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Epidemiology of Sindbis fever in South Africa and development of a real-time pan-alphavirus PCR assay for the detection of Sindbis and other medically important alphaviruses

by

Nadia Storm

Submitted in partial fulfilment of the requirements for the degree

Magister Scientiae (Microbiology)

in the Department of Microbiology and Plant Pathology
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I, Nadia Storm, declare that the dissertation hereby submitted to the University of Pretoria for the degree MSc (Microbiology) and the work contained therein is my own original work and has not previously, in its entirety or in part, been submitted to any university for a degree.

Signed: ______________________ this _______ day of ____________________2013

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SUMMARY

Epidemiology of Sindbis fever in South Africa and development of a real-time pan-alphavirus PCR assay for the detection of Sindbis and other medically important alphaviruses

by

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The genus *Alphavirus* in the family *Togaviridae* consists of approximately 30 single stranded, positive-sense RNA viruses which cause diseases ranging from mild, febrile illness with rash and arthritis to life-threatening encephalitis. Sindbis virus is the

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prototype virus for this genus and is the most widely distributed alphavirus globally. Little epidemiological data exists for this virus due to inadequate surveillance efforts in most countries, and it is suspected that Sindbis and other alphavirus infections are largely underreported. Additionally, changing patterns of alphavirus infections are challenging conventional knowledge of these diseases and have created the need for improved diagnostic methods and increased vigilance and disease surveillance around the globe.

To date, no real-time PCR assay exists that is able to detect all of the medically important (and other) alphaviruses. This study was therefore aimed at developing and evaluating such an assay in order to improve the diagnostic and surveillance activities of these viruses in South Africa. Additionally, this study was aimed at providing novel information on the molecular and epidemiological characteristics of Sindbis virus in South Africa, and to provide insights in the diversity of this virus compared to elsewhere in the world.

This study has revealed that an outbreak of Sindbis fever occurred during 2010, with the Free State and Northern Cape Provinces being most affected. The outbreak coincided with an outbreak of Rift Valley fever. The risk for acquiring a Sindbis virus infection was found to be highest during late summer/early autumn, and was slightly higher among males of increasing age. The phylogenetic results obtained in this study have demonstrated that Sindbis isolates form five separate groups with a considerable amount of genetic variation among these groups. The grouping of the isolates corresponded to the major migratory patterns of birds, suggesting that birds may play a large role in the dispersal of Sindbis viruses between continents. In addition, a real-time reverse transcription PCR assay has been developed which is able to detect Sindbis virus and other medically important alphaviruses with varying sensitivities. This project contributes to the update of epidemiological and phylogenetic data on Sindbis virus in South Africa and delivers potential tools for improved surveillance activities of alphaviruses in future.

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TABLE OF CONTENTS

Acknowledgements **i**
Summary **iv**
List of abbreviations **x**
List of figures **xiii**
List of tables **xvii**
Communications **xix**

CHAPTER 1: LITERATURE REVIEW **1**
1.1 Introduction 1
1.1.1 History of alphavirus infections 2
1.2 Classification of alphaviruses 4
1.3 Epidemiology of alphaviruses 6
1.3.1 Epidemiology of Sindbis virus 8
1.4 Morphology and characteristics of alphaviruses 10
1.5 Genomic structure and genetic diversity of alphaviruses 10
1.6 Replication of alphaviruses 13
1.7 Transmission of alphaviruses 13
1.8 Pathogenesis and virulence of alphaviruses 16
1.9 Diseases caused by alphaviruses 18
1.9.1 Clinical presentation of Sindbis virus infection 19
1.9.2 Treatment of alphavirus infection 21
1.10 Diagnostic identification of alphaviruses 22
1.10.1 Non-molecular techniques for the diagnosis of alphavirus infection 23
1.10.2 Molecular techniques and characterisation assays for alphavirus infection 24
1.10.2.1 Conventional reverse transcription PCR assays for the detection of alphaviruses 24
1.10.2.2 SYBR® Green-based real-time reverse transcription PCR assays for the detection of alphaviruses 25
1.10.2.3 Hydrolysis probe-based real-time reverse tran-

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scription PCR assays for the detection of alphaviruses 28

1.10.2.4 Other molecular assays for the detection of alphaviruses 30

1.11 Prevention, control and surveillance 30

1.12 Significance and aims of this study 31

CHAPTER 2: DESCRIPTIVE EPIDEMIOLOGY OF HUMAN SINDBIS FEVER CASES IN SOUTH AFRICA, 2006-2010 33

2.1 Introduction 33

2.2 Materials and methods 34

2.3 Results 35

2.4 Discussion 40

CHAPTER 3: MOLECULAR IDENTIFICATION AND TYPING OF SINDBIS VIRUS ISOLATES FROM SOUTH AFRICA 45

3.1 Introduction 45

3.2 Materials and methods 46

3.2.1 Virus isolates 46

3.2.2 Nucleic acid extraction 52

3.2.3 Nucleic acid amplification 53

3.2.4 Analysis of PCR reaction products 54

3.2.5 Purification of PCR products 54

3.2.6 Molecular sequencing of the amplicons 55

3.2.7 Phylogenetic analysis 56

3.3 Results 57

3.4 Discussion 59

CHAPTER 4: DEVELOPMENT OF A PAN-ALPHAVIRUS REAL-TIME REVERSE TRANSCRIPTION PCR ASSAY 63

4.1 Introduction 63

4.2 Materials and methods 64

4.2.1 Virus strains 64

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YUNIBESITHI YA PRETORIA

4.2.2 Primer and probe design 65
4.2.3 Real-time RT-PCR and optimisation 69
4.2.4 Production of synthetic controls 72
4.2.4.1 Preparation of DNA 72
4.2.4.2 Cloning of the *nsP4* gene 72
4.2.4.3 Selection of clones 73
4.2.4.4 Plasmid purification 73
4.2.4.5 *In vitro* transcription 74
4.2.4.6 Recovery of RNA 74
4.2.4.7 Determination of RNA concentration 75
4.2.4.8 Construction of standard curves 75
4.2.5 Statistical analysis 76
4.2.6 Determination of analytical sensitivity 76
4.2.7 Determination of analytical specificity 77
4.2.8 Diagnostic and surveillance evaluation 77
4.3 Results 79
4.3.1 Production of standard RNA 79
4.3.2 Construction of standard curves 80
4.3.3 Analytical sensitivity and specificity 84
4.3.4 Diagnostic evaluation 86
4.4 Discussion 87

CHAPTER 5: CONCLUDING REMARKS **92**

REFERENCES **94**

APPENDIX A **122**
Documentation of permission to use material and/or documentation to indicate that material used is in the public domain

APPENDIX B **126**
Confirmed Sindbis and West Nile virus case investigation form

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APPENDIX C **127**

Multiple alignment produced from sequencing information of the *nsP4* gene of alphaviruses (available in the public domain), primers and probes, using the ClustalW subroutine of the BioEdit sequence alignment editor, version 7.

APPENDIX D **128**

Interactions between primers (AlphaF, AlphaR, AlphamodF, AlphamodR, AlphaflapF and AlphaflapR) and probes (AlprobeJC1 and AlprobeJC2) for the pan-alphavirus real-time PCR determined using the OligoAnalyzer 3.1

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List of abbreviations

aa	Amino acid
Ae.	*Aedes*
AMV	Avian myeloblastosis virus
BHQ	Black hole quencher
BLAST	Basic local alignment search tool
bp	Base pair
CCHFV	Crimean-Congo haemorrhagic fever virus
cDNA	Complementary deoxyribonucleic acid
CHIKV	Chikungunya virus
CI	Confidence interval
Cp	Crossing point
Ct	Threshold cycle value
CV	Coefficient of variation
Cx.	*Culex*
ΔG	Change in free energy
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
E	Amplification efficiency
E. coli	*Escherichia coli*
EDTA	Ethylene diamine tetra-acetic acid
EEEV	Eastern equine encephalitis virus
ELISA	Enzyme-linked immunosorbent assay
FABI	Forestry and Agricultural Biotechnology Institute
FAM	Carboxyfluorescein
FRET	Fluorescence resonance energy transfer
g	Gravitational force
HAI	Haemagglutination inhibition
HCl	Hydrochloric acid
HEX	Carboxy-2’,4,4’,5’,7,7’-hexachlorofluorescein
IFA	Immunofluorescent antibody
IgG	Immunoglobulin G

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IgM	Immunoglobulin M
KCl	Potassium chloride
LB	Luria Bertani
LOD	Limit of detection
M	Molar
MAYV	Mayaro virus
MEGA	Molecular evolutionary genetics analysis
MGB	Minor groove binder
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulphate
MIDV	Middelburg virus
ml	Millilitre
mM	Millimolar
m/v	Mass per volume
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NDUV	Ndumu virus
ng	Nanograms
NHLS	National Health Laboratory Service
NICD	National Institute for Communicable Diseases
NJ	Neighbour joining
nt	Nucleotide
NTC	No template control
ONNV	O'nyong-nyong virus
OR	Odds ratio
PCR	Polymerase chain reaction
pfu	Plaque forming units
R^2	Coefficient of determination
RVF	Rift Valley fever
RVFV	Rift Valley fever virus
RNA	Ribonucleic acid
RRV	Ross River virus
RT-LAMP	Reverse transcription loop-mediated isothermal amplification

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RT-PCR	Reverse transcription polymerase chain reaction
SA	South Africa
SD	Standard deviation
SDS	Sodium docedyl sulphate
SEM	Standard error of the mean
SFV	Semliki Forest virus
SIN	Sindbis
SINV	Sindbis virus
SOC	Super optimal broth with catabolite repression
spp.	Species (plural)
TAMRA	Carboxytetramethylrhodamine
Taq	*Thermus aquaticus*
$TCID_{50}$	50% tissue culture infective dose
Tm	Melting temperature
µl	Microlitre
µM	Micromolar
UK	United Kingdom
USA	United States of America
UV	Ultra violet
V	Volt
VEEV	Venezuelan equine encephalitis virus
WEEV	Western equine encephalitis virus
WNV	West Nile virus
w/v	Weight per volume

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List of figures

Chapter 1

Figure 1.1: Phylogenetic tree of the *Alphavirus* genus based on partial *E1* gene sequences. The genetic grouping of the viruses correlates with the serocomplex arrangement of the alphaviruses. Virus abbreviations: MUCV - Mucambo virus; PIXV – Pixuna virus; CABV – Cabassou virus; FMV - Fort Morgan virus; HJV – Highlands J virus; WHAV - Whataroa virus; SINV – Sindbis virus; MIDV - Middelburg virus; SFV - Semliki Forest virus; RRV – Ross River virus; CHIKV – Chikungunya virus; ONNV – O'nyong-nyong virus; BFV - Barmah Forest virus; SAGV- Sagiyama virus; SPDV - Salmon pancreatic disease virus; SDV - Sleeping disease virus; GETV - Getah virus; NDUV – Ndumu virus; BEBV - Bebaru virus; TROCV - Troca virus; AURAV - Aura virus; WEEV – Western equine encephalitis virus; VEEV - Venezuelan equine encephalitis virus; EEEV - Eastern equine encephalitis virus (adapted from Lavergne *et al*., 2006.) See Appendix A for permission to use this material (License number 2918120487233). 5

Figure 1.2: The alphavirus genome and gene products. The *nsP1* gene codes for (-) strand RNA synthesis and capping of RNA, *nsP2* for a helicase and proteinase, *nsP3* for RNA synthesis, *nsP4* for a RNA polymerase, *C* for the capsid, and *E3*, *E2*, *6K* and *E1* for the envelope glycoproteins. 11

Figure 1.3: Map showing the zoogeographic regions and the Sindbis virus genotypes (numbered according to Lundström & Pfeffer, 2010) with corresponding bird flyways occurring in each region (- - - East Asian/Australasian flyway; .... East Atlantic flyway). 12

Figure 1.4: Transmission of alphaviruses. Abbreviations: CHIKV – Chikungunya virus; EEEV - Eastern equine encephalitis virus; MAYV – Mayaro virus; ONNV - O'nyong-nyong

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virus; RRV - Ross River virus; SINV – Sindbis virus; VEEV - Venezuelan equine encephalitis virus, WEEV – Western equine encephalitis virus (adapted from Schmaljohn & McClain, 1996). See Appendix A for permission to use this material. 14

Figure 1.5: The *Culex* mosquito - a vector for both Sindbis and West Nile virus (Gathany, 2003). See Appendix A for documentation that this material is in the public domain. 15

Figure 1.6: Pathogenesis of alphavirus infections (adapted from Schmaljohn & McClain, 1996 - See Appendix A for permission to use this material). Alphaviruses enter the host via a mosquito bite and are spread in the blood. Old World alphaviruses (e.g. SINV, CHIKV) cause a mild, febrile illness with a rash and arthralgia while New World alphaviruses (e.g. EEEV, VEEV, WEEV) cause encephalitis. Rarely, the virus may exit a human host via mosquitoes but usually encounters a dead end. 17

Figure 1.7: Maculopapular rash on the back of a patient infected with Sindbis virus (adapted from Ferguson *et al*., 2005). See Appendix A for documentation that this material is in the public domain. 20

Figure 1.8: The SYBR® Green I real-time PCR detection chemistry: A. Unbound SYBR® Green I dye molecules fluoresce weakly at the beginning of amplification. B. After the primers anneal, some SYBR® Green I molecules bind to the double stranded DNA, which increases the fluorescence. C. During elongation, more SYBR® Green I molecules bind to the newly synthesised DNA strand and fluorescence continues to increase. 27

Figure 1.9: The hydrolysis (TaqMan®) probe real-time PCR chemistry: A. The hydrolysis probe contains a 5’ fluorescent dye (R) and a 3’ quenching dye (Q). When irradiated, the fluorescent dye is quenched and little fluorescence occurs. B. The hydrolysis probe binds to its specific target on the template DNA. C. As amplification occurs, the 5’ nuclease activity of *Taq* DNA polymerase cleaves the probe. This separates the fluorescent

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and the quenching dye, leading to fluorescence. 29

Chapter 2

Figure 2.1: Histogram indicating the number of specimens submitted for arbovirus testing, as well as the anti-SINV IgM detection rate, for 2006 – 2009 and for 2010. 36

Figure 2.2: Distribution of the confirmed cases of SINV and RVFV infection, by epidemiologic week, from 15 January 2010 through 9 September 2010. 37

Figure 2.3: Graph showing the Sindbis virus (SINV) detection rate for each age group from 2006 through 2010. 38

Figure 2.4: Graph showing the Sindbis virus (SINV) detection rate in each Province for the period 2006 to 2010. 40

Chapter 3

Figure 3.1: Map of South Africa indicating the approximate locations where the South African Sindbis virus isolates were collected. 52

Figure 3.2: Phylogenetic tree illustrating the genetic relationships of Sindbis viruses based on sequencing of 312 nucleotides of the *E2* gene. Bootstrap values over 70% are indicated and branch lengths are proportional to genetic relatedness. Midpoint rooting was selected and no out-group was defined. The numbers I to V refer to clusters (corresponding to the different SINV genotypes described in Lundström & Pfeffer, 2010) while a, b and c refer to sub-clusters. 58

Chapter 4

Figure 4.1: Real-time RT-PCR graph showing the increase in fluorescence for serial dilutions of *in vitro* transcribed RNA representing 10^1 to 10^5 copies/µl (in triplicate). 80

Figure 4.2: Standard curve for the real-time RT-PCR assay constructed by plotting crossing point values versus the log concentration of the RNA triplicates. 81

Figure 4.3: Graph showing a comparison of the Cp values for the different serial dilutions in different PCR runs (light grey – run 1, dark

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grey – run 2, black – mean). 82

Figure 4.4: Graph illustrating the relationship between the real-time PCR RNA copy number and the infectious virus titer ($TCID_{50}$). 84

Figure 4.5: Graph illustrating the specificity of the real-time RT-PCR assay by the amplification of the positive control sample (SINV) and the non-amplification of the negative control, Crimean-Congo haemorrhagic fever (CCHF), dengue, Rift Valley fever, rubella, West Nile and yellow fever virus samples. 85

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List of tables

Chapter 1

Table 1.1: Summary of the known medically important alphaviruses 3

Table 1.2: Genus-specific conventional RT-PCR assays for the detection of alphaviruses 25

Chapter 2

Table 2.1: Demographic characteristics of patients with IgM-ELISA-confirmed SINV cases in South Africa from 2006 through 2010 39

Chapter 3

Table 3.1: Sindbis virus isolates included in this study and analysed by partial sequencing of the *E2* gene*. Isolates are grouped based on genotype. 47

Table 3.2: Primer pairs used for the amplification of the partial *E2* gene (Lundström & Pfeffer, 2010) 53

Chapter 4

Table 4.1: Alphavirus strains used in this study for the design and validation* of the pan-alphavirus real-time PCR 65

Table 4.2: Primers and probes designed for the real-time RT-PCR for the detection of alphaviruses 66

Table 4.3: Primer and probe parameters (OligoAnalyzer 3.1) 67

Table 4.4: Mismatches between the alphavirus target region and probes 68

Table 4.5: Cycling conditions for the pan-alphavirus real-time RT-PCR assay 71

Table 4.6: Details of the mosquito pools assayed in this study 77

Table 4.7: Summary and statistical analysis of the data sets for the two independent real-time RT-PCR runs 83

Table 4.8: Virus panel tested using the pan-alphavirus real-time RT-PCR assay and the conventional nested RT-PCR assay 85

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Table 4.9: Results obtained for the diagnostic panel using conventional and real-time PCR 86

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CHAPTER 1: LITERATURE REVIEW

1.1 Introduction

The genus *Alphavirus*, in the family *Togaviridae*, consists of 29 small, spherical and enveloped viruses (International Committee on Taxonomy of Viruses, 2011). These arthropod-borne ribonucleic acid (RNA) viruses (also known as arboviruses) are distributed worldwide and are transmitted to humans and animals by mosquito and other insect vectors (Powers *et al*., 2001; Kurkela *et al*., 2004). Until 2005, alphaviruses such as Sindbis virus (SINV), chikungunya virus (CHIKV), Ross River virus (RRV) and O'nyong-nyong virus (ONNV) were thought to be of minor medical importance as they caused localised outbreaks that affected a relatively small number of people. Additionally, under- and misdiagnoses of these alphavirus infections in dengue-endemic areas lead to the under-appreciation of alphaviruses as a cause of febrile illness in these and other areas (reviewed in Gould *et al*., 2010). The unperturbed view of alphaviruses changed when CHIKV caused a massive and unexpected epidemic throughout Africa, the Indian Ocean Islands, India, Indonesia, Sri Lanka and Malaysia from the end of 2004 (Rezza et al., 2007; Liumbruno *et al*., 2008). Moreover, CHIKV has repeatedly been introduced into new, unanticipated areas of Asia, Europe, Australia and America in recent years in small, autochthonous outbreaks (Rezza *et al*., 2007). In addition to CHIKV, many of the other alphaviruses are agents of significant emerging veterinary and human diseases (reviewed in Weaver *et al*., 2012).

Despite the changing patterns and characteristics of alphavirus infections, epidemiological data for most alphaviruses remain limited. The emergence of certain alphaviruses in unexpected areas has created the need for novel information on the molecular and epidemiological characteristics of these viruses, and for improved methods for disease surveillance around the globe. To date, no comprehensive epidemiological and phylogenetic studies have been done on SINV, the prototype virus in the genus *Alphavirus*, in South Africa. Additionally, no real-time polymerase chain reaction (PCR) assay currently exists that is able to detect all of the medically important African alphaviruses. The studies described in this dissertation will therefore focus on addressing these issues.

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1.1.1 History of alphavirus infections

Alphaviruses were amid the initial arboviruses to be isolated, characterised and classified (Calisher *et al*., 1980; reviewed in Gould *et al*., 2010). Alphaviruses have been shown to cause a wide variety of diseases, ranging from febrile illness and arthritis to life-threatening encephalitis with haemorrhagic and neurological manifestations (Tsai *et al*., 2002; Ryman *et al*., 2010). The medically important alphaviruses are summarised in Table 1.1.

The equine encephalitis viruses were first isolated from the brains of deceased horses that had suffered from encephalitis in as early as 1930 (Meyer *et al*., 1931). By the late 1930s, it was apparent that Western equine encephalitis virus (WEEV) and Eastern equine encephalitis virus (EEEV) were also responsible for cases of encephalitis in humans (Howitt, 1938; Webster & Wright, 1938). Venezuelan equine encephalitis virus (VEEV) has only been associated with human disease since 1952, when human infections occurred together with an equine epizootic in Colombia (Sanmartin-Barberi *et al*., 1954).

Chikungunya virus was isolated for the first time in Tanzania in 1953, where a patient suffered from a febrile illness and severe arthralgia (Robinson, 1955; Ross, 1956). This virus has since been reported in several areas of Asia and Africa and continues to cause sizeable epidemics in those areas (Simon *et al*., 2007). Mayaro virus (MAYV) was initially isolated in Trinidad in 1954 (Anderson *et al*., 1957; Causey & Maroja, 1957) and in French Guiana in 1996 (Talarmin *et al*., 1998). O'nyong-nyong virus was first discovered in 1959 in Uganda when it caused a large outbreak affecting two million people (Williams *et al*., 1965). Within the next 35 years, this virus caused relatively few outbreaks, but reappeared in 1996 to cause a second epidemic in the same area (Rwaguma *et al*., 1997).

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Table 1.1: Summary of the known medically important alphaviruses

Virus	**Major clinical manifestations**	**Reservoir / vectors**	**Known geographic distribution**	**Reference**
Chikungunya	Severe arthritis, rash	Primates (in Africa) and humans (in Asia) / *Aedes* mosquitoes	Africa (including South Africa), India, South East Asia, Italy	Chhabra *et al*., 2008
O'nyong-nyong	Rash and arthritis	Primates and humans / mosquitoes	Africa (Uganda, Kenya, Tanzania, Malawi and Senegal)	Powers *et al*., 2001
Sindbis	Rash and arthritis	Birds / *Culex* mosquitoes	Northern Europe, Africa, Israel, Asia, Philippines, New Zealand and Australia	Laine *et al*., 2004
Semliki Forest	Rash and arthritis	Birds / mosquitoes	Africa	Mathiot *et al*., 1990
Ross River	Rash and arthritis	Mammals including humans / mosquitoes	Australia and the South Pacific	Barber *et al*., 2009 Jacups *et al*., 2008
Mayaro	Rash and arthritis	Primates and humans / mosquitoes	South America	Laine *et al*., 2004
Barmah Forest	Rash, arthritis and muscle tenderness	Humans / ticks	Australia	Jacups *et al*., 2008
Eastern equine encephalitis	Encephalitis	Birds / mosquitoes	Americas	Zacks & Paessler, 2010
Western equine encephalitis	Encephalitis	Birds, mammals / mosquitoes	North America	Zacks & Paessler, 2010
Venezuelan equine encephalitis	Encephalitis	Rodents, horses / mosquitoes	America	Zacks & Paessler, 2010

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Sindbis virus was first isolated from the *Culex* (*Cx.*) mosquito species (spp.) in the Sindbis health district near Cairo, Egypt in 1952 (Taylor *et al.*, 1955). The first cases of human disease was recognised in Uganda in 1961 (Woodall *et al.*, 1962) and South Africa in 1963 (Malherbe *et al.*, 1963). However, this virus was not considered a health threat to humans until after 1974, when epidemics involving thousands of cases occurred in South Africa (McIntosh *et al.*, 1976) and Northern Europe (Lvov *et al.*, 1984). Another outbreak of SINV infections occurred in 1981 in Finland, when a physician diagnosed 73 patients with a syndrome named Pogosta disease (Brummer-Korvenkontio & Kuusisto, 1981). The SINV has since been associated with diseases such as Ockelbo in Sweden and Karelian fever in Russia (Skogh & Espmark, 1982; Lvov *et al.*, 1984).

1.2 Classification of alphaviruses

The *Alphavirus* genus belongs to the family *Togaviridae* and can be classified into seven serocomplexes (Eastern, Venezuelan and Western equine encephalitis, Middelburg, Ndumu, Semliki Forest and Barmah Forest) based on their antigenic properties (Strauss & Strauss, 1994; Powers *et al.*, 2001; Garmashova *et al.*, 2007; International Committee on Taxonomy of Viruses, 2011) (Figure 1.1). The inclusion of an eighth complex, Trocara, has also been suggested based on genetic divergence (Travassos da Rosa *et al.*, 2001).

The members of these complexes can be described as being either Old World or New World viruses based on their geographic distribution and the ailments they cause (Griffin, 2001; Garmashova *et al.*, 2007). Old World viruses include CHIKV, ONNV, RRV, SINV and Semliki Forest virus (SFV) and are associated with a flu-like illness and arthralgia syndrome, while New World viruses include VEEV, EEEV and WEEV and are more commonly associated with encephalitis (Schmaljohn & McClain, 1996; Griffin, 2001). Two non-mosquito-borne species of alphaviruses exist, namely Salmon pancreatic disease virus and Southern elephant seal virus (International Committee on Taxonomy of Viruses, 2011), which are thought to be associated with lice (La Linn *et al.*, 2001; Rodger & Mitchell, 2007).

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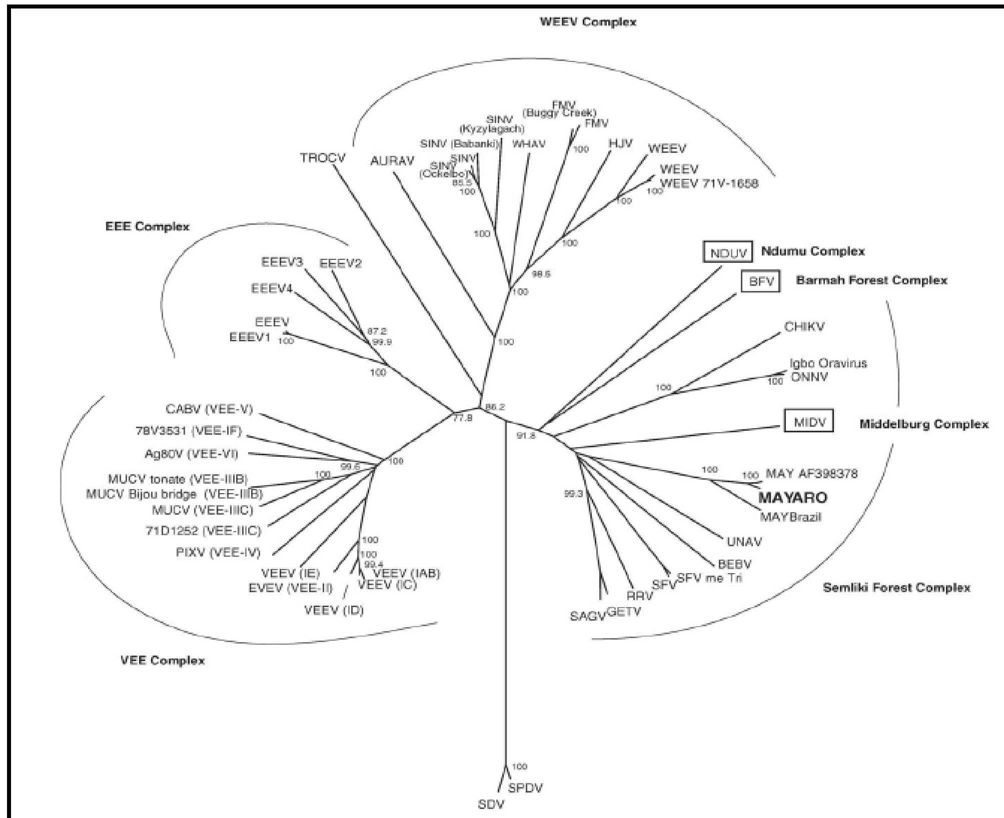

Figure 1.1: Phylogenetic tree of the *Alphavirus* genus based on partial *E1* gene sequences. The genetic grouping of the viruses correlates with the serocomplex arrangement of the alphaviruses. Virus abbreviations: MUCV - Mucambo virus; PIXV - Pixuna virus; CABV - Cabassou virus; FMV - Fort Morgan virus; HJV - Highlands J virus; WHAV - Whataroa virus; SINV - Sindbis virus; MIDV - Middelburg virus; SFV - Semliki Forest virus; RRV - Ross River virus; CHIKV - Chikungunya virus; ONNV - O'nyong-nyong virus; BFV - Barmah Forest virus; SAGV - Sagiyama virus; SPDV - Salmon pancreatic disease virus; SDV - Sleeping disease virus; GETV - Getah virus; NDUV - Ndumu virus; BEBV - Bebaru virus; TROCV - Troca virus; AURAV - Aura virus; WEEV - Western equine encephalitis virus; VEEV - Venezuelan equine encephalitis virus; EEEV - Eastern equine encephalitis virus (adapted from Lavergne *et al*., 2006). See Appendix A for permission to use this material (License number 2918120487233).

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Sindbis virus is the prototype virus for the genus *Alphavirus* and groups with the WEEV complex (Weaver *et al*., 2006; Lundström & Pfeffer, 2010; International Committee on the Taxonomy of Viruses, 2011). The Sindbis complex includes several subtypes such as Ockelbo virus, Karelian fever virus, Babanki virus and Whataroa virus (Lundström & Pfeffer, 2010).

1.3 Epidemiology of alphaviruses

Within the last 50 years, the patterns of emerging alphavirus diseases have transformed considerably (Gould & Higgs, 2009). Climate change has possibly altered the geographic distribution of the arthropod vectors for alphaviruses, the arthropod lifecycles, the evolution of alphaviruses and the effectiveness with which they are transmitted to their vertebrate hosts (Gould & Higgs, 2009). One example is the unexpectedly successful establishment of CHIKV in the northern parts of Italy in 2007 and the adaptation of CHIKV to the tiger mosquito, *Aedes* (*Ae*.) *albopictus*, which led to massive outbreaks of CHIKV infections in the Indian Ocean Islands since 2005 (Rezza *et al*., 2007; de Lamballerie *et al*., 2008a; Liumbruno *et al*., 2008).

Chikungunya virus and ONNV have caused numerous, substantial epidemics of acute, incapacitating arthralgia in Africa and Asia (Johnston & Peters, 1996). The antigenically and clinically comparable MAYV causes frequent infections in the Amazon (Azevedo *et al*., 2009). The latest reported outbreak of ONNV infections occurred in 2003 in the Western Ivory Coast, an area not previously affected by this virus (Posey *et al*., 2005). In South Africa, CHIKV has caused human outbreaks in Mpumalanga in 1956, 1975 and 1977 and a CHIKV epizootic (an outbreak of a disease amongst an animal population) occurred in monkeys in the northern parts of KwaZulu-Natal in 1964 (McIntosh *et al*., 1977; McIntosh, 1986; Jupp & McIntosh, 1990; Jupp, 2005). In the year 2000, CHIKV affected an estimate of almost 50 000 people in the Democratic Republic of Congo (Pastorino *et al*., 2004). Chikungunya virus has also been responsible for a major disease outbreak in 2005, involving India, the Indian Ocean Islands, Malaysia and Sri Lanka, which affected 1.5 million people (Parola *et al*., 2006; Simon *et al*., 2007). An epidemic involving an estimate of 7 474 cases (but no deaths) of CHIKV infections existed in the Democratic Republic of

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Congo (HealthMap, 2011) during June and July of 2011. The vectors for this outbreak were suspected to be *Ae. aegypti* and *Ae. albopictus* (HealthMap, 2011).

