours, C. 1 hour

To optimise the method further, parameters such as primer concentration (50 nM, 75 nM and 100 nM), duration of reverse transcription (2 minutes and 10 minutes) and units of reverse transcriptase (1 unit, 2 units, 4 units and 8 units) were tested. Transcribed MIDV RNA was used as RNA template in these experiments.

Three different primer concentrations – 50 nM, 75 nM and 100 nM were tested, and the results were compared. Due to the intensity of line 2 observed, 75 nM was confirmed as the optimal primer concentration (Figure 2.9).

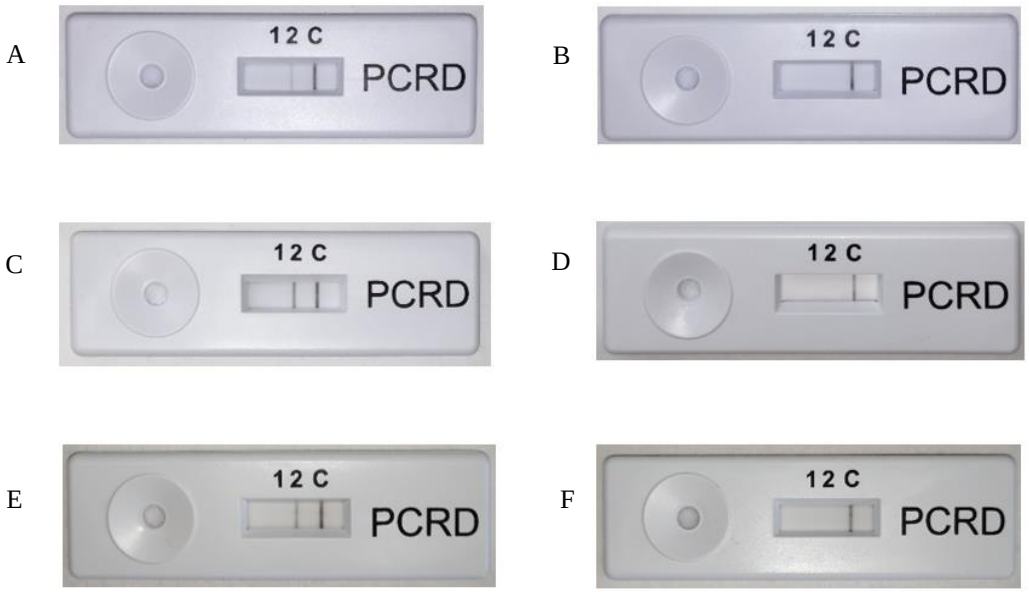

Figure 2.9: Results from One-Step RT-HDA using varying primer concentrations. Testing 50 nM primers using a positive control (A) and a negative control (B), testing 75 nM primers using a positive control (C) and a negative control (D), testing 100 nM primers using a positive control (E) and a negative control (F)

The amount of reverse transcriptase, from 1 to 8 units, and reverse transcription incubation time, 2 minutes and 10 minutes, were also tested. Due to the intensity of line 2 observed, 8 units of reverse transcriptase and 10 minutes of reverse transcription were considered optimal (Figure 2.10 and 2.11).

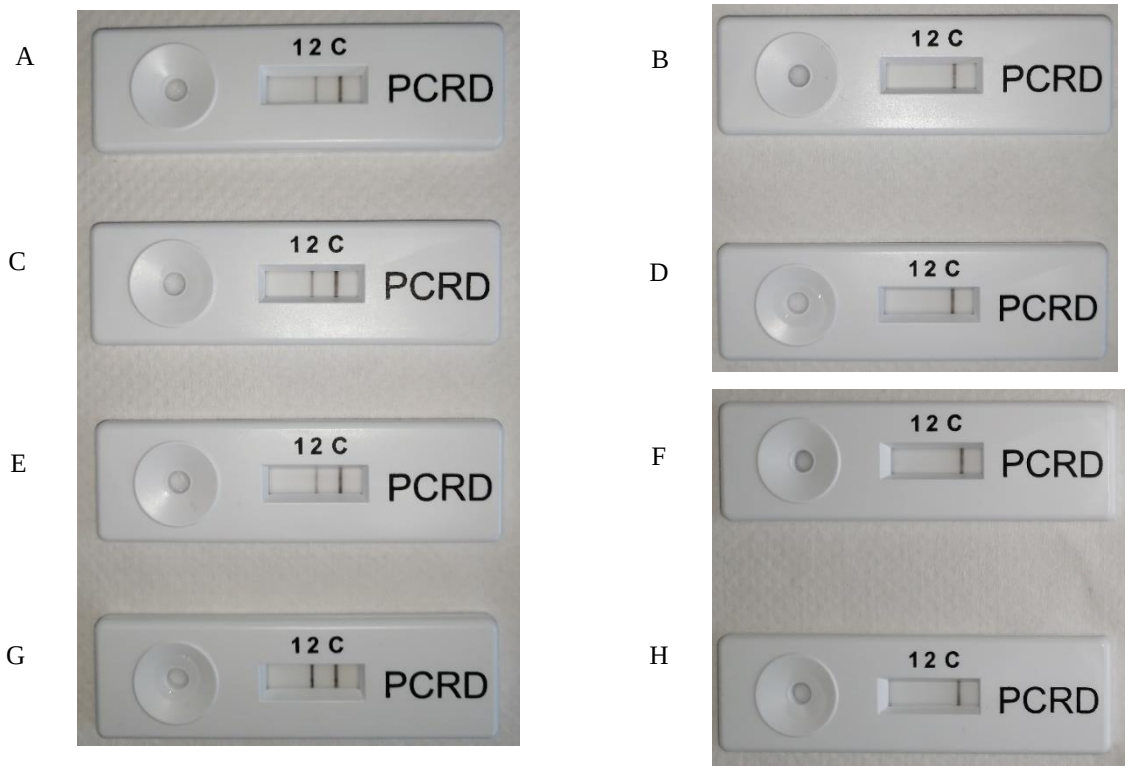

Figure 2.10: Results from One-Step RT-HDA using varying units of reverse transcriptase for 2 minutes. Testing 1 unit of reverse transcriptase using a positive control (A) and a negative control (B), testing 2 units of reverse transcriptase using a positive control (C) and a negative control (D), testing 4 units of reverse transcriptase using a positive control (E) and a negative control (F), testing 8 units of reverse transcriptase using a positive control (G) and a negative control (H)

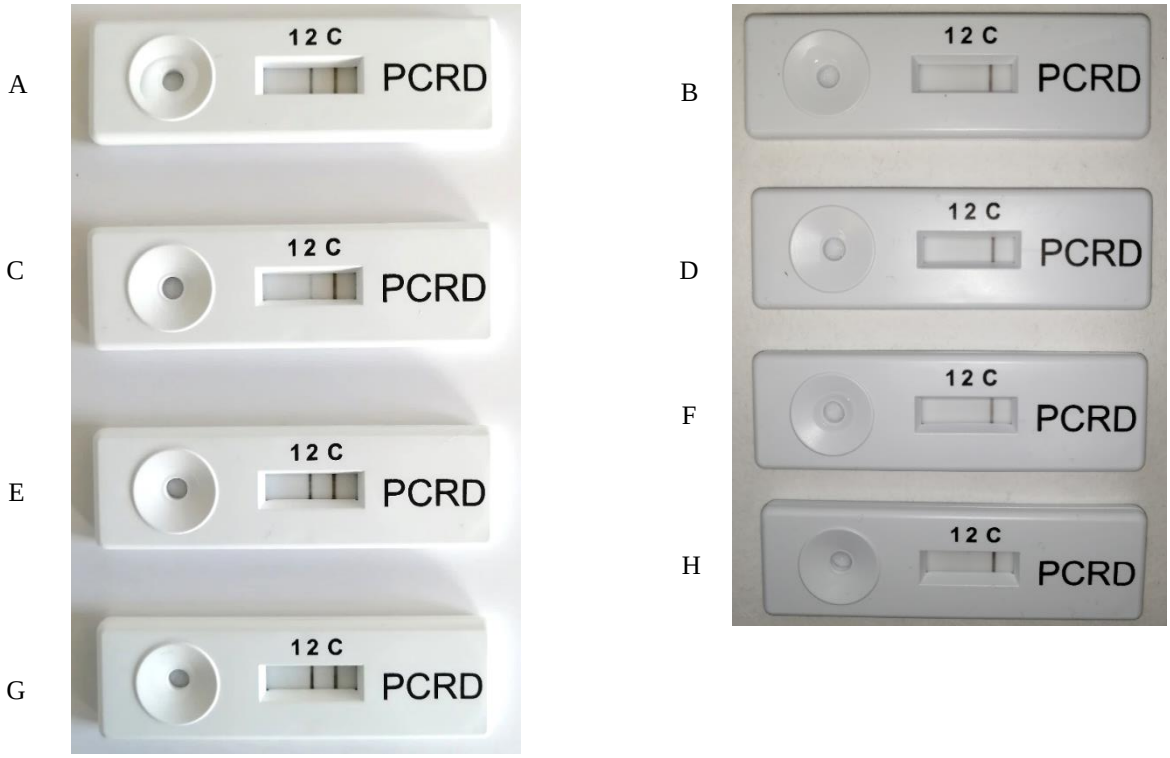

Figure 2.11: Results from One-Step RT-HDA using varying units of reverse transcriptase for 10 minutes. Testing 1 unit of reverse transcriptase using a positive control (A) and a negative control (B), testing 2 units of reverse transcriptase using a positive control (C) and a negative control (D), testing 4 units of reverse transcriptase using a positive control (E) and a negative control (F), testing 8 units of reverse transcriptase using a positive control (G) and a negative control (H)

Using the optimal parameters (Table 2.14), the One-Step RT-HDA method was performed using both SINV and MIDV positive controls as RNA template, with nuclease free water as template for the negative control.

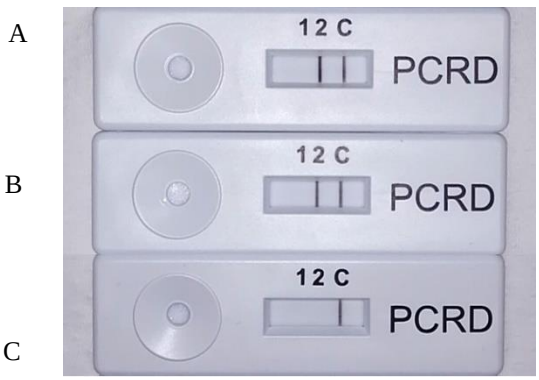

Figure 2.12: Optimised One-Step RT-HDA. A. MIDV positive control, B. SINV positive control, C. Negative control

Table 2.14: Optimal parameters for the One-Step RT-HDA method

Duration of amplification	1 hour
Primer concentration	75 nM
Duration of reverse transcription	10 minutes
Units of reverse transcriptase	8 units

2.3.3.2 Two-Step RT-HDA and optimization

The Two-Step RT-HDA method was performed using the IsoAmp® II Universal tHDA Kit, and HDA Forward (5' biotin) and HDA Reverse (5' 6-FAM) primers to amplify the 116 bp partial nsP4 gene. SINV and MIDV positive controls and a negative control were used. The RT-HDA products were visualised on PCRD test cassettes. As shown in Figure 2.13, a false positive reaction was not indicated for the negative control using the original parameters as seen with the One-Step RT-HDA method.

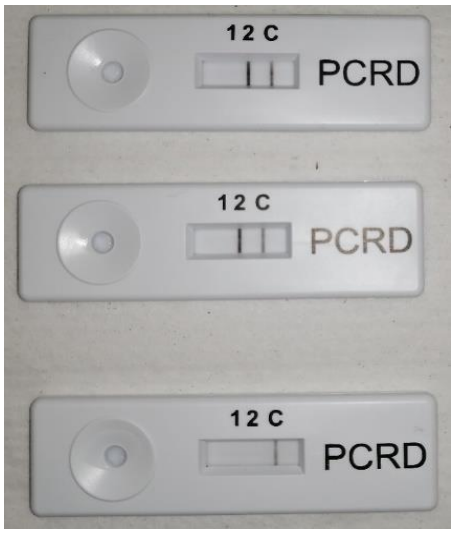

Figure 2.13: Two-Step RT-HDA (using original parameters) products visualised on PCRD test cassettes. A. Transcribed MIDV RNA, B. Total SINV RNA, C. Negative control

To see if the assay could be optimised further, parameters such as primer concentration, units of reverse transcriptase, and duration of reverse transcription were increased, however, the original parameters proved to be optimal (Table 2.15).

Table 2.15: Optimal parameters for the Two-Step RT-HDA method

Duration of amplification	2 hours
Primer concentration	75 nM
Duration of reverse transcription	10 minutes
Units of reverse transcriptase	1 unit

In Figure 2.14, the primer concentration was increased from 75 nM to 100 nM, 200 nM and 400 nM. Due to the intensity of line 2 observed, the original primer concentration, 75 nM, was confirmed as the optimal primer concentration.

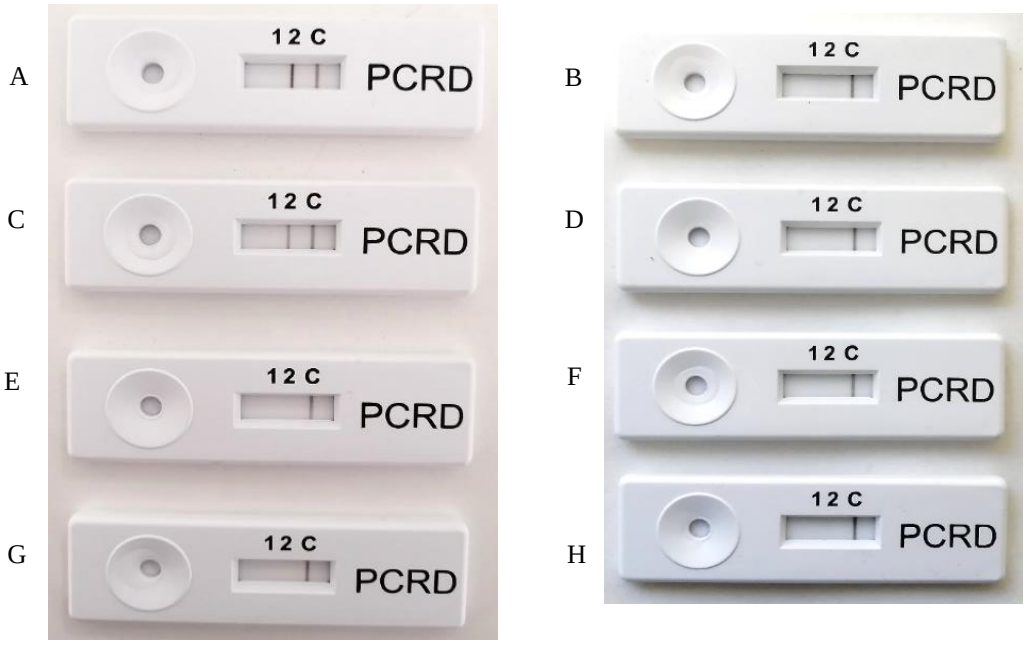

Figure 2.14: Results from Two-Step RT-HDA using varying primer concentrations. Testing 75 nM primers using a positive control (A) and a negative control (B), testing 100 nM primers using a positive control (C) and a negative control (D), testing 200 nM primers using a positive control (E) and a negative control (F), testing 400 nM primers using a positive control (G) and a negative control (H)

In Figure 2.15, the amount of reverse transcriptase was increased from 1 to 8 units. Due to the intensity of line 2 observed, the original number of units for reverse transcriptase, 1 unit of reverse transcriptase was considered optimal.

In Figure 2.16, the duration of reverse transcription was increased to 30 minutes, with varying duration periods for amplification. No amplification was detected by increasing the duration of reverse transcription to 30 minutes, therefore, the original duration period for reverse transcription, 10 minutes, was confirmed optimal.

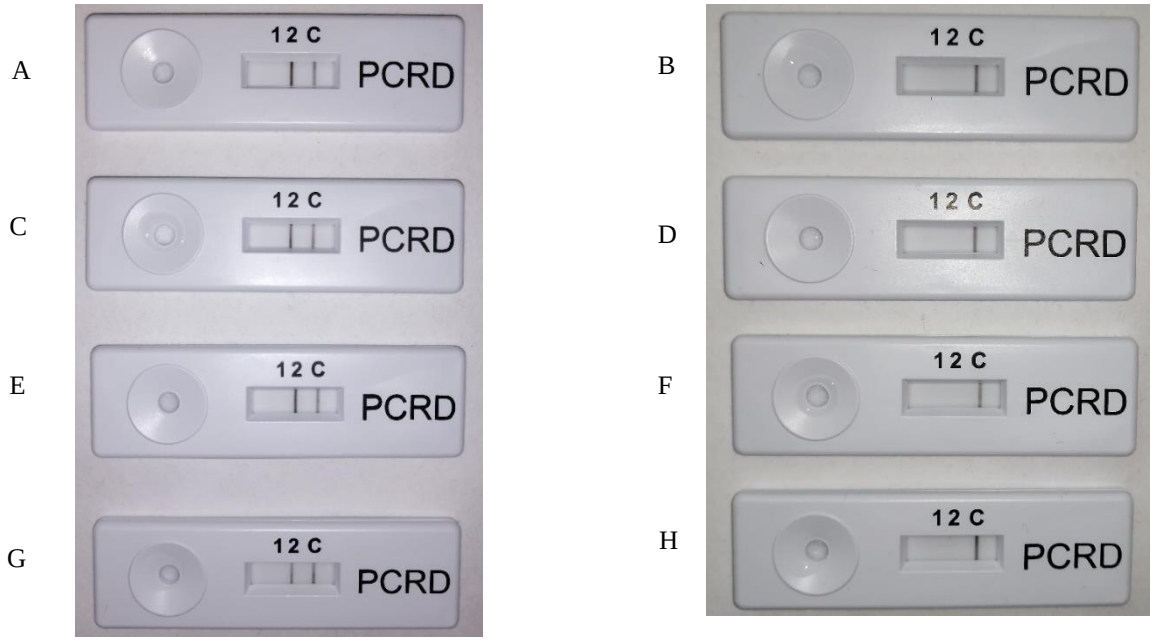

Figure 2.15: Results from Two-Step RT-HDA using varying units of reverse transcriptase. Testing 1 unit of reverse transcriptase using a positive control (A) and a negative control (B), testing 2 units of reverse transcriptase using a positive control (C) and a negative control (D), testing 4 units of reverse transcriptase using a positive control (E) and a negative control (F), testing 8 units of reverse transcriptase using a positive control (G) and a negative control (H)

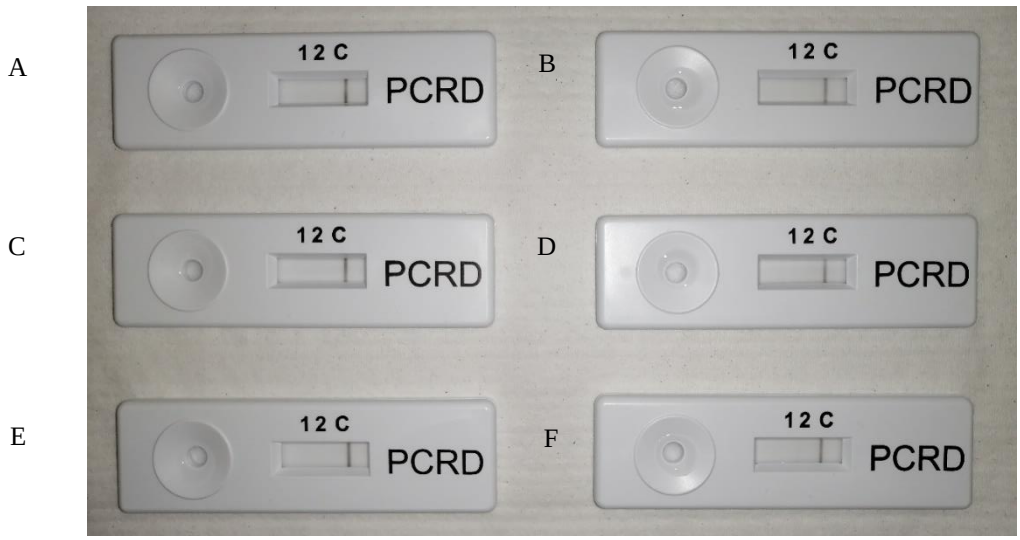

Figure 2.16: Results from Two-Step RT-HDA, extending reverse transcription to 30 minutes, with varying duration periods for amplification. Testing 30 minutes of reverse transcription + 2 hours of amplification using a positive control (A) and a negative control (B), testing 30 minutes of reverse transcription + 1.5 hours of amplification using a positive control (C) and a negative control (D), testing 30 minutes of reverse transcription + 1 hour of amplification using a positive control (E) and a negative control

2.3.4 Minimum detection limit

Three preparations of transcribed MIDV RNA were used and quantified using a Qubit™ 4 Fluorometer (Table 2.16). RNA copy number was determined using the equation described in section 2.2.5. The MIDV RNA controls were serially diluted 10-fold, and the minimum detection limit of the conventional nested RT-PCR assay, the One-Step RT-HDA method and the Two-Step RT-HDA method was determined.