Ross River virus is an alphavirus endemic to Australia and was responsible for a large epidemic of arthralgia that affected 50 000 people on the South Pacific Island of Fiji in 1979 (Aaskov *et al*., 1981). The RRV and Barmah Forest virus (BFV) cause approximately 6 500 cases of epidemic polyarthritis in Australia each year (Mackenzie & Smith, 1996). Infections with RRV and BFV are seldom fatal and hardly ever result in encephalitis (Lewthwaite *et al*., 2009).

The equine encephalitis viruses (EEEV, VEEV and WEEV) are important pathogens in the United States of America (Griffin, 2001). Eastern equine encephalitis virus is transmitted between birds by *Culiseta melanura* mosquitoes and cause isolated cases of disease in humans and horses when *Aedes* spp. mosquitoes, which have fed on infected birds, bite these hosts (Scott & Weaver, 1989; Armstrong & Andreadis, 2010). Western equine encephalitis virus, which has caused many outbreaks of encephalitis in humans and horses, is spread by *Cx. tarsalis* mosquitoes, which are found in numerous areas of America and Canada (Hammon *et al*., 1943; Hayles & Saunders, 1972). Unlike many other alphaviruses, birds have not been found to play an important role as a reservoir for VEEV. Instead, this virus is maintained in mosquito-rodent-mosquito cycles and only occasionally causes severe disease in humans (Carrara *et al*., 2007). Venezuelan equine encephalitis virus is amplified in horses, and in 1969 to 1972, an equine epizootic involving several American countries occurred (Sudia *et al*., 1975). The most recent outbreak of VEEV infections occurred in 1995 in Colombia and Venezuela, affecting 100 000 humans (3 000 with neurological manifestations) and causing 300 encephalitis-related human deaths (Diaz *et al*., 1997). The mortality rate of VEEV in horses may be as high as 83% (Paessler *et al*., 2006).

Alphaviruses are usually maintained in a transmission cycle that involves an arthropod vector and susceptible vertebrate hosts (Kay & Aaskov, 1989, Mackenzie *et al*., 1994). The association between the virus, vector, host and environment contributes to the geographic distribution of these viruses (Tong *et al*., 2001). Alphaviruses may persist in mosquito eggs for extended periods of time and are

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therefore able to cause sudden outbreaks of disease when suitable conditions arise for the hatching of the eggs (Tong *et al*., 2001). Several studies have suggested that alphaviruses are transported over immense distances by migratory birds and that many of these birds contain antibodies to alphaviruses such as SINV (Sammels *et al*., 1999; Buckley *et al*., 2003; Kurkela *et al*., 2008).

1.3.1 Epidemiology of Sindbis virus

Sindbis virus is considered the most extensively distributed arbovirus, occurring widely in the Old World, where it is reported in humans, birds and mosquitoes in South Africa (Jupp *et al*., 1986), Germany (Jost *et al*., 2010), Egypt, Cameroon, Uganda, China, Austria, Bulgaria, Norway, Finland, Greece, Italy, India, Israel, Hungary, Poland, Portugal, Russia, Saudi Arabia, New Zealand, Australia, Spain and Sweden (Gratz, 2004, Lundström & Pfeffer, 2010). Little epidemiological data is available for SINV and other arboviruses due to inadequate surveillance efforts in most endemic countries (Gubler, 2002). In Finland, significant outbreaks of SINV infections in humans (termed Pogosta disease) have occurred every seventh year since 1974 (Kurkela *et al*., 2004; Kurkela *et al*., 2008). During an outbreak of SINV infections in Finland in 1982, a strain was isolated that was closely related to a 1963 SINV strain isolated in South Africa (Shirako *et al*., 1991). The majority of SINV infections in Finland occur during August and September, when the mosquito vectors for this disease are abundant (Sane *et al*., 2010). The last significant outbreak of Pogosta disease in Finland occurred in 2006, when 600 cases were reported (Sane *et al*., 2010). A large SINV epidemic was expected in 2009 as part of the seven year cycle but only 105 cases of Pogosta disease were diagnosed in that year (Sane *et al*., 2010).

Ockelbo disease, clinically identical to Pogosta disease, first occurred in Sweden in 1967 (Skogh & Espmark, 1982). A phylogenetic study done by Kurkela and colleagues (2004) showed that northern European (Finland, Sweden and Russia) isolates of SINV were very closely related, with only a 0.1% to 1.4% difference in the nucleotides of the *nsP3* and *nsP4* genes of these isolates. Furthermore, in a study by Jost and colleagues conducted in 2010, Swedish isolates of SINV were found to be 99.6% identical to SINV isolates found in Germany (Jost *et al*., 2010). South African

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SINV isolates have also been found to be very similar to Swedish SINV isolates and studies suggest that these isolates were transported between South Africa and Sweden by migratory birds (Norder *et al*., 1996; Lundström *et al*., 2001).

A Sindbis-like virus (strain YN87448) was discovered during 1986 in China (Zhou *et al*., 1999). This strain only had a one percent sequence difference from the South African AR86 strain (Wang *et al*., 2008). There is currently no evidence that SINV causes disease in humans in England, although it has been shown that this virus had been introduced into resident United Kingdom birds, probably by migratory birds from Africa (Buckley *et al*., 2003). Sindbis virus has never been found in the Americas (Kurkela *et al*., 2008), however, a very closely related New World alphavirus, Aura virus, is common in South America (Powers *et al*., 2001).

In South Africa, SINV infrequently causes infections in humans during the summer (Jupp *et al*., 1986). Antibody surveys in humans and animals have shown that this virus is widely distributed in the Gauteng, Free State and Northern Cape Provinces of South Africa (McIntosh *et al*., 1976; Jupp *et al*., 1986). Even though mosquitoes are more frequently found in warm and humid regions, studies conducted between 1968 and 1971 in an arid region of the Free State showed that avian infections by *Cx*. *univittatus* frequently occur there during the summer (McIntosh *et al*., 1976). The largest epidemic of SINV infections ever recorded in South Africa occurred in the Karoo and Northern Cape in 1974, where thousands of human cases were reported (McIntosh *et al*., 1976; Jupp *et al*., 1986). It was suggested by Jupp and colleagues (1986) that this epidemic occurred due to unexpectedly high temperatures and rainfall in a normally arid region during the mosquito breeding season. Between December 1983 and April 1984, another outbreak of SINV infections occurred in the Witwatersrand/Pretoria region (Jupp *et al*., 1986), involving hundreds of human cases. In this period, the SINV infection rate for *Cx. univittatus* mosquitoes in the Witwatersrand area was 5.4 per 1 000 mosquitoes, much higher than the infection rate of 0.3 per 1 000 mosquitoes in previous years (1966, 1970) (Jupp *et al*., 1986).

Sindbis virus circulates in the same environmental niche as West Nile virus (WNV) in Africa (Olsen & Trent, 1985; Buckley *et al*., 2003) and both WNV and SINV are endemic to the Highveld and Karoo regions of South Africa (Jupp, 2005). A study by

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Jupp and colleagues (1986) performed in the Karoo and the KwaZulu-Natal Province of South Africa showed that WNV and SINV are transmitted to birds by identical vectors, particularly *Cx. univittatus.* In 2010 an outbreak of Rift Valley fever virus (RVFV), after a quiescence of nearly 30 years, was accompanied by co-circulation of WNV and SINV. More than 100 cases of each of the latter were laboratory confirmed (J Weyer, NICD-NHLS, personal communication).

1.4 Morphology and characteristics of alphaviruses

Alphaviruses are globular, enveloped and have a diameter of approximately 70 nm (Cheng *et al*., 1995). Two hundred and forty copies of capsid protein and a single copy of viral RNA is contained within an icosahedral nucleocapsid with a triangulation number of four (Paredes *et al*., 1992).

The RNA bears the structural characteristics of messenger RNA in that it has a 5' methylated cap and a 3' poly-A tail (Strauss & Strauss, 1986). The lipid-protein envelope is derived from the host plasma membrane (Snyder *et al*., 2011) and has 240 copies of each of two surface glycoproteins (E1 and E2) with the function of attachment and penetration (Bell *et al*., 1984; Snyder *et al*., 2011). The E1 and E2 glycoproteins are situated in close proximity to each other and are arranged on the virion surface as 80 spikes (von Bonsdorff & Harrison, 1978). A third envelope protein (E3) may sometimes be present (Powers *et al*., 2001) but is usually shed from infected cells and does not form part of mature virions (Steele *et al*., 2007).

1.5 Genomic structure and genetic diversity of alphaviruses

The alphavirus genome is between 11 000 and 12 000 nucleotides in length and consists of a positive sense single stranded 42S RNA which codes for non-structural proteins (nsP1-4) and structural proteins (capsid (C), E1-3 and 6K) (Sanchez-Seco, 2001) (Figure 1.2). The arthropod-borne alphaviruses share at least 40% homologous amino acids in the highly variable structural proteins and 60% in the more conserved non-structural proteins (Kuhn *et al*., 1996). The *nsP1* gene codes for an RNA-capping enzyme (Ahola & Kaariainen, 1995) and commences negative strand RNA

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synthesis (Sawicki & Sawicki, 1994). The *nsP2* gene codes for a protease, NTPase, triphosphatase and helicase and *nsP4* for an RNA dependant RNA polymerase (Kamer & Argos, 1984; Ho *et al*., 2010). The function of the *nsP3* gene remains unidentified (Ho *et al*., 2010) but is essential for RNA replication (Wang *et al*., 1994).

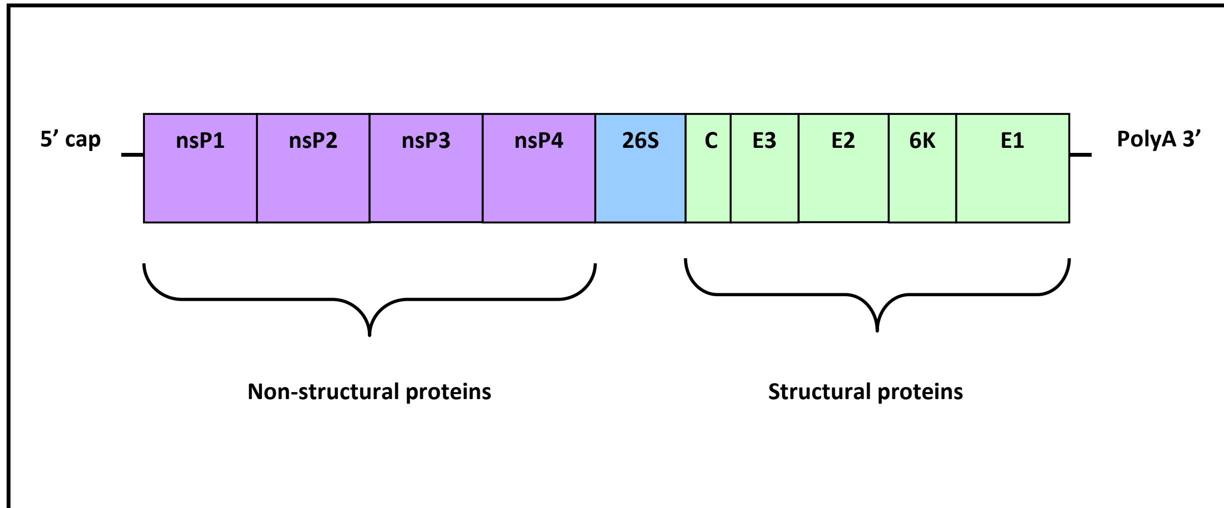

Figure 1.2: The alphavirus genome and gene products. The *nsP1* gene codes for (-) strand RNA synthesis and capping of RNA, *nsP2* for a helicase and proteinase, *nsP3* for RNA synthesis, *nsP4* for a RNA polymerase, *C* for the capsid, and *E3*, *E2*, *6K* and *E1* for the envelope glycoproteins.

The capsid protein encloses the viral RNA and envelope proteins (Ho *et al*., 2010) and provokes the production of antibodies, which are cross-reactive with other alphaviruses by complement-fixation and fluorescent antibody assays (Greiser-Wilke *et al*., 1989; Lloyd, 2009). The function of the 6K protein is not clearly understood but it has been proposed to be involved with the transport and integration of the envelope glycoproteins into progeny virions (Kuhn *et al*., 1996) and with the formation of ion channels (Melton *et al*., 2002).

Cross-reactivity between haemagglutination-inhibiting antibodies of alphaviruses is attributed to the E1 glycoprotein (Schmaljohn & McClain, 1996). The E1 and E2 envelope glycoproteins are the targets of numerous serological tests and previous

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phylogenetic studies of alphaviruses have relied on the partial or complete sequencing of these genes (Powers *et al*., 2001).

Phylogenetic trees based on sequences of the *E1* and *E2* genes of alphaviruses show that SINV groups with the WEEV virus complex and that EEEV and VEEV diverge independently of this complex and of other Old World viruses (Powers *et al*., 2001; reviewed in Gould *et al*., 2010). The relatedness of members of the genus *Alphavirus* was determined by Powers and colleagues (2001) by performing pair wise comparisons using the nucleotide sequences of the *E1* gene. This study showed a nucleotide sequence difference of only three percent between different SINV subtypes for this particular gene (Powers *et al*., 2001). The evolution of SINV in Australia was studied by Sammels and colleagues (1999) using the nucleotide sequences of the *E2* and *C* genes. Analysis of these sequences showed a clear separation of SINV into Palaearctic/Ethiopian and Oriental/Australian types (Figure 1.3) (Sammels *et al*., 1999). In the same study it was concluded that this separation is consistent with the migratory patterns of birds, which are the SINV's major vertebrate host (Sammels *et al*., 1999).

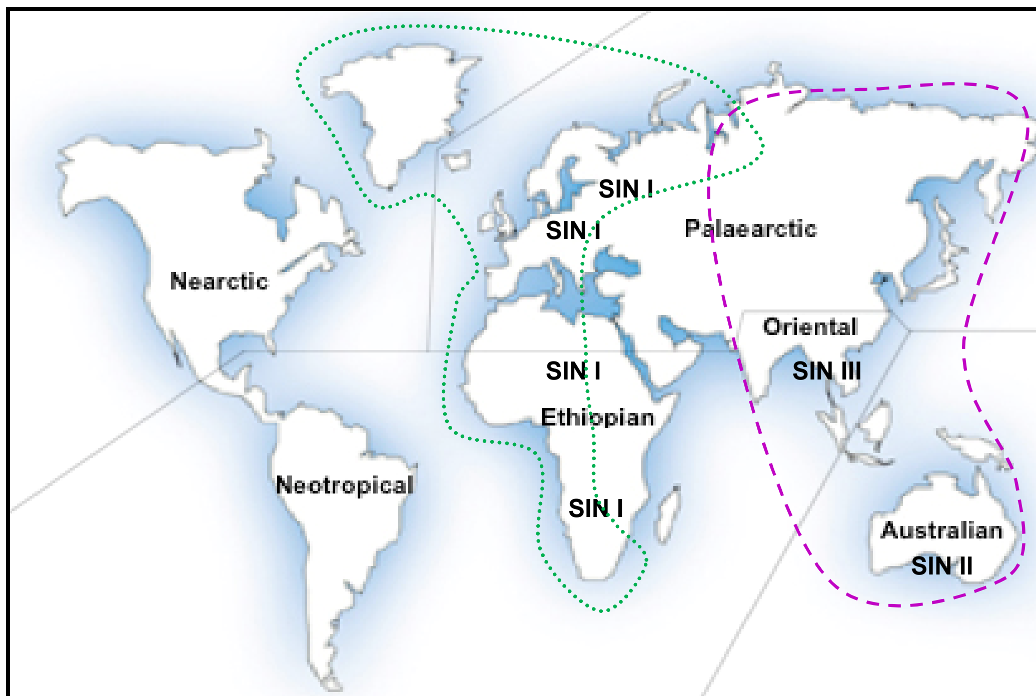

Figure 1.3: Map showing the zoogeographic regions and the Sindbis virus genotypes (numbered according to Lundström & Pfeffer, 2010)

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with corresponding bird flyways occurring in each region (- - - East Asian/Australasian flyway; .... East Atlantic flyway).

It was also suggested by Kurkela and colleagues (2008) that migratory birds may play a role in the spread of SINV, due to the fact that these viruses are widely distributed but still share identical nucleotide sequences. Analyses of SINV strains from Africa and northern Europe suggest that South African SINV strains are continuously imported by birds to northern Europe or vice versa (Norder *et al*., 1996).

1.6 Replication of alphaviruses

Alphaviruses attach to the vector and host cells via the E2 glycoprotein (Kielian, 2010). Entry occurs in slightly acidic endosomes, where the envelope glycoproteins undergo conformational changes and fusion, facilitated by the E1 glycoprotein, transfers viral RNA (42S) to the recipient cell cytoplasm, where replication takes place (Kielian, 2010). An RNA polymerase is produced during initial translation of the viral RNA (Schmaljohn & McClain, 1996). A negative-strand of intermediary viral RNA is then transcribed to produce a 26S positive-strand of mRNA, which is translated to form the structural proteins (C, E1, E2, E3) of the alphavirus (Mukhopadhyay *et al*., 2006). The envelope proteins are translocated via the Golgi apparatus to the plasma membrane and progeny viruses are formed when the pre-formed nucleocapsids bud through areas of the plasma membrane where E1 and E2 glycoproteins occur (Schmaljohn & McClain, 1996; reviewed in Gould *et al*., 2010). The SINV, SFV and RRV have been shown to replicate in the connective tissue, skin and muscle of suckling mice (Heise *et al*., 2000).

1.7 Transmission of alphaviruses

The vertebrate host range of alphaviruses is extensive and these viruses have been isolated from various species of birds, mammals, fish, reptiles and amphibians (Kozuck *et al*., 1978; Chamberlain, 1980; Bell *et al*., 1984). Alphaviruses are transmitted to animals and humans by arthropod vectors such as mosquitoes (Figure 1.4) (Blackburn *et al*., 1982; Kurkela *et al*., 2008). After biting a viraemic host, mosquitoes attain life-long asymptomatic alphavirus infections, characterised by the

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presence of infectious virus in the salivary glands (Frolova *et al*., 2010). The viruses replicate in the vector and are transmitted via the saliva to another vertebrate host (Schmaljohn & McClain, 1996; Girard *et al*., 2007). Humans are not normally involved in the natural zoonotic cycles that preserve alphaviruses and are regarded as incidental and "dead-end" hosts since viraemia rarely reaches high levels in humans and therefore cannot infect mosquitoes (McIntosh *et al*., 1976; McIntosh, 1986). However, it is possible for CHIKV (Schmaljohn & McClain, 1996) and ONNV (Smith *et al*., 2009) to be transmitted in a human-mosquito-human cycle.

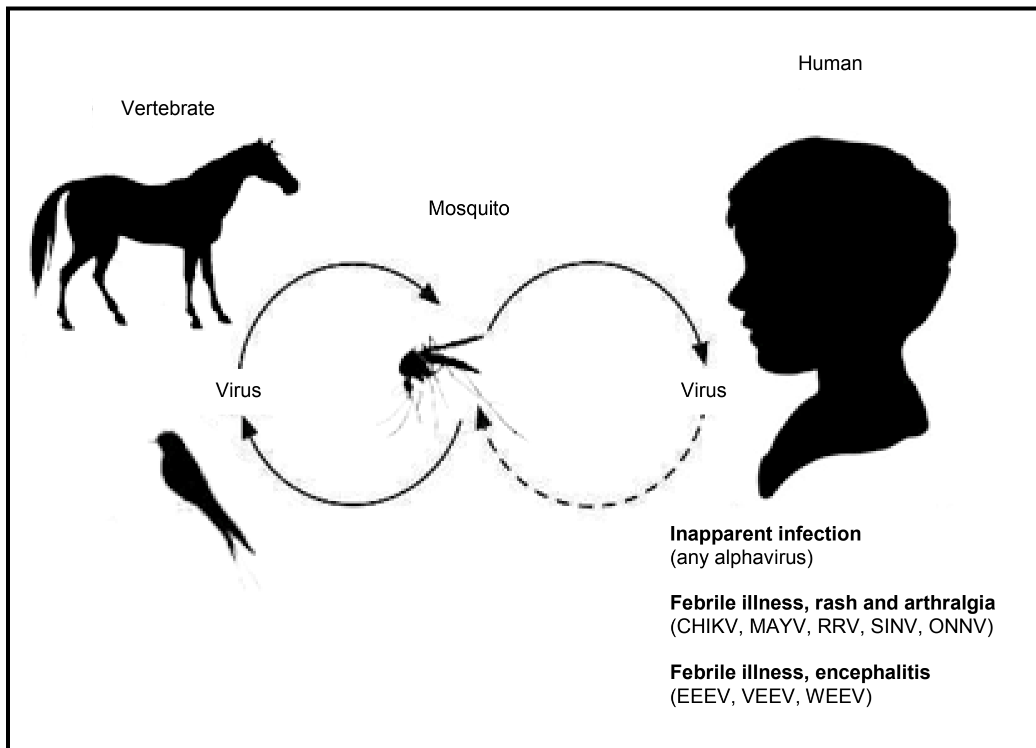

Figure 1.4: Transmission of alphaviruses. Abbreviations: CHIKV – Chikungunya virus; EEEV - eastern equine encephalitis virus; MAYV – Mayaro virus; ONNV - O'nyong-nyong virus; RRV - Ross River virus; SINV – Sindbis virus; VEEV - Venezuelan equine encephalitis virus, WEEV – western equine encephalitis virus (adapted from Schmaljohn & McClain, 1996). See Appendix A for permission to use this material.

Baboons and vervet monkeys are the major vertebrate hosts for CHIKV (McIntosh, 1986). The primary vectors for CHIKV are aedine mosquito species, including *Ae.*

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furcifer and *Ae. cordellieri* (Jupp, 2005). *Aedes* spp. are found in the bushveld, tall woodlands and evergreen forests and viral transmission by these mosquitoes occurs at night (McIntosh, 1986). In South Africa the vectors for CHIKV are restricted to Mpumalanga and the northern regions of KwaZulu-Natal (McIntosh, 1986).

Various wild birds are hosts for SINV and the virus is transmitted among these birds by the ornithophilic species of culicine mosquitoes (Figure 1.5), including *Cx. univittatus* and *Cx. neavei* (McIntosh, 1986; Buckley *et al*., 2003). In South Africa, *Cx. univittatus* is found in the mild inlands while *Cx. neavei* is found in KwaZulu-Natal (McIntosh, 1986). *Culex univittatus* has a low feeding rate on humans and this tends to restrict SINV infections in man (McIntosh, 1986). Transmission of SINV in South Africa occurs between December and April and rarely, infections may be acquired during travel to other endemic countries such as Europe during the European summer (Feigin, 2004).

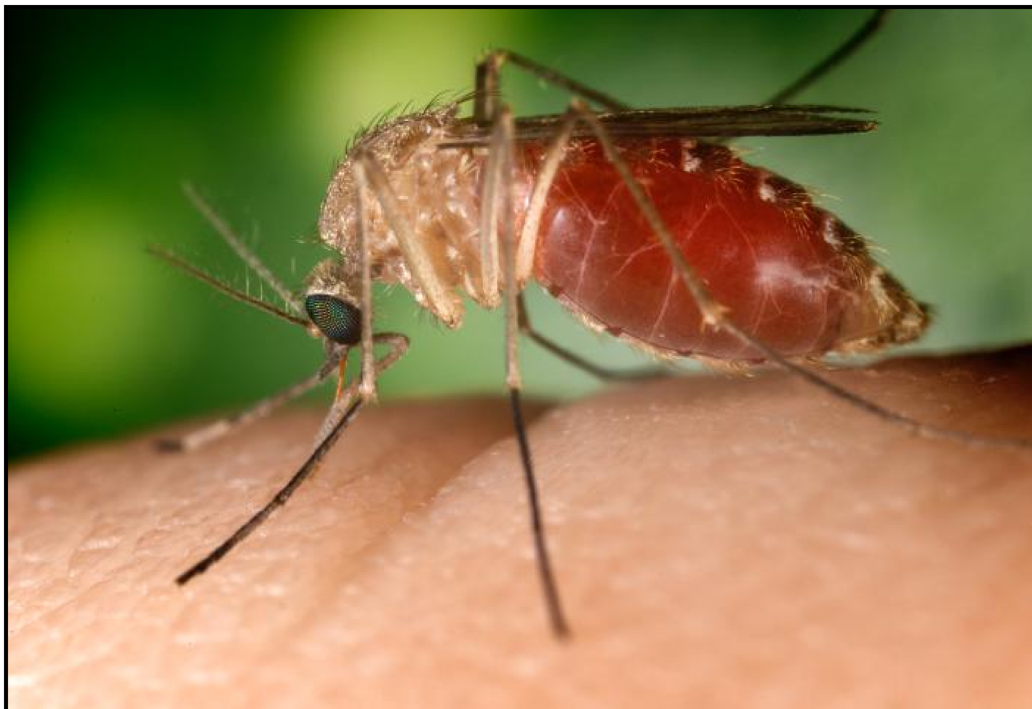

Figure 1.5: The *Culex* mosquito - a vector for both Sindbis and West Nile virus (Gathany, 2003). See Appendix A for documentation that this material is in the public domain.

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In the laboratory, the encephalitic alphaviruses are highly infectious by aerosol transmission and has therefore been proposed to be potential bio-warfare agents (Steele *et al*., 2007). Venezuelan equine encephalitis virus has been responsible for the most laboratory-acquired arbovirus infections in America since it was first isolated (Richmond and McKinney, 1993).

1.8 Pathogenesis and virulence of alphaviruses

The mechanisms involved in alphavirus-induced arthritis and arthralgia are poorly understood (Assunção-Miranda *et al*., 2010), however, viral replication within the affected joints has been proposed to contribute to these conditions (Heise *et al*., 2000). Infection with SINV, RRV and SFV leads to a rapid, fatal disease in neonatal animals (Heise *et al*., 2000). Upon infection, alphaviruses shut down host transcriptional and translational procedures while continuing to produce viral proteins and nucleic acid (Garmashova *et al*., 2007). This shut down leads to a decreased production of interferon-alpha/beta in the host cell, which hampers the ability of the innate immune system to eliminate the infection (Gorchakov *et al*., 2005; reviewed in Jose *et al*., 2009).

Sindbis virus and other alphaviruses cause cytopathic effects (cell rounding, deterioration, lysis and demolition of cell monolayers) in chick-embryo and other tissue cultures (Malherbe *et al*., 1963; Kurkela *et al*., 2005) and leads to cell death by apoptosis within 48 hours post-infection (Frolova *et al*., 2010). The virus is fatal to suckling mice within 3 days of inoculation, probably due to neurolysis, diffuse myositis, encephalitis and skeletal muscle degeneration (Weinbren *et al*., 1956; Malherbe *et al*., 1963). The effects of the SINV AR86 strain (isolated in 1956 in Springs, South Africa by Weinbren and colleagues) in suckling mice include connective tissue necrosis in the skin, joints, bone marrow and skeletal muscle, smooth muscle necrosis and destruction of thymocytes in the thymus (Malherbe *et al*., 1963). It was suggested by Malherbe and colleagues (1963) that some of the symptoms seen in humans may be accounted for by the lesions seen in suckling mice. A study by Assunção-Miranda and colleagues (2010) has shown that macrophages play a role in the development of arthritis associated with alphavirus infections in humans. Macrophages have been shown to produce cytokines and matrix

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metalloproteinases that are implicated in joint damage and inflammation (Assunção-Miranda *et al*., 2010).

The pathogenesis of alphaviruses infections is illustrated in Figure 1.6. In humans and other vertebrate hosts, alphaviruses enter the circulatory system via a mosquito bite and viraemia, dispersal and illness occur as the virus is released from lysed cells at the inoculation site (Schmaljohn & McClain, 1996).

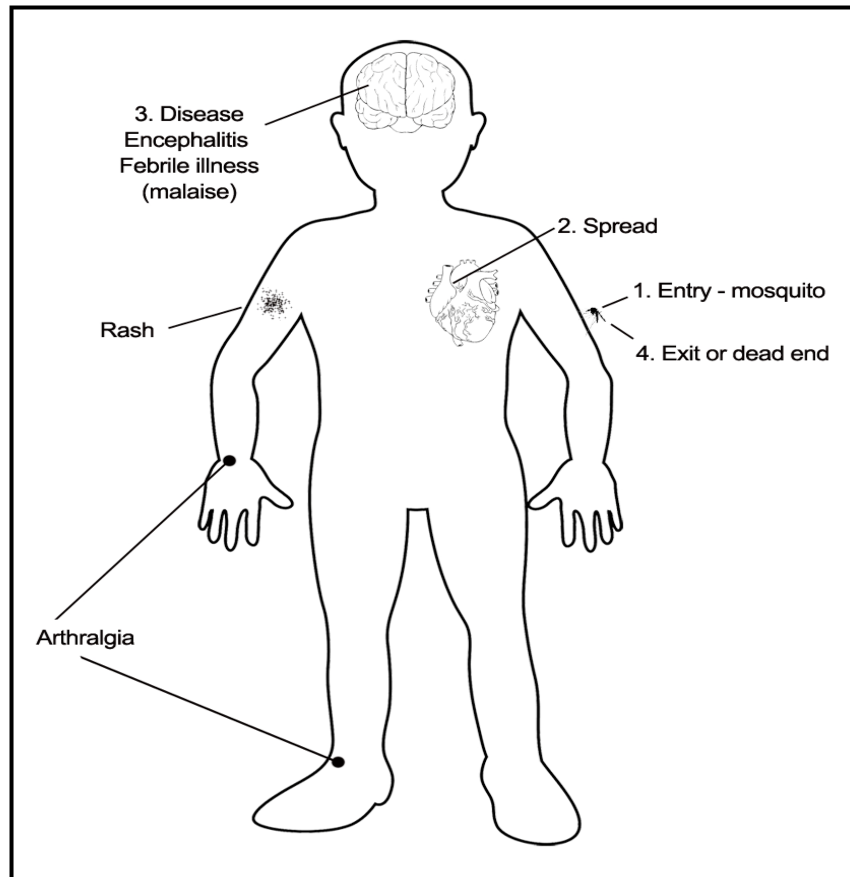

Figure 1.6: Pathogenesis of alphavirus infections (adapted from Schmaljohn & McClain, 1996 - See Appendix A for permission to use this material. Alphaviruses enter the host via a mosquito bite and are spread in the blood. Old World alphaviruses (e.g. SINV, CHIKV) cause a mild, febrile illness with a rash and arthralgia while New World alphaviruses (e.g. EEEV, VEEV, WEEV) cause encephalitis. Rarely, the virus may exit a human host via mosquitoes but usually encounters a dead end.

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Viraemia typically subsides within 5 days and antibodies are usually produced within 4 to 8 days of the onset of symptoms, indicating viral clearance (Johnson *et al*., 1972; Schmaljohn & McClain, 1996; Griffin, 2010). Alphaviruses are eliminated by the immune system but arthralgia may last for weeks (Laine *et al*., 2000).

The pathogenesis of the equine encephalitis viruses in humans and horses is similar to that of other alphaviruses, although the majority of human infections with these viruses are either asymptomatic or cause non-specific febrile illness with or without encephalitis (Brink & Lloyd, 2000). In severe human cases, hepatocellular degeneration, oedema, lymphoid tissue damage and interstitial pneumonia are frequently found upon histological examination (Steele *et al*., 2007). In the brain, polymorphonuclear and mononuclear cells gather in the meningeal areas (Karabatsos & Booss, 2003). The neurons are the main viral targets and neuronal death due to apoptosis and/or necrosis is a major effect of infection with the encephalitic alphaviruses (de la Monte *et al*., 1985).

1.9 Diseases caused by alphaviruses

The majority of alphavirus infections are asymptomatic, but clinically ill patients may present with various manifestations (signs and symptoms) (Laine *et al*., 2003). New World viruses including EEEV, WEEV and VEEV cause fever, malaise, headache, neck stiffness and encephalitis (Griffin, 2001; reviewed in Zacks & Paessler, 2010). Venezuelan equine encephalitis virus has also been associated with abortion and stillbirth in humans (Weaver *et al*., 1996). Neurologic disease (including delirium, seizures, paralysis and coma) occurs in approximately 1 in 1 000 adults, 1 in 58 children and in all infants infected with WEEV (Reeves *et al*., 1962; Steele *et al*., 2007). In cases of encephalitis, alphavirus infections may be fatal within 1 week of infection (Whitley, 1990) and up to 70% of survivors exhibit long-term neurological lesions such as seizures, mental retardation, epilepsy, deafness and spasms (Kurkela *et al*., 2005; Steele *et al*., 2007).

Typical symptoms of Old World alphavirus infections include joint pain, muscle pain, fever, headache, nausea, photophobia, rashes and backache (Brink & Lloyd, 2000;

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reviewed in Jupp, 2005) while bleeding of the gums, petechiae (pin-point haemorrhages), melena (bloody stools) and haematemesis (vomiting of blood) are common in CHIKV infections (Sarkar *et al*., 1965). Glomerulonephritis and lymphadenopathy has been found to occur in cases of RRV (Fraser *et al*., 1988) and ONNV infections and enlarged posterior cervical lymph nodes distinguish ONNV infections from CHIKV infections (Shore, 1961). Infections with the Old World alphaviruses rarely result in encephalitis (Lewthwaite *et al*., 2009).

The joint pain associated with many alphavirus infections may be debilitating and manifests itself as either arthralgia or migrating polyarthritis (Torres *et al*., 2004; reviewed in Toivanen, 2008). In a study by Simon and colleagues (2007), it was shown that all cases of CHIKV infections (occurring in the Indian Ocean Islands) presented with arthritis, although it has been found that joint symptoms do not occur in paediatric cases (Turunen *et al*., 1998; Laine *et al*., 2003). Alphaviruses infect males and females of all ages and the incubation time may range between 2 and 10 days (Laine *et al*., 2003; Simon *et al*., 2007).

1.9.1 Clinical presentation of Sindbis virus infection

A wide range of vertebrate species can be infected with SINV (Spadbrow, 1972; Kozuck *et al*., 1978; McIntosh & Jupp, 1979), and there is evidence of SINV-related disease in humans as well as in horses and rhinoceros (Human *et al*., 2010). In humans, sub-clinical infections with SINV have been found to be more common than clinical ones and the morbidity of SINV disease has been shown to be highest in middle-aged women (45-65 years of age) and lowest in children (Brummer-Korvenkontio *et al*., 2002). The incubation period for SINV has been estimated to be approximately 8 days (Kurkela *et al*., 2005). Infection with SINV usually causes symptoms such as a maculopapular rash (Figure 1.7), mild fever, fatigue, muscle pain, headache and arthralgia (Kurkela *et al*., 2005; Kurkela *et al*., 2007; Kurkela *et al*., 2008).

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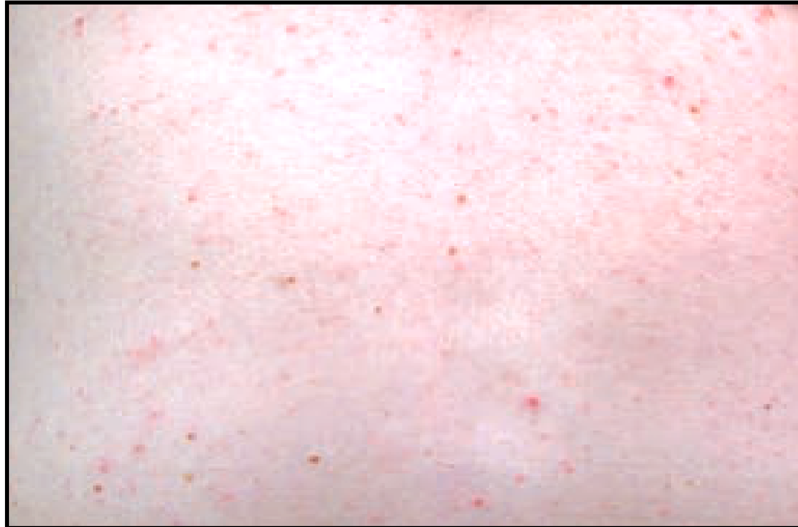

Figure 1.7: Maculopapular rash on the back of a patient infected with Sindbis virus (adapted from Ferguson *et al.*, 2005). See Appendix A for documentation that this material is in the public domain.

The maculopapular rash seen due to infection with SINV usually occurs on the chest, back, arms, legs, palms and soles, may be slightly haemorrhagic and is usually pruritic (McIntosh, 1986; Kurkela *et al.*, 2005). The papules are approximately three to four millimetres in diameter and sometimes circled with a pallid halo (McIntosh, 1986). The rash may disappear and reappear again later as brown stains (Malherbe *et al.*, 1963). The acute stage of SINV infection usually lasts between 3 and 10 days and some infections with SINV and Sindbis-like viruses may last months to years (Turunen *et al.*, 1998; Kurkela *et al.*, 2007). Laine and colleagues (2000) showed that in 26 patients, 50% still experienced musculoskeletal problems almost 3 years after the onset of symptoms.

The arthritis and arthralgia associated with SINV may be quite severe and frequently affects more than one joint, occasionally including major joints such as the shoulder, elbow, knee and hip (Heise *et al.*, 2000). Pain within the tendons associated with joints has also been observed in patients with a SINV infection (Johnston & Peters, 1996). A study conducted by Laine and colleagues (2003) in Finland indicated that only 6.8% of persons infected with a Sindbis-like virus did not present with arthritis.

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Due to the non-specific symptoms of SINV infections, and because most patients only seek medical attention if a rash appears, many cases of the disease may go undetected. Considering that SINV infection may cause long-lasting joint symptoms, the disease could be an impending public health concern (Kurkela *et al*., 2008).

1.9.2 Treatment of alphavirus infection

Human vaccines are available for some alphaviruses but are restricted to individuals that are at a high risk of infection, e.g. laboratory personnel and veterinarians (Schmaljohn & McClain, 1996; Plante *et al*., 2011). During the 1960s and 1970s, the United States of America developed four vaccines against EEEV, VEEV and WEEV as part of a defensive programme against biological warfare (Steele *et al*., 2007). Amongst these vaccines were a formalin-inactivated virus vaccine (C84; 0.5 ml administered subcutaneously) and a live attenuated vaccine (TC83; 0.5 ml administered subcutaneously) which protects against VEEV infection in humans (Steele *et al*., 2007). The live attenuated VEEV vaccine has also been used in horses as an epidemic control measure (reviewed in Zacks & Paessler, 2010). Another attenuated vaccine (V3526) derived from a complemetary deoxyribonucleic acid (cDNA) clone of VEEV has also been developed (Hart *et al*., 2000). Formalin-inactivated vaccines are available for the protection of horses against infection with WEEV and EEEV (Weaver & Paessler, 2009). Second generation vaccines against the encephalitic alphaviruses are under investigation (reviewed in Spurgers & Glass, 2011).

Although chloroquine has been used in the treament of chronic CHIKV infections (Brighton, 1984), a randomised, double-blind, placebo-controlled clinical trial, conducted in the Indian Ocean Islands, has shown no significant difference between placebo and chloroquine groups (http://clinicaltrials.gov/ct/show/NCT00391313; de Lamballerie *et al*., 2008b). Ribavirin in combination with interferon-alpha has been suggested as a treatment for SFV and CHIKV infections (Briolant *et al*., 2004), but this treatment is expensive and not suitable for large-scale use e.g. during epidemics. A live attenuated CHIKV vaccine has been proven useful and safe in investigational human trials (Weaver & Paessler, 2009).

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In general, the symptoms of alphavirus infections are short in duration and patients usually recover completely within a week. There are therefore no specific treatments available or necessary for most alphavirus infections and the treatment for infection is usually supportive and symptomatic. Antihistamines may be prescribed for the pruritic rash and analgesics may be prescribed for the joint pain associated with many alphavirus infections (European Centre for Disease Prevention and Control, 2009). Non-steroidal anti-inflammatory drugs and non-salicylate analgesics have been used for the treatment of painful CHIKV infections (Pialoux *et al*., 2007), although it has been suggested that these drugs may lead to a haemorrhagic form of the disease (HealthMap, 2011).

1.10 Diagnostic identification of alphaviruses

The rapid and early diagnosis of alphavirus infection is important to direct appropriate patient management and to manage outbreaks (Bronzoni *et al*., 2004). The high genetic variability of alphaviruses makes the diagnosis of alphavirus infection technically challenging (Grywna *et al*., 2010). Specific diagnostic tests are essential in providing a definitive diagnosis of alphavirus infection, and no significant pathological indicators are identified. Current diagnostic methods available for alphaviruses include virus isolation in murine neonates and serological tests such as complement fixation and haemagglutination inhibition (HAI) assays (Sanchez-Seco *et al*., 2001). An indirect peroxidase assay may also be employed for the detection of viral antigen in the brains of dead horses that suffered from WEEV infection (reviewed in Zack & Paessler, 2010). These methods are less specific (less able to correctly identify the proportion of healthy individuals that do not have an alphavirus infection) than the molecular methods that have been developed and interpretation of the results of these methods may pose difficulties due to pan-alphavirus cross-reaction (Sanchez-Seco *et al*., 2001). Furthermore, virus isolation usually necessitates biosafety level-3 containment (Kang *et al*., 2010).

In South Africa, medically important alphavirus infections are confirmed by the Centre for Emerging and Zoonotic Diseases (formerly the Special Pathogens Unit) of the National Institute for Communicable Diseases of the National Health Laboratory Service (NICD-NHLS) (National Institute for Communicable Diseases Annual

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Reports, 2005-2008). The NICD-NHLS currently uses various serological tests, including an enzyme-linked immunosorbent assay (ELISA) and an immunofluorescent antibody assay (IFA) for the detection of Immunoglobulin M (IgM) antibody, HAI for the detection of total antibody, *in vitro* and *in vivo* virus isolation techniques, and a conventional reverse transcription polymerase chain reaction (RT-PCR) assay for diagnosis of infections caused by alphaviruses. Despite having high sensitivities and specificities, these conventional assays are time consuming and some run a high risk of contamination. Although serological assays remain the most important diagnostic methods for alphavirus infection (due to the short viraemic period and rapid seroconversion associated with these infections), a rapid and sensitive nucleic acid detection tool would be more practical and ethically sound (as opposed to using murine neonates for isolation) for the identification of alphaviruses in surveillance efforts.

1.10.1 Non-molecular techniques for the diagnosis of alphavirus infection

The diagnosis of alphavirus infections is complex and is most often based on patient history (arthropod exposure e.g. contact with mosquitoes; travel history) and clinical manifestations. Blood, fluid from skin lesions, synovial joint fluid and cerebrospinal fluid may be collected for alphavirus isolation (McIntosh, 1986; Brink & Lloyd, 2000). A paired blood sample may be collected approximately 20 days after infection to demonstrate Immunoglobulin G (IgG) seroconversion and to measure a four-fold rise in antibody titer for confirmatory diagnostic purposes (McIntosh, 1986). The virus antigen may also be demonstrated in autopsy tissue using immunocytochemistry and in serum by means of ELISA (Brink & Lloyd, 2000).

A number of methods available for CHIKV diagnosis was evaluated by Yap and colleagues (2010), including two ELISA assays, a lateral flow rapid test and an IFA assay for the detection of anti-CHIKV IgM. This study found that the ELISAs had a specificity of 95.6%, while the specificities of the lateral flow and IFA assays were 100% and 98.3%, respectively (Yap *et al*., 2010). Even though the gold standard for the diagnosis of alphaviruses is considered to be virus isolation by cell culture (based on the observation of a cytopathic effect) and subsequent quantification of the virus

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by a viral plaque assay (Sam & AbuBakar, 2006; Ho *et al*., 2010), serology is more often used due to the short viraemic period associated with alphaviral infections. The virus isolation method has a further disadvantage in that it is laborious and takes 7 days to complete (Ho *et al*., 2010).

1.10.2 Molecular techniques and characterisation assays for alphavirus infection

No technique has had such a significant impact on the biological sciences as the invention of the polymerase chain reaction (PCR) assay. Initially discovered by Kary Mullis in 1983 (Saiki *et al*., 1985; Mullis, 1990), PCR has evolved to become more sophisticated, reliable and rapid with the introduction of real-time PCR. Real-time PCR has become the norm for detecting and quantifying DNA and RNA and is increasingly being used in diagnostic and research laboratories alike (Bustin & Mueller, 2005). Real-time PCR makes use of different types of fluorescent detection chemistries, which may be either probe-based (also referred to as specific detection chemistries) or non-probe based (also referred to as non-specific detection chemistries) (Bustin & Mueller, 2005). These detection chemistries, as well as their application in the detection of alphaviruses, will be discussed in section 1.10.2.2 and 1.10.2.3.

The routine use of real-time PCR has numerous benefits including a faster turnaround time, an increased sensitivity and specificity and improved patient management (including early diagnosis, prognosis and treatment) (Gunson *et al*., 2006). To date, no real-time PCR assay exists that is able to detect all of the alphaviruses.

1.10.2.1 Conventional reverse transcription PCR assays for the detection of alphaviruses

Several conventional RT-PCR assays have been developed for the detection of alphaviruses. Some species-specific assays include RT-PCRs for the detection of RRV (Sellner *et al*., 1992), EEEV (Vodkin *et al*., 1993), CHIKV (Parida, 2008a) and

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Ockelbo virus (Horling *et al*., 1993). The genus-specific assays are briefly described in Table 1.2.

Table 1.2: Genus-specific conventional RT-PCR assays for the detection of alphaviruses

PCR description	Gene region	Alphaviruses detected	Sensitivity	Reference
Genus-specific, semi-nested RT-PCR	*nsP1*	27 alphaviruses	1 200 plaque-forming units (pfu) for VEEV (Trinidad donkey)	Pfeffer *et al*., 1997
Genus-specific, nested RT-PCR	*nsP4*	EEEV, VEEV, WEEV, SINV, RRV, SFV, CHIKV	25 pfu per tube	Sanchez-Seco *et al*., 2001
Genus-specific RT-PCR followed by multiplex nested PCR	*nsP1*	14 Brazilian alphaviruses (including WEEV, VEEV, EEEV, MAYV and Aura virus)	$10^{0.5}$ $TCID_{50}$/ml	Bronzoni *et al*., 2004
Genus-specific, nested RT-PCR	*nsP4*	CHIKV, ONNV, MAYV, SFV, RRV, SINV, EEEV, VEEV, WEEV, Barmah Forest virus	5 – 100 transcripts per reaction	Grywna *et al*., 2010

As for all conventional nested PCR assays, these genus-specific assays are time-consuming and run a high risk of false-positive results due to possible contamination.

1.10.2.2 SYBR® Green-based real-time reverse transcription PCR assays for the detection of alphaviruses

Higuchi and colleagues (1992) first discovered real-time PCR when ethidium bromide was accidentally added to a PCR reaction prior to amplification. The ethidium

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bromide bound to double stranded DNA, which allowed the visualisation of accumulating PCR product at each amplification cycle using a charge-coupled device camera (Higuchi *et al*., 1993). Since then, a real-time PCR chemistry (known as SYBR® Green I) has been developed that employs this DNA-intercalating principle and is currently the most widely used non-probe based detection chemistry for real-time PCR (Ponchel *et al*., 2003). SYBR® Green I binds to and detects any double-stranded DNA product (Figure 1.8), including primer-dimers and undesired amplicons, and is therefore not very specific (Ponchel *et al*., 2003).

In order to overcome the problem of specificity, the use of SYBR® Green I requires post-PCR processing in the form of a melting curve analysis, in which the melting temperature (Tm) is determined in order to identify the correct product based on the GC content and length of the amplicon (Ririe *et al*., 1997; Espy *et al*., 2006). The melting temperature of target molecules is generally higher than that of non-specific amplicons and primer-dimers (Ririe *et al*., 1997; Espy *et al*., 2006).

Despite the lack of specificity, SYBR® Green I remains popular due to being inexpensive, sensitive and easy to use (Ponchel *et al*., 2003; Yap *et al*., 2010). The design of real-time PCR assays utilising SYBR® Green I is also considerably less difficult than with other detection chemistries, since these assays may make use of longer templates and eliminate the need for probe design (Valasek & Repa, 2005).

In 2010, Ho and colleagues developed a quantitative real-time RT-PCR assay for the detection of CHIKV using a SYBR® Green I DNA dye-binding fluorophore. This study found that the SYBR® Green I-based real-time PCR assay was more sensitive than conventional RT-PCR and IFA (Ho *et al*., 2010). A similar real-time RT-PCR assay developed by Ummul and colleagues (2010) showed that both the sensitivity and specificity of the SYBR® Green I-based real-time PCR assay was 100%.

A SYBR® Green I-based real-time RT-PCR assay was developed by Yang and colleagues (2010) to screen for alphaviruses and flaviviruses in mosquitoes in Taiwan. This method used group-specific primers targeting a consensus region in the *nsP1* gene of alphaviruses and could detect SINV, CHIKV and RRV (Yang *et al*., 2010).

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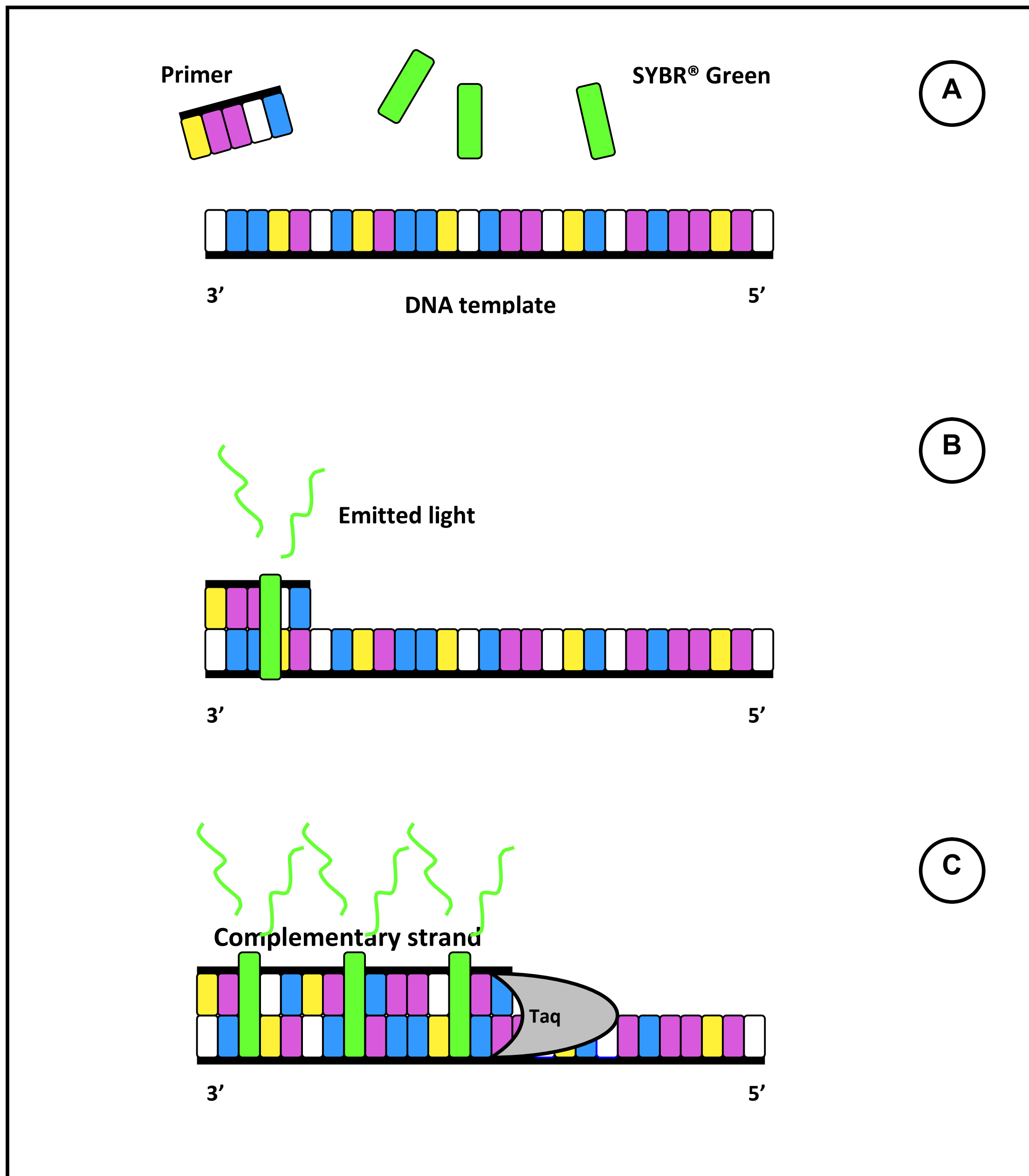

Figure 1.8: The SYBR® Green I real-time PCR detection chemistry: A. Unbound SYBR® Green I dye molecules fluoresce weakly at the beginning of amplification. B. After the primers anneal, some SYBR® Green I molecules bind to the double stranded DNA, which increases the fluorescence. C. During elongation, more SYBR® Green I molecules bind to the newly synthesised DNA strand and fluorescence continues to increase.

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1.10.2.3 Hydrolysis probe-based real-time reverse transcription PCR assays for the detection of alphaviruses

The most commonly used probe-based detection chemistry for real-time PCR is the hydrolysis probe (also known as the TaqMan® probe). Hydrolysis probes are typically short, sequence-specific oligonucleotides with a 5' fluorescent dye and a 3' quenching dye (Ambion, 2001). Hydrolysis probes are dependent on the 5' nuclease activity of *Thermus aquaticus* (*Taq*) DNA polymerase (MacKay, 2004) and rely on fluorescence resonance energy transfer (FRET) for quantification (Ambion, 2001). Fluorescence resonance energy transfer is a process in which energy is transferred between a fluorescent reporter dye and a quencher dye, which are typically separated by 20 to 30 nucleotides specific for the desired product (Epsy *et al*., 2006). When hydrolysis probes are irradiated, FRET occurs and no fluorescent signal is produced (Ambion, 2001) (Figure 1.9). During the annealing and elongation steps of PCR, as amplification of the product to which the hydrolysis probe is bound occurs, the 5' nuclease activity of *Taq* DNA polymerase cleaves the probe, separating the fluorescent dye and the quenching dye (Ambion, 2001; MacKay, 2004). This results in a fluorescent signal, which increases in each cycle as more and more probes are cleaved (Ambion, 2001). The advantage of using hydrolysis probes for real-time PCR lies in their specificity (Ambion, 2001).

Several fluorescent dyes and quenchers are currently available for use with hydrolysis probes, including HEX (carboxy-2',4,4',5',7,7'-hexachlorofluorescein), FAM (carboxyfluorescein) and TAMRA (carboxytetramethylrhodamine) (Ambion, 2001). The selection of a fluorescent dye usually depends on its ability to absorb and emit energy at different wavelengths, and the signal to noise ratio it produces (the association between a fluorescent signal produced by the hybridisation of a probe to a target sequence and a non-specific fluorescent signal) (MacKay *et al*., 2007). Black hole quenchers (BHQ) are non-fluorescent quenchers that produce relatively low noise levels and are increasingly being used in literature (MacKay *et al*., 2007). The most reliable fluorophore and quencher combination for use with hydrolysis probes has been determined to be FAM-BHQ1 (Reynisson *et al*., 2006; Wilson *et al*., 2011).

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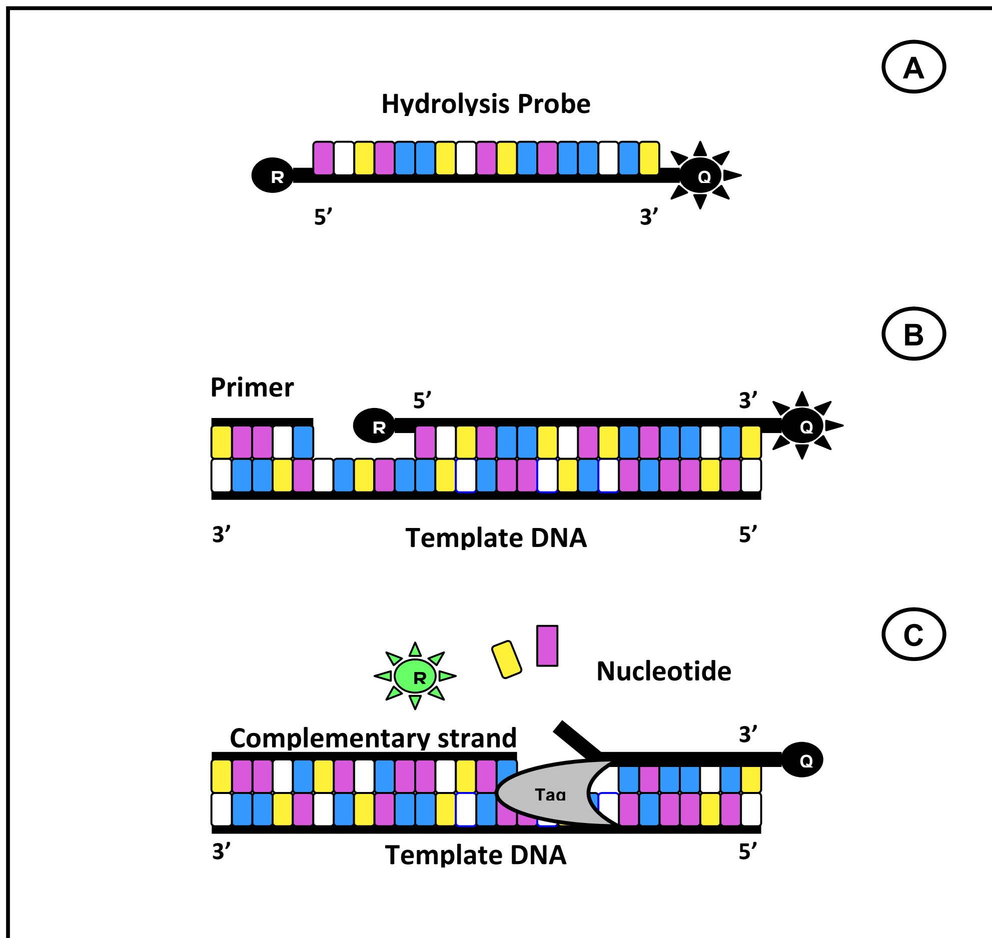

Figure 1.9: The hydrolysis (TaqMan®) probe real-time PCR chemistry: A. The hydrolysis probe contains a 5' fluorescent dye (R) and a 3' quenching dye (Q). When irradiated, the fluorescent dye is quenched and little fluorescence occurs. B. The hydrolysis probe binds to its specific target on the template DNA. C. As amplification occurs, the 5' nuclease activity of *Taq* DNA polymerase cleaves the probe. This separates the fluorescent and the quenching dye, leading to fluorescence.

A hydrolysis probe-based real-time PCR assay was developed by Pastorino and colleagues (2005) for CHIKV diagnosis. A duplex hydrolysis probe-based real-time PCR assay targeting both the *E3* and the *nsP3* genes was developed by Kang and colleagues to detect WEEV and EEEV simultaneously (Kang *et al*., 2010). This PCR assay was found to be ten times more sensitive than virus isolation for the detection of both WEEV and EEEV (Kang *et al*., 2010).

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A one-step hydrolysis probe-based real-time RT-PCR assay was recently developed by Sane and colleagues for the detection of SINV (Sane *et al*., 2012a). The clinical performance of this assay was evaluated using 58 acute-phase serum samples. Of the 58 samples, seven tested positive for SINV using the real-time RT-PCR assay compared to two using conventional RT-PCR and zero using virus isolation techniques (Sane *et al*., 2012). The assay was found to be sensitive and specific, having a 95% detection limit of nine genome copies per reaction (Sane *et al*., 2012a).

1.10.2.4 Other molecular assays for the detection of alphaviruses

A reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay was developed by Parida and colleagues (2007), targeting the *E1* gene of CHIKV. Loop-mediated isothermal amplification makes use of six specific primers which recognise eight different regions on a target gene (reviewed in Parida *et al*., 2008b). The LAMP process involves two steps: a non-cyclic step which involves the construction of DNA with stem loops at either end (with the help of external primers), and a cyclic step in which exponential amplification of the stem looped DNA occurs (with the help of internal primers) (reviewed in Parida *et al*., 2008b). The RT-LAMP assay accurately identified 21 borderline cases of CHIKV infection that were misdiagnosed by conventional RT-PCR (Parida *et al*., 2007).

1.11 Prevention, control and surveillance

The control of mosquito vectors is essential in preventing infections with SINV and other arboviruses (Jupp, 2005). Personal protection methods which may be applied in areas where mosquito vectors are common include mosquito nets, staying indoors between sunset and sunrise, the use of mosquito repellents, the use of fans and wearing long-sleeved clothing (Jupp, 2005). The elimination or reduction of mosquito breeding habitats such as tyres, buckets and water features holding stagnant water is also necessary. In America, the number of human WEEV cases has declined to less than ten per year since 1988 due to changes in irrigation methods and improved mosquito control programmes (reviewed in Zacks & Paessler, 2010). Horses are the major amplifying hosts for VEEV and therefore equine vaccination is the most

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valuable method of preventing epizootics and controlling outbreaks of this virus (Steele *et al*., 2007). Additionally, immunity in the equine population protects humans against infection with the epizootic strains of VEEV (Steele *et al*., 2007). A potential molecular strategy for interrupting alphavirus transmission by mosquitoes has been proposed (Blair *et al*., 2000).

International surveillance of alphaviruses is poor and knowledge is largely based on research performed many years ago. Increased global travel to endemic regions and a wide host range for alphaviruses has amplified the need for surveillance efforts for these viruses worldwide (Bronzoni *et al*., 2004). Intensified surveillance programmes are thus necessary to determine the prevalence and public health impact of alphavirus infections in South Africa and elsewhere in the world. Disease surveillance is also necessary to determine whether hosts, especially horses, should be vaccinated against alphavirus infections (Schmaljohn & McClain, 1996). The development of a generic real-time PCR assay that can detect the different alphavirus species would contribute significantly to diagnostic activities and would boost surveillance efforts for alphaviruses in South Africa and around the world.