Table 2.16 Concentration of the transcribed MIDV RNA controls used to determine minimum limit of detection of the molecular assays

Transcribed MIDV RNA Controls	Concentration (ng/µl)
MIDV control used to test the minimum limit of detection of the conventional nested RT-PCR assay	2
MIDV control used to test the minimum limit of detection of the One-Step RT-HDA method	1.56
MIDV control used to test the minimum limit of detection of the Two-Step RT-HDA method	1.72

2.3.4.1 Minimum detection limit of the conventional nested RT-PCR assay

Transcribed MIDV RNA was serially diluted 10-fold from 6.2 x 10^9 copies to 6.2 x 10^0 copies of RNA to determine the minimum limit of detection of the conventional nested RT-PCR assay. The first round RT-PCR was able to detect 6.2 x 10^5 copies of RNA (Figure 2.17).

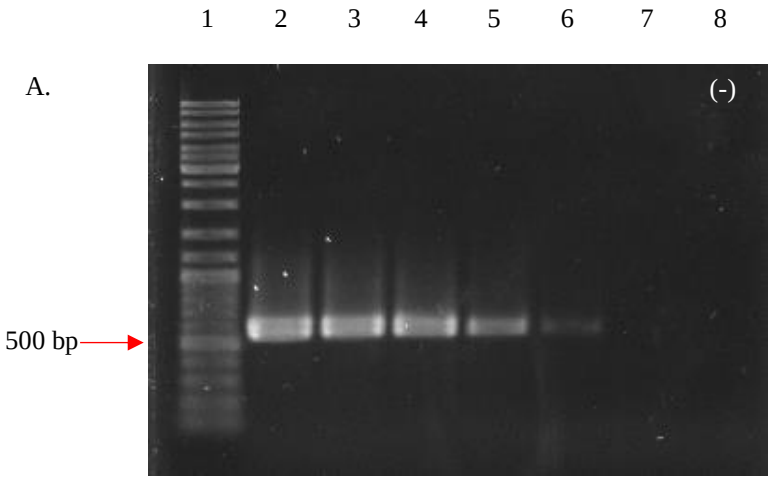

Figure 2.17: Minimum detection limit of the first round RT-PCR. Amplicons of expected size (570 bp) obtained for ~6.2 x 10^9 copies to 6.2 x 10^5 copies of MIDV RNA. Lane 1. DNA ladder, lane 2. ~6.2 x 10^9 RNA copies, lane 3. ~6.2 x 10^8 RNA copies, lane 4. ~6.2 x 10^7 RNA copies, lane 5. ~6.2 x 10^6 RNA copies, lane 6. ~6.2 x 10^5 RNA copies, lane 7. ~6.2 x 10^4 RNA copies (no amplification observed), lane 8. Negative control

Nested PCR was subsequently performed using the amplicons produced from the first round RT-PCR as template, and the assay was able to detect 6.2 x 10^2 copies of RNA (Figure 2.18).

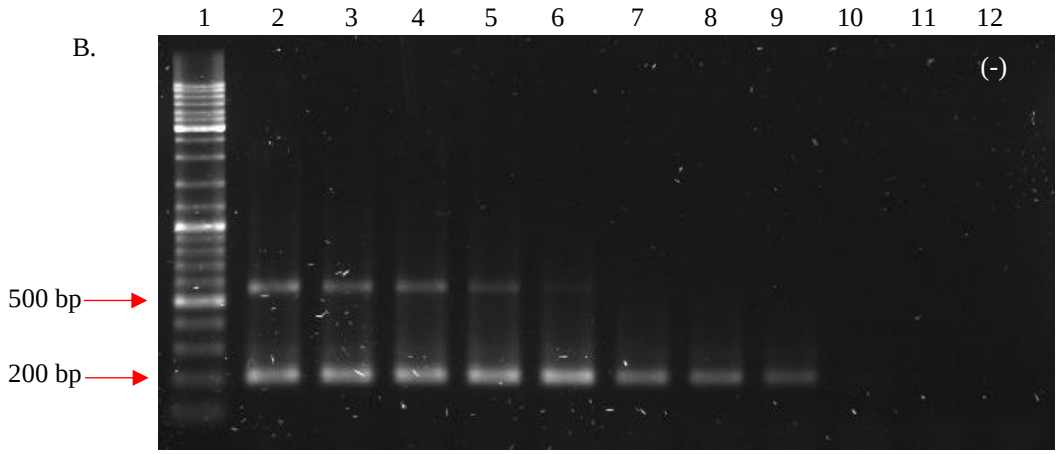

Figure 2.18: Minimum detection limit of second round nested PCR. Amplicons of expected size (200 bp) obtained for PCR products representing ~6.2 x 10^9 copies to 6.2 x 10^2 copies of MIDV RNA. Lane 1. DNA ladder, lane 2. ~6.2 x 10^9 RNA copies, lane 3. ~6.2 x 10^8 RNA copies, lane 4. ~6.2 x 10^7 RNA copies, lane 5. ~6.2 x 10^6 RNA copies, lane 6. ~6.2 x 10^5 RNA copies, lane 7. ~6.2 x 10^4 RNA copies, lane 8. ~6.2 x 10^3 RNA copies, lane 9. ~6.2 x 10^2 RNA copies, lane 10. ~6.2 x 10^1 RNA copies (no amplification observed), lane 11. ~6.2 x 10^0 RNA copies (no amplification observed), lane 12. Negative control

2.3.4.2 Minimum detection limit of the One-Step RT-HDA method

Transcribed MIDV RNA was serially diluted 10-fold from approximately 4.8 x 10^9 copies to 4.8 x 10^0 copies of RNA to determine the minimum limit of detection of the One-Step RT-HDA method. The PCRD FLEX dipsticks were used in place of PCRD test cassettes used in prior RT-HDA experiments due to the lower price of the dipsticks. Alike the test cassettes, Line 1, closest to the sample application port, detects DIG-Biotin labelled amplicons, line 2 detects FAM-Biotin or FITC-Biotin labelled amplicons, and line C, furthest from the sample application port, is a flow-check control line. Since FAM-Biotin labels were used, lines 2 and C indicate the positive result. As shown in Figure 2.19, the One-Step method was able to detect 4.8 x 10^5 copies of RNA.

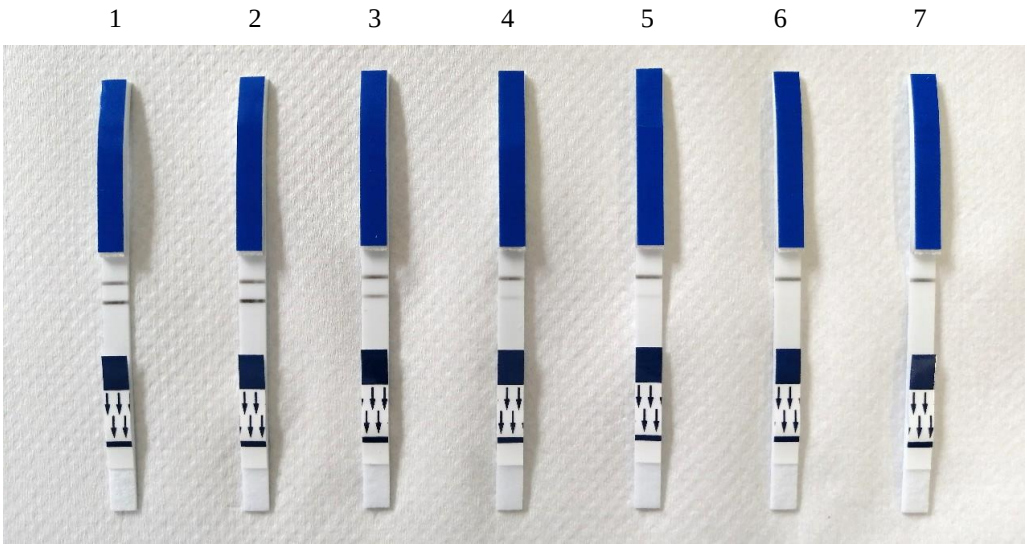

Figure 2.19: Minimum detection limit of the One-Step RT-HDA method. No. 1: ~4.8 x 10^9 RNA copies, no. 2: ~4.8 x 10^8 RNA copies, no. 3: ~4.8 x 10^7 RNA copies, no. 4: ~4.8 x 10^6 RNA copies, no. 5: ~4.8 x 10^5 RNA copies, no. 6: ~4.8 x 10^4 RNA copies (no amplification observed), no. 7: Negative control

2.3.4.2 Minimum detection limit of the Two-Step RT-HDA method

Transcribed MIDV RNA was serially diluted 10-fold from approximately 5.3 x 10^9 copies to 5.3 x 10^0 copies of RNA to determine the minimum limit of detection of the Two-Step RT-HDA method. As shown in Figure 2.20, the Two-Step method was able to detect 5.3 x 10^7 copies of RNA.

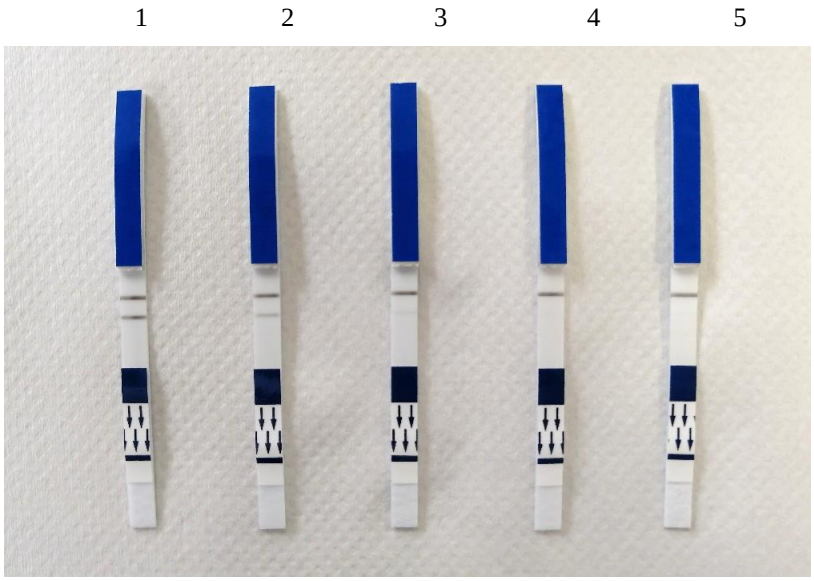

Figure 2.20: Minimum detection limit of the Two-Step RT-HDA method. No. 1: ~5.3 x 10^9 RNA copies, no. 2: ~5.3 x 10^8 RNA copies, no. 3: ~5.3 x 10^7 RNA copies, no. 4: 5.3 x 10^6 RNA copies (no amplification observed), no. 5: Negative control

2.3.5 Theoretical specificity

Theoretical cross-reactivity with other alphaviruses was assessed by multiple alignment of the primer sequences with the partial nsP4 sequences of SINV and CHIKV isolates from other regions and genotypes, and isolates from alphaviruses endemic to other parts of world, such as RRV, BFV, MAYV, EEEV, VEEV and WEEV. The alignment of the primer sequences with the sequences of these isolates is shown in Appendix H, and Table 2.17 indicates the number of mismatches between the conventional nested RT-PCR primers and the target regions of each isolate, and Table 2.18 indicates the number of mismatches between the RT-HDA primers and the target regions of each isolate.

Table 2.17 Number of mismatches between the conventional nested RT-PCR primers and the target regions of each isolate

Alphavirus	Sequence 5' – 3' (Mismatches indicated in red)	No. of mismatches
Alphavirus nsP4 (F1) primer	AARTTYGGVGCNATGATGAA	
SINV isolate 95M116 (SINV-I genotype; Sweden)	AAATTCGGGGCGATGATGAA	0
SINV isolate XJ-160 (SINV-IV genotype; China)	AAATTTGGTGCAATGATGAA	1
SINV isolate M78 (SINV-V genotype; New Zealand)	AAATTTGGATCGATGATGAA	1
SINV isolate SW6562 (SINV-VI genotype; Australia)	AAATTCGGGGCGATGATGAA	0
CHIKV isolate SVUKDP-09 (Asian genotype; India)	AAGTTCGGCGCCATGATGAA	0
RRV isolate PW14	AAGTTCGGCGCCATGATGAA	0
BFV isolate SW94093	AGATTTGGAGCTATGATGAA	1
MAYV isolate TRVL 4675	AAATTTGGTGCTATGATGAA	1
EEEV isolate VT125787	AAGTTCGGGGCAATGATGAA	0
VEEV isolate PE21-0029	AAGTTCGGGGCAATGATGAA	0

Alphavirus	Sequence 5’ – 3’ (Mismatches indicated in red)	No. of mismatches
Alphavirus nsP4 (F1) primer	AARTTYGGVGCNATGATGAA	
WEEV isolate Lake43	AAATTTGGTGCCATGATGAA	1
Alphavirus nsP4 (R1) primer	CWATTTAGGWCCRCCGTASA	
SINV isolate 95M116 (SINV-I genotype; Sweden)	CTATTTAGGACCACCGTAGA	0
SINV isolate XJ-160 (SINV-IV genotype; China)	CTATTTAGGACCACCGTAGA	0
SINV isolate M78 (SINV-V genotype; New Zealand)	CTATTTAGGACCGCCGTAGA	0
SINV isolate SW6562 (SINV-VI genotype; Australia)	CTATTTAGGACCACCGTAGA	0
CHIKV isolate SVUKDP-09 (Asian genotype; India)	CTATTTAGGACCGCCGTACA	0
RRV isolate PW14	CTATTTAGGACCGCCGTAGA	0
BFV isolate SW94093	CTATTTAGGACCACCGTAGA	0
MAYV isolate TRVL 4675	CTATTTAGGACCGCCGTACA	0
EEEV isolate VT125787	CTATTTAGGTCAGCCGTAGA	1
VEEV isolate PE21-0029	CCATTCAGGTTAGCCGTAGA	4
WEEV isolate Lake43	CTATTTAGGTCAGCCGTAGA	1
Alphavirus nsP4 (F2) primer	GCNATGATGAARTCNGGHATG	
SINV isolate 95M116 (SINV-I genotype; Sweden)	GCGATGATGAAATCCGGAATG	0
SINV isolate XJ-160 (SINV-IV genotype; China)	GCAATGATGAAATCCGGAATG	0
SINV isolate M78 (SINV-V genotype; New Zealand)	TCGATGATGAAATCTGGAATG	1
SINV isolate SW6562 (SINV-VI genotype; Australia)	GCGATGATGAAATCCGGAATG	0

Alphavirus	Sequence 5’ – 3’ (Mismatches indicated in red)	No. of mismatches
Alphavirus nsP4 (F2) primer	GCNATGATGAARTCNGGHATG	
CHIKV isolate SVUKDP-09 (Asian genotype; India)	GCCATGATGAAATCAGGTATG	0
RRV isolate PW14	GCCATGATGAAGTCCGGAATG	0
BFV isolate SW94093	GCTATGATGAAGTCCGGAATG	0
MAYV isolate TRVL 4675	GCTATGATGAAGTCAGGCATG	0
EEEV isolate VT125787	GCAATGATGAAATCCGGGATG	1
VEEV isolate PE21-0029	GCAATGATGAAGTCCGGCATG	0
WEEV isolate Lake43	GCCATGATGAAATCCGGTATG	0
Alphavirus nsP4 (R2) primer	TTMACYTCCATGTTSAKCCA	
SINV isolate 95M116 (SINV-I genotype; Sweden)	TTAACTTCCATGTTGAGCCA	0
SINV isolate XJ-160 (SINV-IV genotype; China)	TTAACTTCCATGTTAAGCCA	1
SINV isolate M78 (SINV-V genotype; New Zealand)	TTCACTTCCATGTTAAGCCA	1
SINV isolate SW6562 (SINV-VI genotype; Australia)	TTTACTTCCATGTTAAGCCA	2
CHIKV isolate SVUKDP-09 (Asian genotype; India)	TTCACTTCCATGTTCATCCA	0
RRV isolate PW14	TTTACCTCCATATTAACCCA	4
BFV isolate SW94093	TTGACCTCCATGTTCATCCA	1
MAYV isolate TRVL 4675	TTAACCTCCATGTTAACCCA	2
EEEV isolate VT125787	TTCACTTCCATGTTCAACCA	1
VEEV isolate PE21-0029	TTTACTTCCATGTTCAACCA	1
WEEV isolate Lake43	TTTACTTCCATGTTCAGCCA	0

Table 2.18 Number of mismatches between the RT-HDA primers and the target regions of each isolate

Alphavirus	Sequence 5' – 3' (Mismatches indicated in red)	Number of mismatches
HDA Forward (5' biotin) primer	ATGAAATCYGGMATGTTCCTSACGCT	
SINV isolate 95M116 (SINV-I genotype; Sweden)	ATGAAATCCGGAATGTTCCTCACGCT	0
SINV isolate XJ-160 (SINV-IV genotype; China)	ATGAAATCCGGAATGTTTCTTACATT	4
SINV isolate M78 (SINV-V genotype; New Zealand)	ATGAAATCTGGAATGTTCCTCACCCT	1
SINV isolate SW6562 (SINV-VI genotype; Australia)	ATGAAATCCGGAATGTTCCTCACACT	1
CHIKV isolate SVUKDP-09 (Asian genotype; India)	ATGAAATCAGGTATGTTCCTAACTCT	4
RRV isolate PW14	ATGAAGTCCGGAATGTTCCTGACACT	2
BFV isolate SW94093	ATGAAGTCCGGAATGTTTTTGACGTT	4
MAYV isolate TRVL 4675	ATGAAGTCAGGCATGTTTCTTACATT	6
EEEV isolate VT125787	ATGAAATCCGGGATGTTTCTGACACT	3
VEEV isolate PE21-0029	ATGAAGTCCGGCATGTTTCTTACCCT	4
WEEV isolate Lake43	ATGAAATCCGGTATGTTCTTAACGCT	3
HDA Reverse (5' 6-FAM) primer	TCGCCGATRAAKGCKGCACATTTRGA	
SINV isolate 95M116 (SINV-I genotype; Sweden)	TCGCCGATGAATGCTGCACATTTGGA	0
SINV isolate XJ-160 (SINV-IV genotype; China)	TCGCCGATAAACGCCGCGCATCTCGA	5
SINV isolate M78 (SINV-V genotype; New Zealand)	TCACCGATGAATGCGGCGCATCGCGA	5

Alphavirus	Sequence 5’ – 3’ (Mismatches indicated in red)	Number of mismatches
HDA Reverse (5’ 6-FAM) primer	TCGCCGATRAAKGCKGCACATTTRGA	
SINV isolate SW6562 (SINV-VI genotype; Australia)	TCGCCAATGAACGCTGTACATCTGGA	4
CHIKV isolate SVUKDP-09 (Asian genotype; India)	TCGCCGATGAAGGCCGCGCATGCGGA	4
RRV isolate PW14	TCCCCGATAAACGCGGCGCAGATGGA	5
BFV isolate SW94093	TCTCCTATGAAAGCAGCGCACGGCGA	9
MAYV isolate TRVL 4675	TCGCCGATAAATGCGGCACAGGCTGA	5
EEEV isolate VT125787	TCGCCGATAAATGCTGCGCAAGGGGA	4
VEEV isolate PE21-0029	TCGCCTATGAACGCTGCACACGGTGA	5
WEEV isolate Lake43	TCGCCGATAAAGGCCGCGCACGCTGA	5

The conventional nested RT-PCR assay may be able to detect most alphaviruses due to minimal mismatches detected between the primers and the partial nsP4 sequences of the alphavirus isolates. Some of the mismatches are positioned where there is a degenerate base on the primer, for example, the first nine nucleotides of the Alphavirus nsP4 (F1) primer sequence is AARTTYGGV, while the first nine nucleotides of SINV isolate XJ-160 is AAATTTGGT. The “V” in the ninth position of the Alphavirus nsP4 (F1) primer sequence can be altered to an “N” so that the primer can detect the sequence of SINV isolate XJ-160, without having to add a new degenerate base in the primer sequence. No mismatches were detected with SINV isolate 95M116, an isolate from Sweden classified in the SINV-I genotype.

Conversely, the RT-HDA assay may not be able to detect other alphaviruses due to the many mismatches detected between the primers and the partial nsP4 sequences of the alphavirus isolates, except for SINV isolate 95M116, showing zero mismatches.

2.4 Discussion

Alphaviruses are genetically diverse, presenting challenges for their detection and surveillance in environmental samples such as RNA extracts from mosquitoes. Due to their diversity, numerous separate assays must be performed to ensure their detection. The possibility of the presence of more than one alphavirus in a pool of mosquitoes presents additional challenges (Eshoo et al., 2007). Several genus-specific and multiplex RT-PCR assays have been described for the detection of RNA for various alphavirus members (Bronzoni et al., 2004, 2005; Eshoo et al., 2007; Giry et al., 2017; Grywna et al., 2010; Pfeffer et al., 1997; Romeiro et al., 2016; Sánchez-Seco et al., 2001; Wang et al., 2006), however, these assays may not be well suited to detect the alphavirus isolates circulating in South Africa since more than four base degeneracies are required between the sequences of the primers described in these studies and the sequences of alphavirus isolates from South Africa. It is recommended to limit the number of degenerate bases per primer to no more than four (Li et al., 2012).

One of the more notable outbreaks of human alphavirus infection in South Africa occurred between 1983 and 1984, where hundreds of human SINV infections were reported in the Witwatersrand-Pretoria region. These outbreaks were linked to the increased number of *Cx. univittatus* due to the unusually high temperatures and heavy rainfall recorded in the summer throughout the mosquito season (Jupp et al., 1986).
Hence, developing rapid and reliable assays to detect alphavirus infections in mosquitoes can be used as an early warning system to predict future outbreaks before they arise.