1.12 Significance and aims of this study

The incidence of alphaviruses in South Africa is unknown due to inadequate surveillance efforts. Moreover, little epidemiological data exist on Sindbis virus. A comprehensive molecular characterisation of SINV isolates from South Africa will provide insights in the diversity of this virus compared to elsewhere in the world. Additionally, a descriptive epidemiological study of SINV will be useful in providing the basis for a more complete study in future.

The changing patterns in alphavirus disease has created the need for improved diagnostic methods and increased vigilance and disease surveillance around the globe. To date, no real-time PCR exists that is able to detect all of the alphaviruses. Therefore, the development of a generic real-time pan-alphavirus RT-PCR will contribute considerably to support current diagnostic activities and will be an important tool for future surveillance activities.

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The aims and objectives of this study are:

- To conduct a basic retrospective epidemiological study to describe the characteristics of SINV infection in humans in South Africa:
  - To collect data from questionnaires and specimen requisition forms of suspected arbovirus cases submitted to the Centre for Emerging and Zoonotic Diseases for laboratory verification between 2006 and 2010;
  - To perform a statistical analysis of the data

- To perform molecular characterisation and to investigate the genetic diversity of SINV isolates from mosquito pools and human cases from South Africa based on selected genomic targets:
  - To perform nucleic acid extraction and amplification of a partial *E2* gene target from the SINV isolate bank of the NICD-NHLS;
  - To perform molecular sequencing of the amplified targets and analyse the sequence data;
  - To construct and analyse phylogenetic trees using nucleotide and amino acid sequence information with regards to sequence data available in the public domain

- To develop and evaluate a real-time RT-PCR assay for the detection of medically important and other alphaviruses with possible public health significance:
  - To develop generic primers/probe sets to detect alphavirus RNA (including CHIKV, SINV, MIDV, SFV, MAYV, NDUV, RRV and ONNV);
  - To adapt the generic real-time RT-PCR to the Lightcycler® real-time PCR platform;
  - To produce synthetic RNA standards for positive controls and to optimise the generic real-time PCR using Taguchi parameters;
  - To determine the specificity (cross-reactivity with other related arboviruses and specific detection of each alphavirus) and sensitivity (limit of detection) of the generic RT-PCR;

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- To perform limited validation of the generic PCR using a panel of clinical specimens with known results.

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CHAPTER 2: DESCRIPTIVE EPIDEMIOLOGY OF HUMAN SINDBIS FEVER CASES IN SOUTH AFRICA, 2006-2010

2.1 Introduction

Sindbis virus is widely distributed across Eurasia, Africa and Oceania, but clinical infections with SINV occurring as epidemics have only been reported from a few geographic areas of Finland (Sane *et al*., 2011), Sweden (Espmark & Niklasson, 1984), Russia (Kurkela *et al*., 2004) and South Africa (McIntosh *et al*., 1976; Jupp *et al*., 1986). Little epidemiological data exist for SINV due to poor surveillance efforts in most countries (Gubler, 2002). Sindbis (SIN) fever is characterised by fever, maculopapular rash, arthritis, muscle pain and fatigue, and the joint symptoms may continue for months to years (Laine *et al*., 2004; Kurkela *et al*., 2005; Sane *et al*., 2011).

In Finland, SINV has caused epidemics in seven year cycles involving thousands of cases since 1974 (Kurkela *et al*., 2008). The majority of cases occur there between August and September, when late summer mosquito vectors (*Culex* and *Culiseta* species) are abundant (Sane *et al*., 2010; Sane *et al*., 2011). Studies conducted by Kurkela and colleagues in 2008 on data from the epidemic years of 1995 and 2002 in Finland, showed that the incidence of SINV infections was higher among women and among persons between 50 and 59 years of age. A study conducted in Sweden in 1982 also showed that most SINV infections occurred in August, with the highest incidence of infection being among women between 50 and 59 years of age, and men between 30 and 39 years of age (Espmark & Niklasson, 1984).

South African SINV isolates have been found to be similar to those found in Finland and Sweden, possibly due to the transport of these isolates between these countries by migratory birds (Shirako *et al*., 1991; Norder *et al*., 1996; Kurkela *et al*., 2008). In South Africa, SINV surveillance is largely passive and because the disease often presents in sub-clinical forms in humans, under- and/or misdiagnosis of SINV (and other alphavirus) infection likely exists. Studies conducted by McIntosh and colleagues in the 1970's and by Jupp and colleagues in the 1980's have shown that

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human SINV infections occur infrequently during the summer across the central plateau of South Africa, including the current Gauteng, Free State and Northern Cape provinces (McIntosh *et al*., 1976; Jupp *et al*., 1986). A large epidemic involving an estimate of thousands of cases in the Karoo and Northern Cape occurred in South Africa in 1974 (McIntosh *et al*., 1976; Jupp *et al*., 1986). A second epidemic was recorded in the Pretoria/Witwatersrand region during 1984, involving an estimate of hundreds of cases (Jupp *et al*., 1986).

The knowledge of the epidemiology and public health burden of SIN disease in South Africa remains largely obscure. This study provides an update on the epidemiological aspects of human SIN fever cases in South Africa for a five year period, 2006 -2010. The study was based on a retrospective investigation of suspected arbovirus cases submitted for laboratory testing.

2.2 Materials and methods

The Centre for Emerging and Zoonotic Diseases (formerly the Special Pathogens Unit) of the National Institute for Communicable Diseases of the National Health Laboratory Service (NICD-NHLS) is the reference centre for the laboratory confirmation of human arbovirus, including SINV, infection in South Africa. Specimens are subjected to serological screening using a haemagglutination inhibition assay (HAI), followed by a crude antigen-based SINV Immunoglobulin M Enzyme-Linked Immunosorbent Assay (indirect IgM ELISA). Reverse transcription PCR and virus isolation may also be performed in acute cases. For the time period of this study, from 1 January 2006 through 31 December 2010, data from suspected arbovirus cases, submitted to the laboratory for routine diagnostic evaluation, were collected. Ethical clearance for the retrospective investigation of suspected cases was obtained from the University of the Witwatersrand Human Research Ethics Committee (M10567). Data were collected from test requisition forms and questionnaires, as available in the laboratory's archive. The specimen requisition form was the format used by the different referring laboratories (for example Lancet, Ampath, Pathcare and NHLS) and was not standardised. During the RVFV outbreak in South Africa between 2008 and 2011, a RVF case investigation form was designed at the NICD-NHLS. This case investigation form was adapted for investigation of

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SIN cases (see Appendix B). The SIN disease questionnaire was sent to the referring doctors of SINV IgM-ELISA-confirmed cases retrospectively. The following data points were collected from the requisition forms and questionnaires as available (unverified data): patient date of birth, geographic area, occupation (questionnaire only), gender, symptoms (questionnaire only) and date of specimen collection. Only cases from South Africa were included and duplicates were removed from the data set. Data analysis was performed using a Microsoft Excel database and statistical analysis was performed using EpiInfo software version 3.5.3 (Centres for Disease Control and Prevention, 2011).

2.3 Results

Sindbis cases are confirmed in South Africa annually. Cases were deemed positive for recent SINV infection with the detection of anti-SINV IgM antibodies. From 1 January 2006 to 31 December 2010, a total of 3 631 specimens from patients with suspected arboviral infections were submitted to the Centre for Emerging and Zoonotic Diseases for laboratory investigation. For the period, 2006-2009, a total of 87 specimens out of 1 606 (5.4%) tested positive by HAI. Upon further analysis, anti-SINV IgM antibodies were detected in only 21 of the 87 HAI-positive cases, relating to an overall SINV detection rate of 1.3% (21/1 606).

In 2010, a rise in the number of cases was noted compared to the preceding period (odds ratio (OR): 8.64; confidence interval (CI) 95%: 5.39 – 13.99; $p < 0.001$), with 243 HAI-positive cases out of 2 025 investigated. Of the 243 HAI-positive cases, 208 cases were anti-SINV IgM positive (208/2 025, detection rate of 10%). The pattern of cases during the 2010 outbreak year compared to the low preceding period, displaying the cumulative specimens tested for SIN-disease from 2006 to 2009 and the number of specimens tested for SIN-disease in 2010, is shown on Figure 2.1. The graph shows higher detection rates in the late summer/early autumn months (March and April).

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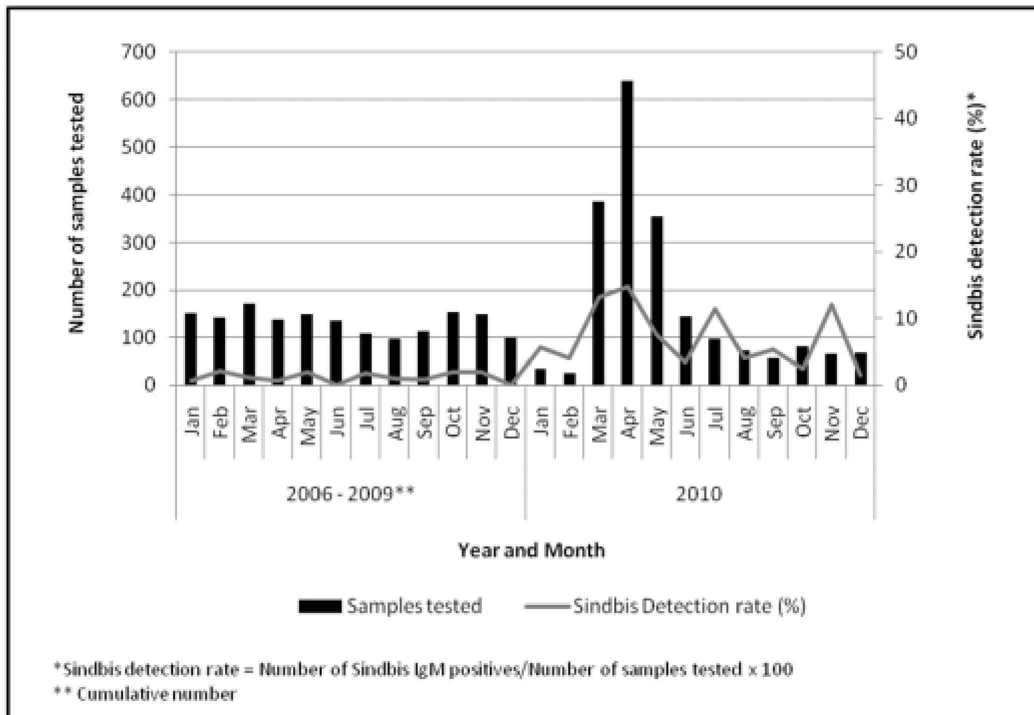

Figure 2.1: Histogram indicating the number of specimens submitted for arbovirus testing, as well as the anti-SINV IgM detection rate, for 2006 - 2009 and for 2010.

During the SINV outbreak in 2010, the date of onset for the first laboratory-confirmed SINV case in South Africa was the 15th of January 2010. The date of onset of the last case of SINV infection was the 7th of December 2010. The date of onset for the first RVFV case was the 25th of February 2010 and that of the last case was the 10th of December 2010. The epidemic curve of laboratory-confirmed SINV and RVFV cases by weekly periods, starting in the week of the date of onset of the first confirmed case and ending in the 37th epidemiological week, is illustrated in Figure 2.2. Some sporadic cases occurred in the 38th to the 50th epidemiological week and were not included in the graph. Laboratory-confirmed cases of SINV infection included specimens that tested positive for anti-SINV IgM, while laboratory confirmed cases of RVFV infection included specimens that tested positive with either IgM-ELISA, PCR or virus isolation.

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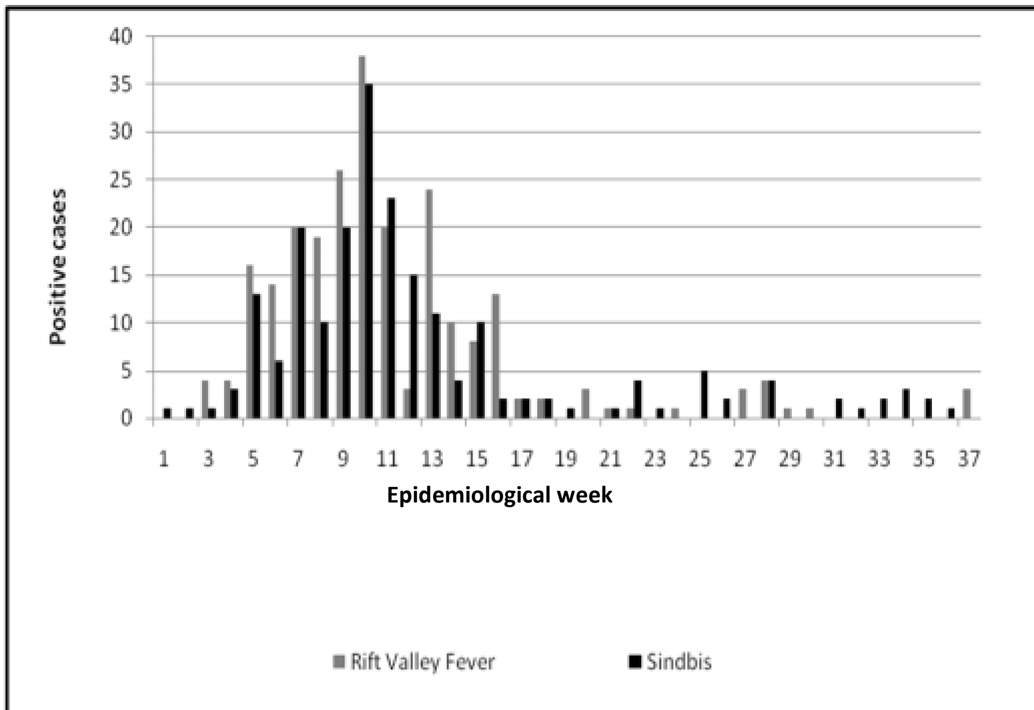

Figure 2.2: Distribution of the confirmed cases of SINV and RVFV infection, by epidemiological week, from 15 January 2010 through 9 September 2010.

The majority of SINV and RVFV cases occurred in the 5th to the 16th epidemiologic week of 2010 (February to April 2010). The detection rate for SINV during 2010 was 10% (208/2 025), whereas the detection rate for RVFV was 12% (241/2 025).

Of the 3 631 specimens that were submitted from 2006 through 2010, almost twice as many specimens were received from men (64%) as from women (35%). The anti-SINV IgM antibody detection rate was higher among men (167/2 334, 7%) than among women (62/1 265, 5%) (OR for gender: 1.49; CI 95%: 1.09 – 2.03; p-value: 0.009).

The majority of the specimens submitted for arbovirus investigation between 2006 and 2010 were received from persons aged between 20 and 49 years of age (2 279/3 631), while the least number of specimens were received from persons younger than 10 (176/3 631) and older than 70 (86/3 631) years of age.

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The average age for persons from whom specimens were received for arbovirus testing was 37 years (standard deviation (SD): 12.9), while the median age was 36 years. The risk for acquiring a SINV infection increased linearly with age, as is shown in Figure 2.3 ($p < 0.001$).

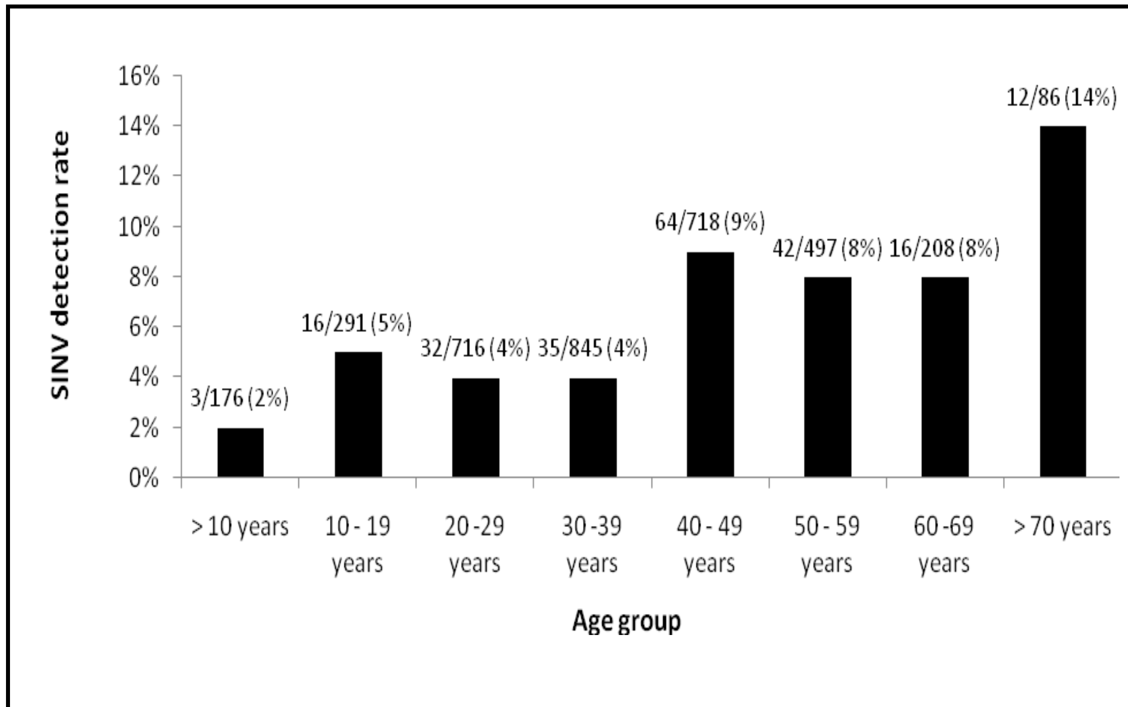

Figure 2.3: Graph showing the Sindbis virus detection rate for each age group from 2006 through 2010.

Of the 3 631 specimens received for arbovirus testing between 2006 and 2010, 229 tested positive for SINV using IgM-ELISA. The demographic features for these 229 patients are shown in Table 2.1. Only 7% (15/229) of persons infected with SINV were under the age of 18.

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Table 2.1: Demographic characteristics of patients with IgM-ELISA-confirmed SINV cases in South Africa from 2006 through 2010

Characteristics	Value
Age (years)[a]	
Range	7 - 85
Mean ± SD	42.38 ± 12.09
Median	44
Age group[a]	
< 10 years	3 (1%)
11 – 20 years	18 (8%)
21 – 30 years	30 (14%)
31 – 40 years	38 (17%)
41 – 50 years	68 (31%)
51 – 60 years	43 (20%)
61 – 70 years	11 (5%)
> 70 years	9 (4%)
Sex	
Male	167 (73%)
Female	62 (27%)

[a] Information was available for 220 patients

The majority of the specimens submitted to the diagnostic arbovirus laboratory and for which the geographic data was available (2 197/3 631), originated from the Gauteng Province (709/2 197; 32%), followed by specimens sent from the Free State and the Northern Cape Provinces (572/2 197; 26% and 251/2 197; 11%, respectively). The remaining 31% of specimens were sent from the other six Provinces of South Africa: Eastern Cape (153/2 197; 7%), North West (153/2 197; 7%), Western Cape (208/2 197; 10%), KwaZulu-Natal (75/2 197; 3%), Limpopo and Mpumalanga (76/2 197; 3%). During the study period (2006 to 2010), the SINV detection rates were higher in the Free State and Northern Cape Provinces compared to the detection rates of the other Provinces, as shown in Figure 2.4.

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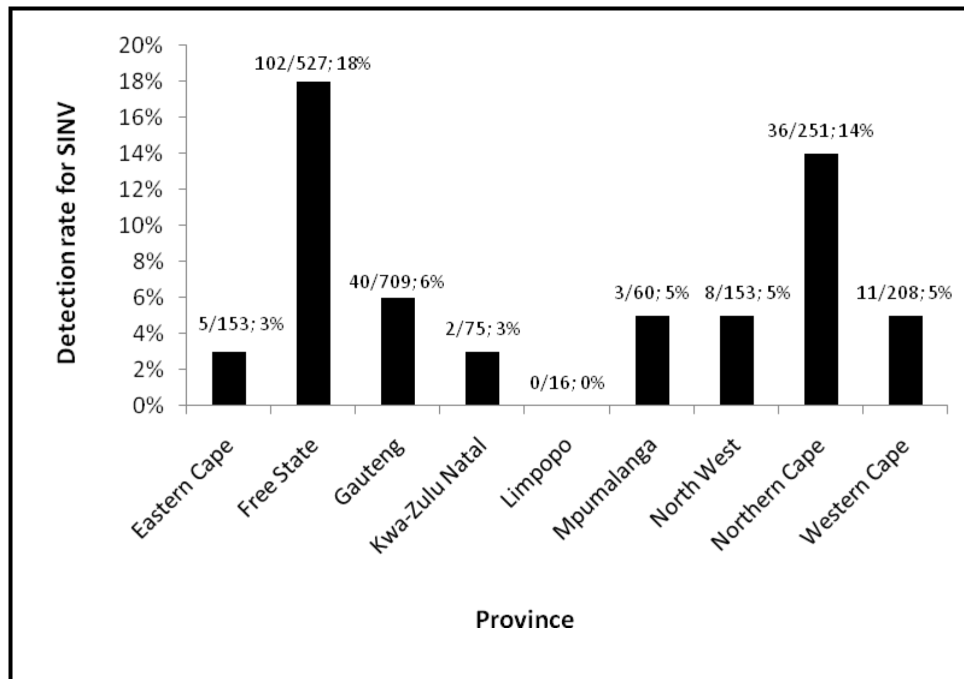

Figure 2.4: Graph showing the Sindbis virus (SINV) detection rate in each Province for the period 2006 to 2010

Based on an analysis of questionnaires on SINV laboratory confirmed cases, which were sent out to clinicians retrospectively, the most frequently observed symptoms for SINV infection included fever (39/58; 67%), myalgia (45/58; 78%), arthralgia (20/58; 35%), headache (40/58; 69%) and fatigue (20/58; 35%). The questionnaires showed that SINV infections were common among farmers (16/58; 28%) and farm workers (8/58; 14%).

2.4 Discussion

This retrospective study briefly describes the epidemiology of SINV infection in humans in South Africa for a 5 year period from 2006 through 2010. Previous restricted descriptions of SIN fever in South Africa were limited to historical studies in the 1970s and 1980s.

In this study, the majority of specimens were received from men (during the 2010 outbreak year as well as during the preceding years), possibly due to an increased frequency of mosquito bites among men due to employment in the farming sector and

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outdoor-associated labour (Statistics South Africa, 2007). This study has indicated that men are 1.5 times more likely to acquire a SINV infection than women (p-value: 0.009). However, it should be noted that the results obtained for gender and occupation in this study are biased since most of the specimens tested for SINV were originally submitted from farmers and farm workers (who are at the highest risk of RVFV infection) for RVFV investigation. The results obtained for gender in this study differed from results reported in studies conducted in Finland by Brummer-Korvenkontio and colleagues (2002) and Kurkela and colleagues (2008), which showed that women were more frequently infected with SINV, possibly because women are more likely to be exposed to mosquito bites due to taking walks in the Finnish countryside to pick mushrooms and berries (Brummer-Korvenkontio *et al*., 2002).

The risk for acquiring a SINV infection increased linearly with age ($p < 0.001$), while the average age of persons infected with SINV was 42 years. This is comparable to results found by Kurkela and colleagues (2005) in a study conducted in Finland, which showed that the average age of persons infected with SINV was 41 years, and to a second study by Kurkela and colleagues (2008), which showed that SINV infection was most common in persons between 50 and 59 years of age.

The detection rate for RVFV was slightly higher than for SINV during 2010. Rift Valley fever virus is usually transmitted to humans through contact with the blood or tissues of infected animals, while SINV is mainly transmitted through mosquito bites. A small number of human RVFV infections may result from the bites of infected mosquitoes (Pourrut *et al*., 2010). The mosquito vectors for RVFV are usually of the *Aedes* spp., which do not readily feed on humans. However, *Aedes* mosquitoes may transmit RVFV to their offspring via eggs and, during periods of high rainfall, larval habitats may flood causing the eggs to hatch and the mosquito population to expand. This causes an increased spread of RVFV to the animals on which the mosquitoes feed. Other mosquitoes, such as *Culex* spp., may later feed on these animals and then feed on humans, leading to the transmission of RVFV to humans. *Culex univittatus* mosquitoes are the main vectors for SINV, therefore co-infections with RVFV and SINV may occur during these periods (Jupp, 2005; World Health Organisation, 2010).

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The months during which the majority of SINV infections were diagnosed (March and April) correspond to the period during which *Culex univittatus* mosquitoes (important vectors for arboviral diseases) are abundant in South Africa (Rautenbach, 2011). Although rainfall and temperature investigations did not fall within the objectives of this study, the high number of cases observed in the Free State and Northern Cape Provinces should be attributed to the high rainfall and, in turn, the increased amount of mosquito breeding areas, in these Provinces during this period. Uejio and colleagues (2012) showed that simultaneous summer rainfall and a change in summer rainfall patterns from the preceding to the present summer were strongly related to SINV transmission and human outbreaks. The distribution of SINV cases in this study corresponds to the distribution for SINV proposed by earlier studies (McIntosh *et al*., 1976; Jupp *et al*., 1986).

The major symptoms of SINV disease included fever, myalgia, arthralgia, headache and fatigue. These findings are consistent with those of other studies conducted in South Africa and Finland regarding the major symptoms of SINV infection (Malherbe *et al*., 1963; McIntosh *et al*., 1976; Turunen *et al*., 1998; Laine *et al*., 2000; Kurkela *et al*., 2005; Sane *et al*., 2011). To date, no fatal cases of Sindbis fever have been reported. Likewise, no fatalities or complicated cases were noted for this cohort of cases.

Some shortcomings exist with the current data set and will be discussed briefly. Firstly, the data set is not based on a single case definition. During the RVFV outbreak in 2010, the majority of the cases were routinely submitted as suspected RVFV cases. A suspected RVFV case was defined as any person within a high risk category or residing within an area where RVFV may occur, presenting with influenza-like illness (fever, myalgia, arthralgia and backache), and/or encephalitis, haemorrhage, hepatitis and retinitis. The high risk categories included contact with livestock or game animals in or from RVFV-affected areas, residence within areas where RVFV is known to occur, with recent exposure to mosquito bites, and consumption of unpasteurised milk from RVFV-affected areas (National Institute for Communicable Diseases, 2011).

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Secondly, there exists submission bias. When interpreting the data, there is likely to be some bias in the manner that the specimens are submitted for laboratory testing. Tests are conducted for routine screening as well as for clinical investigation purposes, and specimens are mainly submitted via private laboratories. Laboratory tests are therefore influenced by financial constraints and consequently, the data do not necessarily reflect the true disease frequency and epidemiology of SINV infection within the human population of South Africa.

Thirdly, the diagnoses were based on single submissions. Follow up specimens were not available to test for rise in IgG titer in HAI-positive cases that tested IgM negative. These cases may either present previous infection with SINV or acute cases that can only be confirmed by indicating rise in IgG titer. From previous studies, it is known that approximately 60% of SINV patients do not show anti-SINV IgM antibodies within the first week of illness, therefore, a negative serology test is common during this period (Kurkela *et al*., 2005). Further to this, the persistence of anti-SINV IgM is not known. Kurkela and colleagues showed that anti-SINV IgM antibodies persisted up to 6 months after the onset of disease in 36% of patients (Kurkela *et al*., 2005). Vene and colleagues studied the IgM and IgG antibody patterns of SINV infection in 16 patients presenting with symptoms typical for SINV disease. Fifteen of these patients developed IgM antibodies against SINV within 2 weeks of the onset of symptoms (Vene *et al*., 1994). After 30 months, all of the patients tested negative for anti-SINV IgM antibodies (Vene *et al*., 1994). Detection of virus specific IgM together with the clinical presentation of the patient is, however, interpreted as a diagnostic result.

Lastly, the epidemiological data used in this study may be skewed in terms of time, place and occupation. The use of questionnaires is subject to certain limitations, for example errors of recall (Kurkela *et al*., 2005). Consequently, limitations exist in the interpretation of results. Additionally, the interpretation of the data set proved to be difficult due to incomplete questionnaires, missing specimen data and/or specimens being sent to a central laboratory instead of where the patient acquired the infection.

In conclusion, Sindbis fever appears to be an infrequent but continuous occurrence in the Northern Cape, Free State and Gauteng Provinces of South Africa. An increase in

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the number of cases reported annually was recorded during 2010. This coincided with an outbreak of Rift Valley fever in South Africa and was ascribed to above average rainfall which in turn provided favourable breeding grounds for mosquito vectors. Sindbis fever affects mostly middle aged men and has not been associated with severe presentations. Further investigation to clarify the burden of SINV-related disease in South Africa should address the prevalence and incidence of the disease in endemic areas. Morbidity may also be underestimated considering that patients included in this study did require medical consultation.

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CHAPTER 3: MOLECULAR IDENTIFICATION AND TYPING OF SINDBIS VIRUS ISOLATES FROM SOUTH AFRICA

3.1 Introduction

Sindbis virus (SINV) belongs to the family *Togaviridae* and is the prototype virus within the genus *Alphavirus* (Lundström & Pfeffer, 2010; International Committee on Taxonomy of Viruses, 2011). The virus is mosquito-borne and has the most extensive geographic distribution amongst all of the alphaviruses (Weaver *et al.*, 2006), with reports of the virus from Africa (Egypt, Cameroon, Uganda and South Africa), Eurasia (Sweden, Finland, Germany, United Kingdom, Italy, Israel, India, Saudi Arabia, China and Russia) and Oceania (Malaysia, the Philippines, New Zealand and Australia) (Sammels *et al.*, 1999; Jost *et al.*, 2010; Lundström & Pfeffer, 2010) . The SINV complex has been postulated to consist of several subtypes including Ockelbo virus from Sweden, Karelian fever virus from Russia and Whataroa virus from Australia and New Zealand (Weaver *et al.*, 1997). However, more recent studies have suggested that the SINV complex consists of only two species, namely Aura virus and SINV (Powers *et al.*, 2001; Lundström & Pfeffer, 2010). Birds are the reservoirs for SINV, while certain *Culex* (*Cx.*) mosquito species serve as vectors and occasionally transmit the virus to humans and other vertebrates (McIntosh *et al.*, 1986). Sindbis virus infection is associated with febrile rash-arthritis syndromes in humans (reviewed in Laine *et al.*, 2004), with no fatal cases reported to date.

Phylogenetic studies of SINV, mostly based on *E2* and *C* gene sequences, have shown a separation of isolates into Palaearctic/Ethiopian and Oriental/Australian groups (Sammels *et al.*, 1999; Jost *et al.*, 2010; Lundström & Pfeffer, 2010). This arrangement correlates with the geographic distribution of mosquito vectors and follows the known migratory bird flyways (Powers *et al.*, 2001). Kurkela and colleagues (2008) suggested that migratory birds may play a role in the spread of SINV, due to the fact that these viruses are widely distributed but still share identical nucleotide sequences. Additionally, analyses of SINV isolates from Africa and northern Europe have suggested that South African SINV strains are continuously imported by birds to northern Europe and vice versa (Norder *et al.*, 1996).

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In South Africa, SINV infrequently causes infections in humans during the summer (McIntosh *et al*., 1964; Jupp *et al.*, 1986). This virus is widely distributed across the central plateau of the country (including the current Gauteng, Free State and Northern Cape Provinces) (Jupp *et al*., 1986), but sporadic cases have also been reported from the Mpumalanga, North West, Eastern Cape, Western Cape and KwaZulu-Natal Provinces of South Africa (refer to Chapter 2). Presently, limited information exists on the genetic diversity among the South African SINV isolates. In previous phylogenetic studies, only a few South African isolates (AR86, Girdwood, AR6071, AR18141 and AR18132) were analysed (Jost *et al*., 2010; Lundström & Pfeffer, 2010). This investigation includes a more comprehensive phylogenetic inference of SINV isolates and provides insights in the diversity of this virus in South Africa, compared to elsewhere in the world. The *E2* gene was chosen since it is the most variable alphavirus gene and has been widely used in previous phylogenetic studies (Norder *et al*., 1996; Lundström & Pfeffer, 2010).

3.2 Materials and methods

3.2.1 Virus isolates

Ethics approval for the use of patient clinical material for the purpose of this study was obtained from the University of the Witwatersrand Human Research Ethics Committee (M060449). Twenty six SINV isolates from mosquito pools and one isolated from patient serum, were available from the Centre for Emerging and Zoonotic Diseases, of the National Institute for Communicable Diseases of the National Health Laboratory Service (NICD-NHLS) (Table 3.1 and Figure 3.1). The material was available in the form of lyophilised mouse brain preparations. The lyophilised material was reconstituted in sterile phosphate buffered saline before nucleic acid extraction. A total of 65 published SINV *E2* gene sequences from elsewhere in the world, available from NCBI GenBank, were also included in this study. No out-group was defined.

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Table 3.1: Sindbis virus isolates included in this study and analysed by partial sequencing of the *E2* gene*. Isolates are grouped based on genotype.

Isolate	Date collected	Place of collection	Source of isolate[a]	Passage[b]	Accession number	Reference
Ia						
AR86	1954	Springs, Gauteng, South Africa	*Cx. pipiens; Cx. theileri; Cx. univittatus*	Unknown	U62447	Weinbren *et al*., 1956
AR169	1954	Isis Estates, Babsfontein, Gauteng, South Africa	*Cx. annulioris; Cx. tigripes*	p3	JX682535	This study
AR1627	1959	Site 4, Ndumu, KwaZulu-Natal, South Africa	*Cx. univittatus*	p4	JX682536	This study
AR2471	1959	Site 18, Ndumu, KwaZulu-Natal, South Africa	*Cx. univittatus*	p3	JX682538	This study
AR166	1959	Isis Estates, Babsfontein, Gauteng, South Africa	*Cx. univittatus*	p7	JX682539	This study
AR4084	1962	Olifantsvlei, Gauteng, South Africa	*Cx. pipiens; Cx. fatigans, Cx. simpsoni*	p2	JX682541	This study
Girdwood	1962	Johannesburg, Gauteng, South Africa	*Homo sapiens*	Unknown	JX682548	Re-sequenced in this study
AR5078	1963	Lake Chrissie, Mpumalanga, South Africa	*Cx. univittatus*	p5	JX682542	This study
AR4952	1963	Olifantsvlei, Gauteng, South Africa	*Cx. univittatus*	p2	JX682543	This study
AR5186	1963	Olifantsvlei, Gauteng, South Africa	*Cx. univittatus*	p3	JX682545	This study
AR5850	1963	Olifantsvlei, Gauteng, South Africa	*Cx. pipiens*	p3	JX682546	This study
AR5857	1963	Olifantsvlei, Gauteng, South Africa	*Cx. univittatus*	p3	JX682550	This study
AR5907	1963	Olifantsvlei, Gauteng, South Africa	*Cx. univittatus*	p6	JX682560	This study
AR6071	1964	Olifantsvlei, Gauteng, South Africa	*Cx. univittatus*	p3	JX682544	Re-sequenced in this study
AR7046	1964	Olifantsvlei, Gauteng, South Africa	*Cx. univittatus*	p4	JX682547	This study
AR6232	1964	Olifantsvlei, Gauteng, South Africa	*Cx. univittatus*	p2	JX682549	This study
AR6194	1964	Olifantsvlei, Gauteng, South Africa	*Cx. univittatus*	p4	JX682551	This study

(Continued)

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Table 3.1 continued

Isolate	Date collected	Place of collection	Source of isolate	Passage	Accession number	Reference
AR7638	1965	Boksburg, Gauteng, South Africa	*Cx. univittatus*	p5	JX682552	This study
AR7489	1965	Olifantsvlei, Gauteng, South Africa	*Cx. univittatus*	p4	JX682553	This study
AR9698	1967	Olifantsvlei, Gauteng, South Africa	*Cx. univittatus*	p4	JX682557	This study
AR10962	1969	Damfontein, Free State, South Africa	*Cx. univittatus*	p3	JX682554	This study
AR10847	1969	Constantia; Northern Cape, South Africa	*Cx. theileri*	p5	JX682555	This study
AR12868	1971	Bethulie, Free State, South Africa	*Culicoides spp.*	p3	JX682556	This study
AR12859	1971	Bethulie, Free State, South Africa	*Cx. theileri*	p4	JX682558	This study
1038	1964	Rubin, Israel	*Streptopelia turtur*	Unknown	U62443	Nir *et al.*, 1967
Y-251	1969	Cameroon	*Mansonia africana*	sm2, bhk1	AF339477	Karabatsos, 1985
Edsbyn 82/5	1982	Edsbyn, Sweden	*Culiseta spp.*	Unknown	M69205	Niklasson *et al.*, 1984
83M107	1983	Sässman, Sweden	*Culi. morsitans*	sm1, v1	U62451	Francy *et al.*, 1989
83M108	1983	Sässman, Sweden	*Cx. pipiens*	v2	U62476	Francy *et al.*, 1989
84M137	1984	Sässman, Sweden	*Culi. morsitans*	v2	U62459	Francy *et al.*, 1989
84M140	1984	Sässman, Sweden	*Culi. morsitans*	v1	U62468	Francy *et al.*, 1989
85M68	1984	Sässman, Sweden	*Culi. morsitans*	v1	U62468	Francy *et al.*, 1989
85M78	1985	Sässman, Sweden	*A. cinereus*	v1	U62460	Francy *et al.*, 1989
85M94	1985	Sässman, Sweden	*Cx. pipiens*	v1	U62469	Francy *et al.*, 1989
85M134	1985	Sässman, Sweden	*Cx. pipiens*	v1	U62444	Norder *et al.*, 1996
86-520	1985	Sässman, Sweden	*Cx. pipiens*	v2	U62445	Norder *et al.*, 1996
86-737	1985	Sässman, Sweden	*A. cinereus*	v2	U62453	Norder *et al.*, 1996
86-743	1985	Bergsaker, Sweden	*A. cinereus*	v2	U62462	Norder *et al.*, 1996
86-752	1985	Bergsaker, Sweden	*Cx. pipiens*	v2	U62470	Norder *et al.*, 1996
86-486	1985	Bergsaker, Sweden	*Cx. pipiens*	v2	U62477	Norder *et al.*, 1996

(Continued)

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Table 3.1 continued

Isolate	Date collected	Place of collection	Source of isolate	Passage	Accession number	Reference
86-828	1985	Sässman, Sweden	*Culi. Morsitans*	v2	U62478	Norder *et al.*, 1996
90B146	1990	Boda Bruk, Sweden	*Anas plathyrhynchos*	v1	U62446	Norder *et al.*, 1996
95M116	1995	Tärnsjö, Sweden	*A. cinereus*	v2	FJ744539	Lundstrom & Pfeffer, 2010
LEIV-9298	1983	Karelia, Russia	*Aedes spp.*	sm2, v2	U62465	Lvov *et al.*, 1984
YN87448	1992	Yunnan, China	*Homo sapiens*	Unknown	AF103734	Liang *et al.*, 2000
Vemork 1/92	1992	Rjukan, Norway	*Aedes spp.*	v4	U62467	Norder *et al.*, 1996
Johannes-2002	2002	-	-	Unknown	JQ771797	Sane *et al.*, 2012
Kiihtelysvaara-2002	2002	Kiihtelysvaara, Finland	-	Unknown	JQ771798	Sane *et al.*, 2012
Ilomantsi-2002B	2002	Ilomantsi, Finland	-	Unknown	JQ771795	Sane *et al.*, 2012
Ilomantsi-2002C	2002	Ilomantsi, Finland	-	Unknown	JQ771796	Sane *et al.*, 2012
Ilomantsi-2002A	2002	Ilomantsi, Finland	-	Unknown	JQ771794	Sane *et al.*, 2012
Ilomantsi-2005M	2005	Ilomantsi, Finland	-	Unknown	JQ771793	Sane *et al.*, 2012
3.3	2009	Weinheim, Germany	*Culex spp.*	Unknown	GU361115	Jost *et al.*, 2010
5.3	2009	Weinheim, Germany	*Culex spp.*	Unknown	GU361116	Jost *et al.*, 2010
21.3	2009	Weinheim, Germany	*Culex spp.*	Unknown	GU361117	Jost *et al.*, 2010
28.9	2009	Weinheim, Germany	*Culex spp.*	Unknown	GU361118	Jost *et al.*, 2010
32.1	2009	Weinheim, Germany	*Culex spp.*	Unknown	GU361119	Jost *et al.*, 2010
34.4	2009	Weinheim, Germany	*Culex spp.*	Unknown	GU361120	Jost *et al.*, 2010
Ib						
MP684	1960	Entebbe, Uganda	*Mansonia fuscopennata*	Unknown	U62480	Woodall *et al.*, 1964
AR18141	1976	Upington, Northern Cape, South Africa	*Cx. univittatus*	p5	JX682540	Re-sequenced in this study
AR18132	1976	Upington, Northern Cape, South Africa	*Cx. univittatus*	p4	JX682559	Re-sequenced in this study

(Continued)

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Table 3.1 continued

Isolate	Date collected	Place of collection	Source of isolate	Passage	Accession number	Reference
Ic						
AR339	1952	Cairo, Egypt	*Cx. pipiens; Cx. univittatus*	p8	JX682537	Re-sequenced in this study
M 1855	1967	Hadera, Israel	*Cx. molestus*	Unknown	U62473	Nir *et al.*, 1972
R 33	1971	Slovak Republic	*Acrocephalus scirpaceus*	v4	U62457	Ernek *et al.*, 1973
SK2	1972	Slovak Republic	*Cricetus cricetus*	sm7, v1	U62458	Norder *et al.*, 1996
R 2	1975	Slovak Republic	*Rana ridibunda*	sm7, v1	U62449	Kozuck *et al.*, 1978
Gresikova	1975	Sicily, Italy	*Hyalomma marginatum*	Unknown	U62456	Gresikova *et al.*, 1978
AZ-16	1977	Azerbaijan, Russia	*Nycticorax nycticorax*	p1, v1	U62464	Gaidamovich *et al.*, 1978
SA80-394	1980	Saudi Arabia	*Cx. univittatus*	d1, v1	FJ744535	Wills *et al.*, 1985
SA80-480	1980	Saudi Arabia	*Cx. univittatus*	d1, v1	FJ744536	Wills *et al.*, 1985
SA80-66	1980	Saudi Arabia	*Cx. univittatus*	d1, v1	U62450	Wills *et al.*, 1985
SA80-231	1980	Saudi Arabia	*Cx. univittatus*	d1, v1	U62466	Wills *et al.*, 1985
SA80-370	1980	Saudi Arabia	*Cx. univittatus*	v2	FJ744534	Wills *et al.*, 1985
SA80-62	1980	Saudi Arabia	*Cx. tritaeniorhynchus*	d1, v1	U62461	Norder *et al.*, 1996
SA80-287	1980	Saudi Arabia	*Cx. univittatus*	v2	U62474	Norder *et al.*, 1996
Acrocephalus	-	-	-	Unknown	U62454	Norder *et al.*, 1996
AS-2A1	-	-	-	Unknown	U62455	Norder *et al.*, 1996
Cricetus	-	-	*Cricetus cricetus*	Unknown	U62472	Norder *et al.*, 1996
II						
RR2215	1955	Kuantan, Malaysia	*Cx. tritaeniorhynchus*	Unknown	FJ744532	Maguire *et al.*, 1967
MRM 39	1960	Queensland, Australia	*Culex spp.*	Unknown	FJ44527	Doherty *et al.*, 1963
C-377	1960	Queensland, Australia	*Mansonia septempunctata*	sm1, v1	FJ44537	-

(Continued)

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Table 3.1 continued

Isolate	Date collected	Place of collection	Source of isolate	Passage	Accession number	Reference
C-263	1961	Queensland, Australia	*Aedes normanensis*	Unknown	FJ744526	Doherty *et al.*, 1963
MRM 18520	1975	Queensland, Australia	*Culicidae* spp.	sm4, v1	FJ744538	Olson & Trent, 1985
CH-19470	1976	Queensland, Australia	*Culicidae* spp.	Unknown	FJ744525	Rentier Delrue & Young, 1980
III						
A 1036	1953	Mysore state, India	*Bdellonyssus bursa*	Unknown	FJ744531	Shah *et al.*, 1960
B322/23/24	1953	Bombay state, India	*Motacilla alba*	Unknown	FJ744530	Shah *et al.*, 1960
631310	1963	Madras, India	*Culex* spp.	Unknown	FJ744528	-
654999-2	1965	Poona, India	*Culex* spp.	Unknown	FJ744529	-
P-886	1956	Luzon, Philippines	*Culex* spp. *bitaeniorhynchus*	Unknown	FJ744533	Rudnick *et al.*, 1962
IV						
LEIV 65A	1969	Azerbaijan, Russia	*Cx. modestus*	p8, sm1, bhk1	AF339478	Lvov *et al.*, 1979
XJ-160	1970	Xinjiang, China	*Culicidae* spp.	Unknown	AF103728	Liang *et al.*, 2000
V						
M78	1962	North Westland, New Zealand	*Cx. pervigilans*	p8, sm1, bhk1	AF339479	Ross *et al.*, 1964

* Not all isolates listed in this table were included in the final phylogenetic tree due to space constraints

[a] Abbreviations: Cx. – *Culex*, Culi. – *Culiseta*, A. – *Amblonyx*

[b] Abbreviations: p – passage, v – vero, bhk – baby hamster kidney

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Figure 3.1: Map of South Africa indicating the approximate locations where the South African Sindbis virus isolates were collected.

3.2.2 Nucleic acid extraction

The RNA from the SINV specimens was extracted using the RNeasy® Lipid Tissue Mini Kit (QIAGEN, USA) according to the manufacturer's instructions. Briefly, the SINV specimens were disrupted and homogenised in one millilitre QIAzol lysis reagent (QIAGEN, USA). The specimens were kept at room temperature (25°C) for 5 minutes. Two hundred microlitres of chloroform (Merck, SA) was added to each specimen and then shaken for 15 seconds. The specimens were kept at 25°C for 3 minutes and centrifuged (Eppendorf Centrifuge 5415D, Merck, SA) at 12 000 x *g* for 10 minutes at 25°C. The upper, aqueous phase of each specimen was transferred to new, sterile 1.5 ml microcentrifuge tubes and 600 µl of 70% ethanol (Merck, SA) was added. The specimens were shaken and centrifuged (Eppendorf Centrifuge 5415D, Merck, SA) briefly. A volume of 700 µl of the specimens were transferred to RNeasy® columns (QIAGEN, USA) in two millilitre tubes and were centrifuged

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(Eppendorf Centrifuge 5415D, Merck, SA) at 12 000 x *g* for 30 seconds at 25°C. The flow-through was discarded and 700 µl of buffer RW1 (QIAGEN, USA) was added to the RNeasy® columns. The specimens were centrifuged (Eppendorf Centrifuge 5415D, Merck, SA) at 12 000 x *g* for 30 seconds at 25°C. The flow-through was discarded and 500 µl of buffer RPE (QIAGEN, USA) was added to each RNeasy® column. The specimens were centrifuged (Eppendorf Centrifuge 5415D, Merck, SA) at 12 000 x *g* for 30 seconds at 25°C and the flow-through was discarded. A volume of 500 µl of buffer RPE (QIAGEN, USA) was added to each RNeasy® column and the specimens were centrifuged (Eppendorf Centrifuge 5415D, Merck, SA) for 2 minutes at 12 000 x *g* at 25°C. The flow-through was discarded. The RNeasy® columns were placed in new two millilitre tubes and centrifuged (Eppendorf Centrifuge 5415D, Merck, SA) at full speed for 1 minute. The RNeasy® columns were placed in new, sterile 1.5 ml microcentrifuge tubes and 50 µl of RNase-free water was added. The specimens were centrifuged (Eppendorf Centrifuge 5415D, Merck, SA) at 12 000 x *g* for 1 minute at 25°C. The extracted RNA was stored at -20°C until processed.

3.2.3 Nucleic acid amplification

A reverse transcription polymerase chain reaction (RT-PCR) assay was performed using the oligonucleotides (Table 3.2) and adapted from conditions described in Lundström and Pfeffer (2010) for the amplification of a 527 base pair region of the *E2* gene (positions 8919–8938 and 9426–9445 of the SINV genome, respectively) (Strauss *et al*., 1984).

Table 3.2: Primer pairs used for the amplification of the partial *E2* gene (Lundström & Pfeffer, 2010)

Gene	Primer (5' – 3')	Oligonucleotide sequence (5'-3')
Partial *E2* gene	A1 Forward primer (8880 – 8899*)	AAA GGA TAC TTT CTC CTC GC
	A2 Reverse primer (9445 - 9426*)	TGG GCA ACA GGG ACC ATG CA

* Position numbered according to SINV reference strain NC_001547.1 (Strauss *et al*., 1984)

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A conventional RT-PCR reaction mixture was prepared to a total of 50 µl using the following reagents: 23.8 µl of nuclease-free water, 0.75 µl of a 20 pmol working solution of forward primer (University of Cape Town, South Africa), 0.75 µl of a 20 pmol working solution of reverse primer (University of Cape Town, South Africa), one microlitre of 10 mM dNTP, 2.5 µl of 100 mM DTT, 0.25 µl of RNase inhibitor, 10 µl of 5x RT-PCR buffer and one microlitre of enzyme mix (Titan One Tube RT-PCR System, Roche Applied Science, Germany). A volume of ten microlitres of template RNA was added. The RT-PCR reaction was performed in a thermal cycler (2720 Thermal Cycler, Applied Biosystems, USA) using the following cycling conditions: cDNA synthesis by AMV reverse transcriptase at 50°C for 30 minutes (one cycle); initial denaturation at 94°C for 2 minutes (one cycle); denaturation at 94°C for 30 seconds, annealing at 47°C for 30 seconds and extension at 68°C for 30 seconds (35 cycles); and a final extension step at 68°C for 7 minutes (one cycle).

3.2.4 Analysis of PCR reaction products

Amplification products were analysed using standard molecular biology techniques as described by Sambrook and Russell (2001) (results not shown). Briefly, ten microlitres of RNA with 2.5 µl of loading dye (Promega, USA) containing Gel Red (Biotium, USA) was loaded onto a 1% (m/v) agarose (Seakem LE agarose, Whitehead Scientific, SA) gel. A 100 bp molecular marker (DNA Molecular Weight Marker XIV, Roche, Germany) was included as a reference for each gel. The samples were electrophoresed (Power Pac 300, Biorad, SA) for 45 minutes at 100 V. The amplicons of the RT-PCR reactions were visualised using an ultra violet light box (White/UV Transilluminator, UVP, USA). The expected band size was 566 bp for the partial *E2* gene.

3.2.5 Purification of PCR products

The PCR products were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, USA) according to the manufacturer's instructions. Briefly, an equal volume (40 µl) of membrane binding solution was added to the PCR amplicons. One SV minicolumn was placed in a collection tube for each PCR product. The prepared PCR product was transferred to the SV minicolumn assembly and kept at

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25°C for 1 minute. The SV minicolumn assembly was centrifuged (Zentrifugen Mikro 200, Hettich, Germany) at 16 000 x *g* for 1 minute at 25°C. The SV minicolumn was removed from the spin column assembly and the liquid in the collection tube was discarded. The SV minicolumn was returned to the collection tube. The column was washed by adding 700 µl of membrane wash solution (diluted with 95% ethanol (Merck, SA)) to the SV minicolumn. The SV minicolumn assembly was centrifuged (Zentrifugen Mikro 200, Hettich, Germany) for 1 minute at 16 000 x *g* at 25°C. The collection tube was emptied and the wash was repeated with 500 µl of membrane wash solution. The SV minicolumn assembly was centrifuged (Zentrifugen Mikro 200, Hettich, Germany) for 5 minutes at 16 000 x *g* at 25°C. The collection tube was emptied and the column assembly was centrifuged (Zentrifugen Mikro 200, Hettich, Germany) for 1 minute at 16 000 x *g* at 25°C. The SV minicolumn was transferred to a new, sterile 1.5 ml microcentrifuge tube. Fifty microlitres of nuclease-free water was added and the column assembly was kept at 25°C for 1 minute. The column assembly was centrifuged (Zentrifugen Mikro 200, Hettich, Germany) at 16 000 x *g* for 1 minute at 25°C. The SV minicolumn was discarded. The concentration of the purified deoxyribonucleic acid (DNA) was estimated by performing electrophoresis (Power Pac 300, Biorad, SA) for 45 minutes at 110 V as follows: four microlitres of a 100 bp molecular marker (DNA Molecular Weight Marker XIV, Roche, Germany) and four microlitres of each DNA sample was loaded onto a 1% (m/v) agarose (Seakem LE agarose, Whitehead Scientific, SA) gel. The band densities of the DNA samples were compared to the molecular marker using an ultra violet light box (White/UV Transilluminator, UVP, USA). The rest of the DNA was stored at -20°C until required for further processing.

3.2.6 Molecular sequencing of the amplicons

A ten microlitre sequencing reaction was prepared for each specimen using the BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems, USA) as follows: one microlitre of sequencing buffer, one microlitre of either the forward primer (A1 (Table 3.2), 3.2 mM) or the reverse primer (A2 (Table 3.2), 3.2 mM), two microlitres of BigDye v3.1 mix, five microlitres of nuclease-free water and one microlitre of template DNA (approximately 500 ng/µl) was added to each tube. Sequencing cycles were performed in a thermal cycler (2720 Thermal Cycler, Applied

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Biosystems, USA) with the following conditions: one cycle at 94°C for 1 minute, followed by 25 cycles of 94°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes. To each tube, one microlitre of 125 mM EDTA (Merck, SA), one microlitre of 3 M sodium acetate (pH 5.2) (Merck, SA) and 25 µl of 100% ethanol (Merck, SA) was added. The tubes were shaken and incubated at 25°C in the dark for 15 minutes. The samples were centrifuged (Eppendorf MiniSpin, Merck, SA) for 30 minutes at 12 225 x *g*. The supernatant was removed by pipetting and 100 µl of 70% ethanol (Merck, SA) was added. The samples were centrifuged (Eppendorf MiniSpin, Merck, SA) for 15 minutes at 12 225 x *g* and the supernatant was removed. Another volume (100 µl) of 70% ethanol (Merck, SA) was added and the samples were centrifuged (Eppendorf MiniSpin, Merck, SA) at 12 225 x *g* for 15 minutes. The supernatant was removed and the samples were air-dried in the dark. The samples were submitted to the Faculty of Natural and Agricultural Sciences sequencing core facility at the University of Pretoria for sequencing.

3.2.7 Phylogenetic analysis

Sequences were analysed using BioEdit Sequence Alignment Editor Version 7 (Hall, 1999). The sequences were trimmed to a final size of 312 nucleotides, corresponding to position 8968 to 9288 of the Sindbis virus reference strain, NC_001547.1 (Strauss *et al*., 1984). The reverse sequence was reverse complemented and imported to the forward sequence alignment. A multiple sequence alignment was created using the ClustalW subroutine of the BioEdit software (Hall, 1999). The aligned sequences were opened using the Molecular Evolutionary Genetics Analysis (MEGA) version 4 software and a neighbour-joining (NJ) phylogenetic tree was constructed. The genetic distances were calculated using the Kimura two-parameter method (Tamura & Kumar*,* 2007). The reliability of the branching pattern was assessed by a bootstrap analysis of 1 000 replications and mid-point rooting was selected. An amino acid analysis was performed by comparing the E2 protein sequences of the SINV strains investigated in this study with the deduced amino acid sequences of the SINV reference strain described by Strauss and colleagues (1984), using MEGA version 4 software.

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3.3 Results

The partial *E2* gene sequences of 27 African Sindbis virus isolates were aligned with published sequences of SIN viruses isolated in Europe, Asia, Africa, Australia and New Zealand. One cluster (Figure 3.2, corresponding to SINV genotype I) resolved into three sub-clusters (Ia, Ib and Ic), of which the first consisted of SINV isolates originating mainly from South Africa (n=24), Cameroon (n=1), Germany (n=3), Sweden (n=11), Finland (n=4), Norway (n=1), Russia (n=1) and Israel (n=1). A single isolate from eastern China (YN87448) also grouped within this sub-cluster (Figure 3.2, Ia).

Two isolates from Upington, South Africa and one isolate from Uganda formed a second sub-cluster (Figure 3.2, Ib). A third sub-cluster consisted of isolates from Saudi Arabia (n=4), Israel (n=1), Italy (n=1), Egypt (n=1), Azerbaijan, Russia (n=1) and the Slovak Republic (n=3) (Figure 3.2, Ic).

A second cluster (Figure 3.2, corresponding to SINV genotype II) consisted of isolates from Australia (n=4) and one isolate from Malaysia. A third cluster (Figure 3.2, corresponding to SINV genotype III) included isolates from India (n=4) and the Philippines (n=1). The fourth cluster (Figure 3.2, corresponding to SINV genotype IV) included isolates from Azerbaijan, Russia (n=1) and China (n=1). A single isolate from New Zealand grouped separately, forming a final cluster designated SINV genotype V (Figure 3.2). Apart from the Whataroa virus M78 isolate from New Zealand, the division of SINV isolates into these distinct groups were supported by high bootstrap values of between 70% and 100%.

Maximum diversity among the SINV groups in this study was observed between SINV genotypes III and V, with a 30.1% difference in nucleotides (94/312) and a 23.1% difference in amino acids (24/104), while the least amount of divergence was observed between the South African and European SINV isolates (sub-cluster Ia), having a maximum divergence of 36 nucleotides (11.5%) and 11 amino acids (10.6%). South African SINV isolates were highly homogenous, having a maximum divergence of four nucleotides (1.3%) and four amino acids (3.8%).

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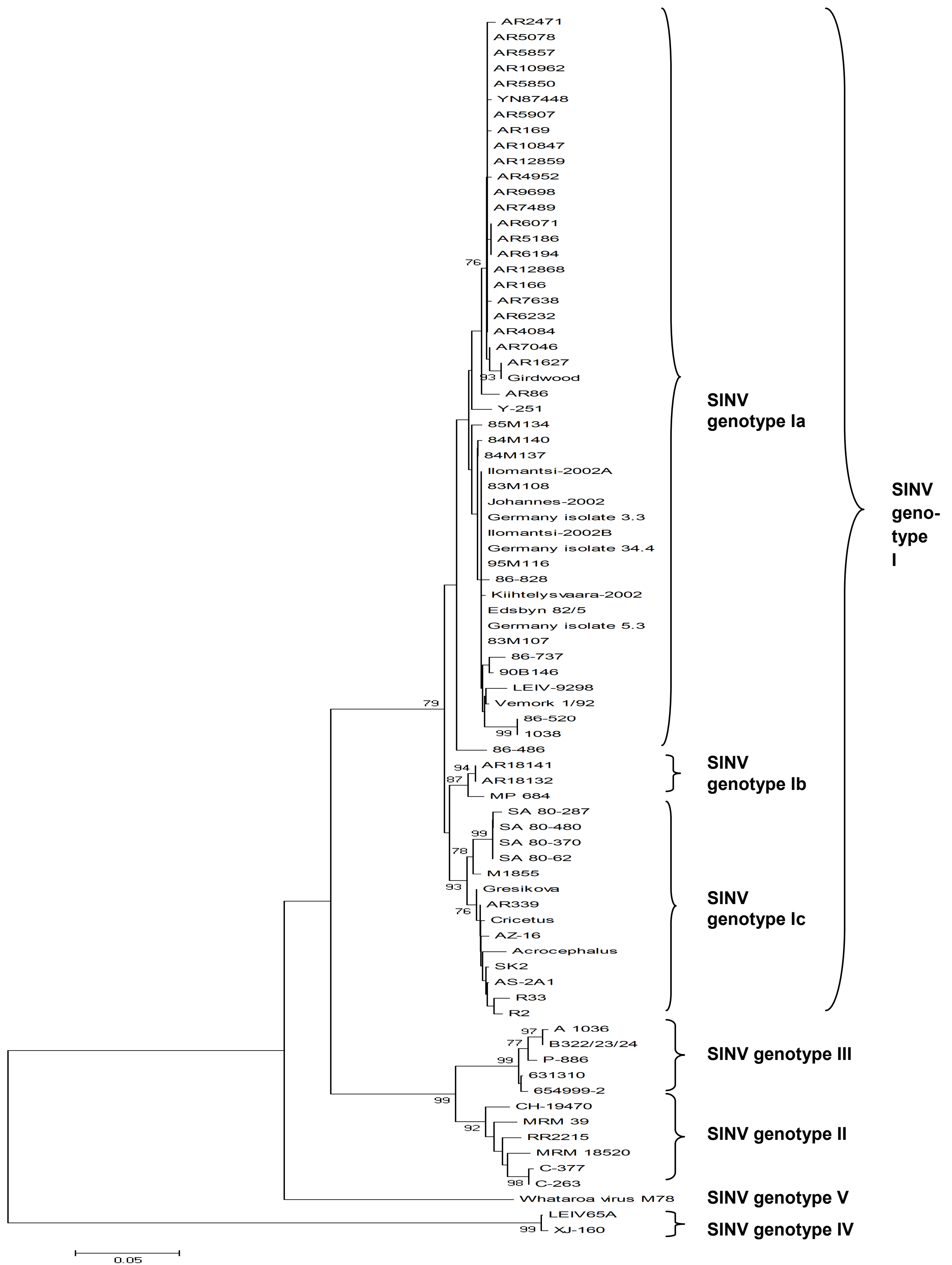

Figure 3.2: Phylogenetic tree illustrating the genetic relationships of Sindbis viruses based on sequencing of 312 nucleotides of the *E2* gene.

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Bootstrap values over 70% are indicated and branch lengths are proportional to genetic relatedness. Midpoint rooting was selected and no out-group was defined. The numbers I to V refer to clusters (corresponding to the different SINV genotypes described in Lundström & Pfeffer, 2010) while a, b and c refer to sub-clusters.