In this study, a conventional nested RT-PCR assay was developed to simultaneously detect alphaviruses such as SINV, MIDV, CHIKV, SFV, NDUV and ONNV in mosquito vectors in South Africa. Although recent studies suggest that only SINV and MIDV circulate in South Africa (Steyn et al., 2020; Storm et al., 2013; van Niekerk et al., 2015), alphaviruses historically reported in South Africa such as CHIKV and NDUV, and alphaviruses reported in other regions of Africa such as ONNV and SFV, were included in the design of the assay to consider the possibility of resurgence or cross-border infections.

The nsP4 gene is the most conserved region among the alphaviruses and therefore appropriate for identifying regions for primer design which aligns with the findings from previous studies

(Brightwell et al., 1998; Giry et al., 2017; Grywna et al., 2010; Sánchez-Seco et al., 2001; J. H. Strauss & Strauss, 1994). South African and other African alphavirus isolates with available nsP4 sequences in GenBank were identified. Multiple alignment of the 40 chosen isolates allowed for identification of suitable consensus primer regions for assay development. Degenerate primers allow for amplification of a wide range of viral isolates, however, high degeneracy increases the possibility of primers binding to non-target regions (Elbrecht et al., 2018; Linhart & Shamir, 2002), thus no more than four degenerate bases were included per primer designed for this assay.

One isolate per virus – CHIKV, MIDV, NDUV, ONNV and SINV – was chosen to prepare a positive control to represent each member. Although a positive control was not prepared for SFV, the primers designed should in theory detect SFV African isolates. The positive controls include one total RNA control (SINV) and four transcribed RNA controls (CHIKV, MIDV, NDUV and ONNV). SINV total RNA, extracted from isolate S.A.A.R86 cultured in the laboratory in a previous study, was used as a positive control to represent SINV circulating in South Africa. Many of these viruses required biocontainment that was not available at that time, hence non-infectious RNA was prepared for development and optimization of the assays.

Nested PCR-based methods are advantageous over traditional or semi-nested PCR based methods, due to improved sensitivity and specificity of the former (Sánchez-Seco et al., 2001). Nested PCR/ RT-PCR involves the use of two primer sets and two successive amplification reactions. The first pair of primers (external primers) is used to amplify a target sequence to yield a primary amplicon. Subsequently, the second pair of primers (internal or nested primers) is used to amplify a smaller target sequence positioned within the primary amplicon.

The limit of detection of the first round of the conventional nested RT-PCR assay in this study, prior to nested amplification, was determined to be approximately 6.2×10^5 RNA copies. Subsequent to nested amplification, the limit of detection of the assay was determined to be approximately 6.2×10^2 RNA copies, or 620 copies of RNA. Nested amplification has indeed improved the sensitivity of the assay, which may be used to detect alphavirus in mosquitoes with low viral loads.

More sensitive RT-PCR assays were developed in the past for the simultaneous detection of more than one alphavirus member. A conventional nested RT-PCR assay developed by Sánchez-Seco et al., 2001 was able to detect up to approximately 1 – 10 copies of RNA, and a real-time TaqMan RT-PCR assay developed by Giry et al., 2017 was able to detect up to approximately 40 copies of RNA. These assays incorporate the use of inosine (I) containing oligonucleotides as an alternative to degenerate base codes to decrease primers degeneracy and thus improve sensitivity. Therefore, incorporation of inosine bases in the primers designed in this study should be considered as an option to further improve the sensitivity of the assay.

Theoretical cross-reactivity with other alphaviruses was assessed by multiple alignment of the conventional nested RT-PCR primer sequences with the partial nsP4 sequences of SINV and CHIKV isolates from other regions and genotypes, and isolates from alphaviruses endemic to other parts of world, such as RRV, BFV, MAYV, EEEV, VEEV and WEEV. SINV isolates include a northern European isolate from the SINV-I genotype to consider transcontinental transmission of the virus from northern Europe to South Africa and vice versa by migratory birds, and an isolate from other SINV genotypes which include SINV-IV, SINV-V and SINV-VI. There are no nsP4 sequence data currently available for the isolates SINV genotypes II and III. CHIKV isolates from the ECSA genotype, which include two Kenyan isolates from the 2004 Indian Ocean Outbreak, and an isolate from the West African genotype were included in the alignment when designing the primers. Therefore, to test theoretical cross-reactivity, a CHIKV isolate from the Asian genotype was compared. The conventional nested RT-PCR assay may be able to detect most alphaviruses due to minimal mismatches detected between the primers and the partial nsP4 sequences of the alphavirus isolates. No mismatches were detected with SINV isolate 95M116, an isolate from Sweden classified in the SINV-I genotype, therefore, the assay may be able to detect SINV infection brought from northern Europe to South Africa and vice versa by migratory birds.

A rapid fieldable isothermal assay was developed in this study as an alternative to the conventional nested RT-PCR assay for application in low resource settings, and to potentially screen for alphaviruses that are currently known to circulate in South Africa such as MIDV and SINV in mosquito vectors. The development and proof of concept of the assay was focused on alphaviruses known to occur in South Africa.

Two RT-HDA methods were compared – a One-Step RT-HDA and a Two-Step RT-HDA, and the more sensitive assay will be used to screen for MIDV and SINV in mosquito samples. Degenerate consensus primers were designed using conserved regions identified in the nsP4 gene following the multiple alignment of sequence data retrieved for South African and other African SINV and MIDV isolates which contain the nsP4 region in NCBI GenBank. A total of five SINV isolates and five MIDV isolates were identified. The primers were designed using the recommended specifications for the isothermal technique (Barreda-garcía & Miranda-castro, 2018).

The occurrence of false positive results was a challenge initially when performing the One-Step RT-HDA method. To overcome this challenge, the duration of amplification was decreased from two hours to one hour. Despite the simplicity of the method, HDA is often vulnerable to template-independent primer interactions that induce nonspecific amplification, therefore causing false positive results (Barreda-garcía & Miranda-castro, 2018). Although careful primer design may reduce the formation of primer-dimers, this process and nonspecific amplification are more pronounced in HDA-based assays than in PCR-based assays (Mahalanabis et al., 2010), and essentially occur because no hot-start polymerase is currently available for HDA (Barreda-garcía & Miranda-castro, 2018). Yang et al. (2015) describes the use of a self-avoiding molecular recognition system (SAMRS) to eliminate these artifacts. The One-Step RT-HDA was further optimised by increasing the units of reverse transcriptase in the rection from 1 unit to 8 units, and by increasing the duration of reverse transcription to 10 minutes. The limit of detection of this method was determined to be approximately 4.8×10^5 copies of RNA, which is similar to the limit of detection of the first round of the conventional nested RT-PCR assay.

Subsequently, the Two-Step RT-HDA method was tested. The occurrence of false positive outcomes was not a challenge with this assay, which could be due to the initial denaturation step at a high temperature of 95 °C. To see if the assay could be further optimised, parameters such as primer concentration, units of reverse transcriptase and duration of reverse transcription were increased, however, the original parameters proved to be most optimal. However, the limit of the detection of the Two-Step RT-HDA assay is very low, which was determined to be approximately 5.3×10^7 copies of RNA, which is about 100 times lower than that of the One-

Step RT-PCR assay. The Two-Step RT-HDA assay is also more laborious to perform, and the amplification step is an hour longer than the One-Step RT-HDA assay.

Although the One-Step RT-HDA method is more sensitive than the Two-Step RT-HDA method and will therefore be chosen as the RT-HDA assay to screen for MIDV or SINV in pooled mosquito samples, the assay is not as sensitive as the conventional nested RT-PCR assay and may therefore not be able to detect viral RNA in mosquitoes with low viral loads. Improvement on the One-Step RT-HDA is therefore needed for application in alphavirus detection in mosquitoes circulating in South Africa, or development of RT-RPA or RT-LAMP should be considered in future.

Jang et al., 2021 compares the sensitivities of three isothermal amplification lateral flow assays, which include RT-RPA, RT-HDA and RT-LAMP developed for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) detection. The RT-RPA assay had a limit of detection of 8.12×10^3 RNA copies, compared to 2.59×10^4 RNA copies for RT-HDA assay, and 2.33×10^2 RNA copies for the RT-LAMP assay. The RT-HDA assay has proven to be the least sensitive of the three isothermal amplification assays.

An asymmetric RT-HDA assay developed by Tang et al., 2010, was able to detect approximately 50 copies of RNA. In this assay, a labelled probe was incorporated, and the labelled primer was used in excess (compared to the non-labelled primer) in order for the probe to bind to the remaining unbound labelled DNA strands (thus asymmetric). This approach should be considered to improve the RT-HDA designed in this study.

Another possibility to improve the sensitivity of the RT-HDA is to employ gold nanoparticles (AuNPs) in the reaction (Barreda-garcía & Miranda-castro, 2018; Kolm et al., 2019; Sedighi et al., 2017). AuNPs bind to ssDNA with higher affinity than to dsDNA, and consequently they may improve the denaturation efficiency of helicases. In this way sensitivity of HDA-based assays can be improved (Sedighi et al., 2017). In addition, incorporation of inosine bases in the primer design as previously described may also be considered as an option to improve the sensitivity of the RT-HDA assay.

Theoretical cross-reactivity with other alphaviruses was assessed by multiple alignment of the RT-HDA primer sequences with the partial nsP4 sequences of SINV and CHIKV isolates from other regions and genotypes, and isolates from alphaviruses endemic to other parts of world, such as RRV, BFV, MAYV, EEEV, VEEV and WEEV as described above. In contrast to the conventional nested RT-PCR assay, the RT-HDA assay may not be able to detect other alphaviruses due to the many mismatches detected between the primers and the partial nsP4 sequences of the alphavirus isolates, except for SINV isolate 95M116, showing zero mismatches. Therefore, the RT-HDA assay, if sensitivity is improved, may also be able to detect SINV infection brought from northern Europe to South Africa and vice versa by migratory birds.

CHAPTER 3: DETECTION AND PHYLOGENY OF ALPHAVIRUS ISOLATES FROM WILD CAUGHT MOSQUITOES IN THE FREE STATE

3.1 Introduction

Surveillance of suspected arbovirus infection in South Africa between 2006 and 2010 reported the annual occurrence of SINV infections in humans throughout most of the country but more frequently in Gauteng, the Free State and Northern Cape provinces (Storm et al., 2013). IgM antibodies for human SINV infection were detected in all provinces except for Limpopo. From 2006 – 2009, 1.3% of the samples submitted tested positive for IgM antibody. In 2010, 10.3% of the samples submitted tested positive to IgM antibody. The increase in reported cases in 2010, coinciding with an outbreak of Rift Valley fever in South Africa, was accounted for by the above average rainfall providing favourable environments for mosquito breeding (Storm et al., 2013).

In a surveillance study conducted between January 2008 – December 2013, 623 horses presenting with unexplained febrile and acute neurologic infections throughout South Africa were investigated (van Niekerk et al., 2015). Of the 623 samples submitted, eight tested positive for SINV, of which three died from neurologic disease, and 44 tested positive for MIDV, of which 12 died from neurological disease. The viruses were isolated in the Northern Cape, Western Cape, KwaZulu-Natal, Gauteng and Free State provinces (van Niekerk et al., 2015).

In a related study, 608 samples were collected from animal species other than horses that had undiagnosed neurologic, febrile, and respiratory disease or sudden unexpected death during February 2010 – September 2018 (Steyn et al., 2020). Nine samples tested positive for SINV, with sudden unexpected deaths reported in a buffalo and a blesbuck, and 23 samples tested positive for MIDV, with a sudden unexpected death reported in a waterbuck. The viruses were

isolated in the Free State, North West, Gauteng, Limpopo, Mpumalanga, KwaZulu-Natal provinces (Steyn et al., 2020).

The geographic distribution of alphaviruses detected in surveillance studies in South Africa from 2006 until present is shown in Figure 3.1.

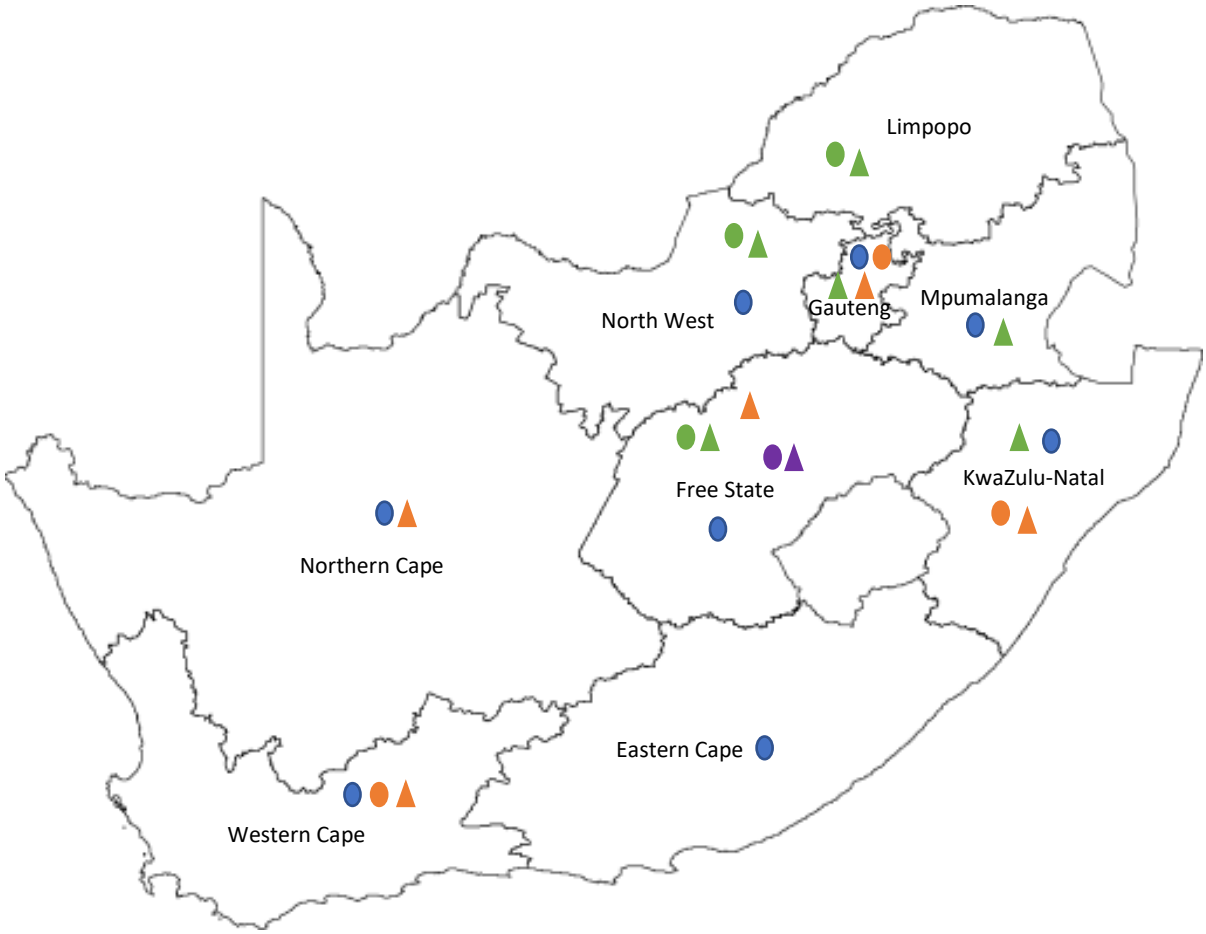

Figure 3.1: Geographic distribution of alphavirus detected in surveillance studies in South Africa from 2006 until present. Provinces with SINV ● infection detected in human cases by Storm et al. (2013) between 2006 – 2010, provinces with SINV ● and MIDV ▲ infection detected in horses by van Niekerk et al. (2015) between 2008 – 2013, provinces with SINV ● and MIDV ▲ infection detected by Steyn et al. (2020) in animals, and province with SINV ● and MIDV ▲ infection detected in this study in wild mosquitoes caught between 2019 – 2020.

This chapter describes the detection and identification of alphaviral RNA detected in wild mosquitoes collected in the Free State, and the genetic relationship of these isolates with other alphavirus isolates circulating in South Africa and around the world.

3.2 Materials and methods

3.2.1 Sample collection

RNA was extracted from a total of 42 mosquito pools in a related study and stored at -80 °C. Briefly, a total of 456 mosquitoes were caught between 2019 and 2020 in Bloemfontein, Free State in a related ongoing PhD study (Sekee, 2021). Mosquitoes were caught using Shannon traps and a CDC light trap baited with CO_2 using dry ice (Appendix I). The mosquitoes were identified morphologically and pooled according to species, collection site and date of collection (Appendix I). The number of mosquitoes in each pool ranged from 1 to 50 mosquitoes.

RNA was extracted using the TRIzol Reagent kit (Thermo Scientific, USA) and purified using the RNeasy Mini Kit (Qiagen, Germany) according to the manufacturers' instructions. Briefly, the mosquito samples were homogenised using beads. The samples were homogenised using DMEM and centrifuged at 4 °C. A 600 µl volume of TRIzol was added, and the mixture was incubated at room temperature for 5 minutes. A 220 µl volume of chloroform was added, followed by vigorous shaking for 15 seconds, and the mixture was incubated at room temperature 5 minutes. The samples were centrifuged at 12,000 × g for 15 minutes at 4 ˚C. The upper aqueous layer (~650 µl) was transferred to a 1.5 ml microcentrifuge tube, and equal volume (~650 µl) of 70% ethanol was added to tube. Following gentle mixing by inverting and quick centrifugation, 700 µl of the mix was transferred to a Qiagen RNeasy mini column inserted into a collection tube, and the tube was centrifuged at
14,000 × g for 15 seconds. The remaining 700 µl of the mix was added to the same RNeasy mini column, and the tube was centrifuged at 14,000 × g for 15 seconds. A 350 µl volume of Buffer RW1 was added to the column, and the tube was centrifuged for 15s at 14,000 × g for 15 seconds. In a separate microcentrifuge tube, 10 µl of DNase I was added to 70 µl Buffer RDD. The DNase I mix (80 µl) was added directly to the column membrane and the tube was incubated at room temperature for 15 min. A 350 µl volume of Buffer RW1 was added to the column, and the tube was centrifuged at 14,000 × g for 15 seconds. A 500 µl of the RPE buffer was added to the column, and the tube was centrifuged at 14,000 × g for 15 seconds. This step was repeated to wash the column membrane. The RNeasy spin column was placed in a new
2 ml collection tube, and the tube was centrifuged at

14,000 × g speed for 1 minute. The spin column was placed in a new collection tube, and 30 µl RNAse-free water was added directly to the spin column membrane. The tube was centrifuge for 1 min at 14,000 × g to elute the RNA.

3.2.2 Screening for alphaviruses

3.2.2.1 Conventional nested RT-PCR

To screen mosquito pools for evidence of alphavirus infection, the extracted RNA was tested using the conventional nested RT-PCR described in section 2.2.3, and the primer pairs targeting the nsP4 gene, designated Alphavirus nsP4 (F1) and Alphavirus nsP4 (R1) which amplify a 570 bp partial nsP4 region. The RT-PCR was performed using the Transcriptor One-Step RT-PCR Kit (Roche, USA) according to the manufacturer's instructions as previously described in section 2.2.3. Transcribed RNA for CHIKV and ONNV was used as positive controls and nuclease free water was used as a negative control. A nested reaction was performed using the primer pair Alphavirus nsP4 (F2) and Alphavirus nsP4 (R2) primers targeting a 200 bp region, and using the GoTaq Hot Start Polymerase Kit (Promega, USA) according to the manufacturer's instructions. The PCR products were visualised by gel electrophoresis as previously described in section 2.2.4 using a 1.5% agarose gel. See Table G1 in Appendix G regarding the % agarose gel used.

3.2.2.2 RT-HDA

The mosquito pools that tested positive for alphavirus infection using the conventional nested RT-PCR assay were tested using the RT-HDA assay One-Step method as described in section 2.2.4.1. The primer pair designated HDA Forward (5' biotin) and HDA Reverse (5' 6-FAM) was used to amplify the 116 bp partial nsP4 region. The RT-HDA was performed using the IsoAmp® II Universal tHDA Kit (New England BioLabs, USA) according to the optimised One-Step RT-HDA protocol as previously described in section 2.2.4.1. Transcribed RNA for MIDV was used as a positive control and nuclease free water was used as a negative control. The RT-HDA products were visualised on PCRD FLEX dipsticks as previously described in section 2.2.5.

3.2.3 Nucleotide sequence determination

RT-PCR amplicons were excised from the agarose gel and purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, USA) according to manufacturer's instructions as previously described in section 2.2.5. DNA concentrations and purity ratios were determined using a NanoDrop™ 2000 Spectrophotometer. The purified amplicons were subsequently cloned into pGEM®-T easy vectors, and the nucleotide sequences of the inserts were determined to identify the alphavirus species for each positive sample.

3.2.3.1 Cloning the purified amplicons into pGEM®-T easy

Ligation

The 200 bp purified amplicons were ligated into pGEM®-T easy (see Appendix J for vector map) by TA cloning using the pGEM®-T Easy Vector Systems kit (Promega, USA) according to the manufacturer's instructions. Ligation reaction mixtures were prepared using the components listed in Table 3.1:

Table 3.1 Reaction components for ligation

Components	Volume (µl)
2X Rapid Ligation Buffer, T4 DNA ligase	5
pGEM®-T easy vector (50 ng)	1
Purified amplicon	3
T4 DNA ligase (3 Weiss units/µl)	1
Nuclease Free Water	-
Total	**50**

The ligation reactions were incubated at 4 °C overnight.

Transformation

A 25 µl aliquot of One-Shot Top10 chemically competent *Escherichia coli* cells was transferred into each ligation reaction tube and the tube incubated on ice for 30 minutes. The cells were subsequently heat-shocked for 30 seconds in a 42 °C water bath. A 250 µl aliquot of pre-warmed super optimal broth with catabolite repression (SOC) media was added to each ligation reaction. The reaction tubes were incubated for an hour at 37 °C while shaking at 220 rpm. A 175 µl aliquot of each transformation culture was plated on Luria Bertani (LB) plates containing ampicillin (amp) at a final concentration of 100 µg/ml. After 24 hours, a single colony was selected for each ligation reaction from the LB/amp plates. Each colony was inoculated into a 5 ml LB/amp liquid culture and grown overnight at 37 °C while shaking at 220 rpm.

Plasmid DNA was subsequently purified from each culture using PureYield™Plasmid Miniprep system (Promega, USA) according to the manufacturer's instructions. Briefly, for each sample, the bacterial culture was centrifuged at 14,000 × g for 30 seconds. A 600 µl aliquot of TE buffer (pH 8.0) was added to the cell pellet and resuspended completely. Subsequently, 100 µl of cell lysis buffer and 350 µl of neutralization solution were added to the bacterial culture. The reaction mixture was inverted six times and centrifuged at 14,000 × g for three minutes. The supernatant was transferred to a Pure Yield™ Minicolumn and centrifuged at 14,000 × g for 15 seconds. Wash steps using endotoxin removal and column wash solution were performed to eliminate residual debris. Finally, plasmid DNA was eluted in 30 µl elution buffer. The concentration of the plasmid DNA was measured using a NanoDrop™ 2000 Spectrophotometer. The purified plasmid DNA was stored at -20 °C.

Verification of successful cloning

To verify that the amplicons were successfully ligated into pGEM®-T easy and inserted in the correct orientation, PCR was performed using the Alphavirus nsP4 (F2) forward primer (Table 2.1) and the SP6 reverse primer (5'-ATTTAGGTGACACTATAG-3'), and GoTaq Hot Start Polymerase Kit (Promega, USA) according to the manufacturer's instructions. Each reaction mixture was prepared in a total of 50 µl as follows:

Table 3.2 Reaction components for plasmid DNA PCR to verify successful cloning

Components	Volume (µl)	Final concentration
5X Green or Colorless GoTaq® Flexi Buffer	10	1X
$MgCl_2$ Solution, 25 mM	4	2 mM
PCR Nucleotide Mix, 10 mM each	1	0.2 mM each dNTP
Alphavirus nsP4 (F2) forward primer (20 µM)	1	0.4 µM
SP6 reverse primer (20 µM)	1	0.4 µM
GoTaq® Hot Start Polymerase (5 U/µl)	0.25	1.25 U
Template DNA	2	-
Nuclease Free Water	30.75	-
Total	**50**	**-**

The PCR reactions were performed in a ProFlex™ PCR system thermocycler (ThermoScientific, USA) using the following cycling conditions: initial denaturation at 95 °C for 2 minutes, followed by 30 cycles of denaturation at 95 °C for 30 seconds, annealing at 45 °C for 30 seconds and elongation at 72 °C for 35 seconds, ending with one final elongation cycle at 72 °C for 5 minutes. The reactions were subsequently held at 4 °C indefinitely. The PCR products were visualised by gel electrophoresis as previously described in section 2.2.4, using a 1.5% agarose gel.

3.2.3.2 Determination of the nucleotide sequences of the cloned inserts

The nucleotide sequence of each gene fragment cloned into pGEM®T-Easy was determined using the BigDye™ Terminator v3.1 Cycle Sequencing kit (Thermo Scientific, USA) according to the manufacturer's instructions. Two reaction mixtures were prepared per sample, each containing a primer that flank the gene, the SP6 reverse primer (5'-ATTTAGGTGACACTATAG-3') or the T7 forward primer (5'-TAATACGACTCACTATAGG-3'). Each reaction mixture was prepared using the components listed in Table 3.3:

Table 3.3 Reaction components for sequencing PCR

Components	Volume (µl)
BigDye™ Terminator v3.1 Ready Reaction Mix	1
Sequencing primer (T7/ SP6) forward primer (0.8pmol/µl)	4
BigDye™ Terminator v3.1 5X Sequencing Buffer	2
Template DNA	3
NFW	-
Total	**10**

The sequencing reactions were cycled in a ProFlex™ PCR system thermocycler using the following cycling conditions: polymerase activation at 96 °C for 1 minute, followed by 25 cycles of denaturation at 96 °C for 10 seconds, annealing at 50 °C for 5 seconds, and an extension step at 60 °C for 4 minutes. The reactions were subsequently held at 4 °C indefinitely.

The sequencing reactions were subsequently purified using EDTA/ Ethanol precipitation. Briefly, 10µl of nuclease free water were added to each reaction to adjust the volume to 20 µl. A 5µl aliquot of 125 mM EDTA, prepared by diluting a 0.5 M solution of EDTA (pH 8.0), and 60 µl of absolute ethanol were added to each reaction tube. The tubes were vortexed for five seconds and incubated at room temperature for 15 minutes to allow precipitation. The tubes were centrifuged at 14,000 × g for 20 minutes at 4 °C. The supernatant was completely aspirated without disturbing the pellet. A 500 µl volume of 70% ethanol was added to each tube. The reaction tubes were centrifuged at 14,000 × g for 10 minutes at 4 °C, and the supernatant was completely aspirated without disturbing the pellet. The reaction tubes were incubated at 37 °C overnight with the lids open to air-dry. The samples were stored in the dark at 4 °C until submission at the Division of Virology at University of the Free State for sequencing.

Lastly, sequence data was edited using Geneious Prime software version 2021.2.2, and the identity of the alphavirus was determined using BLASTn.

3.2.4 Phylogenetic analysis

Phylogenetic relationships of the positive mosquito pools, identified as MIDV and SINV isolates, were determined using sequence data retrieved from GenBank and MEGA software version 11. Briefly, sequence data from 28 SINV isolates (Table 3.4) with nsP4 sequences available in GenBank were multiple aligned with the sequences obtained for the three SINV isolates identified in this study, and 12 MIDV isolates (Table 3.5) with nsP4 sequences available in GenBank were multiple aligned with the sequences obtained for the two MIDV isolates identified in this study. The 28 SINV isolates represent SINV genotypes I, IV, V and VI (SINV-I, -IV, V and -VI). There are currently no nsP4 sequences available in GenBank for isolates grouped under SINV-II and SINV-III, therefore, these sequences were not included in the analysis. Multiple alignments were performed using the MUSCLE algorithm in MEGA v11, and the sequences were trimmed to a final size of 200 bp. Neighbour-joining phylogenetic trees were constructed and pairwise distances were computed using the aligned sequences. Bootstrap confidence intervals were calculated by 1 000 replications to assess reliability of the branching pattern, and the p-distance model was used to calculate genetic distances.

Table 3.4: SINV isolates with nsP4 sequences available in GenBank used for phylogenetic tree construction

Genotype	Isolate	Country of isolation	Year of isolation	Source	Accession number
SINV-I	AR18132	South Africa	1974	*Culex univittatus*	MK045247
	SAAR_18141	South Africa	1976	*Culex univittatus*	MK045246
	Girdwood	South Africa	1962	*Homo sapiens*	U38304
	SAAR_6071	South Africa	1964	*Culex univittatus*	MK045250
	S.A.AR86	South Africa	1954	*Culex* spp.	U38305
	DakAry 251	Cameroon	1969	*Mansonia africana*	AF339477

Genotype	Isolate	Country of isolation	Year of isolation	Source	Accession number
	Edsbyn	Sweden	1982	*Culiseta* spp.	M69205
	83M107	Sweden	1983	*Culiseta morsitans*	MK045228
	83M108	Sweden	1983	*Culiseta pipiens*	MK045227
	84M140	Sweden	1984	*Culiseta morsitans*	MK045226
	85M68	Sweden	1985	*Culiseta morsitans*	MK045236
	85M78	Sweden	1985	*Amblonyx cinereus*	MK045233
	85M94	Sweden	1985	*Culex pipiens*	MK045234
	85M134	Sweden	1985	*Culex pipiens*	MK045244
	95M116	Sweden	1995	*Amblonyx cinereus*	MK045231
	LEIV-9298	Russia	1983	*Aedes* spp.	MG679381
	YN87448	China	1992	*Homo sapiens*	AF103734
	Johannes-2002	Finland	2002	*Homo sapiens*	JQ771797
	Kiihtelysvaara-2002	Finland	2002	*Homo sapiens*	JQ771798
	Ilomantsi-2002A	Finland	2002	*Homo sapiens*	JQ771794
	lomantsi-2002B	Finland	2002	*Homo sapiens*	JQ771795
	Ilomantsi-2002C	Finland	2002	*Homo sapiens*	JQ771796
	Ilomantsi-2005M	Finland	2002	Mosquito	JQ771793
	MP684	Uganda	1960	*Coquillettidia*	MK045248

Genotype	Isolate	Country of isolation	Year of isolation	Source	Accession number
SINV-IV	LEIV 65A	Russia	1969	*Culex modestus*	AF339478
	XJ-160	China	1970	*Culicidae* spp.	AF103728
SINV-V	M78	New Zealand	1962	*Culex pervigilans*	AF339479
SINV-VI	SW6562	Australia	1990	*Culex annulirostris*	AF429428

Table 3.5: MIDV isolates with nsP4 sequences available in GenBank used for phylogenetic tree construction

Isolate	Country of isolation	Year of isolation	Source	Accession number
SaAr 749	South Africa	1957	*Aedes caballus*	AF339486
ArB-8422	Central African Republic	1977	*Aedes vittatus*	KM115530
ArTB-5290	Central African Republic	1984	*Amblyomma variegatum*	KM115531
SAE25_11	South Africa	2011	Horse	KF680222
MIDV857	Zimbabwe	1993	Horse	EF536323
ZRU080/14	South Africa	2014	Horse	MT015693
ZRU089/14	South Africa	2014	Horse	MT015692
ZRU044/17	South Africa	2017	Horse	MT015691
ZRU059/17/1	South Africa	2017	Horse	MT015690

Isolate	Country of isolation	Year of isolation	Source	Accession number
ZRU075/17	South Africa	2017	Horse	MT015689
ZRU103/17	South Africa	2017	Horse	MT015688
ZRUH399/17	South Africa	2017	*Homo sapiens*	MN967313

3.3 Results

3.3.1. Screening for alphaviruses

3.3.1.1 Conventional nested RT-PCR

The conventional nested RT-PCR developed in this study was used to screen RNA extracted from 42 mosquito pools. RNA was previously extracted in a related study (Sekee, 2021), from 456 mosquitoes collected in Bloemfontein, Free State between 2019 and 2020. RT-PCR was performed using the Transcriptor One-Step RT-PCR Kit, using the 42 RNA samples as template, and Alphavirus nsP4 (F1) and Alphavirus nsP4 (R1) primers to target the 570 bp partial nsP4 region. Transcribed CHIKV and ONNV RNA were used as positive controls and nuclease free water was used as a negative control. Subsequently, nested PCR was performed using the GoTaq Hot Start Polymerase Kit. The RT-PCR amplicons were used as template, and Alphavirus nsP4 (F2) and Alphavirus nsP4 (R2) primers were used to target the 200 bp partial nsP4 region. The nested PCR products were visualised by gel electrophoresis using a 1.5% agarose gel. A total of 5 out of the 42 pools tested positive for alphaviruses and produced DNA bands of the expected size (approximately 200 bp). Positive pools included pool 6 (Figure 3.2), pools 14b and 25 (Figure 3.3), pools 29 and 37 (Figure 3.4).

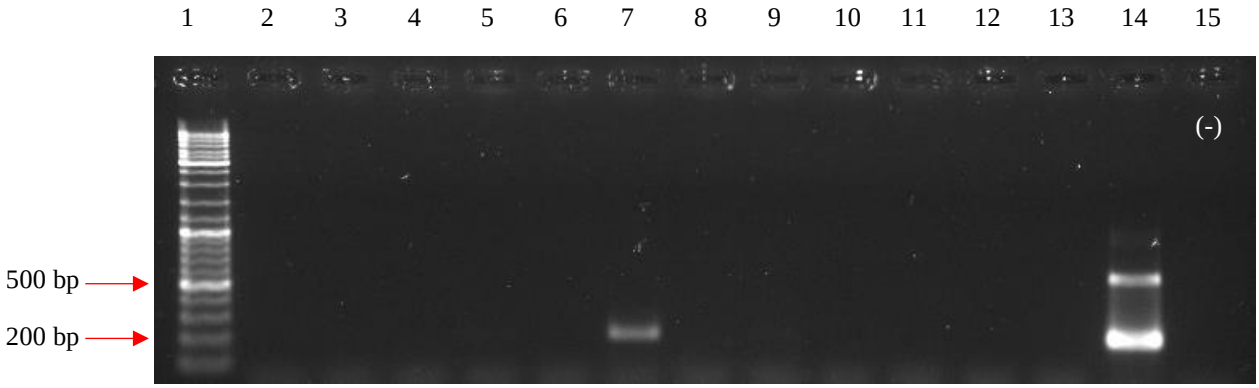

Figure 3.2: Screening for alphaviruses in pools 1 to 12 using conventional nested RT-PCR. Lane 1. DNA ladder, lane 2. Pool 1, lane 3. Pool 2, lane 4. Pool 3, lane 5. Pool 4, lane 6. Pool 5, lane 7. Pool 6, lane 8. Pool 7, lane 9. Pool 8, lane 10. Pool 9, lane 11. Pool 10, lane 12. Pool 11, lane 13. Pool 12, lane 14. CHIKV positive control, lane 15. Negative control

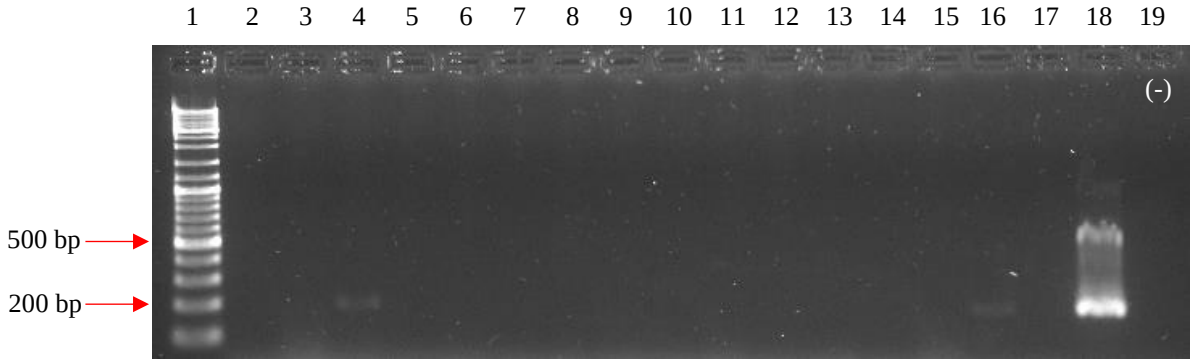

Figure 3.3: Screening for alphaviruses in pools numbered 13 to 26 using conventional nested RT-PCR
Lane 1. DNA ladder, lane 2. Pool 13, lane 3. Pool 14a, lane 4. Pool 14b, lane 5. Pool 15, lane 6. Pool 16, lane 7. Pool 17, lane 8. Pool 18, lane 9. Pool 19, lane 10. Pool 20, lane 11. Pool 21, lane 12. Pool 22a, lane 13. Pool 22b, lane 14. Pool 23, lane 15. Pool 24, lane 16. Pool 25, lane 17. Pool 26, lane 18. ONNV positive control, lane 19. Negative control

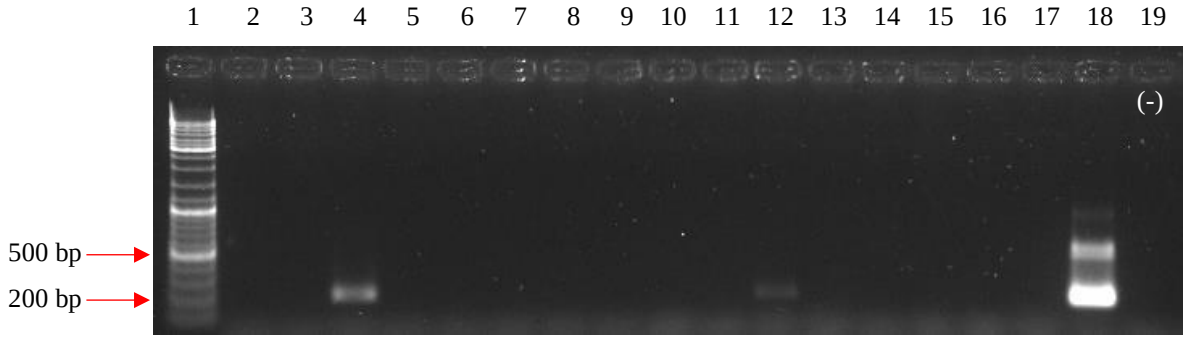

Figure 3.4: Screening for alphaviruses in pools numbered 27 to 42 using conventional nested RT-PCR. Lane 1. DNA ladder, lane 2. Pool 27, lane 3. Pool 28, lane 4. Pool 29, lane 5. Pool 30, lane 6. Pool 31, lane 7. Pool 32, lane 8. Pool 33, lane 9. Pool 34, lane 10. Pool 35, lane 11. Pool 36, lane 12. Pool 37, lane 13. Pool 38, lane 14. Pool 39, lane 15. Pool 40, lane 16. Pool 41, lane 17. Pool 42, lane 18. ONNV positive control, lane 19. Negative control

3.3.1.2 RT-HDA

To test the application of the RT-HDA assay to detect SINV and/ or MIDV infection in mosquito samples, the 5 positive pools were tested using the RT-HDA assay (One-Step method). The RT-HDA was performed using the IsoAmp® II Universal tHDA Kit using the optimised One-Step protocol, and the primer pair designated HDA Forward (5' biotin) and HDA Reverse (5' 6-FAM) to amplify the 116 bp target region. Transcribed RNA for MIDV was used as a positive control and nuclease free water was used as a negative control. The RT-HDA products were visualised on PCRD FLEX dipsticks (Figure 3.5). All pools tested negative.

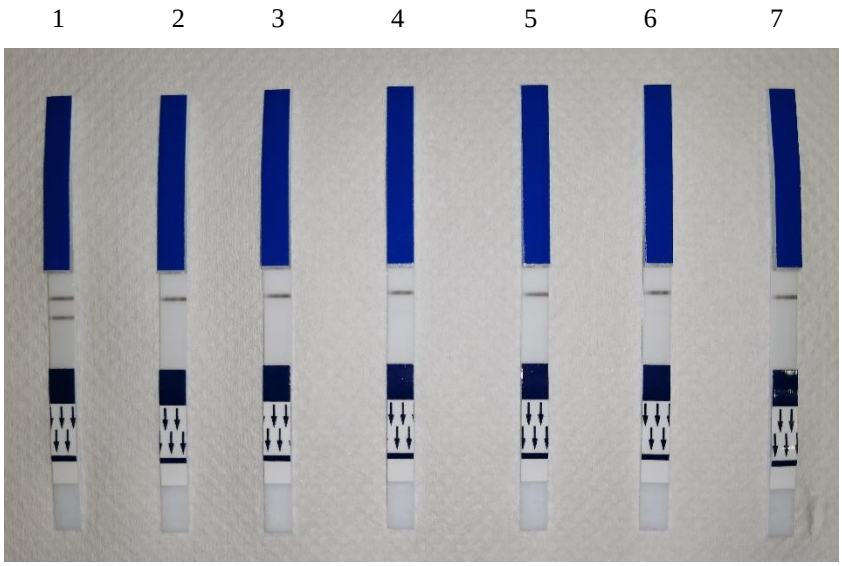

Figure 3.5: Screening for SINV and/ or MIDV infection in pools 6, 14b, 25, 29 and 37 using the RT-HDA. No. 1: transcribed MIDV RNA control, no. 2: pool 6, no. 3: pool 14b, no. 4: pool 25, no. 5: pool 29, no. 6: pool 37, no. 7: Negative control

3.3.2 Cloning positive amplicons into pGEM®-T easy

The alphavirus positive RT-PCR amplicons were purified using the Wizard® SV Gel and PCR Clean-Up System, and the purified amplicons were subsequently cloned into pGEM®-T easy vectors. To verify that the amplicons were successfully ligated into pGEM®-T easy, PCR was performed using Alphavirus nsP4 (F2) forward primer and SP6 reverse primer, using the GoTaq Hot Start Polymerase Kit (Promega, USA). The PCR products were visualised on a 1% agarose gel (Figure 3.6). The expected size of the PCR products was 299 bp: partial nsP4 gene

(200 bp) + SP6 promotor region (20 bp) + restriction sites on plasmid (77 bp) + 3'-T overhangs at cloning site (2 bp).