Similarly to results reported by Lundström and Pfeffer (2010), amino acid analysis revealed that SINV isolates belonging to genotypes II and III shared the following amino acid substitutions: Tyr153, Ile154, Ser162, Thr178, Ala213, Lys223 and Ile228 (results not shown due to space constraints). South African Sindbis isolates, as well as the majority of the other SINV isolates belonging to genotype I, had Thr153, Val154, Thr162, Ser178, Thr213, Ile223 and Thr228 in the same positions. Except for the SINV Girdwood isolate recovered from patient serum, all of the South African SINV isolates shared the amino acid substitution Gly188, along with the majority of the SINV isolates belonging to SINV genotype I. The SINV reference strain, the Girdwood isolate and all of the SINV isolates belonging to genotypes II to V had Tyr188 in this position (results not shown due to space constraints).

3.4 Discussion

The results obtained in this study have shown that the SINV isolates belong to five distinct phylogenetic groups and confirm the existence of considerable genetic variants. The genetic variation for this gene region is very similar to those reported in previous studies by Kinney and colleagues in 1998 (30.4% at the nucleotide level and 22.5% at the amino acid level) and by Lundström and Pfeffer in 2010 (28.2% at the nucleotide level and 22.2% at the amino acid level). The amino acid substitutions were similar to results previously reported by Lundström and Pfeffer (2010) and were comparable for SINV strains from the same genotype and with related geographic distributions.

In this study, SINV genotype I formed three sub-clusters, namely Ia, Ib and Ic, with Sindbis viruses originating mainly from South Africa, Sweden, Finland and Germany grouping within one sub-cluster (Ia). Even though SINV has a wide geographic distribution, clinical outbreaks occurring as epidemics have mainly occurred in parts

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of northern Europe and South Africa, indicating that humans may be more susceptible to infection with SINV strains from this particular genotype (Lundström & Pfeffer, 2010). These results further support the hypothesis that these viruses may be transported between South Africa and Europe by migratory birds. The Palaearctic/Ethiopian spread of the SINV isolates belonging to genotype I corresponds to the two African-Eurasian avian flyways (Broere *et al*., 2006). Similar results were reported by Norder and colleagues in 1996 and by Lundström and Pfeffer in 2010. The isolate from China (YN87448) grouping within cluster I along with the North European and South African isolates, has been suggested to be a contamination due to its striking similarity with the commonly used lab strain, AR86 (isolated in South Africa) (Lundström & Pfeffer, 2010). A very low diversity was noted between South African SINV isolates (four nucleotides (1.3%) and four amino acids (3.8%)). This is to be expected since the majority of the South African isolates used in this study originated from the same geographic area (Olifantsvlei, Gauteng). A phylogenetic study including isolates from other regions of South Africa and Africa would provide more comprehensive information on the phylogeography of the South African and African SINV strains.

The second sub-cluster (Ib) for SINV genotype I was composed of isolates from Upington in the Northern Cape Province of South Africa, and Uganda. These isolates differed from the South African isolates in sub-cluster Ia by 15 nucleotides (4.8%) and two amino acids (1.9%). Considering that none of the SINV isolates in this cluster were isolated from humans, the dispersal of these isolates by means of human travel may be ruled out. The most likely explanation for the close relationship between these isolates would therefore appear to be their transmission between these locations through the migration of birds. Given that the Northern Cape Province is one of the least ornithologically studied areas in South Africa, it is difficult to pinpoint exactly which bird species could be involved in transporting SINV between Upington and Uganda (Brooke, 1984). Birds such as the Barn Swallow *Hirundo rustica*, the Red-Backed Shrike *Lanius collurio* and the Willow Warbler *Phylloscopus trochilus* have a preference for migrating to South Africa through Uganda and the Lake Victoria basin (Pearson & Lack, 1992; Birdlife International, 2012). Additionally, some species of birds such as the Rock pratincole *Glareola nuchalis* are intra-African migrants that breed in southern Africa and migrate to Uganda in non-breeding seasons

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(Hockey *et al*., 2005). The migration of these and several other birds, which are potential carriers for SINV, might explain the separate grouping of the SINV isolates from Upington with the SINV isolate from Uganda.

The third sub-cluster for SINV genotype I consisted of isolates from the Middle East, the South of Europe, the northern parts of Africa and Saudi-Arabia. The grouping of these SINV isolates corresponds to the East Asian/East African flyway (part of the African-Eurasian flyway), in which birds migrate south-westwards from the mid-Palaearctic towards Africa in order to avoid the obstructions presented by the Himalayas and Tibetan plateau (Birdlife International, 2012).

Sindbis virus genotype II consisted of isolates from Australia and Malaysia, while SINV genotype III was composed of isolates from India and the Philippines. These isolates had an Oriental/Australian spread corresponding to the East Asian/Australasian flyway of birds (Broere *et al*., 2006; Birdlife International, 2012). The SINV isolates belonging to genotype III were the most different (based on a nucleotide and amino acid comparison) from the other genotypes. These results are similar to results reported by Olson and Trent (1985), Sammels and colleagues (1999) and Lundström and Pfeffer (2010). As previously reported by Lundström and Pfeffer (2010), amino acid analysis results revealed that SIN viruses belonging to genotype II and III may easily be distinguished from SIN viruses belonging to genotype I based on amino acid substitutions in positions 213, 197 and 223. To date, no human SINV infections have been reported from the Oriental/Australian areas, suggesting that the strains belonging to group II and III in this study are either the least infectious, or are largely misdiagnosed or under-reported.

Sindbis virus genotype IV consisted of one isolate from Russia and one isolate from China. Birds such as the white stork (*Ciconia ciconia*) may use the Central Asian flyway to migrate between Russia and China (Birdlife International, 2012) and this might explain the grouping of these isolates within one genotype. The New Zealand isolate formed a separate branch designated SINV genotype V. It has been suggested that New Zealand SINV isolates might be distinctly different from other SINV isolates as New Zealand hosts a large variety of endemic birds that rarely migrate between New Zealand and other countries (Heather *et al*., 1997; Lundström & Pfeffer,

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2010). Studies including more isolates from these areas would be necessary to better elucidate the phylogeography of the genotype IV and genotype V strains.

In conclusion, this study considered the relationship of a panel of SINV isolates from South Africa and Africa with a global selection of SINV and has confirmed considerable genetic diversity and distinct phylogenetic configuration. The phylogenetic groups were associated with some of the major migratory patterns of birds and suggested that birds play a major role in the distribution of SINV. These findings are consistent with and complimentary to previous studies that considered the phylogeography of SINV.

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CHAPTER 4: DEVELOPMENT OF A PAN-ALPHAVIRUS REAL-TIME REVERSE TRANSCRIPTION PCR ASSAY

4.1 Introduction

Several members of the genus *Alphavirus* cause febrile illnesses frequently including arthralgia or encephalitis (reviewed in Laine *et al*., 2004). Due to the rising number of alphavirus infections and the movement of alphavirus vectors into new areas, the development of improved diagnostic and surveillance methods are essential (Grywna *et al*., 2010). In the past decade, molecular methods such as RT-PCR have become imperative tools for detecting and quantifying various ribonucleic acid (RNA) targets and are becoming increasingly popular in diagnostic laboratories (Bustin & Mueller, 2005). Some molecular assays have been developed for the detection of certain alphaviruses and of these, conventional RT-PCR is the most widely used at present. Reverse-transcription PCR differs from PCR by the addition of an initial reverse transcription step, during which RNA is converted to deoxyribonucleic acid (DNA) by the enzyme, reverse transcriptase (Bustin & Mueller, 2005). A genus-specific conventional RT-PCR assay was recently developed by Grywna and colleagues (2010) for the detection of all alphaviruses, but this assay has the disadvantage of being very time-consuming and runs a high risk of contamination since it relies on nested PCR principles. Real-time RT-PCR assays have been developed for the detection of CHIKV (Ho *et al*., 2010; Ummul *et al*., 2010), EEEV and WEEV (Kang *et al*., 2010), but currently, no real-time PCR assay exists that can detect all of the alphaviruses simultaneously.

Real-time RT-PCR technologies have several advantages over conventional RT-PCR assays, including speed, reliability and reproducibility, improved dynamic range, quantitative measurement, a lower risk of contamination, increased sensitivity and specificity and easy standardisation (Bustin & Mueller, 2005; Ho *et al*., 2010). Real-time RT-PCR assays rely on detection chemistries that can be either specific (probe-based) or non-specific (non-probe based) (Bustin & Nolan, 2004; Bustin & Mueller, 2005). Non-probe based chemistries such as SYBR® Green I involves the binding of fluorescent dyes to double stranded DNA, which then emits a fluorescent signal

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(Bustin & Nolan, 2004). Advantages of these types of chemistries are that they are cost-effective and easy to develop and adapt from an optimised conventional RT-PCR assay (Yap *et al*., 2010). The disadvantages of non-probe based chemistries are that the specificity of these assays relies on the specificity of the primers and melting curve analyses need to be performed post-amplification in order to identify the correct target (Mergny & Lacroix, 2003). Probe-based chemistries involve sequence-specific fluorescent probes which generate a fluorescent signal only when these probes bind to their specific targets (Bustin & Mueller, 2005). Assays employing these chemistries therefore possess an added level of specificity, although the primer and probe design for these assays may be difficult (Bustin & Mueller, 2005).

In this chapter, the development of a fast and reliable pan-alphavirus real-time RT-PCR assay was attempted using hydrolysis probe PCR technology. The development of such an assay would be important in enhancing clinical diagnostic procedures. Additionally, such a rapid and sensitive nucleic acid detection tool would be more practical and ethically sound (as opposed to using murine neonates or cell culture for virus isolation) for the identification of alphaviruses in surveillance and would therefore improve alphavirus surveillance activities in the future. The *nsP4* gene region was chosen for the development of primers and probes since this gene is the most conserved amongst all of the alphavirus species (Strauss & Strauss, 1994).

4.2 Materials and methods

4.2.1 Virus strains

The alphavirus isolates and their corresponding GenBank accession numbers, used for the design of the primers and the probes in this study are listed in Table 4.1. Alphavirus specimens available at the Centre for Emerging and Zoonotic Diseases (formerly Special Pathogens Unit) of the National Institute for Communicable Diseases of the National Health Laboratory Service (NICD-NHLS) for the testing and validation of the real-time RT-PCR assay included Sindbis virus (SINV), CHIKV, Mayaro virus (MAYV), Ndumu virus (NDUV), Middelburg virus (MIDV), O'nyong-nyong virus (ONNV) and Semliki Forest virus (SFV).

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Table 4.1: Alphavirus strains used in this study for the design and validation* of the pan-alphavirus real-time PCR

Virus	Isolate	Year of isolation	Accession numbers
Aura	BeAR 10315	Unknown	NC_003900, AF126284
Barmah Forest	BH2193	Unknown	NC_001786, U73745
Chikungunya	H817	2001	NC_004162, AF369024
EEEV	Not available	Unknown	NC_003899, X63135
Highlands J	585-01	2001	NC_012561, FJ827631
Mayaro	Not available	1959	NC_003417, AF237947
Middelburg	SA AR749	1974	J02246, AF339486
Ndumu	SA AR2211	1980	NC_016959
O'nyong'nyong	Gulu strain	Unknown	NC_001512, M20303, M33999
Ross River	NB5092	1988	NC_001544, M20162
Semliki Forest	Uganda	1989	NC_003215, AY112987
Sindbis	SA AR86	1954	NC_001547, J02363, U38305
VEEV	Not available	1992	NC_001449, L04653
WEEV	71V-1658	1971	NC_003908, AF214040

*Only Sindbis virus, Chikungunya virus, Mayaro virus, Ndumu virus, Middelburg virus, O'-nyong-nyong virus and Semliki Forest virus were available to be assayed at the NICD-NHLS

4.2.2 Primer and probe design

Degenerate primers (AlphaF, AlphaR, AlphaModF, AlphaModR, AlphaFlap F and AlphaFlapR) and hydrolysis probes (AlprobeJC1 and AlprobeJC2) (Table 4.2) were designed from a multiple alignment of the *nsP4* gene of 14 different alphaviruses, generated using sequence information (Appendix C and Table 4.1) available in the public domain (GenBank, www.ncbi.nlm.nih.gov). Additionally, a primer set (Alpha 2+ and Alpha 2-) developed by Sanchez-Seco and colleagues (2001) and currently used by the NICD-NHLS in a conventional RT-PCR assay to confirm positive alphavirus infections, was included for comparison. Corresponding probes specific for the African alphaviruses were designed for this primer set (AlphaProbeNS1 and AlphaProbeNS2).

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Table 4.2: Primers and probes designed for the real-time RT-PCR for the detection of alphaviruses

Primer or probe[a]	Sequence (5'-3')	Position[b] (5' – 3')
AlphaF	TRT GYG GNA TWC AYM GRG A[c]	6733-6751
AlphaR	AAN GAG GCD ATR TCH GTY TC[c]	6889-6870
AlphamodF	TAC CTA TGC GGS ATW CAC AG[c]	6729-6748
AlphamodR	AAK GAK GCK ATG TCC GTY TC[c]	6889-6870
AlphaflapF	AAT AAA TCA TAA TAT GCG GSA TWC ACA G[c]	6721-6748
AlphaflapR	AAT AAA TCA TAA TAT GCK ATG TCC GT[c]	6886-6861
AlprobeJC1	(FAM) ATA TGT CGG CAG AGG ATT TCG A (BHQ)	6805-6826
AlprobeJC2	(FAM) ACA TGT CGG CAG AAG ACT TTG A (BHQ)	6805-6826
Alpha 2+ (Sanchez-Seco *et al*., 2001)	GIA AYT GYA AYG TIA CIC ARA TG[c]	6379-6401
Alpha 2- (Sanchez-Seco *et al*., 2001)	GCR AAI ARI GCI GCI GCY TYI GGI CC[c]	6574-6549
AlphaProbeNS1	(FAM) TGG ACT CAG CAG TGT TTA ACG TGG (BHQ)	6418-6441
AlphaProbeNS2	(FAM) TGG ACT CAG CGA CAT TCA ATG TCG (BHQ)	6418-6441

[a] Primer and probe sets used were as follows: AlphaF and AlphaR, or AlphaModF and AlphaModR, or AlphaFlapF and AlphaFlapR with AlprobeJC1 and AlprobeJC2, and Alpha 2+ and Alpha 2- with AlphaProbeNS1 and AlphaProbeNS2

[b] Position numbered according to Sindbis virus reference sequence, GenBank Accession number NC_001547 (Strauss *et al*., 1984)

[c] R indicates A or G; Y indicates C or T; N indicates any nucleotide; W indicates A or T; M indicates A or C; D indicates A, G or T; H indicates A, C or T; K indicates G or T; S indicates C or G, I indicates inosine

The primers and probes were analysed for primer-probe interactions (Gibbs free energy change and secondary structures) using OligoAnalyzer 3.1 (Integrated DNA Technologies, http://eu.idtdna.com) (Table 4.3) and Beacon Designer Free Edition (PremierBiosoft, www.premierbiosoft.com). These interactions aid in the prediction of how successfully the primers and probes will hybridise to the target.

The secondary structures (hairpin structures and self dimers) are shown in Appendix D. The Gibbs free energy change (ΔG) is a measure of the spontaneity of development of the most stable secondary structure (OligoArchitect, www.sigmaaldrich.com) - the more negative the change in free energy is, the more stable the secondary structure and thus the more energy is required to break the structure. In general, a ΔG of -3.0 kcal/mol at the 3' end, and a ΔG of -5.0 kcal/mol

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Table 4.3: Primer and probe parameters (OligoAnalyzer 3.1)

	AlphaF	AlphaR	AlprobeJC1	AlprobeJC2	AlphamodF	AlphamodR	AlphaflapF	AlphaflapR	Alpha 2+	Alpha 2-	Alpha ProbeNS1	Alpha ProbeNS2
Length (bp)	19	20	22	22	20	20	28	26	23	24	26	21
Tm (°C)	53.1	52.5	55.9	56.4	54.2	54.8	54	51.8	56.3	73.4	59.2	59.3
GC Content (%)	47.4	45.8	45.5	45.5	50	50	32.1	28.8	34.8	76.9	50	50
GC Clamp	0	0	1	0	1	1	1	0	0	1	0	1
Cross Dimer (ΔG) (kcal/mol)	With AlphaR: -9.2	With AlphaF: -8.22 With AlprobeJC2: -7.3	With AlphaF: -8.26	With AlphaF: -8.26	With AlphamodR: -6.68	With AlphamodF: -7.19	With AlphaflapR: -7.8	With AlphaflapF: -7.53	With AlphaprobeNS1: -8.03 With AlphaprobeNS2: -8.29	With AlphaprobeNS1: -5.92 With AlphaprobeNS2: -7.68	With AlphaprobeNS2: -3.61 With Alpha 2+: -8.03 With Alpha 2-: -3.55	With AlphaprobeNS1: -3.61 With Alpha2-: -5.92 With Alpha2+: -8.29
Self Dimer (ΔG) (kcal/mol)	-8.28	-7.41	-6.76	-8.07	-7.8	-4.64	-7.8	-4.7	-18.98	-10.31	-6.3	-11.9
Hairpin (ΔG) (kcal/mol)	2.67	1.86	-0.2	-0.78	-0.28	1.86	0.22	0.22	2.67	-0.36	-0.15	-4.68

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in the internal region of the primer or probe are deemed acceptable (OligoArchitect, www.sigmaaldrich.com).

The number of mismatches between the target region of each alphavirus and the probes were determined manually from the multiple sequence alignment. Mismatches are shown in Table 4.4.

Table 4.4: Mismatches between the alphavirus target region and probes

Virus	Sequence (Mismatches indicated in red)	Mismatches
AlprobeJC1	ATA TGT CGG CAG AGG ATT TCG A	
Aura	ACA TGT CTG CCG AGG ATT TCG A	3
Barmah Forest	ATA TGT CCG CAG AGG ATT TCG A	1
Chikungunya	ACA TGT CTG CCG AGG ATT TCG A	3
EEEV	ACA TGT CTG CAG AAG ATT TTG A	3
Highlands J	ATA TGT CGG CAG AAG ATT TTG A	2
Mayaro	ACA TGT CTG CCG AAG ACT TCG A	5
Middelburg	ACA TGT CGG CGG AAG ACT TTG A	5
O'nyong-nyong	ATA TGT CAG CCG AAG ACT TCG A	4
Ross River	ATA TGT CGG CAG AAG ATT TTG A	2
Semliki Forest	ATA TGT CGG CCG AAG ACT TTG A	4
Sindbis	ACA TGT CGG CGG AGG ATT TTG A	3
VEEV	ACA TGT CGG CTG AAG ACT TTG A	5
WEEV	ACA TGT CAG CGG AAG ATT TTG A	5
AlprobeJC2	ACA TGT CGG CAG AAG ACT TTG A	
Aura	ACA TGT CTG CCG AGG ATT TCG A	4
Barmah Forest	ATA TGT CCG CAG AGG ATT TCG A	4
Chikungunya	ACA TGT CTG CCG AGG ATT TCG A	4
EEEV	ACA TGT CTG CAG AAG ATT TTG A	2
Highlands J	ATA TGT CGG CAG AAG ATT TTG A	2
Mayaro	ACA TGT CTG CCG AAG ACT TCG A	2
Middelburg	ACA TGT CGG CGG AAG ACT TTG A	1
O'nyong-nyong	ATA TGT CAG CCG AAG ACT TCG A	3
Ross River	ATA TGT CGG CAG AAG ATT TTG A	2

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Table 4.4 continued

Virus	Sequence (Mismatches indicated in red)	Mismatches
Semliki Forest	ATA TGT CGG CCG AAG ACT TTG A	2
Sindbis	ACA TGT CGG CGG AGG ATT TTG A	3
VEEV	ACA TGT CGG CTG AAG ACT TTG A	1
WEEV	ACA TGT CAG CGG AAG ATT TTG A	3
AlphaProbeNS1	TGG ACT CAG CAG TGT TTA ACG TGG	
Chikungunya	TGG ACT CAG CAG TAT TCA ACG TGG	2
Mayaro	TGG ACT CAG CCG TGT ACA ATG TAG	5
Middelburg	TGG ATT CAG CGG TGT TTA ATG TGG	3
Ndumu	TGG ACT CAG CAG TGT TTA ATG TGG	1
O'nyong-nyong	TGG ACT CCG CAG TGT TTA ACG TGG	1
Ross River	TAG ACT CAG CAG TGT TTA ACG TGG	1
Semliki Forest	TGG ACT CGG CAG TGT TCA ACG TGG	2
Sindbis	TGG ACT CAG CGA CAT TCA ATG TCG	7
AlphaProbeNS2	TGG ACT CAG CGA CAT TCA ATG TCG	
Chikungunya	TGG ACT CAG CAG TAT TCA ACG TGG	5
Mayaro	TGG ACT CAG CCG TGT ACA ATG TAG	6
Middelburg	TGG ATT CAG CGG TGT TTA ATG TGG	6
Ndumu	TGG ACT CAG CAG TGT TTA ATG TGG	6
O'nyong-nyong	TGG ACT CCG CAG TGT TTA ACG TGG	8
Ross River	TAG ACT CAG CAG TGT TTA ACG TGG	8
Semliki Forest	TGG ACT CGG CAG TGT TCA ACG TGG	9
Sindbis	TGG ACT CAG CGA CAT TCA ATG TCG	0

4.2.3 Real-time RT-PCR and optimisation

The primers and probes (Roche Diagnostics, Germany) described in section 4.2.2 (Table 4.2), along with the LightCycler® HybProbe RNA Amplification Kit (Roche Diagnostics, Germany), were used to perform real-time RT-PCR. A separate reaction mix was prepared for each primer set and corresponding probes according to the

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manufacturer's instructions. Briefly, 12.2 µl of nuclease-free water, four microlitres of reaction mix, one microlitre of each primer (20 µM working solution), 0.2 µl of each probe (20 µM working solution) and 0.4 µl of enzyme was pipetted into a sterile real-time PCR capillary. One microlitre of template RNA (SINV) was added. The capillary was capped and centrifuged for 5 seconds at 3 000 x *g*. Cycling conditions were set according to the manufacturer's recommendations, with a reverse transcription cycle at 55°C for 10 minutes, a denaturation cycle at 95°C for 30 seconds, 45 cycles of 95°C for 30 seconds, 40°C for 30 seconds (for primers AlphaF, AlphaR, AlphaModF, AlphaModR, AlphaFlapF and AlphaFlapR) or 50°C for 30 seconds (for Alpha 2+ and Alpha 2-) and 72°C for 30 seconds, and a final cooling cycle at 30°C for 30 seconds.

The real-time RT-PCR assay was optimised by setting up a Taguchi array (Rao *et al*., 2003) for each primer set with variables being the primer concentration (0.3 µM, 0.6 µM and 1 µM), probe concentration (0.2 µM, 0.3 µM and 0.4 µM) and magnesium chloride concentration (3 mM, 5 mM and 7 mM). After the optimal reagent concentrations were obtained, reactions were tested at different annealing temperatures ranging between 40°C and 50°C, as well as reverse transcription temperatures of 45°C, 47°C and 50°C and reverse transcription incubation times of 5, 20 and 35 minutes, respectively.

Using the optimal reagent concentrations and temperatures, a real-time RT-PCR run was set up for each primer and probe set to test the assay on RNA of different alphaviruses using the LightCycler® HybProbe RNA Amplification Kit (Roche Diagnostics, Germany). Briefly, a master mix for eight reactions was prepared as follows: 76.5 µl of nuclease-free water, 34 µl of reaction mix, 8.5 µl of each primer (from a 20µM working solution; final concentration 1 µM), 1.7 µl of each probe (from a working solution of 20 µM; final concentration of 0.2 µM), 3.4 µl enzyme and 27.2 µl of $MgCl_2$ (from a stock solution of 25 mM; final concentration of 7 mM) were pipetted into a sterile 1.5 ml Eppendorf tube. The tube content was mixed by pipetting and 19 µl of master mix was pipetted into each capillary. One microlitre of nuclease-free water was added to the negative control capillary, while one microlitre of template (SINV, CHIKV, MAYV, MIDV, SFV, ONNV and NDUV, respectively)

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was added to the other seven capillaries. The capillaries were capped and centrifuged for 5 seconds at 3 000 x *g* (Eppendorf Centrifuge 5415D, Merck, SA). The capillaries were placed in the LightCycler® thermocycler (Roche Diagnostics, Germany). Cycling conditions were set as described in Table 4.5.

Table 4.5: Cycling conditions for the pan-alphavirus real-time RT-PCR assay

Analysis mode	Cycles	Segment	Temperature	Time	Acquisition mode
Reverse Transcription					
None	1		47°C	35 min	None
Denaturation					
None	1		95°C	30 s	None
Amplification					
Quantification	50	Denaturation	95°C	5 s	None
		Annealing	42°C for primers designed in this study, or 50°C for primers designed by Sanchez-Seco *et al.*, 2001	20 s	Single
		Elongation	70°C (slope - 2°C/s)	8 s	None
Cooling					
None	1		30°C	30 s	None

The LightCycler® version 3.5 software was used to analyse the fluorescence, using the second derivative maximum method. The most efficient primer and probe set (AlphamodF and AlphamodR with Alprobe JC1 and AlprobeJC2) was selected for use in further applications.

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4.2.4 Production of synthetic controls

4.2.4.1 Preparation of DNA

Primers AlphamodF and AlphamodR, described in section 4.2.2, were used to produce a 160 bp amplicon representing the *nsP4* gene of alphaviruses using the Sindbis AR 339 (see section 3.2.1) strain as template. This strain was chosen as it is universally available and has a similar amount of mismatches with the primers and probes as the other alphaviruses used in this study. The PCR product was examined using agarose gel electrophoresis as described in section 3.2.4. The PCR product was purified using the Wizard® SV Gel and PCR Clean-up System (Promega, USA) as described in section 3.2.5.

4.2.4.2 Cloning of the *nsP4* gene

The purified 160 bp fragment of the *nsP4* gene of the alphavirus genome was cloned using the pCRII TOPO® TA cloning kit (Invitrogen, USA) according to the manufacturer's instructions. Briefly, one microlitre of the TOPO® vector (10 ng/µl in 50% glycerol, 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 MM DTT, 0.1% Triton X-100, 100 µg/ml BSA and phenol red) was added to one microlitre of a salt solution (1.2 M NaCl, 0.06 M $MgCl_2$) and four microlitres of purified PCR product (10 ng/µl, determined by means of spectrophotometry using the Nanodrop 1 000 (Thermo Fisher Scientific, USA)) in a sterile Eppendorf tube. The tube was incubated at 25°C (room temperature) for 30 minutes and then placed on ice. Three microlitres of the contents of the tube was added to one vial (50µl) of TOP10® *Escherichia coli* (*E. coli*) cells. A negative control containing only *E. coli*, as well as a positive control containing *E. coli* and pUC 19 (10 pg/µl in 5 mM Tris-HCl, 5 mM EDTA, pH 8) was included. The ligation reaction and the two controls were incubated on ice for 5 minutes. The reactions were then heat-shocked at 42°C for 1 minute and immediately placed on ice. Two hundred and fifty microlitres of super optimal broth with catabolite repression (SOC) (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose) was added to each reaction. The reactions were incubated for 1 hour at 37°C in a shaking incubator (Labcon, SA) at 200 rotations per minute. Fifty microlitres of each reaction was plated onto Luria Bertani (LB) agar

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(Merck, SA) plates (1% tryptone, 1% NaCl, 0.5% yeast extract, pH 7.4) containing 50 µg/ml Kanamycin (Invitrogen, UK). Forty microlitres of X-gal (Promega, USA) was added to the ligation plate and the plates were incubated overnight at 37°C.

4.2.4.3 Selection of clones

Clones were selected using blue-white screening through α-complementation. Five white colonies were selected for additional characterisation. These colonies were placed in LB broth (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.4) (Merck, SA) containing 50 µg/ml Kanamycin (Invitrogen, UK) and incubated overnight at 37°C in a shaking incubator (Labcon, SA) at 200 rotations per minute.

4.2.4.4 Plasmid purification

Plasmid DNA was purified using the Wizard® Plus SV Minipreps DNA Purification System (Promega, USA) according to the manufacturer's instructions. Briefly, five millilitres of overnight culture (section 4.2.4.3) was pelleted by centrifugation (Zentrifugen Mikro 200, Hettich, Germany) at 10 000 x *g* for 5 minutes. The pellet was resuspended in 250 µl cell resuspension solution (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 100 ug/ml RNase A). Two hundred and fifty microlitres of cell lysis solution (0.2 M NaOH, 1% SDS) was added to each sample and the tubes were inverted four times to mix the reagents. Ten microlitres of alkaline protease solution was added to each sample and the tubes were inverted four times to mix the reagents. The tubes were incubated at room temperature (25°C) for 5 minutes. Three hundred and fifty microlitres of neutralising solution (4.09 M guanidine hydrochloride, 0.759 M potassium acetate, 2.12 M glacial acetic acid, pH 4.2) was added to each sample and the tubes were inverted four times to mix the reagents. The tubes were centrifuged (Zentrifugen Mikro 200, Hettich, Germany) at 14 000 x *g* for 10 minutes. A spin column was inserted into a collection tube for each reaction and 850 µl of cleared lysate was added. The spin columns were centrifuged (Zentrifugen Mikro 200, Hettich, Germany) at 14 000 x *g* for 1 minute and the flow through was discarded. Seven hundred and fifty microlitres of column wash solution (60% ethanol, 60 mM potassium acetate, 8.3 mM Tris-HCl, pH 7.5, 0.04 mM EDTA, pH

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8.0) was added and the spin columns were centrifuged (Zentrifugen Mikro 200, Hettich, Germany) at 14 000 x *g* for 1 minute. The flow through was discarded and 250 µl of column wash solution was added. The spin columns were centrifuged (Zentrifugen Mikro 200, Hettich, Germany) at 14 000 x *g* for 2 minutes and the flow through was discarded. The spin columns were transferred to new sterile 1.5 ml Eppendorf tubes and 100 µl of nuclease free water was added. The tubes were centrifuged (Zentrifugen Mikro 200, Hettich, Germany) at 14 000 x *g* for 1 minute and the eluted DNA was stored at -20°C until required. The recombinant clones were characterised by performing sequencing to determine the orientation of the insert in relation to the SP6 promoter of the vector, using the M13 forward and reverse primers included in the kit, as described in section 3.2.6.

4.2.4.5 *In vitro* transcription

One recombinant clone containing the insert in the correct orientation with reference to the SP6 promoter was selected. The insert was *in vitro* transcribed using the MegaScript® kit (Ambion, Applied Biosystems, USA) according to the manufacturer's instructions. Briefly, eight microlitres of DNA template (0.5 µg/µl) (obtained in section 4.2.4.4) was added to a sterile PCR tube containing two microlitres of each dNTP (50 mM), two microlitres of a 10 x reaction buffer and two microlitres of SP6 enzyme mix (Ambion, Applied Biosystems, USA). The reaction was centrifuged briefly and incubated at 37°C for 3 hours. One microlitre of TURBO® DNase (2U/µl) was added to remove any DNA and the reaction was incubated at 37°C for 15 minutes.

4.2.4.6 Recovery of RNA

The *in vitro* transcribed RNA was purified using the RNeasy® RNA cleanup kit (QIAGEN, Germany) according to the manufacturer's instructions. Briefly, 80 µl of nuclease-free water and 350 µl of buffer RLT were added to the *in vitro* transcription reaction and mixed. Two hundred and fifty microlitres of 96% ethanol (Merck, SA) was added and the reaction was transferred to an RNeasy® mini column in a two millilitre collection tube. The mini column assembly was centrifuged (Eppendorf

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Centrifuge 5415D, Merck, SA) for 30 seconds at 8 000 x *g* and the flow-through was discarded. The RNeasy® mini column was transferred to a sterile collection tube and 500 µl of buffer RPE was added. The mini column assembly was centrifuged (Eppendorf Centrifuge 5415D, Merck, SA) for 30 seconds at 8 000 x *g*. The wash step was repeated and the mini column assembly was centrifuged (Eppendorf Centrifuge 5415D, Merck, SA) for 2 minutes at 8 000 x *g*. The flow-through was discarded. The RNA was eluted by transferring the mini column to a sterile 1.5 ml Eppendorf tube, adding 50 µl of nuclease-free water and placing it in a centrifuge (Eppendorf Centrifuge 5415D, Merck, SA) for 1 minute at 8 000 x *g*.

4.2.4.7 Determination of RNA concentration

The concentration of the RNA was determined by means of spectrophotometry using the Nanodrop 1 000 (Thermo Fisher Scientific, USA). The RNA copy number was subsequently calculated using the following formula:

$$\text{Copy number} = \frac{\text{Concentration (g/µl)}}{\text{(transcript length x molecular weight of ssRNA)}} \times (6.022\times10^{23})$$

4.2.4.8 Construction of standard curves

The *in vitro* transcribed RNA (obtained in section 4.2.4.6) was serially diluted with nuclease-free water representing dilutions of 10^1 to 10^5 copies per microlitre. The LightCycler® HybProbe RNA Amplification Kit (Roche Diagnostics, Germany) was used to test each of the dilutions in triplicate in a single run. Optimised PCR cycling conditions, as described in section 4.2.3 were used. A standard curve was constructed using the LightCycler® software version 3.5 (Roche Diagnostics, Germany) by plotting the crossing point (Cp) values against the log concentration of the target.

A second standard curve was constructed by serially diluting a second *in vitro* transcribed RNA aliquot to represent 10^1 to 10^5 copies per microlitre, and repeating the run described above using reagents from a different LightCycler® HybProbe RNA Amplification Kit (Roche Diagnostics, Germany).

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4.2.5 Statistical analysis

Data sets of the standard curves constructed in section 4.2.4.8 were compared and analysed with regards to PCR amplification efficiency (E) (determined from the slope of the log-linear phase of the standard curves using the LightCycler® version 3.5 software), limit of detection (LOD) (the lowest concentration where 95% of the positive specimens are detected (Bustin *et al*., 2009)), precision (intra-assay variability based on the standard deviation (SD) and coefficient of variation (CV) (Bustin *et al*., 2009)) and reproducibility (inter-assay variability based on SD and CV (Bustin *et al*., 2009)). The CV was determined by dividing the SD with the mean and converting the value to a percentage. The linear dynamic range (the highest and lowest measureable copy number where the reaction is linear (Bustin *et al*., 2009)) was determined by measuring the coefficient of determination (R^2) for each of the two standard curves constructed in section 4.2.4.8, using the "Pearson correlation" function of Microsoft Excel. Confidence intervals (95%) were constructed for the complete dynamic range using the "Confidence" function of Microsoft Excel.

The student t-test was performed using Microsoft Excel in order to determine whether the variation in copy numbers observed between the two runs described in section 4.2.4.8 were statistically significant. The null hypothesis (H_0) was defined as having no significant difference in copy numbers between the two runs, and the alternative hypothesis (H_1) was defined as having a significant difference in copy numbers between the two runs.

4.2.6 Determination of analytical sensitivity

The analytical sensitivity of the real-time RT-PCR assay was determined for each of the available alphaviruses (SINV, CHIKV, ONNV, NDUV, MIDV, SFV and MAYV) by preparing a serial dilution of each virus (representing a tissue culture infective dose ($TCID_{50}$/ml) of 1 x 10^7 to 1 x 10^1) and performing real-time RT-PCR as described in section 4.2.3. Additionally, the serial dilutions were assayed using the conventional nested RT-PCR assay currently used by the NICD-NHLS to confirm positive alphavirus infections, published by Sanchez-Seco and colleagues (2001).

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4.2.7 Determination of analytical specificity

The analytical specificity of the real-time RT-PCR assay was determined by testing rubella virus (a rubivirus related to alphaviruses in the family *Togaviridae*) as well as a panel of arboviruses including yellow fever virus (10^{6} $TCID_{50}$/ml), dengue virus I ($10^{5.5}$ $TCID_{50}$/ml), Crimean-Congo haemorrhagic fever virus ($10^{4.3}$ $TCID_{50}$/ml), West Nile virus ($10^{7.8}$ $TCID_{50}$/ml) and Rift Valley fever virus ($10^{6.3}$ $TCID_{50}$/ml) using the PCR conditions described in section 4.2.3. These virus isolates were all obtained from the Centre for Emerging and Zoonotic Diseases of the NICD-NHLS and were amplified in suckling mice or in Vero cells (CCL-81) using standard laboratory procedures (World Organisation for Animal Health, 2004).

4.2.8 Diagnostic evaluation

A panel of clinical specimens (n = 19) submitted for arbovirus diagnosis was tested using the PCR protocols as described in section 4.2.3, as well as with the conventional RT-PCR assay developed by Sànchez-Seco and colleagues (2001) currently used by the NICD-NHLS for alphavirus screening. Additionally, 59 mosquito pools were screened for alphavirus RNA using the same PCR assays (Table 4.6).

Table 4.6: Details of the mosquito pools assayed in this study

Specimen number	Specimen origin	Mosquito species	Mosquito sex	Pool size
69/12/1	Etsha1, Botswana	*Anopheles pharoensis*	Female	5
69/12/2	Etsha1, Botswana	*Anopheles squamosus*	Female	20
69/12/3	Etsha1, Botswana	*Coquillettidia flavocincta + Cq. Microannulata*	Female	2
69/12/4	Etsha1, Botswana	*Coquillettidia fuscopennata*	Female	3
69/12/5	Etsha1, Botswana	*Culex pipiens*	Female	76
69/12/6	Etsha1, Botswana	*Culex quinquefasciatus*	Female	3
69/12/7	Etsha1, Botswana	*Culex spp.*unidentifiable	Female	11
69/12/8	Etsha1, Botswana	*Culex horridus*	Female	11
69/12/9	Etsha1, Botswana	*Mansonia Africana*	Female	17
69/12/10	Etsha1, Botswana	*Mansonia uniformis*	Female	51

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Table 4.6 continued

Specimen number	Specimen origin	Mosquito species	Mosquito sex	Pool size
69/12/11	Etsha1, Botswana	*Anopheles arabiensis+implexus+pretoriensis+ziemanni namibiensis*	Female	17
69/12/12	Etsha1, Botswana	*Anopheles argenteolobatus*	Female	9
69/12/13	Etsha1, Botswana	*Anopheles pharoensis*	Female	49
69/12/14	Etsha1, Botswana	*Anopheles squamosus*	Female	50
69/12/15	Etsha1, Botswana	*Anopheles tenebrosus*	Female	5
69/12/16	Etsha1, Botswana	*Coquillettidia fuscopennata*	Female	6
69/12/17	Etsha1, Botswana	*Culex pipiens*	Female	100
69/12/18	Etsha1, Botswana	*Culex pipiens*	Female	179
69/12/19	Etsha1, Botswana	*Culex pipiens*	Female	100
69/12/20	Etsha1, Botswana	*Mansonia Africana*	Female	17
69/12/21	Etsha1, Botswana	*Mansonia uniformis*	Female	50
69/12/22	Etsha1, Botswana	*Culex pipiens*	Female	100
69/12/23	Etsha1, Botswana	*Culex pipiens*	Female	100
69/12/24	Etsha1, Botswana	*Culex pipiens*	Female	88
69/12/25	Etsha1, Botswana	*Mansonia uniformis*	Female	50
69/12/26	Etsha1, Botswana	*Mansonia uniformis*	Female	50
69/12/27	Etsha1, Botswana	*Anopheles pharoensis*	Female	38
69/12/28	Etsha1, Botswana	*Anopheles squamosus*	Female	83
69/12/29	Etsha1, Botswana	*Coquillettidia fuscopennata*	Female	2
69/12/30	Etsha1, Botswana	*Culex pipiens*	Female	32
69/12/31	Etsha1, Botswana	*Culex quinquefasciatus*	Female	1
69/12/32	Etsha1, Botswana	*Mansonia Africana*	Female	24
69/12/33	Etsha1, Botswana	*Mansonia uniformis*	Female	46
69/12/34	Etsha1, Botswana	*Mansonia uniformis*	Male	26
69/12/35	Etsha1, Botswana	*Anopheles spp.*unidentifiable	Female	61
69/12/36	Etsha1, Botswana	*Anopheles argenteolobatus*	Female	2
69/12/37	Etsha1, Botswana	*Anopheles tenebrosus*	Female	9
69/12/38	Etsha1, Botswana	*Culex spp.*unidentifiable	Female	15
166/12-1	Utha diptank, Mpumalanga, South Africa (SA)	*Aedes ochraceus*	Female	1
166/12-2	Utha diptank, Mpumalanga, SA	*Aedes aegypti*	Female	4

(Continued)

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Table 4.6 continued

Specimen number	Specimen origin	Mosquito species	Mosquito sex	Pool size
166/12-3	Utha diptank, Mpumalanga, SA	*Aedes aurovenatus*	Female	5
166/12-4	Utha diptank, Mpumalanga, SA	*Aedes mcintoshi*	Female	22
166/12-5	Utha diptank, Mpumalanga, SA	*Aedes leesoni/alboventralis*	Female	8
166/12-6	Utha diptank, Mpumalanga, SA	*Aedes mcintoshi*	Female	11
166/12-7	Utha diptank, Mpumalanga, SA	*Culex neavei*	Female	9
166/12-8	Utha diptank, Mpumalanga, SA	*Aedes circumluteolus*	Female	1
166/12-9	Utha diptank, Mpumalanga, SA	*Aedes vexans* ssp. *Arabiensis*	Female	1
166/12-10	Utha diptank, Mpumalanga, SA	*Aedes fryeri*	Female	1
166/12-11	Utha diptank, Mpumalanga, SA	*Aedes aegypti*	Female	3
166/12-11	Utha diptank, Mpumalanga, SA	*Aedes aegypti*	Female	3
166/12-12	Utha diptank, Mpumalanga, SA	*Aedes sudanensis*	Female	2
166/12-13	Utha diptank, Mpumalanga, SA	*Aedes* spp. unidentifiable	Female	1
166/12-14	Utha diptank, Mpumalanga, SA	*Aedes unilineatus*	Female	1
166/12-15	Utha diptank, Mpumalanga, SA	*Culex quinquefasciatus*	Female	3
166/12-16	Utha diptank, Mpumalanga, SA	*Aedes Eritrea*	Female	9
166/12-17	Utha diptank, Mpumalanga, SA	*Aedes aurovenatus*	Female	1
166/12-18	Utha diptank, Mpumalanga, SA	*Aedes quasiunivittatus/aerarius*	Female	9
166/12-19	Utha diptank, Mpumalanga, SA	*Anopheles arabiensis (gambiae* complex)	Female	17
166/12-20	Utha diptank, Mpumalanga, SA	*Anopheles coustani*	Female	2

4.3 Results

4.3.1 Production of standard RNA

The 160 bp target region of SINV isolate AR 339 was effectively cloned and characterised by subsequent sequencing. The concentration of the *in vitro* transcribed RNA was measured and calculated to be 300 ng/µl, which is equal to 1.80 x 10^{12} molecules per microlitre.

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4.3.2 Construction of standard curves

Serial dilutions of the SINV RNA were tested in triplicate in two separate runs using the optimised PCR assay. Results of the first run are shown in Figure 4.1. Subsequently, a standard curve was constructed using the LightCycler® software version 3.5 (Figure 4.2).

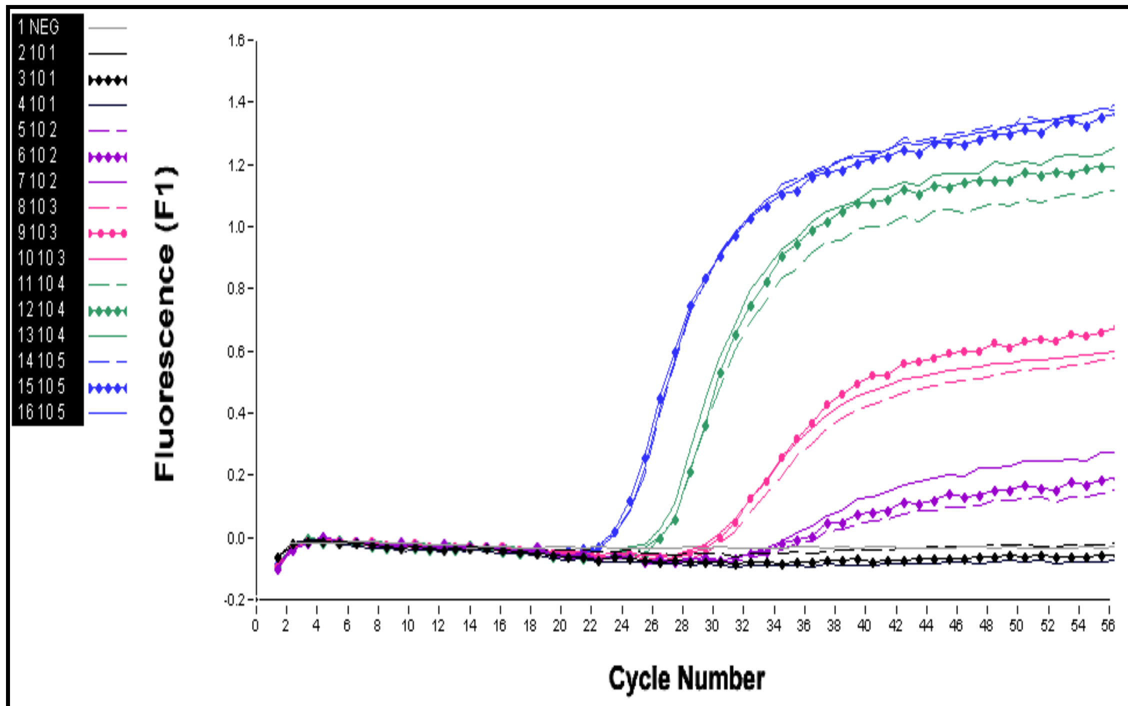

Figure 4.1: Real-time RT-PCR graph showing the increase in fluorescence for serial dilutions of *in vitro* transcribed RNA representing 10^1 to 10^5 copies/µl (in triplicate)

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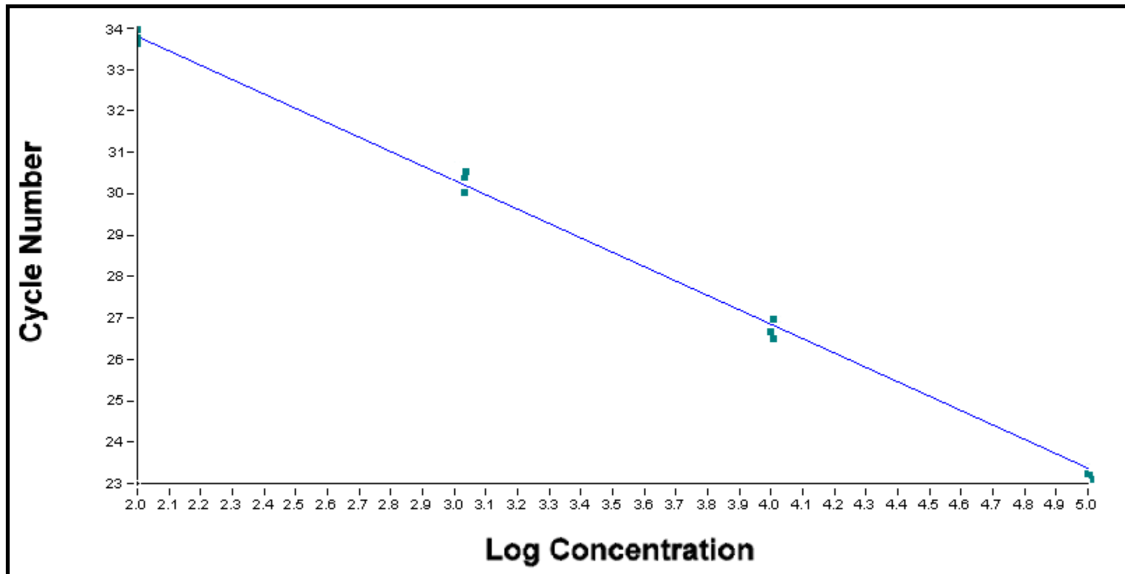

Figure 4.2: Standard curve for the real-time RT-PCR assay constructed by plotting crossing point values versus the log concentration of the RNA triplicates

The PCR assay was able to detect 100 copies (10^2) of synthetic SINV RNA in the first run (LOD), and 1 000 copies in the second run (10^3). The characteristics of the standard curve for the first run were as follows:

Error rate: 0.2

E: 94.02%

Slope: -3.474

R^2: 0.99

Intercept: 41.21

$y = -3.474x + 41.21$

The characteristics of the second standard curve were comparable to those of the first run. Table 4.7 and Figure 4.3 summarises the data of the two real-time RT-PCR runs with regards to the SD, CV and mean.

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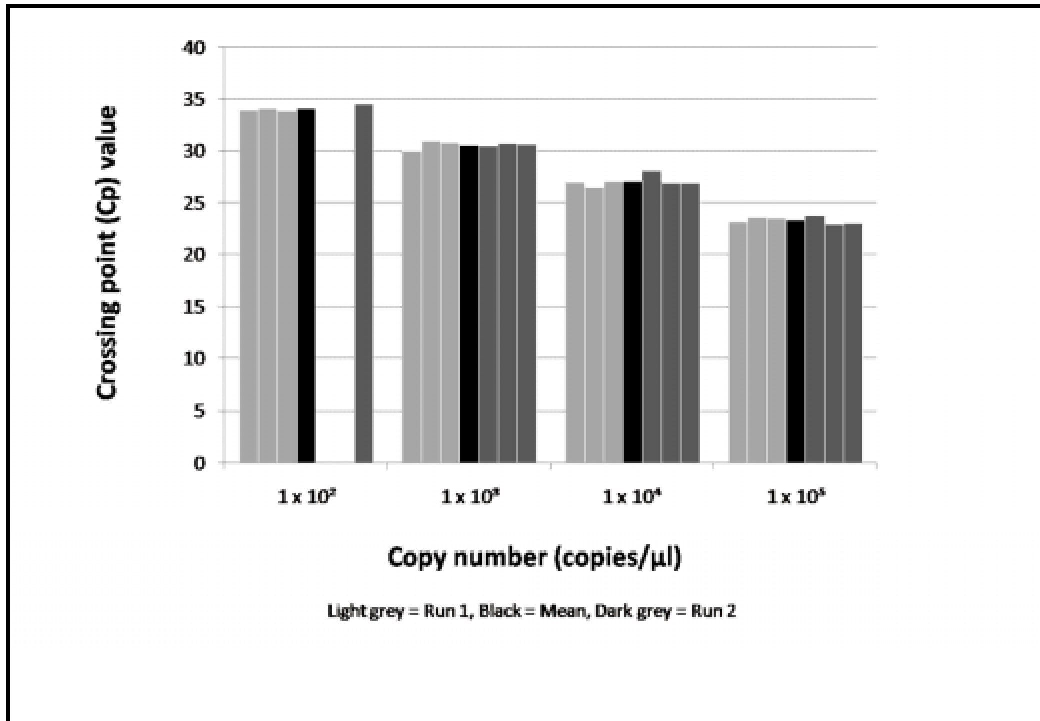

Figure 4.3: Graph showing a comparison of the Cp values for the different serial dilutions in different PCR runs (light grey – run 1, dark grey – run 2, black – mean)

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Table 4.7: Summary and statistical analysis of the data sets for the two independent real-time RT-PCR runs

Copy number	CP value			Mean	SD	CV (%)	Copy number	CP value			Mean	SD	CV (%)
10^1	ND	ND	ND				10^1	ND	ND	ND			
10^2	33.96	34.12	33.84	33.973333	0.1404754	0.4134872	10^2	ND	ND	34.52	34.52		
10^3	30.02	30.96	30.78	30.586667	0.4989322	1.6312081	10^3	30.36	30.73	30.64	30.576667	0.1929594	0.6310675
10^4	26.96	26.54	27.12	26.873333	0.2995552	1.1146932	10^4	27.98	26.93	26.88	27.263333	0.6211548	2.2783525
10^5	23.22	23.58	23.56	23.453333	0.2023199	0.8626487	10^5	23.67	22.96	23.01	23.213333	0.3962743	1.7070979
Average					0.2853207	1.0055093	Average					0.4034629	1.5388393

Run 1 and 2 combined

Copy number	Mean	SD	CV (%)	CI (95%)	Min Cp	Max Cp	SEM	P-value
10^2	34.11	0.2964231	0.8690212	0.2371835	33.84	34.52	0.1482116	0.15
10^3	30.581667	0.3383736	1.1064589	0.2707503	30.02	30.96	0.1381404	
10^4	27.068333	0.4856508	1.7941658	0.3885944	26.54	27.98	0.1982661	
10^5	23.333333	0.3105908	1.3311036	0.2485199	22.96	23.67	0.1267982	
Average		0.3577596	1.2751873				0.1528541	

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The CV ranged from 0.41% to 2.29%. The recommended range for CV is between 2% and 4% (Pfaffl, 2001). The standard deviations for the first and the second run were 0.29 and 0.4, respectively.

4.3.3 Analytical sensitivity and specificity

The analytical sensitivity of the real-time RT-PCR assay was determined by testing serial dilutions of each available alphavirus. Figure 4.4 shows the relationship between the SINV synthetic control RNA copies/µl and the SINV titer of infectious virus ($TCID_{50}$).

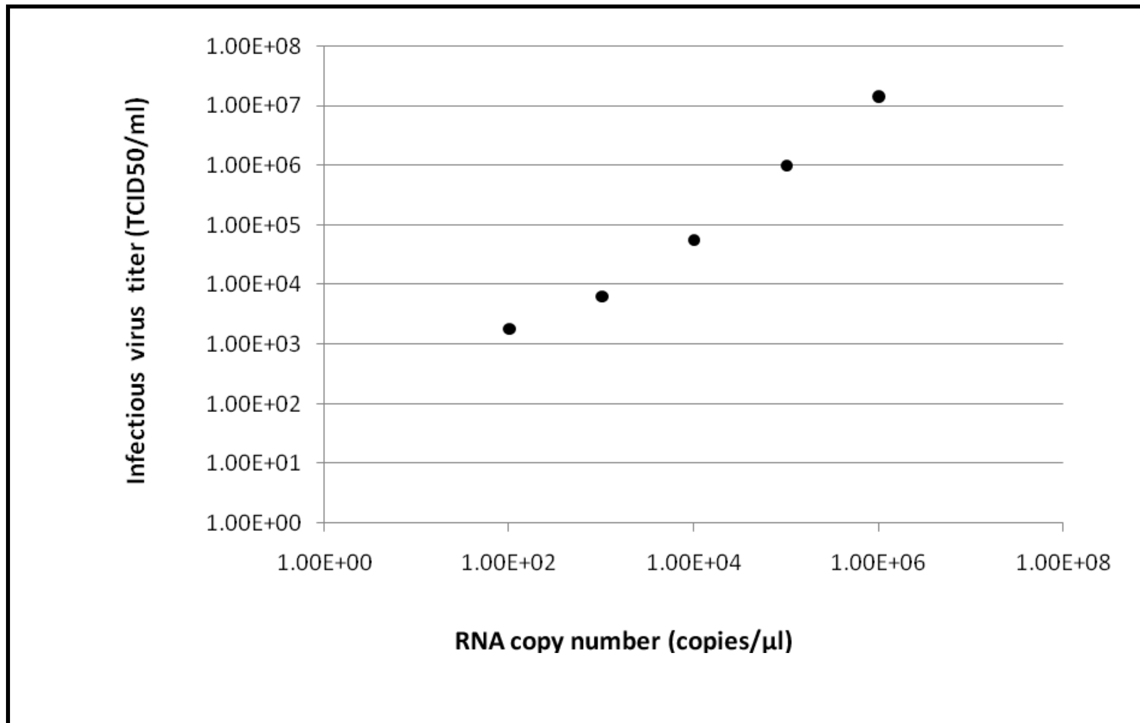

Figure 4.4: Graph illustrating the relationship between the real-time PCR RNA copy number and the infectious virus titer ($TCID_{50}$)

Table 4.8 summarises the PCR result and limit of detection of the assay for each different alphavirus tested using both conventional and real-time RT-PCR. The limit of detection for the conventional RT-PCR assay was better than the limit of detection for the real-time PCR assay for most of the alphaviruses assayed.

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Table 4.8: Virus panel tested using the pan-alphavirus real-time RT-PCR assay and the conventional nested RT-PCR assay

Alphavirus	PCR result	Limit of detection for real-time RT-PCR assay (approximate)	Limit of detection for conventional nested RT-PCR assay
Chikungunya virus	Positive	1 x $10^{3.25}$ $TCID_{50}$/ml	1 x $10^{1.25}$ $TCID_{50}$/ml
Mayaro virus	Positive	1 x $10^{6.25}$ $TCID_{50}$/ml	Not detected
Middelburg virus	Positive	1 x $10^{3.75}$ $TCID_{50}$/ml	1 x $10^{3.75}$ $TCID_{50}$/ml
Ndumu virus	Positive	1 x 10^{6} $TCID_{50}$/ml	1 x 10^{4} $TCID_{50}$/ml
O'nyong-nyong virus	Positive	1 x 10^{5} $TCID_{50}$/ml	1 x 10^{1} $TCID_{50}$/ml
Semliki Forest virus	Positive	1 x $10^{2.25}$ $TCID_{50}$/ml	1 x $10^{1.25}$ $TCID_{50}$/ml
Sindbis virus	Positive	1 x 10^{4} $TCID_{50}$/ml	1 x 10^{1} $TCID_{50}$/ml

The real-time RT-PCR assay had a high level of analytical specificity, as illustrated by the lack of amplification products when using the alphavirus-specific primers and probes (Table 4.2) with RNA extracted from greatly concentrated stocks of arboviruses and rubella virus (Figure 4.5).

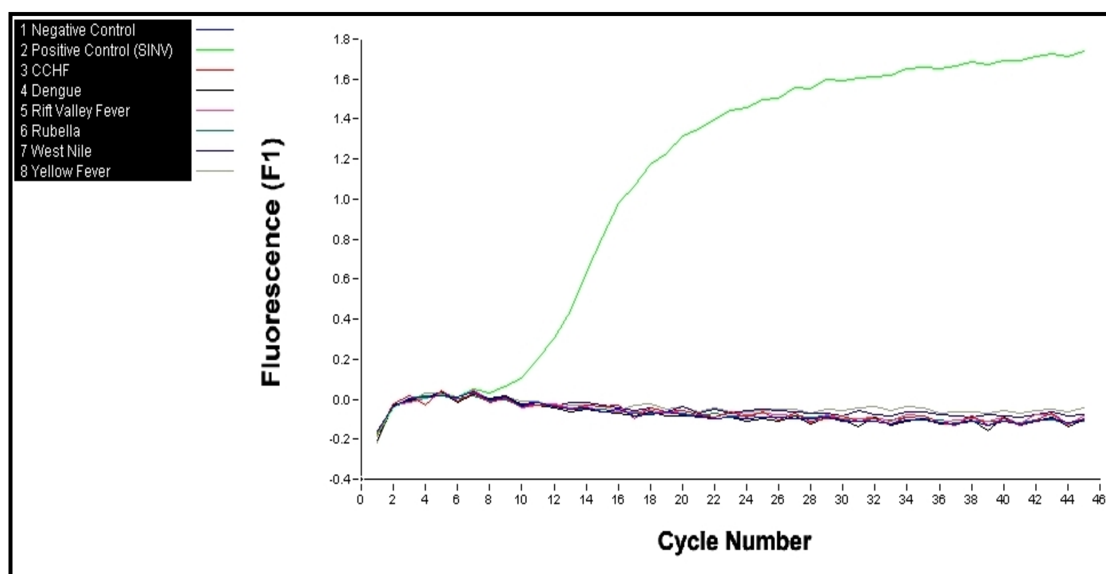

Figure 4.5: Graph illustrating the specificity of the real-time RT-PCR assay by the amplification of the positive control sample (SINV) and the non-amplification of the negative control, Crimean-Congo haemorrhagic fever (CCHF), dengue, Rift Valley fever, rubella, West Nile and yellow fever virus samples.

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4.3.4 Diagnostic evaluation

Table 4.9 summarises the results for the diagnostic specimens screened in this study, using the conventional RT-PCR currently used by the NICD-NHLS for alphavirus diagnosis, and the novel real-time RT-PCR developed in this study. All of the mosquito pools screened in this study tested negative using both the conventional and real-time PCR assays (results not shown).

Table 4.9: Results obtained for the diagnostic panel using conventional and real-time PCR

Specimen number	Expected result (from previous diagnostic testing)	Real-time PCR result	Conventional PCR result
MAF2:2010 2B (SA 278/12)	Negative (Kunjin virus)	Negative	Negative
MAF3: 2010 3B	Negative (Japanese encephalitis virus)	Negative	Negative
MAF2: 2010 2A	Negative	Negative	Negative
MAF3: 2009 3A	Negative (Dengue virus 3)	Negative	Negative
MAF2: 2010 3C	Negative	Negative	Negative
MAF3: 2009 3B	Negative (Dengue virus 1)	Negative	Negative
MAF2: 2010 2B	Negative (Kunjin virus)	Negative	Negative
MAF2: 2012 2A (SA 277/12)	Negative (Japanese encephalitis virus)	Negative	Negative
MAF3: 2009 3C	Negative	Negative	Negative
MAF2: 2012 2C (SA 279/12)	Negative	Negative	Negative
MAF 2011 1B	Positive (Ross River virus)	Positive	Positive
MAF 2011 2B	Negative (Dengue virus)	Negative	Negative
MAF 2011 3C	Negative (Dengue virus 3)	Negative	Negative
MAF 2011 1C	Negative (Murray Valley encephalitis virus)	Negative	Negative
MAF 2011 3B	Negative	Negative	Negative
MAF 2011 2C	Negative	Negative	Negative
MAF 2011 1A	Negative (Dengue virus 2)	Negative	Negative
MAF 2011 3A	Positive (Chikungunya virus)	Positive	Positive
MAF 2011 2A	Negative (Japanese encephalitis virus)	Negative	Negative

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4.4 Discussion

Substantial possibilities of misdiagnosis exist when Sindbis and other alphavirus infections are diagnosed based on clinical symptoms alone (Kurkela *et al*., 2005; Manni *et al*., 2008). It is therefore of the utmost importance to confirm these diagnoses with up-to-date assays and techniques such as real-time PCR (Manni *et al*., 2008). Additionally, the surveillance of alphaviruses using the latest assays is essential in preventing major outbreaks such as the CHIKV that occurred in the Indian Ocean islands in 2005 (Gould & Higgs, 2009). Real-time RT-PCR has several advantages over the conventional methods (virus isolation, serology and conventional RT-PCR) currently used for alphavirus diagnosis. These advantages include a faster turnaround time, increased sensitivity, a lower risk for contamination and being a more ethically appropriate approach to alphavirus screening as compared to virus isolation in murine neonates.