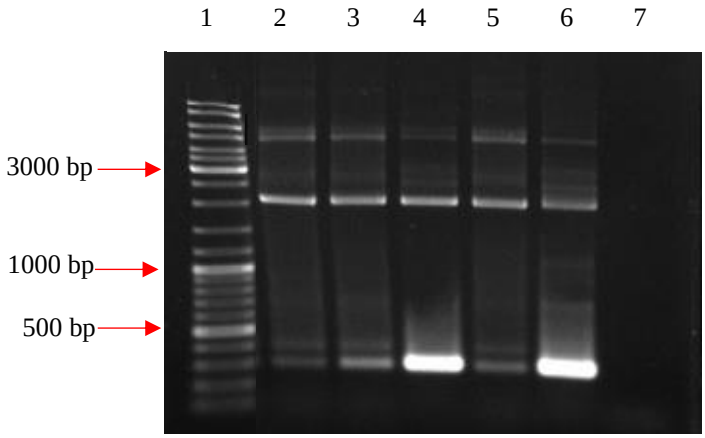

Figure 3.6: Verification that the RT-PCR amplicons were successfully ligated into pGEM®-T easy. Lane 1. DNA ladder, lane 2. pGEM®-T easy containing RT-PCR amplicon from pool number 6, lane 3. pGEM®-T easy containing RT-PCR amplicon from pool number 14b, lane 4. pGEM®-T easy containing RT-PCR amplicon from pool number 25, lane 5. pGEM®-T easy containing RT-PCR amplicon from pool number 29, lane 6. pGEM®-T easy containing RT-PCR amplicon from pool number 37, lane 7. Negative control

3.3.3 Determination of the nucleotide sequences of the cloned inserts

The nucleotide sequence of each gene fragment cloned into pGEM®T-Easy was determined using the BigDye™ Terminator v3.1 Cycle Sequencing kit, using T7 and SP6 primers. The sequencing reactions were purified using EDTA/ Ethanol precipitation and subsequently submitted to the Division of Virology at University of the Free State for sequencing.

The sequence data was edited using Geneious Prime software version 2021.2.2, and the identity of the alphavirus was determined using BLASTn (Appendix K). SINV was identified in pools 6, 14b and 29, and MIDV was identified in pools 25 and 37.

3.3.4 Phylogenetic analysis

A phylogenetic tree was generated from sequence data retrieved from 28 SINV isolates which contain nsP4 sequences in GenBank, plus partial nsP4 sequences obtained for pools 6, 14b and 29 that were identified as SINV isolates, by neighbour-joining distance analysis with node values generated by 1 000 bootstrap replications (Figure 3.7). Pools 6, 14b and 29, marked in

yellow in Figure 3.7, correspond with SINV genotype I (SINV-I), and the branching patterns indicate that these isolates are most closely related genetically to three Sindbis-like virus South African isolates, namely Girdwood, SAAR 6071 and S.A.AR86, and a Sindbis-like Babanki virus strain (DakAry 251) from Cameroon. A pairwise distance analysis was performed to assess the genetic diversity between these isolates and all the SINV isolates from Africa (Table 3.3).

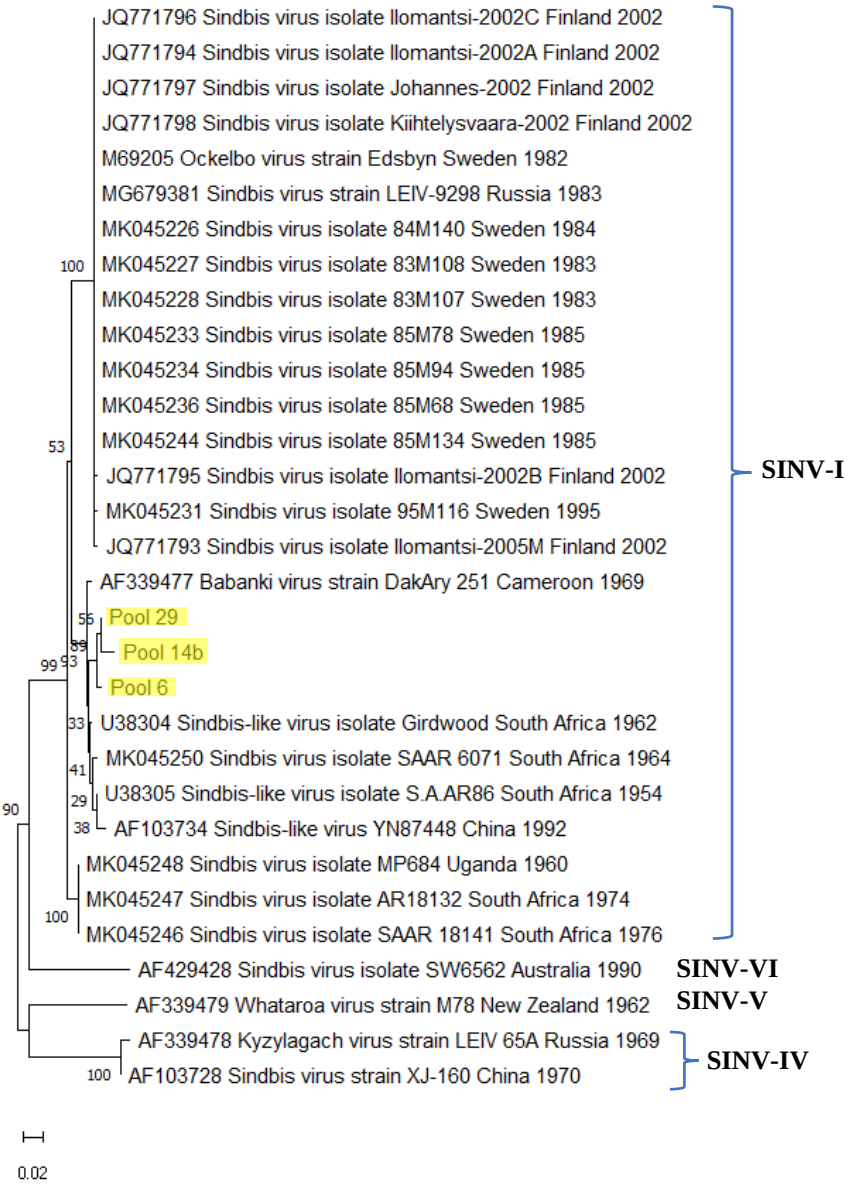

Figure 3.7: Phylogenetic relationships of 28 SINV isolates using data retrieved from GenBank and three SINV isolates identified in this study, based on neighbour-joining analysis applying a p-distance model. Isolates from SINV genotypes I, IV, V and VI (SINV-I, -IV, -V and -VI) were included. Node values indicate the level (%) of bootstrap support from 1 000 replicates. Scale bar indicates 0.02 base substitutions per site. Isolates marked in yellow indicates sequences determined in this study.

Pool 6 showed highest nucleotide similarity to pool 29 and South African isolate S.A.AR86, with 98.5 % similarity (Table 3.6). Pool 14b showed highest nucleotide similarity to pool 29, with 98.5% similarity, followed by South African isolate S.A.AR86, with 97% similarity (Table 3.6). Pool 29 showed highest nucleotide similarity to pool 6, pool 14b and South African isolate S.A.AR86, with 98.5% similarity (Table 3.6).

Table 3.6 Estimates of evolutionary divergence between sequences of SINV isolates based on partial nucleotide sequence data

	1	2	3	4	5	6	7	8	9	10
1										
2	2.0%									
3	1.5%	1.5%								
4	1.5%	3.0%	1.5%							
5	2.0%	3.5%	2.0%	0.5%						
6	2.5%	4.0%	2.5%	1.0%	1.5%					
7	5.0%	6.5%	5.0%	4.5%	4.0%	5.5%				
8	5.0%	6.5%	5.0%	4.5%	4.0%	5.5%	0.0%			
9	5.0%	6.5%	5.0%	4.5%	4.0%	5.5%	0.0%	0.0%		
10	2.0%	3.5%	2.0%	1.5%	1.0%	2.5%	4.0%	4.0%	4.0%	

[1] Pool_6
[2] Pool_14b
[3] Pool_29
[4] U38305_Sindbis-like_virus_isolate_S.A.AR86_South_Africa_1954
[5] U38304_Sindbis-like_virus_isolate_Girdwood_South_Africa_1962
[6] MK045250_Sindbis_virus_isolate_SAAR_6071_South_Africa_1964
[7] MK045248_Sindbis_virus_isolate_MP684_Uganda_1960
[8] MK045247_Sindbis_virus_isolate_AR18132_South_Africa_1974
[9] MK045246_Sindbis_virus_isolate_SAAR_18141_South_Africa_1976
[10] AF339477_Babanki_virus_strain_DakAry_251_Cameroon_1969

Similarly, a phylogenetic tree was generated from partial nsP4 sequence data retrieved from GenBank for 12 MIDV isolates and partial nsP4 sequence data obtained for pools 25 and 37 that were identified as MIDV isolates, by neighbour-joining distance analysis with node values generated by 1 000 bootstrap replications (Figure 3.8). Pools 25 and 29, marked in yellow in

Figure 3.8, clustered in a different lineage compared with previous isolates reported. Pairwise distances were determined to assess genetic diversity (Table 3.7).

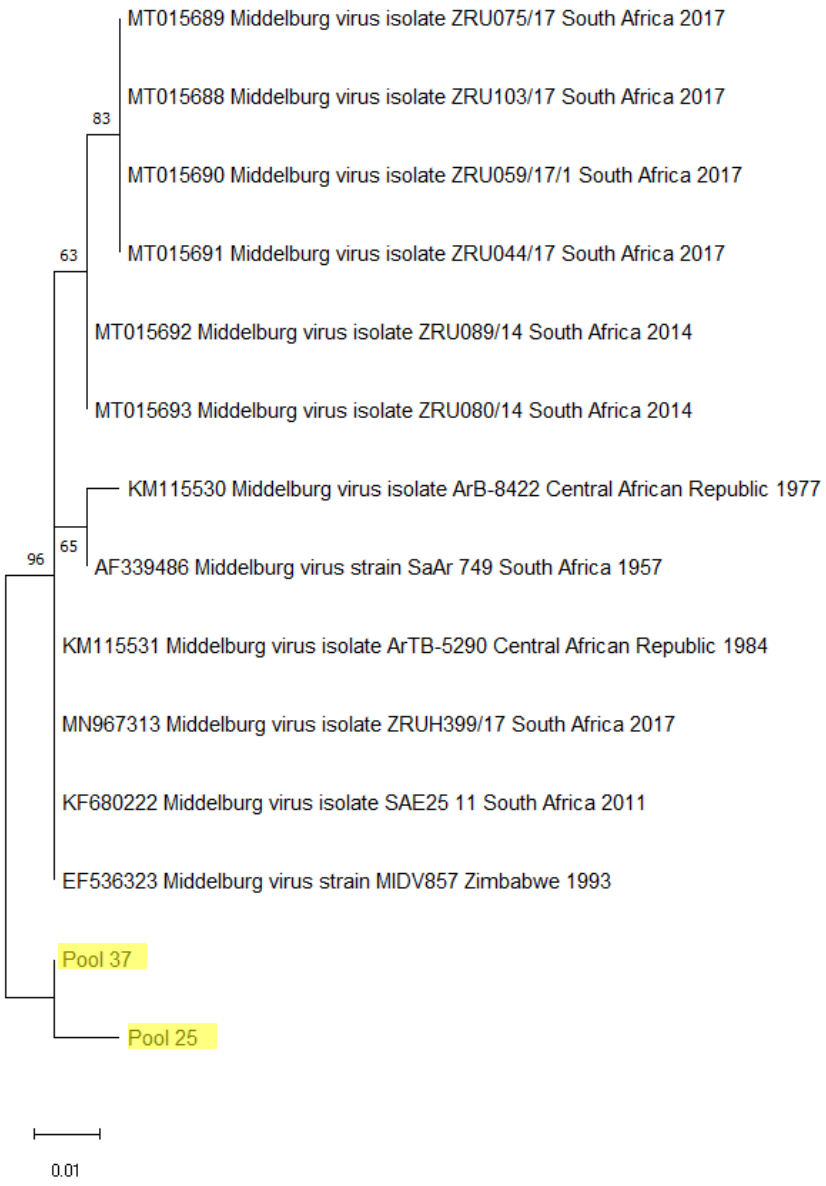

Figure 3.8: Phylogenetic relationships of 12 MIDV isolates using data retrieved from GenBank and two MIDV isolates identified in this study, based on neighbour-joining analysis applying a p-distance model. Node values indicate the level (%) of bootstrap support from 1 000 replicates. Scale bar indicates 0.01 base substitutions per site. Isolates marked in yellow indicates sequences determined in this study.

Pools 25 and 37 showed highest nucleotide similarity to each another, with 99% similarity (Table 3.7), and of the 12 MIDV isolates documented from previous studies, pools 25 and 37 showed highest nucleotide similarity to two isolates from South Africa, SAE25_11 and ZRUH399/17, one isolate from Zimbabwe, MIDV857, and one isolate from Central African Republic, ArTB-5290, with 97.5% nucleotide similarity between the sequences of these isolates with pool 25, and 98.5% nucleotide similarity between the sequences of these isolates with pool 37 (Table 3.7).

Table 3.7 Estimates of evolutionary divergence between sequences of MIDV isolates based on partial nucleotide sequence data

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1														
2	1.0%													
3	2.0%	3.0%												
4	2.0%	3.0%	0.0%											
5	2.5%	3.5%	0.5%	0.5%										
6	2.5%	3.5%	0.5%	0.5%	0.0%									
7	2.5%	3.5%	0.5%	0.5%	0.0%	0.0%								
8	2.5%	3.5%	0.5%	0.5%	0.0%	0.0%	0.0%							
9	1.5%	2.5%	0.5%	0.5%	1.0%	1.0%	1.0%	1.0%						
10	1.5%	2.5%	0.5%	0.5%	1.0%	1.0%	1.0%	1.0%	0.0%					
11	2.5%	3.5%	1.5%	1.5%	2.0%	2.0%	2.0%	2.0%	1.0%	1.0%				
12	1.5%	2.5%	0.5%	0.5%	1.0%	1.0%	1.0%	1.0%	0.0%	0.0%	1.0%			
13	1.5%	2.5%	0.5%	0.5%	1.0%	1.0%	1.0%	1.0%	0.0%	0.0%	1.0%	0.0%		
14	2.0%	3.0%	1.0%	1.0%	1.5%	1.5%	1.5%	1.5%	0.5%	0.5%	0.5%	0.5%	0.5%	

[1] Pool_37

[2] Pool_25

[3] MT015693_Middelburg_virus_isolate_ZRU080/14_South_Africa_2014

[4] MT015692_Middelburg_virus_isolate_ZRU089/14_South_Africa_2014

[5] MT015691_Middelburg_virus_isolate_ZRU044/17_South_Africa_2017

[6] MT015690_Middelburg_virus_isolate_ZRU059/17/1_South_Africa_2017

[7] MT015689_Middelburg_virus_isolate_ZRU075/17_South_Africa_2017

[8] MT015688_Middelburg_virus_isolate_ZRU103/17_South_Africa_2017

[9] MN967313_Middelburg_virus_isolate_ZRUH399/17_South_Africa_2017

[10] KM115531_Middelburg_virus_isolate_ArTB-5290_Central_African_Republic_1984

[11] KM115530_Middelburg_virus_isolate_ArB-8422_Central_African_Republic_1977

[12] KF680222_Middelburg_virus_isolate_SAE25_11_South_Africa_2011

[13] EF536323_Middelburg_virus_strain_MIDV857_Zimbabwe_1993

[14] AF339486_Middelburg_virus_strain_SaAr_749_South_Africa_1957

3.4 Discussion

In chapter 2, an in-house conventional nested RT-PCR assay and an in-house RT-HDA assay to detect mosquito-borne alphaviruses in South Africa was developed. To test the application of these assays to detect these alphaviruses in wild, caught mosquitoes, the conventional nested RT-PCR assay, which is able to detect a wider range of alphaviruses and which is more sensitive than the RT-HDA assay, was used as the primary assay to screen for potential alphaviruses. RNA extracted in a related study (Sekee, 2021), from 42 mosquito pools consisting of 456 mosquitoes collected in the Free State between 2019 and 2020 were tested. Five of the pools tested positive for alphaviruses, of which three were identified as SINV isolates and two were identified as MIDV by nucleotide sequence determination and BLASTn analysis. The alphaviruses detected in this study are consistent with the findings obtained in previous alphavirus surveillance studies (Steyn et al., 2020; Storm et al., 2013; van Niekerk et al., 2015), as illustrated in Figure 3.1.

SINV has an extensive geographic distribution and has been identified in Europe, Africa, Asia and Oceania. The SINV complex includes several named viruses or virus subtypes, including Karelian fever virus (Russia), Ockelbo virus (Sweden), Babanki virus (Cameroon), Kyzylagach virus (Russia), and Whataroa virus (New Zealand) (Chen et al., 2018; Weaver et al., 1997). There are six SINV genotypes (SINV-I – SINV-VI) identified from previous phylogenetic analyses of the partial E2 gene, each restricted to a specific geographic region (Ling et al., 2019; Lundström & Pfeffer, 2010; Saleh et al., 2003). SINV-I is restricted to Europe, Africa, and the Middle East. Although one SINV strain from China (YN87448) was identified as genotype SINV-I, the strain was almost indistinguishable from the widely used laboratory strain S.A.AR86, indicating that YN87448 is a contamination (Lundström & Pfeffer, 2010). SINV-II and SINV-VI are restricted to Australia; SINV-III is restricted to

Southeast Asia; SINV-IV is restricted to Asia and the Middle East; and SINV-V (also referred to as Whataroa virus) is restricted to New Zealand.

In this study, isolates from all SINV genotypes were included in the multiple alignment for phylogenetic analysis, except for SINV-II and SINV-III since currently, nsP4 sequence data is not available on GenBank for the isolates in these genotypes. The phylogenetic tree shows that the three SINV isolates identified in this study from pools 6, 14b and 29, identify as SINV-I isolates. The branching patterns indicate that these isolates are most genetically similar to three SINV South African isolates, namely Girdwood, SAAR 6071 and S.A.AR86, and a Sindbis-like Babanki virus strain (DakAry 251) from Cameroon. A pairwise distance analysis was also performed to assess the genetic diversity between these isolates and all the SINV isolates from Africa. The estimate percentage of base differences per site between sequences shows that the isolates identified in this study are genetically similar to other South Africa isolates within the SINV-I genotype.

SINV-I is the only genotype that has been associated with outbreaks of human infection, which were reported in South Africa and northern Europe. Hence the continual surveillance of SINV-I infection is needed. In South Africa, significant outbreaks of human infection occurred in 1963, 1974, 1983-1984, and between 2006 and 2010 (Jupp et al., 1986; McIntosh et al., 1976; Storm et al., 2013). In northern Europe, significant outbreaks of human infections, represented by Ockelbo disease (Sweden), Pogosta disease (Finland), and Karelian fever (Russia), were reported in 1981-1982, 1988, 1995, 2002, and 2013 (Bergqvist et al., 2015; Brummer-Korvenkontio et al., 2002; Kurkela et al., 2005; Lundström, 1999; Niklasson & Espmark, 1984). There is evidence suggesting that migratory birds are responsible for the transcontinental dissemination of SINV from South Africa to northern Europe (Kurkela et al., 2008). In Finland between 1974 and 2002, outbreaks of Pogosta disease occurred every 7 years, for reasons that may have to do with the behaviour of migratory birds playing a role in dispersal of virus between continents (Bergqvist et al., 2015; Brummer-Korvenkontio et al., 2002). Clinical signs and symptoms include fever, rash, myalgia, and cases of arthritis that can remain for several years (Gylfe et al., 2018). Previous studies indicated that the transcontinental dissemination of SINV-I was caused by northward-migrating birds, connecting South Africa to northern Europe (Kurkela et al., 2008). The driving forces behind SINV-I outbreaks and SINV-I transcontinental or transregional movements are still not well known (Ling et al.,

2019). SINV infections typically occur during late summer or early autumn, particularly after periods of heavy rainfall which favour mosquito breeding (Jupp et al., 1986; Lundström, 1999)..

Less is known about MIDV. First isolated in 1957 from mosquitoes in South Africa, subsequent surveys include the identification of the virus in two humans, cattle, sheep and goats, from serological tests conducted in the KwaZulu-Natal Province of South Africa (Kokernot et al., 1961; Smithburn et al., 1959), in horses with neurological disease in South Africa and Zimbabwe (Attoui et al., 2007; van Niekerk et al., 2015), and in wildlife and nonequine domestic animals showing signs of febrile, neurologic disease or unexplained deaths (Steyn et al., 2020). Apart from South Africa and Zimbabwe, MIDV has also been identified in Central African republic, Cameroon, Kenya, and Senegal (Hubálek et al., 2014; Tricou et al., 2014), limiting its distribution to Africa. In this study, two MIDV isolates were identified in pools 25 and 27. To assess the genetic relationship of these isolates with other MIDV isolates that were previously reported, a neighbour-joining phylogenetic tree was constructed to by aligning the 200 bp partial nsP4 sequences obtained for pools 25 and 27 with the sequence data retrieved for 12 MIDV isolates that contain the nsP4 region in GenBank. Of the 12 MIDV isolates, nine were isolated from South Africa, one was isolated from Zimbabwe, and two were isolated from Central African Republic. Phylogenetic and pairwise distance analyses indicate that the MIDV isolates identified in this study showed highest nucleotide similarity to each another, with 99% similarity. Of the 12 MIDV isolates documented from previous studies, the isolates from this study showed highest nucleotide similarity to two isolates from South Africa, SAE25_11 and ZRUH399/17, one isolate from Zimbabwe, MIDV857, and one isolate from Central African Republic, ArTB-5290, with 97.5% nucleotide similarity between the sequences of these isolates with pool 25, and 98.5% nucleotide similarity between the sequences of these isolates with pool 37. Sequence data from additional isolates will be required to determine if there are different genotypes or lineages of MIDV.

The in-house conventional nested RT-PCR assay was successfully used to detect SINV and MIDV isolates from wild mosquitoes collected in the Free State. The five positive samples were tested using the in-house RT-HDA assay, however, the assay lacks sensitivity as shown in Chapter 2 when the sensitivity was investigated using known copies of RNA and was therefore unable to detect the RNA in these samples. Cost effective and rapid assays which are

highly sensitive and specific to screen for alphaviruses are ideal in low resource settings. As described in chapter 2, improvements are needed in the design of the RT-HDA assay developed in this study to increase the sensitivity assay, enough to use for routine surveillance of SINV and MIDV in mosquitoes. Otherwise, alternative rapid isothermal amplification methods should be considered such as RT-RPA and RT-LAMP, or even modernised PCR-based methods such as droplet RT-PCR and insulated isothermal PCR (iiPCR).

CHAPTER 4: CONCLUDING REMARKS

Arboviruses from the *Flavivirus* and *Alphavirus* genera are responsible for many important mosquito-borne diseases in humans and animals including dengue, yellow fever, chikungunya, and Ross River disease (Hall et al., 2012). One of the main elements of the control of arbovirus transmission is arbovirus surveillance. Surveillance is used to estimate the abundance of vector populations, to detect viral activity in vectors, and to document cases of human and animal infections (Hall et al., 2012). In the last decade, surveillance of alphaviral infections in South Africa were conducted on humans and animals suspected with arboviral diseases (Steyn et al., 2020; Storm et al., 2013; van Niekerk et al., 2015). Currently, there is a lack of active vector surveillance and monitoring programs in South Africa for most arboviral diseases. Frequent surveillance programmes, proper control strategies, and the continual development of rapid and sensitive assays to detect and diagnose arbovirus infections is needed (Mensah & El Zowalaty, 2018). Screening mosquitoes for circulating alphaviruses can be used as an early warning system to predict future outbreaks before they arise.

In this study, a conventional nested RT-PCR assay and a RT-HDA assay were developed to detect mosquito-borne alphaviruses in South Africa, and the assays were tested for application to detect alphaviral RNA in wild, caught mosquitoes. Using the conserved nsP4 gene of alphaviruses to identify amplification primers, the conventional nested RT-PCR assay was designed to detect alphaviruses known to occur in South Africa, such as SINV and MIDV, including alphaviruses historically reported in South Africa such as CHIKV and NDUV, and alphaviruses reported in other regions of Africa such as ONNV and SFV, to consider the possibility of resurgence and cross-border infections. The RT-HDA was designed to detect alphaviruses currently known to circulate in South Africa such as MIDV and SINV in mosquito vectors. The development and proof of concept of the assay was focused on alphaviruses known to occur in South Africa.

While both assays were able detect alphaviral RNA controls in the laboratory, only the conventional nested assay was able to detect alphaviral RNA in the mosquito samples tested, due to its higher sensitivity. The assay was able to detect 620 copies of RNA. Although RT-HDA provides are more rapid and simple alternative to conventional nested RT-PCR, the assay lacks in sensitivity and is thus incapable of detecting alphaviral RNA in infected organisms

with less than approximately 4.8 x 10^5 copies of RNA. Isothermal amplification assays usually only require a simple heating device (e.g., a heating block) for amplification and lateral flow devices for end point detection, and is therefore suitable for application in low resource or field settings. Improvement is needed on the development of the RT-HDA assay, such as replacing degenerate bases with inosine bases in the primer sequences should be considered as an option to further improve the sensitivity of the assay. Alternative rapid and fieldable NAATs should also be considered in the future for the application of alphaviral surveillance or diagnosis in low resource settings.

Both assays were tested for theoretical cross-reactivity with other alphaviruses, which include SINV and CHIKV isolates from other regions and genotypes, and isolates from alphaviruses endemic to other parts of world, such as RRV, BFV, MAYV, EEEV, VEEV and WEEV to determine whether they are capable of detecting these viruses. Alignment of the primers with the sequences of these isolates show that both assays in theory would be able to detect SINV isolates from northern Europe, considering the transcontinental movement of the virus between South Africa and northern Europe by migratory birds. The alignment also shows that the conventional nested RT-PCR assay may be able to detect most alphaviruses due to minimal mismatches detected between the primers and the partial nsP4 sequences of the alphavirus isolates, and theoretically, this assay could be used to detect alphaviruses from around the world, perhaps with minor adjustments to the degenerate bases. On the other hand, RT-HDA assay may not be capable to detect other alphaviruses due to the many mismatches detected between the primers and the partial nsP4 sequences of the alphavirus isolates. Nonetheless, this shows that the RT-HDA is theoretically more specific that the conventional nested RT-PCR assay.

The conventional nested assay was used to screen 42 mosquito pools from 456 mosquitoes collected in the Free State due to its high sensitivity, and the assay was able to detect alphaviral RNA in five of the pools. The positive isolates were confirmed to be SINV isolates and MIDV isolates by nucleotide sequence determination and BLASTn analysis. These viruses are already circulating in South Africa (Steyn et al., 2020; Storm et al., 2013; van Niekerk et al., 2015). Phylogenetic analysis confirmed that the isolates are most closely related to isolates previously identified from South Africa and other isolates from Africa, and that the MIDV isolates identified in this study may possibly form part of a different or new lineage. Sequence data

from additional isolates is needed to determine if there are different genotypes or lineages of MIDV. This assay would be useful in future studies to screen for alphaviruses in other regions of South Africa to keep up to date with the alphaviruses circulating in the country.

The prevalence of arboviruses is not well known or infrequently updated due to the lack of surveillance studies, particularly in Africa (Ushijima et al., 2021). Numerous countries in Africa, where most of the recognised arboviruses have been detected, have experienced sporadic outbreaks of arbovirus diseases (Gould et al., 2017; Marchi et al., 2018; Ushijima et al., 2021). The conventional nested RT-PCR assay developed in this study would also be useful as a surveillance tool to detect circulating alphaviruses with the potential to cause outbreaks such as CHIKV and ONNV in other African countries. In future, a sensitive and field-friendly alternative to the conventional nested RT-PCR assay should be developed for application in low resource settings where the virus is more prevalent.

With a changing global climate, the rise in temperatures in conjunction with heavy rainfall are such conditions in which mosquitoes thrive and may play a role in causing future outbreaks. In addition, these viruses could spread to non-endemic regions, provided there are competent vectors present. Hence, continual development of rapid and reliable tools for the surveillance and detection of alphaviruses is important, and will aid in the understanding of which viruses are currently circulating with the potential to cause outbreaks.

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Appendix A: Ethics approval of application and amendment

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

UFS·UV
RESEARCH DEVELOPMENT
NAVORSINGSONTWIKKELING

Environmental & Biosafety Research Ethics Committee

11-Jul-2019

Dear **Ms Micah Dimaculangan**

Project Title: **Development of molecular assays for the detection of mosquito-borne alphaviruses in South Africa.**

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

Campus) APPLICATION APPROVED

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

Your ethical clearance number, to be used in all correspondence is: **UFS-ESD2019/0014**

Please note the following:

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

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

Yours Sincerely

Prof. RR (Robert) Bragg

Chairperson: Environment & Biosafety Research Ethics Committee
University of the Free State

Environment & Biosafety Research Ethics Committee

18-Apr-2021

Dear **Ms Micah Dimaculangan**

Project Title: **Development of molecular assays for the detection of mosquito-borne alphaviruses in South Africa.**

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

Campus) AMENDMENT APPROVED

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

Your ethical clearance number, to be used in all correspondence is: **UFS-ESD2019/0014**

Please note the following:

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

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

Yours Sincerely

Prof. RR (Robert) Bragg

Chairperson: Environment & Biosafety Research Ethics Committee
University of the Free State

Appendix B: Section 20

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: HerryG@daff.gov.za
Reference: 12/11/1/4 (1152)

Prof Felicity Jane Burt
Professor/ Med Scientist: Division of Virology
Faculty of Health Sciences
University of the Free State
Tel: 051 405 3348
Email: BurtFJ@ufs.ac.za

Dear Prof Burt

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

Your application dated 9 May 2019 requesting an amendment of the Section 20 permit (12/11/1/4) dated 01 August 2016 (attached), refers. I am pleased to inform you that the following amendments are hereby granted:

1. The title of the study is amended to *"Arboviruses in the Free State Province and Northern Cape Province, South Africa"*
2. Mosquitoes may also be collected from the University of the Free State in Bloemfontein and in the areas for which a state veterinary letter of no restriction has been supplied to DAFF, i.e. Kroonstad state veterinary area in Free State Province and Kimberley state veterinary area in Northern Cape Province. It is the responsibility of the researcher to consult with the relevant state veterinarian to ensure no restrictions have been placed on the above mentioned areas prior to sampling and to facilitate the issuing of movement permits as may be required;

3. Mosquitoes must be killed by freezing at the site of collection. These must be packaged and transported in compliance with the National Road Traffic Act 1996 (Act no 93 of 1996);

Kind regards,

DR. MPHO MAJA

DIRECTORO NIMALHEALTH

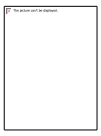
Date: 2019 -6\- 2 2

SUBJECT: AMENDMENT ON PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THEANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984)

Appendix C: Documentation indicating that material is available in the public domain and/ or permission to use material

Figure 1.1

File: Ijms-20-04657-g003.webp (3,354 × 1,750 pixels, file size: 454 KB, MIME type: image/webp)



This file is from Wikimedia Commons, the free media repository

Source: https://www.mdpi.com/1422-0067/20/18/4657/htm

Author(s): Madiiha Bibi Mandary, Malihe Masomian, and Chit Laa Pooh

Date: 19 September 2019

Figure 1.2

From: Hannah Kris Arcenio <help@hindawi.com>
Sent: Thursday, 25 November 2021 07:56
To: micah.dmac@gmail.com
Subject: Re: Permission to use figure in academic dissertation

Dear Dr. Dimaculangan,

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I hope this information is useful, and please don't hesitate to contact me with any further questions.

Best regards,

Hannah

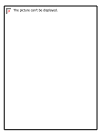
_____

Hannah Arcenio

Support Specialist

Figure 1.3

File: CHIK-World-Map-09-17-2019.jpg (1,430 × 858 pixels, file size: 262 KB, MIME type: image/jpeg)



This file is from Wikimedia Commons, the free media repository

Title: Structure and genome of the alphavirus

Source: https://www.cdc.gov/chikungunya/pdfs/Chik_World_Map_09-17-19-P.pdf

Author(s): CDC

Date: 17 September 2019

Figure 1.4 and Figure 1.5

From: PLOS Neglected Tropical Diseases <plosntds@plos.org>
Sent: Thursday, 25 November 2021 10:53
To: micah.dmac@gmail.com
Subject: Re: Permission to use figure in academic dissertation

Dear Micah,

Thank you for your email. You are welcome to re-use the content from any PLOS articles.

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All of our articles are available (through our websites) for anyone to download, re-use, reprint, modify, distribute, and/or copy so long as the original authors and source are cited.

Please let me know if you require any further assistance.

Kind regards,

Eoin O'Connor
Journal Assistant, Editorial Office Ltd.
On behalf of PLOS Neglected Tropical Diseases

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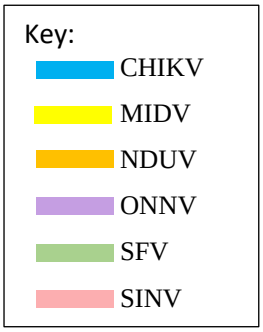
1160 Battery Street, Suite 225, San Francisco, CA 94111

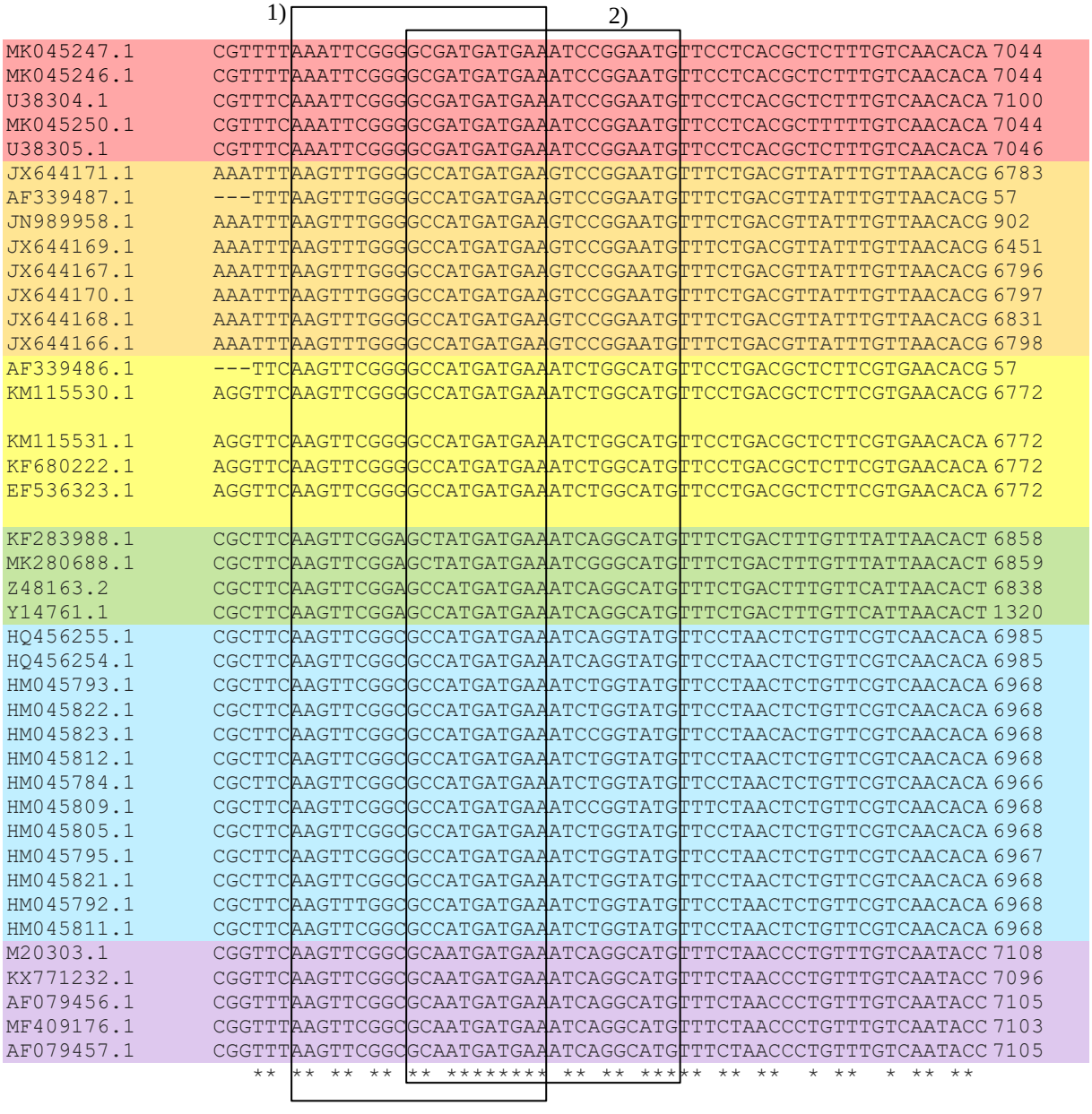
Case Number: 07393665
ref:_00DU0Ifis._5004P1iJgTQ:ref

Appendix D: Multiple alignment of the partial nsP4 gene of South African and other African alphavirus isolates used for primer design for the conventional nested RT-PCR assay

Annotations:

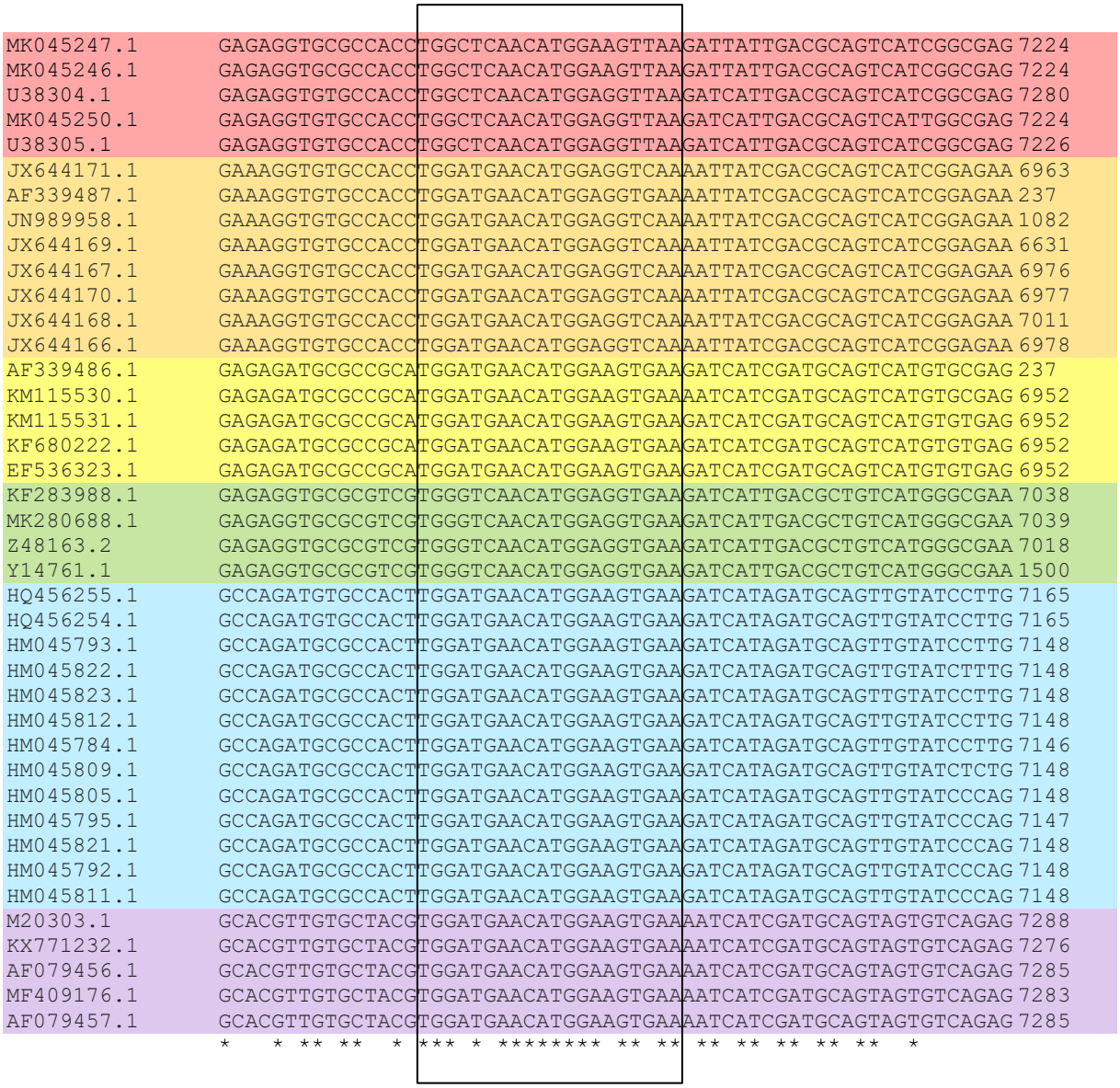
1) Alphavirus nsP4 (F1) primer region
2) Alphavirus nsP4 (F2) primer region
3) Alphavirus nsP4 (R2) primer region
4) Alphavirus nsP4 (R1) primer region





180 bp later

3)



341 bp later

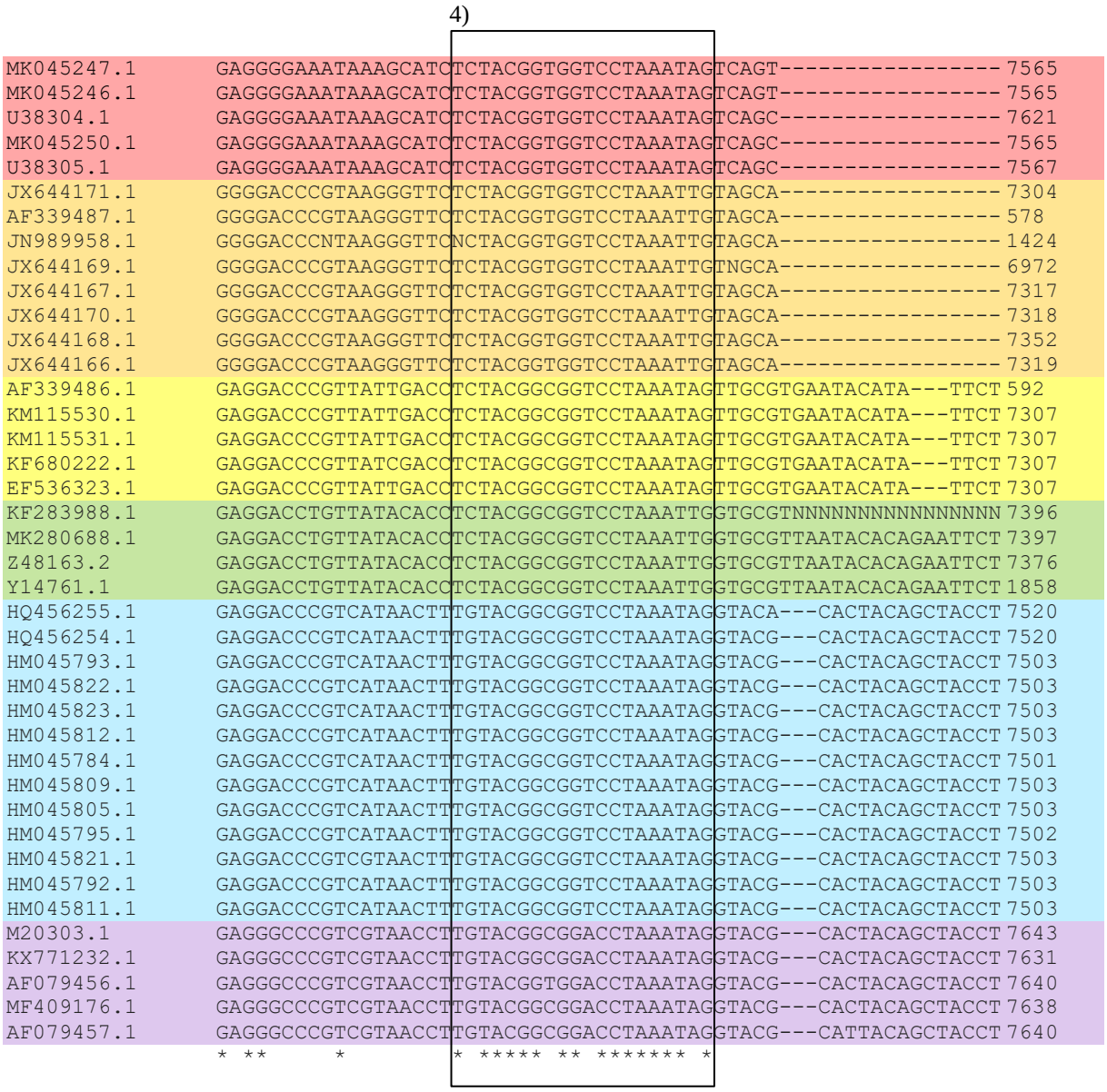


Table D1 Position of the conventional nested RT-PCR primers on the gene relative to the isolates used as positive controls

Alphavirus	Isolates	Alphavirus nsP4 (F1) primer 5'to 3'	Alphavirus nsP4 (F2) primer 5'to 3'	Alphavirus nsP4 (R2) primer 5'to 3'	Alphavirus nsP4 (R1) primer 5'to 3-
SINV	S.A.A.R 86	6993 – 7012	7002 – 7022	7201 – 7182	7264 – 7245
MIDV	SAE25_11	6719 – 6738	6728 – 6748	6927 – 6908	6990 – 6971
CHIKV	AR 18211	6915 – 6934	6924 – 6944	7123 – 7104	7186 – 7167
NDUV	SaAr 2204	4 – 23	13 – 33	212 – 193	275 – 256
ONNV	SG650	7052 – 7071	7061 – 7081	7260 – 7241	7323 – 7304

Appendix E: Multiple alignment of the partial nsP4 gene of South African and other African MIDV and SINV isolates used for primer design for the RT-HDA assay

Annotations:

1) HDA Forward (5’ biotin) primer region
2) HDA Reverse (5’ 6-FAM) primer region

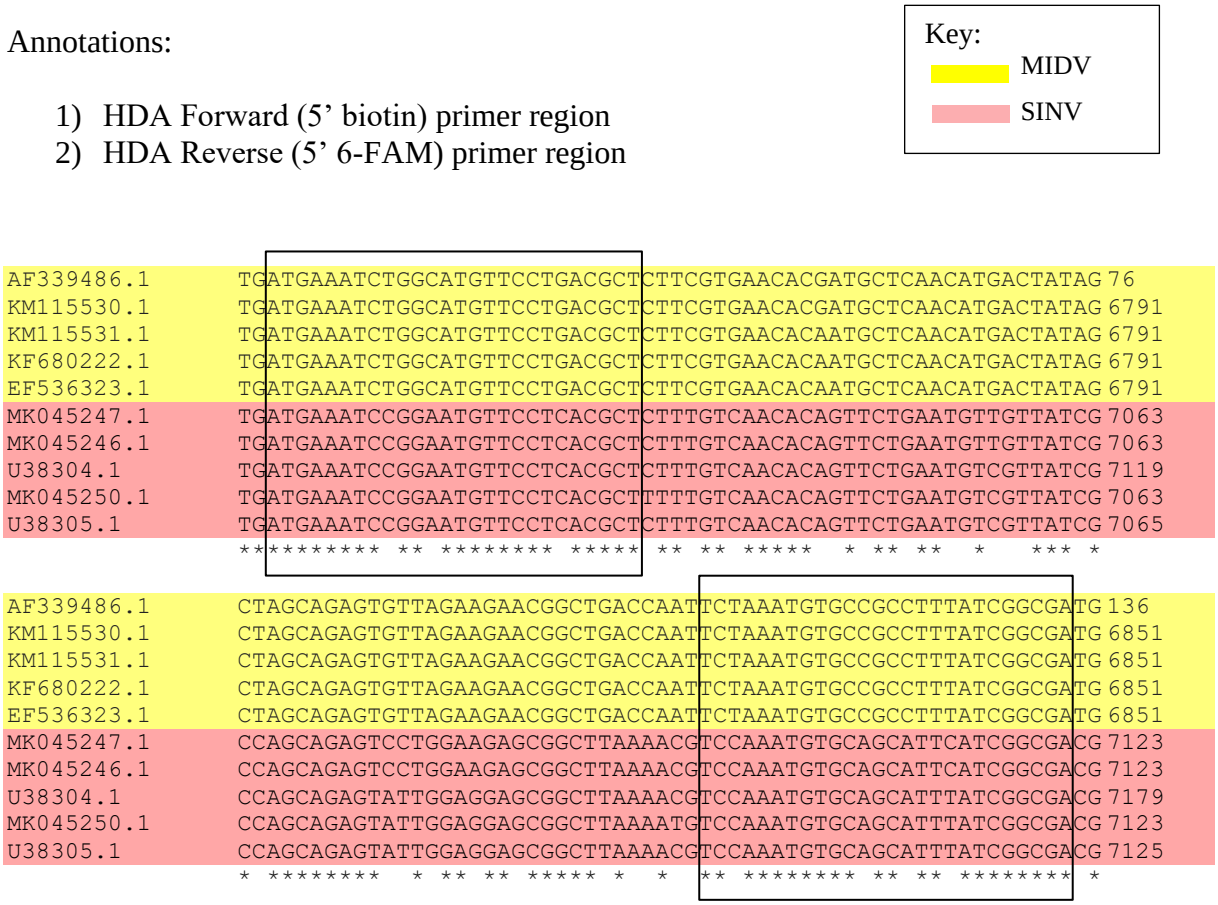


Table E1 Position of the RT-HDA primers on the gene relative to the isolates used as positive controls

Alphavirus	Isolates	HDA Forward (5’ biotin) primer 5’to 3’	HDA Reverse (5’ 6-FAM) primer 5’to 3’
SINV	S.A.A.R 86	7008 – 7033	7123 – 7098
MIDV	SAE25_11	6734 – 6759	6849 – 6824

Appendix F: Synthetic Genes

MIDV (isolate SAE25_11) partial nsP4 sequence (570 bp) + T7 promotor region (20 bp) added to 5' end and SP6 promoter region (20 bp) added to 3' end

5'TAATACGACTCACTATAGGGAAGTTCGGGGCCATGATGAAATCTGGCATGTTCCTGACGCTCTTCGTGAACACAATGCT
CAACATGACTATAGCTAGCAGAGTGTTAGAAGAACGGCTGACCAATTCTAAATGTGCCGCCTTTATCGGCGATGATAACA
TTGTGCATGGAGTGAAATCTGATAAACTGCTGGCTGAGAGATGCGCCGCATGGATGAACATGGAAGTGAAGATCATCGA
TGCAGTCATGTGTGAGCGCCCCCCCTACTTCTGCGGAGGGTTTATCGTGTTTGACCAAGTTACAGGTACCTGTTGCAGAGT
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GGCATTGGCCGATGAGGCACAACGGTGGAACCGCGTAGGTATCCAAGCAGACTTGGAGGCCGCAATGAGCAGCCGTTAC
GAGGTCGAGGGGATCCGAAACGTCATCACGGCGTTAACCACGCTGTCACGGAATTACCACAATTTCCGGCATTTAAGAGG
ACCCGTTATCGACCTCTACGGCGGTCCTAAATAGTTCTATAGTGTCACCTAAAT3'

CHIKV (isolate AR 18211) partial nsP4 sequence (570 bp) + T7 promotor region (20 bp) added to 5' end and SP6 promoter region (20 bp) added to 3' end

5'TAATACGACTCACTATAGGGAAGTTCGGCGCCATGATGAAATCTGGTATGTTCCTAACTCTGTTCGTCAACACACTGCTA
AATATCACCATCGCCAGCCGAGTGCTGGAAGATCGTCTGACAAAATCCGCGTGCGCAGCCTTCATCGGCGACGACAACAT
AATACATGGAGTCGTCTCCGATGGATTGATGGCAGCCAGATGCGCCACTTGGATGAACATGGAAGTGAAGATCATAGAT
GCAGTTGTATCCCAGAAAGCCCCTTACTTTTGTGGAGGGTTTATACTGCACGATACCGTGACAGGTACAGCTTGCAGAGT
GGCAGACCCGCTAAAAAGGCTATTTAAACTGGGCAAACCGCTAGCGGCAGGTGACGAACAAGATGAGGATAGAAGACG
AGCGCTGGCTGACGAAGTGGTCAGATGGCAACGAACAGGGCTAATTGATGAGTTGGAGAAAGCGGTATACTCTAGGTAT
GAAGTGCAGGGTATATCAGTTGTGGTAATGTCCATGGCCACCTTTGCAAGCTCCAGATCCAACTTCGAGAAGCTCAGAGG
ACCCGTCATAACTTTGTACGGCGGTCCTAAATAGTTCTATAGTGTCACCTAAAT3'

ONNV (isolate SG650) partial nsP4 sequence (570 bp) + T7 promotor region (20 bp) added to 5' end and SP6 promoter region (20 bp) added to 3' end

5'TAATACGACTCACTATAGGGAAGTTCGGCGCAATGATGAAATCAGGCATGTTTCTAACCCTGTTTGTCAATACCCTCCTG
AACATCACCATTGCTAGTCGGGTGCTAGAGGAGCGATTGACTACTTCAGCCTGTGCAGCATTCATTGGGGACGACAACAT
AATACATGGAGTTGTCTCTGACGCACTAATGGCTGCACGTTGTGCTACGTGGATGAACATGGAAGTGAAAATCATCGATG
CAGTAGTGTCAGAGAAGGCGCCATACTTCTGCGGGGGATTTATCTTACACGACACGGTGACAGGCACGTCGTGCAGAGT
AGCAGACCCTTTAAAGAGACTGTTCAAGTTAGGCAAACCTCTGGCAGCTGGAGACGAACAGGATGAGGACAGAAGACGT
GCTCTGGCAGATGAGGTTACTAGATGGCAAAGAACCGGCTTAATCACAGAATTAGAAAAAGCAGTATACTCCAGGTATG
AAGTACAAGGAATAACAGCCGTAATAACATCAATGGCTACCTTTGCGAGTAGCAAAGAAAACTTTAAAAAACTAAGAGG
GCCCGTCGTAACCTTGTACGGTGGACCTAAATAGTTCTATAGTGTCACCTAAAT3'

NDUV (isolate SaAr 2204) partial nsP4 sequence (570 bp) + T7 promotor region (20 bp) added to 5' end and SP6 promoter region (20 bp) added to 3' end

5'TAATACGACTCACTATAGGGAAGTTTGGGGCCATGATGAAGTCCGGAATGTTTCTGACGTTATTTGTTAACACGCTGCTT
AACGTCGTTATAGCTAGTCGCGTCCTAGAATCCAAATTGACGGGGTCGCGATGTGCCGCCTTCATTGGGGACGATAACAT
CGTGCATGGCGTGGTCTCAGATAAGTTGATGGCAGAAAGGTGTGCCACCTGGATGAACATGGAGGTGAAAATTATCGAC
GCAGTCATCGGAGAAAAACACCCGTATTTCTGCGGCGGGTTCATCCTACAGGATGCTGTGACCGGCACGGCGTGCCGAG
TATCCGACCCATTGAAGAGACTGTTTAAGTTGGGTAAACCACTGCCTGCGGACGATGAGCAGGATGAGGACCGCAGACG
AGCACTCCGTGACGAGGTGATGAGATGGTTTAGGGTAGGTCTGCGGTCTGAGGTGTGTGCTGCAGTTTATTCCAGGTAC
GGCGTGCAGGGGCTGGATGTTGCTTTGATGGCTATGGCAACCCTGTCGAAGACTAGGAAGCACTTCGACATGATTAGGG
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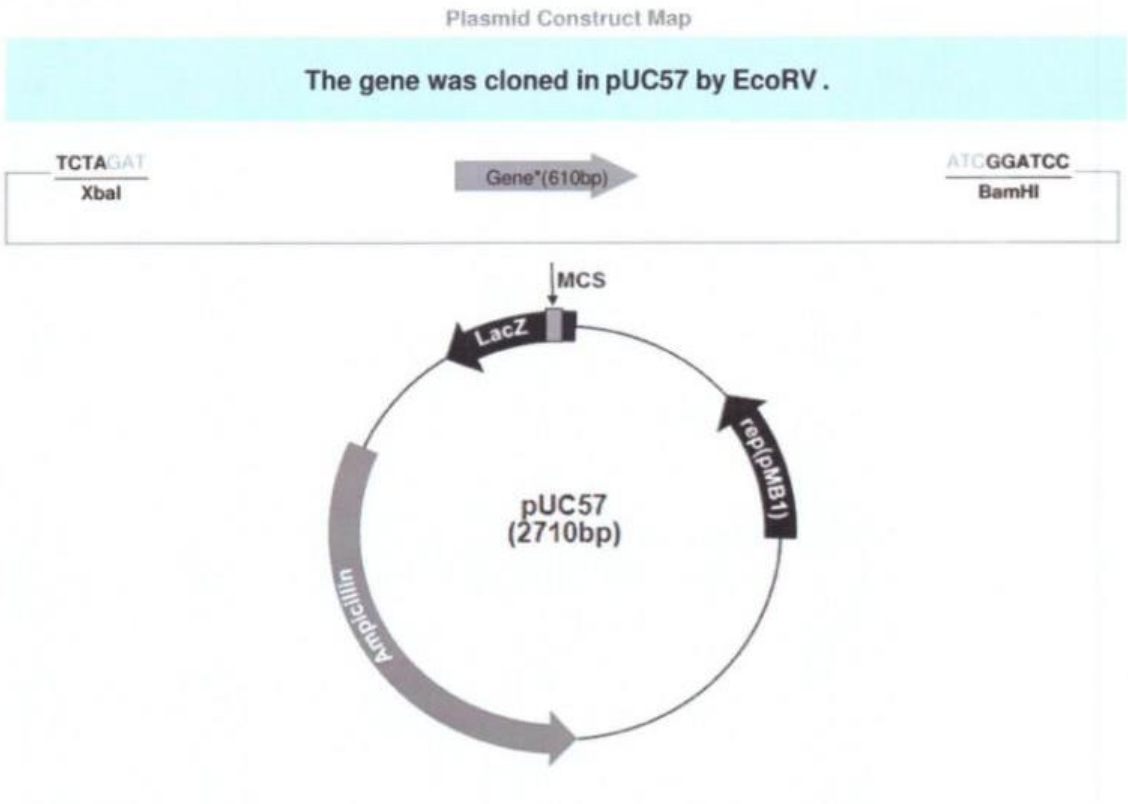


Figure F1: Synthetic genes synthesised in pUC57 vectors by GenScript, containing partial nsP4 gene (570 bp) + T7 region (20 bp) + SP6 region (20 bp)

Appendix G: Buffers and solutions used

50 X Tris-acetate-EDTA (TAE) stock (pH 8.0)

Tris-base: 242g

Acetate (100% acetic acid): 57.1 ml

Ethylene-diamine-tetra-acetic acid (EDTA): 100ml (0.5M sodium EDTA)

Add dH_2O to one litre

1X TAE (pH 8.0)

Dilute 20ml of 50X stock into 980 ml dH_2O

(x)% Agarose gel

Weigh (x) gram(s) of Seakem®LE agarose powder (Lonza, USA)

Add to 100ml 1X TAE buffer (pH 8.0)

Heat mixture in a microwave oven until dissolved

Cool liquid down before pouring (pour before gel solidifies)

GelRed solution

45 ml dH_2O

5 ml 0.1% NaCl

10µl GelRed™ Nucleic Acid Gel Stain, 10000X in Water (Biotium, Hayward, USA)

Ratio of sample to 6X Orange DNA Loading Dye (Thermo Scientific, USA)

5 µl sample: 1 µl 6X loading dye

Table G1: Recommended % agarose gel for resolving DNA fragments

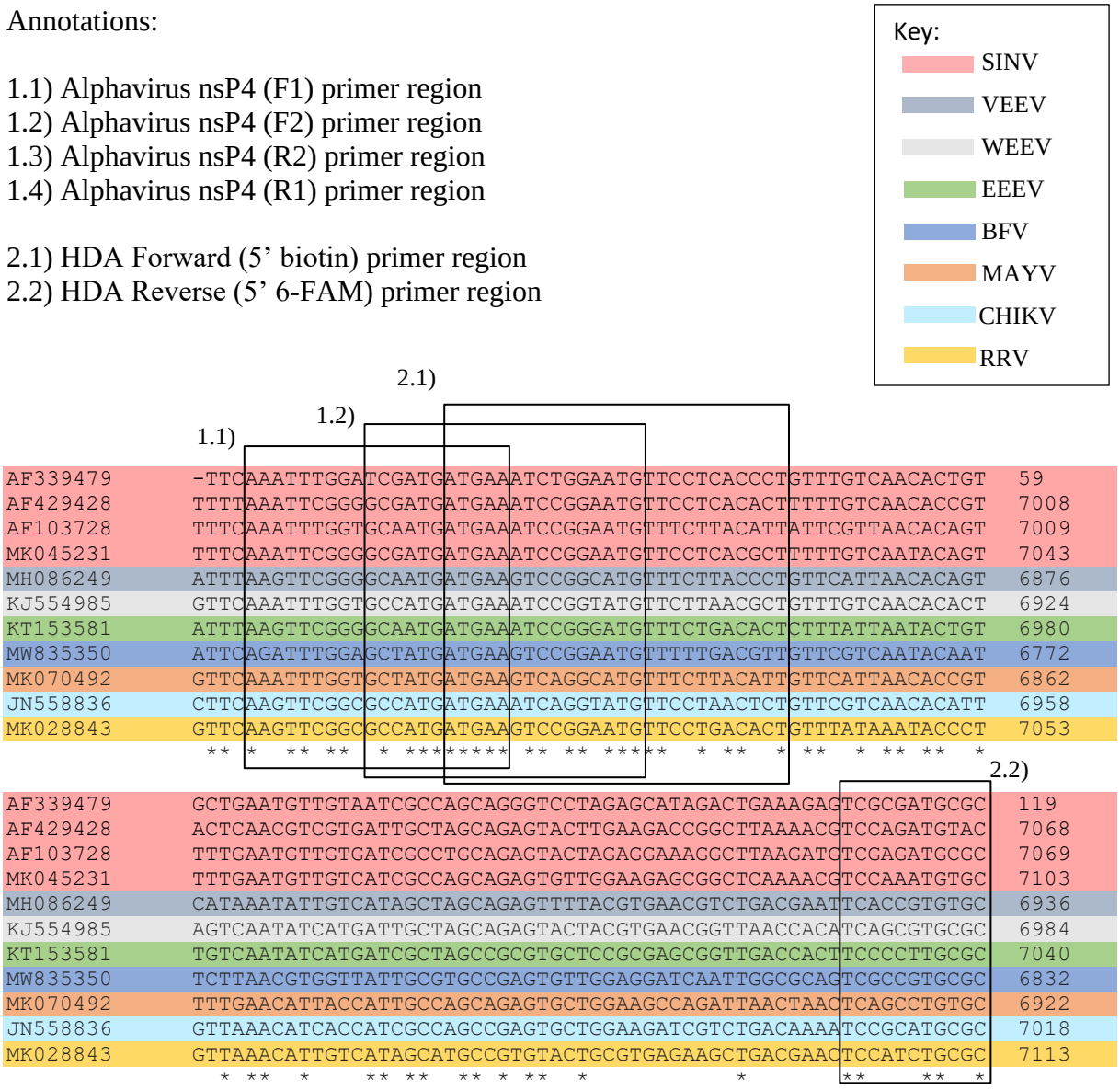
% Agarose Gel	DNA Size Resolution (bp)
0.5	1000 - 30000
0.7	800 - 12000
1.0	500 - 10000
1.2	400 - 7000
1.5	200 - 3000
2.0	50 - 2000

Appendix H: Multiple alignment of the primer sequences with the partial nsP4 sequences of SINV and CHIKV isolates from other regions and genotypes, and isolates from alphaviruses endemic to other parts of world, such as RRV, BFV, MAYV, EEEV, VEEV and WEEV

Annotations:

1.1) Alphavirus nsP4 (F1) primer region
1.2) Alphavirus nsP4 (F2) primer region
1.3) Alphavirus nsP4 (R2) primer region
1.4) Alphavirus nsP4 (R1) primer region

2.1) HDA Forward (5' biotin) primer region
2.2) HDA Reverse (5' 6-FAM) primer region



2.2)

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AF103728    GGCGTTTATCGGCGACGATAACATTATACACGGGGTAGTGTCTGATAAGGTGATGGCAGA  7129
MK045231    AGCATTCATCGGCGACGACAACATCATACACGGAGTAGTATCTGACAAAGAAATGGCTGA  7163
MH086249    AGCGTTCATAGGCGACGACAATATTGTCAAAGGTGTCAAATCTGACAAATTAATGGCAGA  6996
KJ554985    GGCCTTTATCGGCGACGATAACATAGTGCATGGTGTCGTCTCCGACACCTTGATGGCGGA  7044
KT153581    AGCATTTATCGGCGACGACAACATCGTGAAAGGGGTTACATCTGACGCGCTGATGGCAGA  7100
MW835350    TGCTTTCATAGGAGATGACAACATAATCCATGGTATAATATCAGACAAATTGATGGCGGA  6892
MK070492    CGCATTTATCGGCGACGACAACGTGGTTCACGGAGTCGTCTCCGATAAACTGATGGCAGA  6982
JN558836    GGCCTTCATCGGCGACGACAACATAATACATGGAGTCGTCTCCGATGAATTAATGGCAGC  7078
MK028843    CGCGTTTATCGGGGATGACAACATAGTGCACGGGGTAAGATCTGACCCGTTGATGGCTGA  7173
                ** ** ** ** ** ** **  *  *  * **  *    ** **      ***** *
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1.3)

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AF339479    AAGATGCGCTACCTGGCTTAACATGGAAGTGAAGATCATCGACGCCGTCATAGGCATCAG  239
AF429428    CCGATGCGCCACGTGGCTTAACATGGAAGTAAAAATCATCGATGCGGTCATCGGTGAAAG  7188
AF103728    GAGGTGCGCCACCTGGCTTAACATGGAAGTTAAGATTATCGACGCCATTATCGGTGAGAG  7189
MK045231    GAGGTGTGCCACCTGGCTCAACATGGAAGTTAAGATCATTGACGCAGTCATCGGCGAGAG  7223
MH086249    CAGGTGTGCGACATGGTTGAACATGGAAGTAAAAATCATTGATGCCGTAGTCGGAGAAAA  7056
KJ554985    GAGATGCGCCACTTGGCTGAACATGGAAGTAAAAATTATTGATGCAGTTATTGGTATCAA  7104
KT153581    GCGGTGCGCCACGTGGTTGAACATGGAAGTGAAGATCATCGATGCAGTAGTCGGAGTAAA  7160
MW835350    TAGATGTGCCACCTGGATGAACATGGAGGTCAAGATACTGGATTCCATAGTTGGAATACG  6952
MK070492    TAGATGTGCCACATGGGTTAACATGGAGGTTAAAATAATAGATGCAGTCATGTGTGCAAA  7042
JN558836    CAGATGTGCCACTTGGATGAACATGGAAGTGAAGATCATAGATGCAGTTGTATCCTTGAA  7138
MK028843    AAGGTGCGCCAGCTGGGTTAATATGGAGGTAAAGATAATTGATGCTACCATGTGCGAGAA  7233
              * ** ** *  *** * ** ***** ** **  **  * **  *     *
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AF339479    ACCTCCATATTTTTGTGGTGGATTCATCCTTCAAGATGAGACGACATTAACCACATGTCG  299
AF429428    TCCGCCGTATTTTTGCGGCGGCTTCATACTGCAAGATTCAGTCACACTTACCGCATGCCG  7248
AF103728    ACCACCGTATTTTTGCGGTGGATTTATTCTGCAAGACTCAGTCACTTCCACAGCGTGCCG  7249
MK045231    ACCACCCTACTTCTGCGGCGGATTCATCTTGCAAGACTCGGTTACCTCCACAGCGTGTCG  7283
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KJ554985    AGCACCCTACTTCTGTGGGGGATTTATCCTGGTGGACCAGATAACAGGCACAGCCTGCAG  7164
KT153581    GGCACCGTACTTTTGCGGAGGGTTCATCGTAGTCGATCAGATCACAGGAACTGCGTGCAG  7220
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AF103728    CGTGGCCGACCCACTGAAAAGATTATTTAAGCTTGGTAAACCGCTACCAGCCGACGACGA  7309
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KT153581    AGTCGCCGACCCCCTGAAGAGACTGTTTAAGCTAGGTAAGCCGCTTCCACTGGACGATGA  7280
MW835350    CGTCGCAGACCCACTGAAGAGACTGTTCAAGCTAGGTAAGCCATTGCCACTTGACGATGG  7072
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JN558836    AGTGGCAGACCCGCTAAAAAGGCTTTTTAAACTGGGCAAACCGCTAGCGGCAGGTGACGA  7258
MK028843    AGTGGCCGACCCGCTGAAAAGGTTATTTAAATTAGGTAAACCTTTACCCGCCGGAGACAC  7353
              * ** ** **  * ** **  * ** **  * ** ** **  *  *    *  **
```

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AF429428        TCAAGACGAGGATAGGAGACGCGCCCTCCTGGATGAGACCAAAGCGTGGTTTAGAGTCGG  7368
AF103728        GCAAGACGAAGATAGAAGGCGAGCTTTATTGGACGAAACAAAGGCGTGGTTCAGAGTGGG  7369
MK045231        GCAAGACGAAGACAGAAGACGCGCTCTGCTAGATGAAACGAAGGCGTGGTTTAGAGTAGG  7403
MH086249        GCACGATGACGACCGTCGTAGAGCTCTGTACGAGGAGTCGACTCGCTGGAACCGCGTAGG  7236
KJ554985        CCAAGACTGCGACCGCCGCCGGGCACTGCATGATGAAGCAATGCGATGGAACAGAATTGG  7284
KT153581        CCAGGACGTCGACAGGCGCAGAGCTCTGCATGATGAAGCGGCACGTTGGAACAGAATTGG  7340
MW835350        CCAAGATGAAGACAGAAGACGTGCATTGCATGATGAAGTGAAAACCTGGTCGCGCGTAGG  7132
MK070492        CCAAGATGAAGACCGCCGTAGGGCATTGCACGACGAAGTTAAAAAATGGTTTAGATCAGG  7222
JN558836        ACAAGATGAAGATAGAAGACGAGCGCTGGCCGACGAAGTGATCAGATGGCAACGAACAGG  7318
MK028843        CCAAGATGAAGATCGTAGGCGTGCATTGAAGGATGAGACGGATAGATGGGCACGAGTAGG  7413
                 ** **    **  *  *  * **  *    ** **          ***    *    **

AF339479        AATTAGGAACACTCTCGCAGTTGCCGTATCGACCAGGTACGAGGTAGAAGATATTACACC  479
AF429428        TATTACTCTGACACTAGACTCCGCCGTGGCGACTAGGTACGAGGTGGATAACATCGCACC  7428
AF103728        CATTACTGATACTTTAGCAACTGCTGTAGCAACCCGGTACGAGGTAGATAATATCACACC  7429
MK045231        TATAACAGACACCTTAGCAGTGGCCGTGGCGACTCGGTATGAGGTAGATAACATTACACC  7463
MH086249        TATATTCCACGAGTTGTGCAAGGCCGTGGAGTCACGGTATGAGACGTCAGGAACGGCCGT  7296
KJ554985        AATTACGGACGAGTTGGTGAAGGCCGTAGAATCCAGATACGAGATCATACTGGCAGGCCT  7344
KT153581        CATCACTGAAGAGCTGGTGAAAGCAGTTGAATCACGCTACGAGGTGAACTATGTGTCACT  7400
MW835350        GCTGCGACACAGAGTGTGTGAAGCCATCGAAGACCGTTATGCCGTCCATTCATCAGAACT  7192
MK070492        CTTGGGTTCGGAGATCGAGGTCGCCCTCGCCACCAGATACGAGGTGGAAGGGGGTTACAA  7282
JN558836        GCTAATTGATGAGCTGGAGAAAGCGGTATACTCTAGGTATGAAGTGCAGGGTATATCAGT  7378
MK028843        GTTGAAGTCTGAACTGGAAATAGCACTAAGTTCCCGGTATGAGGTGAACGGGACCGGCAA  7473
                   *          *       **  *        * ** *

AF339479        CGTTCTATACGCGCTTAGAACATTCGCTCAAAGCAAAAAGGCCTTCCAGACTATACGAGG  539
AF429428        CGTATTGTTGGCACTACGGACGTTTGCCTCGAGCAAAAAGGCATTTCAGGCCATCAGAGG  7488
AF103728        TGTCTTATTGGCATTGAGAACGTTTGCCCAGAGCAAAAATTCGTTTCAAGCCATTAGAGG  7489
MK045231        TGTCCTGCTGGCATTGAGAACTTTTGCCCAGAGCAAACGAGCATTCCAAGCCATAAGAGG  7523
MH086249        GATTATAACCGCCATGTCTACTTTGGCGAAGAATGTTTCATCATTTAAATACCTAAGAGG  7356
KJ554985        GATCATCACGTCTCTGTCCACGTTAGCCGAAAGCGTTAAGAACTTCAAGAGCATAAGAGG  7404
KT153581        AATCATCACAGCGTTGACTACATTAGCATCTTCAGTTAGCAACTTTAAACACATAAGAGG  7460
MW835350        AGTTTTATTGGCACTGACTACTCTGTCTAAGAACTTGAAGTCCTTCAGAAACATAAGAGG  7252
MK070492        CCTATTGTTGGCTATGTCCACCTTTGCACACAGTATGAAGAATTTTTCTGCATTGAGGGG  7342
JN558836        TGTGGTAATGTCCATGGCCACCTTTGCAAGCTCCAGATCCAACTTCGAGAAGCTCAGAGG  7438
MK028843        CATAGTGCGAGCAATGGCCACACTGGCCAAGAGCTTGAAGAATTTTAAAAAGCTGCGTGG  7533
                   *  *     *  *    **  *  *              **        *  * **

                                                                  1.4)
AF339479        AGAAATAAGACAGCTCTACGGCGGTCCTAAATAGTCAGTGCATTACACATTTTTATCTGA  599
AF429428        AGAAGTAAAGCAGCTCTACGGTGGTCCTAAATAGTGCACTATCAT-ACACAGTATATGAT  7547
AF103728        AGAAATAAAGCAACTCTACGGTGGTCCTAAATAGTCAGCATAGCATATTTTATCTGACTA  7549
MK045231        TGAAATAAAGCATCTCTACGGTGGTCCTAAATAGTCAGCATAGTACATTTCATCTGACTA  7583
MH086249        GAACCCGGTGACCCTCTACGGCTAACCTGAATGGACCGTGACGTAGTCCAGTCCGCCACC  7416
KJ554985        GAGCCCAATCACCCTCTACGGCTGACCTAAATAGGTGACGTAGTAGAAACGCACCTACCC  7464
KT153581        TCACCCCATAACCCTCTACGGCTGACCTAAATAGGTTGTGCATTAGTACCTAACCTATTT  7520
MW835350        GAAACCAATACATCTCTACGGTGGTCCTAAATAGTTGCCGTTAGACAACT----------  7302
MK070492        ACCCGTCATACACTTGTACGGCGGTCCTAAATAGGTGCTCTACACGACACCTATACCA--  7400
JN558836        ACCCGTCATAACTTTGTACGGCGGTCCTAAATAGGTACGCACTACAGCTA-CCTATTTTG  7497
MK028843        ACCCATTGTTCACCTCTACGGCGGTCCTAAATAGATGCAGAGACACACCTTCATCTAATA  7593
                              * *****    *** *** *
```

Appendix I: Sample collection

A

B

Figure I1 A and B: Shannon traps with Styrofoam box containing dry ice (to release CO_2 to attract mosquitoes) placed in the centre of the traps

Figure I2: CDC light trap near punctured container containing dry ice (to release CO_2 to attract mosquitoes)

Table I1: Forty-two mosquito pools from 456 mosquitoes caught between 2019 and 2020 in Bloemfontein, Free State (co-ordinates: (-29.069338, 26.20183)

Pool number	Number of mosquitoes	Mosquito species	Year
1	3	*Culex theileri*	2019
2	19	*Culex theileri*	2020
3	3	*Aedes dalzielli*	2020
4	1	*Aedes galloisi*	2020
5	17	*Culex univitattus*	2020
6	6	*Aedes mcitoshi*	2020
7	1	*Aedes cumminsii*	2020
8	1	*Ochlerotatus caballus*	2020
9	18	*Anopheles spp*	2020
10	20	*Culex pipiens*	2020
11	1	*Culex zombiensis*	2020
12	5	*Aedes galloisi*	2020
13	13	*Culex pipiens*	2020
14a 14b	60 (subdivided into 2 pools)	*Culex theileri*	2020 2020
15	5	*Aedes vexans*	2020
16	5	*Aedes cumminsii*	2020
17	1	*Culex zombiensis*	2020
18	28	*Anopheles spp*	2020
19	2	*Aedes vexans*	2020
20	18	*Anopheles spp*	2020
21	12	*Aedes galloisi*	2020
22a 22b	67 (subdivided into 2 pools)	*Culex theileri*	2020 2020
23	4	*Aedes cumminsii*	2020

Pool number	**Number of mosquitoes**	**Mosquito species**	**Year**
24	26	*Culex univitattus*	2020
25	1	*Aedes spp*	2020
26	14	*Anopheles spp*	2020
27	47	*Culex theileri*	2020
28	7	*Aedes galloisi*	2020
29	8	*Aedes mcitoshi*	2020
30	5	*Aedes cumminsii*	2020
31	1	*Aedes spp*	2020
32	2	*Culex zombiensis*	2020
33	1	*Culex pipiens*	2020
34	1	*Aedes vexans*	2020
35	1	*Ochlerotatus caballus*	2020
36	1	*Aedes galloisi*	2020
37	23	*Culex theileri*	2020
38	4	*Anopheles spp*	2020
39	2	*Aedes galloisi*	2020
40	1	*Culex univitattus*	2020
41	1	*Culex pipiens*	2020
42	1	*Culex spp*	2020

Appendix J: pGEM®T-Easy Vector (Promega, USA)

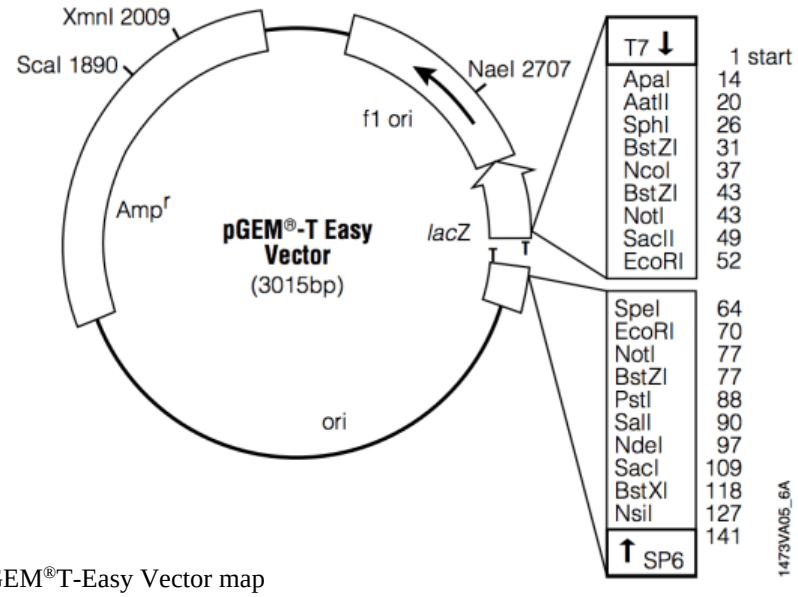

Figure J1: pGEM®T-Easy Vector map

Table J1: pGEM®T-Easy sequence reference points

Vector sequence reference points	Position on vector
T7 RNA polymerase transcription initiation site	1
multiple cloning region	10–113
SP6 RNA polymerase promoter (–17 to +3)	124–143
SP6 RNA polymerase transcription initiation site	126
pUC/M13 Reverse Sequencing Primer binding site	161–177
lacZ start codon	165
lac operator	185–201
β-lactamase coding region	1322–2182
phage f1 region	2365–2820
lac operon sequences	2821–2981, 151–380
pUC/M13 Forward Sequencing Primer binding site	2941–2957
T7 RNA polymerase promoter (–17 to +3)	2984–3

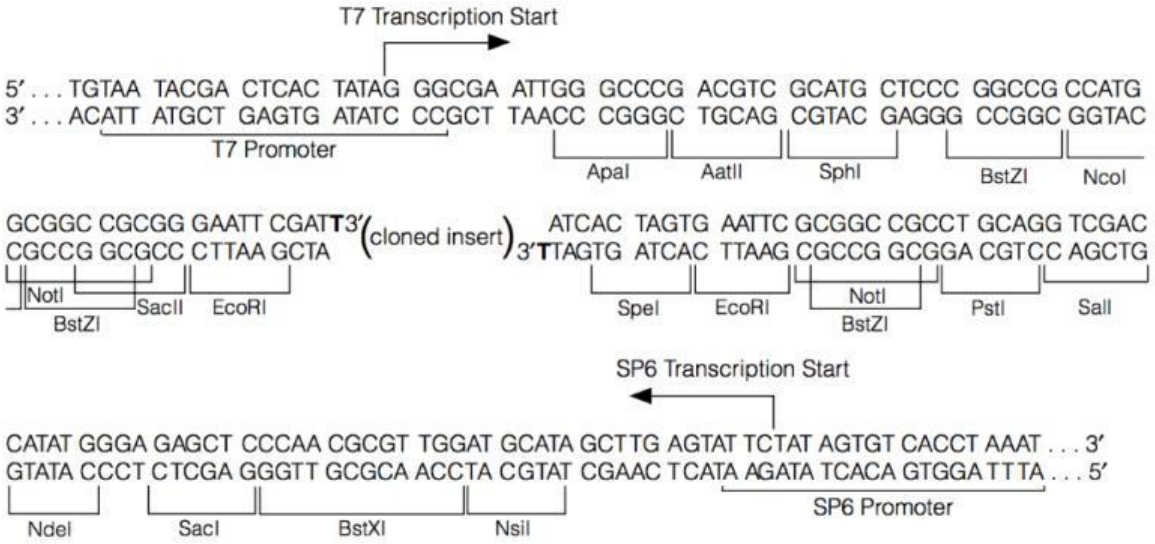

Figure J2: pGEM®T-Easy Sequence and Multi-Cloning Site

Appendix K: Nucleotide sequence determination and BLASTn results of positive alphavirus samples

Shown below are the nucleotide sequences obtained for each pool using bi-directional sequencing, and the BLASTn results obtained for each sequence. "Query coverage describes the percent of the query length that is included in the aligned segments. Expect (E) Value describes the number of alignments expected by chance with the calculated score. The lower the E-value, the better the score and alignment. Percentage identity describes the highest percent identity for a set of aligned segments to the same subject sequence" (Uniformed Services University, 2020).

Pool 6:

Nucleotide sequence:

5'GCGATGATGAAGTCCGGCATGTTCCTCACGCTCTTTGTCAACACAGTTCTGAAT
GTCGTTATCGCCAGCAGAGTATTGGAGGAGCGGCTTAAAACGTCCAAATGTGCA
GCATTTATCGGCGACGACAACATTATACACGGAGTAGTATCTGACAAAGAAATG
GCTGAGAGGTGTGCCACCTGGCTCAACATGGAAGTTAA3'

BLASTn results:

SINV isolate S.A.AR86

Query cover: 100%

E value: $3e^{-93}$

Percentage identity: 98.50%

Pool 14b:

Nucleotide sequence:

5'GCCATGATGAAGTCTGGTATGTTCCTCACGCTCTTTGTCAACACAGTTCTGAAT
GTCGTTATCGCCAGCAGAGTATTGGAGGAGCGGCTTAAAACGTCCAAATGTGCA
GCATTTATCGGCGACGACAACATTATACACGGAGTAGTATCTGACAAAGAAATG
GCTGAGAGGTGTGCCACCTGGCTCAACATGGAAGTGAA3'

BLASTn results:

SINV isolate S.A.AR86

Query cover: 97%

E value: $3e^{-88}$

Percentage identity: 97.94%

Pool 25:

Nucleotide sequence:

5’GCGATGATGAAGTCTGGCATGTTCCTGACGCTCTTCGTGAACACAATGCTCAAC
ATGACTATAGCTAGCAGAGTGTTAGAAGAACGGCTGACCAATTCTAAATGTGCC
GCCTTTATCGGCGGTGATAACATTGTGCATGGAGTGAAATCTGATAAACTGCTGG
CTGGAGATGCGCCGCATGGCTCAACATGGAAGTGAA3’

BLASTn results:

MIDV isolate SAE25_11

Query cover: 100%

E value: $1e^{-87}$

Percentage identity: 97.00%

Pool 29:

Nucleotide sequence:

5’GCGATGATGAAATCTGGTATGTTCCTCACGCTCTTTGTCAACACAGTTCTGAAT
GTCGTTATCGCCAGCAGAGTATTGGAGGAGCGGCTTAAAACGTCCAAATGTGCA
GCATTTATCGGCGACGACAACATTATACACGGAGTAGTATCTGACAAAGAAATG
GCTGAGAGGTGTGCCACCTGGCTCAACATGGAAGTTAA3’

BLASTn results:

SINV isolate S.A.AR86

Query cover: 100%

E value: $3e^{-93}$

Percentage identity: 98.50%

Pool 37:

Nucleotide sequence:

5’GCGATGATGAAGTCTGGCATGTTCCTGACGCTCTTCGTGAACACAATGCTCAAC
ATGACTATAGCTAGCAGAGTGTTAGAAGAACGGCTGACCAATTCTAAATGTGCC
GCCTTTATCGGCGATGATAACATTGTGCATGGAGTGAAATCTGATAAACTGCTGG
CTGAGAGATGCGCCGCATGGCTGAACATGGAAGTGAA3’

BLASTn results:

MIDV isolate SAE25_11

Query cover: 100%

E value: $3e^{-93}$

Percentage identity: 98.50%