In this chapter, primers and probes were designed in the *nsP4* gene region of the alphavirus genome. The *nsP4* gene region was chosen since this gene is the most conserved amongst all of the alphavirus species (Strauss & Strauss, 1994). The primers and probes were designed with degenerate bases filling the non-preserved positions so that the primer sequences matched the sequences of the alphaviruses available in the public domain (GenBank, www.ncbi.nlm.nih.gov). For this reason, all of the alphaviruses from all of the antigenic complexes should theoretically be able to be detected with varying sensitivities using the real-time RT-PCR assay developed in this study.

Three different sets of primers were designed for the purposes of this study: AlphaF and AlphaR, AlphamodF and AlphamodR, and AlphaflapF and AlphaflapR. The AlphaF and AlphaR primers showed a lower sensitivity as compared to the AlphamodF and AlphamodR primers, as was expected since the Alphamod primers contained less degenerate bases. Some studies have suggested the use of a 12 bp AT-rich flap at the 5' end of the primers in order to improve the fluorescent signal and/or sensitivity and specificity of the PCR assay (Afonina *et al*., 2007; Wei & Clover,

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2008). However, in the current study, the Alphaflap primers were not able to detect any of the alphaviruses effectively (very low sensitivity). This was probably because the primer sequence after the flap was short and contained a number of degenerate bases. Primers Alpha2+ and Alpha 2- showed excellent sensitivity in a study conducted by Sanchez-Seco and colleagues (2001) when used in a nested conventional RT-PCR (25 plaque-forming units per tube), but did not work effectively with their corresponding probes (designed in the current study). For this reason, AlphamodF and AlphamodR were selected for all further applications.

The probes designed in this study contained a number of mismatches with the alphavirus strains used due to the high diversity and limited sequence similarity amongst the members of the *Alphavirus* genus. Hughes and colleagues (2004) recommended that the number of mismatches between the target and the hydrolysis probe not exceed four nucleotide bases. Additionally, a previous study conducted by Nadin-Davis and colleagues (2009) has indicated that even a single mismatch between the target and the probe may lead to reduced sensitivity and false-negative results. Hydrolysis probes may therefore be of restricted use in real-time PCR assays when limited sequence homology between strains exists. The high number of mismatches between the probes designed in this study and some of the alphavirus targets may explain the limited sensitivity obtained for some of the alphaviruses assayed in this study. Furthermore, the occurrence of unavoidable secondary structures such as hairpins and cross-dimers could be an added explanation for the low sensitivities observed.

In the development of a real-time PCR assay, the optimisation of the reagents and temperature settings is crucial for obtaining dependable and reproducible results. Real-time PCR assays can be optimised with regards to cycling conditions, primer and probe concentrations, $MgCl_2$ concentration and template concentration. During optimisation of the assay developed in this study, primers AlphamodF and AlphamodR with corresponding probes AlprobeJC1 and AlprobeJC2 proved to yield the best results. The real-time RT-PCR assay was adapted to the Lightcycler® 480 platform using the same optimised conditions as described for the Lightcycler® version 3.5 platform and yielded similar results. In all PCR runs performed during this study, a negative or no template control (NTC) was included in order to detect

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contamination (Muska *et al*., 2007), as well as a synthetically constructed positive control in order to determine whether the primer-probe combination was able to detect the desired nucleotide fragment. The chances of contamination were reduced by good laboratory practices and none of the negative controls were amplified. RNA integrity was critical in obtaining reliable results using this novel PCR assay, therefore freshly extracted RNA should be used whenever possible.

When PCR assays are used for quantification purposes, consistent amplification efficiency is essential, particularly when an external standard is used. The two separate standard curves constructed in this study both had efficiencies of approximately 94% and the standard error was 0.2, indicating fair precision (acceptable: <0.25, good precision: <0.167) (Invitrogen, 2012). The linear dynamic range for this assay was determined over five orders of magnitude (10^1 - 10^5), and the R^2 value of 0.99 indicates a good confidence for correlation between the concentration of the starting material and the Cp value (higher concentrations of synthetic virus were not included in the standard curves as they repressed the detection of the lowest concentrations). The precision (intra-assay variability) (1.3%) and reproducibility (inter-assay variability) (1 – 1.54%) for the assay developed in this study fell outside of the recommended range of 2% to 4%. A two-tailed student t-test was performed to determine whether the difference in Ct values between the two different runs was statistically significant. A p-value of 0.15 (> 0.05) indicated that the differences were not statistically significant.

In order to determine the analytical sensitivity of the PCR assay, seven alphaviruses, namely SINV belonging to the WEEV complex, CHIKV, ONNV, MAYV and SFV belonging to the Semliki Forest complex, NDUV belonging to the Ndumu complex and MIDV belonging to the Middelburg complex, were assayed in this study using both conventional and real-time RT-PCR. For the real-time PCR assay developed in this study, the limit of detection for these viruses ranged from 1 x $10^{2.25}$ $TCID_{50}$/ml to 1 x $10^{6.25}$ $TCID_{50}$/ml, indicating poor analytical sensitivity for certain alphaviruses. The limit of detection for these viruses using conventional PCR was considerably better, indicating that the conventional PCR assay is superior in sensitivity and therefore of higher value in diagnostics and surveillance despite the slow turn-around

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time. Nevertheless, the real-time PCR assay had an excellent specificity, as indicated by the non-amplification of non-alphaviral target genes.

The results of the mosquito and diagnostic data were identical to results obtained using the conventional nested RT-PCR assay currently used by the NICD-NHLS for the confirmation of positive alphavirus infections. It is possible that all of the mosquito pools tested were negative due to the small number of mosquitoes in each pool, the mosquito species and the site of collection of the mosquito pools. The majority of the mosquito pools were collected in either Botswana or Mpumalanga, where the incidence for SINV and many other alphaviruses is predominantly low.

Some conventional RT-PCR assays exist that is able to detect all of the alphaviruses with fair sensitivities and specificities (Pfeffer *et al*., 1997; Sanchez-Seco *et al*., 2001; Grywna *et al*., 2010), but these methods all rely on nested PCR principles and therefore remain time-consuming. Several species-specific real-time RT-PCR assays exist, including SYBR® Green I-based assays for the detection of CHIKV (Ho *et al*., 2010; Ummul *et al*., 2010) and SINV (He *et al*., 2005), and hydrolysis probe-based assays for the detection of CHIKV (Pastorino *et al*., 2005) and SINV (Sane *et al*., 2012a). To our knowledge, this is the first real-time RT-PCR assay that has been developed which is able to detect all of the medically important African alphaviruses, albeit with moderate to low sensitivities.

The assay developed in this study may possibly be improved upon by developing separate primer and probe sets for each different alphavirus due to the limited sequence similarity between the viruses, although this would considerably add to the cost and complexity of the assay. Alternatively, a SYBR® Green I assay might yield better results, but these assays require a representative panel of viruses (which was not available for this study) to determine the correct melting temperature required to perform a melting curve analysis. Additionally, real-time PCR assays employing SYBR® Green generally lack specificity and may therefore also not be desirable.

Due to the limited sequence homology across the *Alphavirus* genus, no sensitive real-time PCR assay has successfully been developed that is able to detect all of the alphaviruses. Such an assay would be an important surveillance tool and has potential

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to be used for active surveillance, e.g. screening alphaviruses in mosquitoes and wildlife. This type of surveillance is essential in obtaining early warnings of possible outbreaks, so that efficient and timely control measures may be put into action (Rautenbach, 2011). However, real-time PCR, as with other molecular diagnostic techniques, is of restricted value for the diagnosis of alphavirus infections in humans due to the short periods of viraemia associated with some alphavirus infections e.g. SINV (Sane *et al*., 2012a). Nonetheless, while serology remains the most important assay for alphavirus diagnosis, a real-time RT-PCR assay such as the one that was developed in this study would be an important component of a comprehensive diagnostic approach, and attempts at improving such assays should be continued.

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CHAPTER 5: CONCLUDING REMARKS

Alphaviruses are distributed globally and are considered to have significant public health impacts. Nevertheless, systematic and informative surveillance efforts are not frequently conducted to determine the true burden of the disease. This situation is further compounded by the concerns about the effects that global climate change could have on mosquito and other vector populations, and the ease and speed of global travel. These factors are heralded as driving forces for the emergence and re-emergence of arboviral diseases. Sindbis fever is considered the mostly widely spread arbovirus infection and has been reported from many Old World countries. Vigilance for the disease is most profound in Scandinavia where it is associated with considerable morbidity in certain communities.

This study provided an update of human SINV cases that were laboratory confirmed in South Africa for a five year period. An outbreak of Sindbis fever occurred during 2010, which coincided with an outbreak of Rift Valley fever. During the outbreak, the Free State and Northern Cape Provinces were most affected, with the highest risk for acquiring a SINV infection being during the late summer and early autumn months. Sindbis fever was more common among males of increasing age, while the main symptoms reported included fever, arthritis, headache, muscle pain and fatigue. Further systematic seroprevalence studies will provide information regarding the incidence and prevalence of SINV and will provide a more clear appreciation of the burden associated with SIN disease.

This study also investigated the genetic diversity of SINV isolates collected from mosquito pools and a human case in South Africa. The results obtained demonstrate that SINV isolates form five separate groups with a considerable amount of genetic variation among these groups. The grouping of the isolates corresponds to the major migratory patterns of birds, suggesting that birds may play a large role in the dispersal of Sindbis viruses between continents.

In support of the suggestion for further systematic surveillance, a rapid real-time RT-PCR assay was developed. Such an assay would be useful for screening mosquito

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specimens for surveillance purposes, and would be a more ethically sound approach compared to the conventional method of virus isolation in murine neonates. Additionally, this assay will assist in the diagnosis of suspected cases of SINV and other alphaviruses in the African setting. The real-time RT-PCR assay developed in this study could detect all of the alphaviruses assayed in this study with varying sensitivities (ranging from 1 x $10^{2.25}$ $TCID_{50}$/ml to 1 x $10^{6.25}$ $TCID_{50}$/ml), and, to our knowledge, is the first assay of its kind.

Alphaviruses are likely to continue to be a public health threat around the globe for many years in the future, as most of these viruses have become established in various geographic locations and have adapted to a wide range of competent mosquito vectors. Active surveillance programmes for alphavirus infections, using the tools developed in this study, have the potential to assist in monitoring the spread and control of these viruses.

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APPENDIX A

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Figure 1.1

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Schmaljohn AL and McClain D (1996). Alphaviruses (Togaviridae) and Flaviviruses (Flaviviridae). In: Baron S, editor. Medical Microbiology, 4th edition. University of Texas Medical Branch: Galveston, Texas.

Figure 54-1 and figure 54-3

Kind regards,

Nadia Storm

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Figure 1.5

CulexNil.jpg (700 × 478 pixels, file size: 30 KB, MIME type: image/jpeg)

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Culex mosquito

***Culex quinquefasciatus*, vecteur du virus du Nil occidental**

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Title : Known as a vector for the West Nile virus, this Culex quinquefasciatus mosquito has landed on a human finger.

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Figure 1.7

Characteristics of the Rash Associated with West Nile Virus Fever

Dayna D. Ferguson,[1,a] Ken Gershman,[2] Adrienne LeBailly,[3] and Lyle R. Petersen[4]

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Presented in part: 2005 National Conference on West Nile Virus, San Jose, California, February 2005.

[a] Assigned to the Colorado Department of Public Health and Environment, Denver, Colorado.

Reprints or correspondence: Dr. Ken Gershman, Communicable Disease Epidemiology, Colorado Department of Public Health and Environment, 4300 Cherry Creek Dr. South, Denver CO 80246 (ken.gershman@state.co.us).

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APPENDIX B

CONFIRMED SINDBIS AND WESTNILE VIRUS CASE INVESTIGATION FORM

FAX/E-MAIL COMPLETED FORM TO: +27118823741 / jacquelinew@nicd.ac.za,
OR SEND TO: NICD, Special Pathogens Unit, Private bag X4, Sandringham, 2131

PATIENT DETAILS

SURNAME, FIRST NAME:

AGE/DOB: GENDER: ☐ M ☐ F

CONTACT NUMBER:

OCCUPATION:

TOWN: DISTRICT: PROVINCE:

CONSULTATION/ADMISSION DETAILS

NAME OF THE CLINICIAN: CELL/TEL NUMBER:

DATE OF FIRST CONSULTATION:

ADMITTED TO HOSPITAL? ☐ FACILITY: REQUIRED ICU CARE? ☐

DURATION OF HOSPITAL ADMISSION: If yes, DURATION OF ICU CARE?

CLINICAL DETAILS ON FIRST PRESENTATION/ADMISSION

PAST MEDICAL HISTORY:
UNDERLYING ILLNESS? ☐ … If yes, WHAT?
IMMUNOSUPRESSION? ☐ … If yes, PLEASE GIVE DETAILS, if possible

DATE OF ONSET OF SINV/WNV ILLNESS:

SYMPTOMS:

☐ FEVER HISTORY
- BI-PHASIC? ☐ Yes ☐ No

☐ MYALGIA
☐ ARTHRALGIA
☐LOCALISED, SPECIFY SITE(S): ____________________
☐GENERALISED
☐ MACULOPAPULAR RASH
☐ NECK STIFFNESS
☐ ABDOMINAL PAIN
☐ LOSS OF APPETITE

☐ VOMITING
☐ SORE THROAT
☐ PHOTOPHOBIA
☐ OCULAR PAIN
☐ BLURRED VISION
☐ LOSS OF VISION
☐ HEADACHE
☐ FATIGUE
☐ DIZZINESS

EXAMINATION ON PRESENTATION:

☐ FEVER, PATIENT'S TEMPERATURE=____________°C
☐ SHOCK (↓BP)
☐ DEHYDRATION
☐ JAUNDICE
☐ PALLOR
☐ LYMPHADENOPATHY
☐ PHARYNGITIS
☐ MENINGISM

☐ ENCEPHALITIS
☐ CONFUSION / DELIRIUM / DISORIENTATION
☐ RETINITIS
☐ HEPATOMEGALY
☐ ABDO TENDERNESS
☐ MACULOPAPULAR RASH
☐ LOCALISED, SPECIFY SITE(S):

☐ **HAEMORRHAGE**
If yes …
☐ EPISTAXIS
☐ HAEMATEMESIS
☐ MELAENA
☐ MENORRHAGIA
☐ PETECHIAE BLEEDING FROM VENEPUNCTURE SITES

☐ BLEEDING ELSEWHERE?
If yes, SPECIFY SITE(S):

OTHER CLINICAL FINDINGS?

CLINICAL PROGRESSION

☐ UNEVENTFUL RECOVERY
☐ DEVELOPED COMPLICATIONS … If yes, WHAT?

OUTCOME: ☐ ALIVE ☐ DIED … If yes, DATE OF DEATH:

EXPOSURE

☐ MOSQUITO EXPOSURE
☐ RECENT TRAVEL INSIDE/OUTSIDE OF SOUTH AFRICA?
☐ IF YES, WHERE TO? ____________________
☐ DATE: ____________________

DATE OF EXPOSURE: ____________

DETAILS: ____________________

LABORATORY INFORMATION: OTHER SPECIMENS

WERE CLINICAL LAB TESTS (E.G. FBC, LFT, U/E) ORDERED?

IF YES, PLEASE ATTACH COPIES OF RESULTS, OR PROVIDE LAB NAME AND CONTACT DETAILS

NATIONAL HEALTH LABORATORY SERVICE

National Institute for Communicable Diseases

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APPENDIX C

Multiple alignment produced from sequencing information of the *nsP4* gene of alphaviruses (available in the public domain), primers and probes, using the ClustalW subroutine of the BioEdit Sequence Alignment Editor, version 7.

```
                         10        20        30        40        50        60
                ....|....|....|....|....|....|....|....|....|....|....|....|
Chikungunya     GGCGACAGCATACCTATGTGGGATTCACAGAGAGCTGGTTAGGAGGCTGAACGCCGTCCT
Mayaro          ...C..C..T...T....C..A..................C.CC.C..C..T..T..GT.
Middelburg      A..C..C..C.....G..C..T..A..T.....A........A...T.A..T..A..T..
Onyongnyong     ...A...........G.....C..A.........T....G..A..A..A..T..A..T..
Ross_River      A..T.....T..TT.......C........G...T.A..CC.CC.C.....G........
Semliki_Forest  ......C..T.....G..C..C..C.....G..AT.A..A.....A..A..T..T..GT.
Sindbis         ......T..T...T....C.........C.G..AT.A..GC.T.....T.CG......T.
AlphaF          --------------.R..Y..N..W..YM.R..---------------------------
AlphaR          ------------------------------------------------------------
AlprobeJC1      ------------------------------------------------------------
Alprobe JC2     ------------------------------------------------------------
AlphamodF       ----------........C..S..W.....------------------------------
AlphamodR       ------------------------------------------------------------

                         70        80        90       100       110       120
                ....|....|....|....|....|....|....|....|....|....|....|....|
Chikungunya     CCTACCCAATGTACATACACTATTTGACATGTCTGCCGAGGATTTCGATGCCATCATAGC
Mayaro          G..T..G..CA.C.....C..G.................A..C........T......T.
Middelburg      G..G..G..C..C..C..C..G...........G..G..A..C..T........A...T.
Onyongnyong     G.....G.....C..C.....G..C..T.....A.....A..C........A..T.....
Ross_River      GGCC..G..CA........T.G..C..T.....G..A..A.....T..C...........
Semliki_Forest  A.GC..T..C..G..C...T.G.....T.....G.....A..C..T..C..G.....C..
Sindbis         G..T..A..CA.T..C..G..T...........G..G........T.....A........
AlphaF          ------------------------------------------------------------
AlphaR          ------------------------------------------------------------
AlprobeJC1      --------------------------.T.....G..A...........------------
Alprobe JC2     --------------------------.......G..A..A..C..T..------------
AlphamodF       ------------------------------------------------------------
AlphamodR       ------------------------------------------------------------

                        130       140       150       160       170       180
                ....|....|....|....|....|....|....|....|....|....|....|....|
Chikungunya     CGCACACTTTAAGCCAGGAGACACTGTTTTGGAAACGGACATAGCCTCCTTTGATAAGAG
Mayaro          G.AG.....C.....T..G...CAC..CC....G........T..T..T........A..
Middelburg      ..AG.....CCGC..T..G...G....AC.A.....A.....C..A..G..C..C.....
Onyongnyong     .A.C..T..C..A..G..C..TG....AC.A.....T...........A...........
Ross_River      ......T..CC.A.....T...G.A..............................C.....
Semliki_Forest  .T.T.....CC.C.........C.G...C.A..G........T..A..A..C..C..A..
Sindbis         A.A......C....A...C...C.G..AC....G.....T..C..A..A..C..C..A..
AlphaF          ------------------------------------------------------------
AlphaR          -------------------------------..R..D..Y..H.....N..---------
AlprobeJC1      ------------------------------------------------------------
Alprobe JC2     ------------------------------------------------------------
AlphamodF       ------------------------------------------------------------
AlphamodR       -------------------------------..R........M..M..M..---------
```


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APPENDIX D

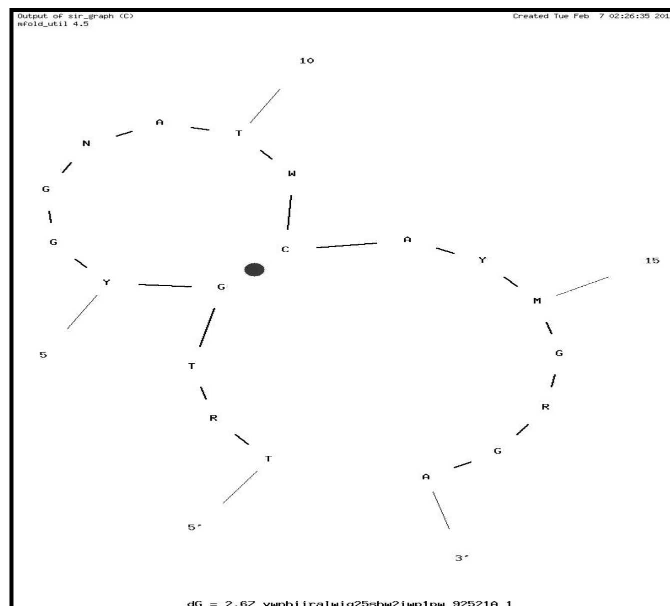
Interactions between primers (AlphaF, AlphaR, AlphamodF, AlphamodR, AlphaflapF and AlphaflapR) and probes (AlprobeJC1 and AlprobeJC2) for the pan-alphavirus real-time PCR determined using the OligoAnalyzer 3.1

Secondary structures for AlphaF

1. Self dimer:
   Delta G -8.28 kcal/mol
   Base Pairs 6

   ```
   5'    TRTGYGGNATWCAYMGRGA
               :     || | ||| |      :
   3' AGRGMYACWTANGGYGTRT
   ```

2. Hairpin:
   Delta G 2.67 kcal/mol



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Secondary structures for AlphaR

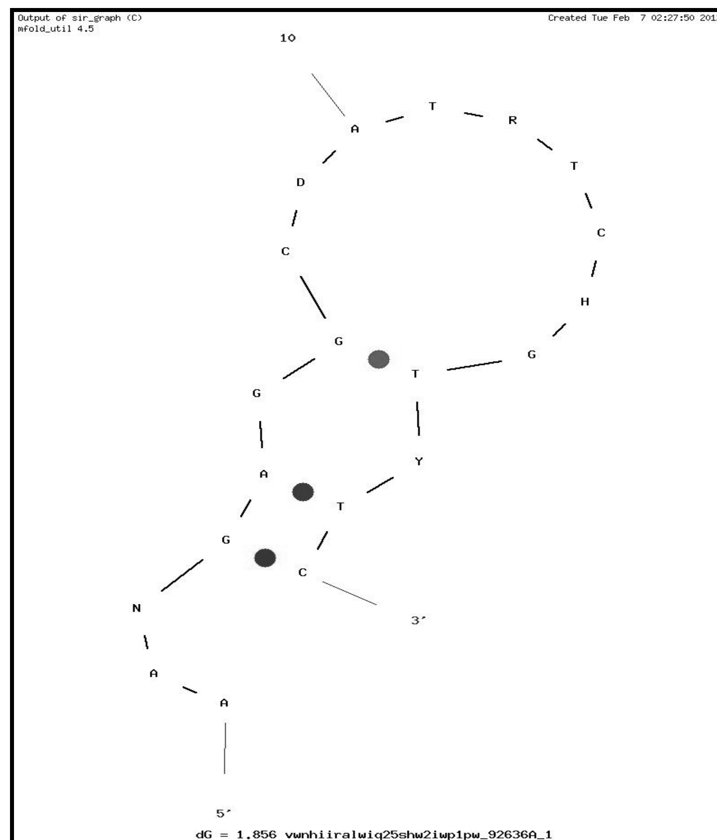
1. Self dimer:

   Delta G -7.41 kcal/mol

   Base Pairs 6

2. Hairpin:

   Delta G 1.86 kcal/mol



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Secondary structures for AlphamodF

1. Self dimer:

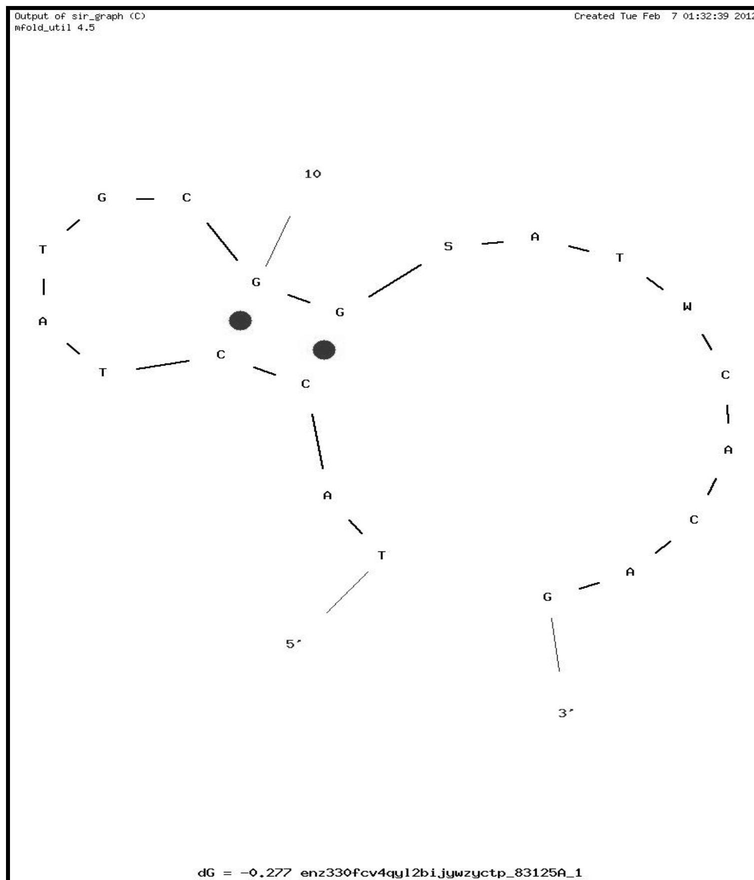
   Delta G -7.8 kcal/mol

   Base Pairs 5

```
5'    TACCTATGCGGSATWCACAG
      :      : :: ::  |||||     :
3' GACACWTASGGCGTATCCAT
```

2. Hairpin:

   Delta G -0.28 kcal/mol



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Secondary structures for AlphamodR

1. Self dimer:

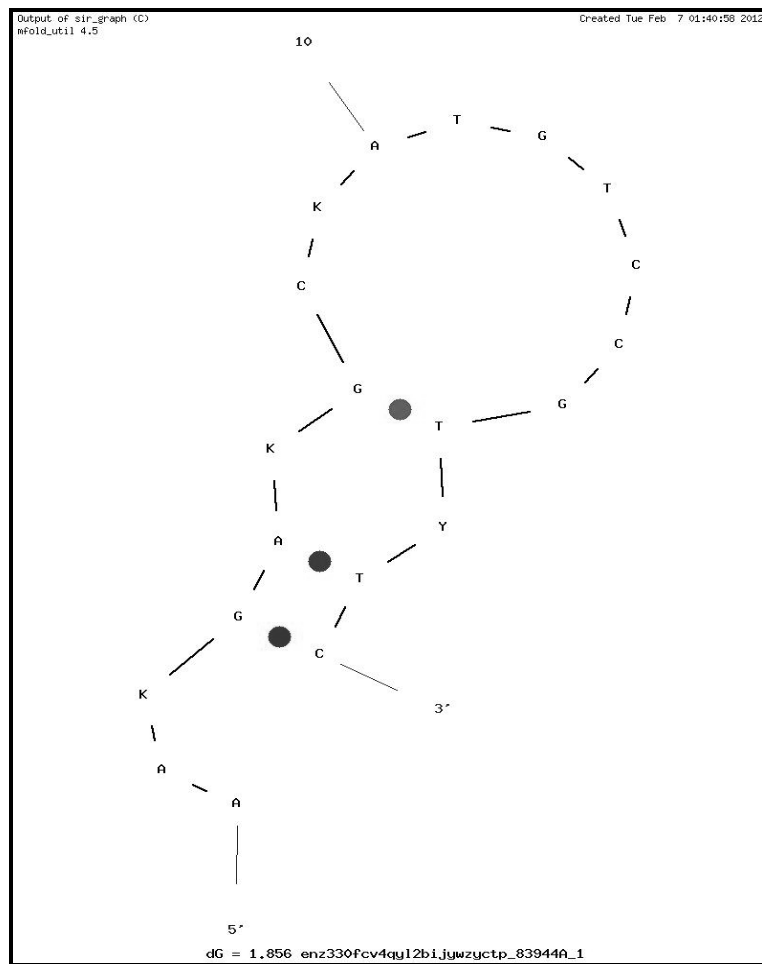
   Delta G -4.64 kcal/mol

   Base Pairs 3

```
5'      AAKGAKGCKATGTCCGTYTC
        :   : : :          | | |   :
3' CTYTGCCTGTAKCGKAGKAA
```

2. Hairpin:

   Delta G 1.86 kcal/mol



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Secondary structures for AlphaflapF

1. Self dimer:

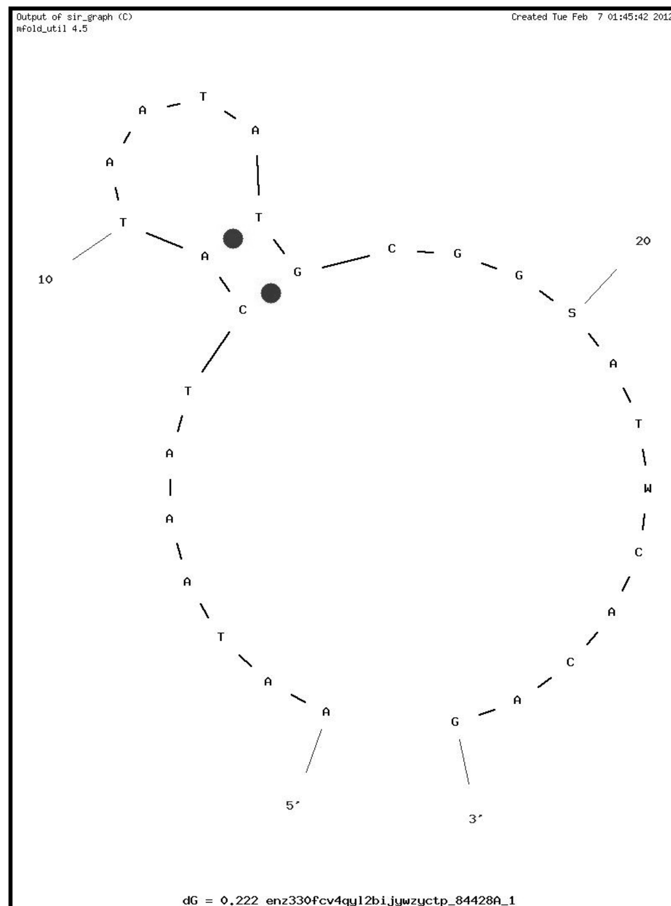
   Delta G -7.8 kcal/mol

   Base Pairs 5

```
5' AATAAATCATAATATGCGGSATWCACAG
                :    : :: ::   |||||   :
3'              GACACWTASGGCGTATAATACTAAATAA
```

2. Hairpin:

   Delta G   0.22 kcal/mol



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Secondary structures for AlphaflapR

1. Self dimer:

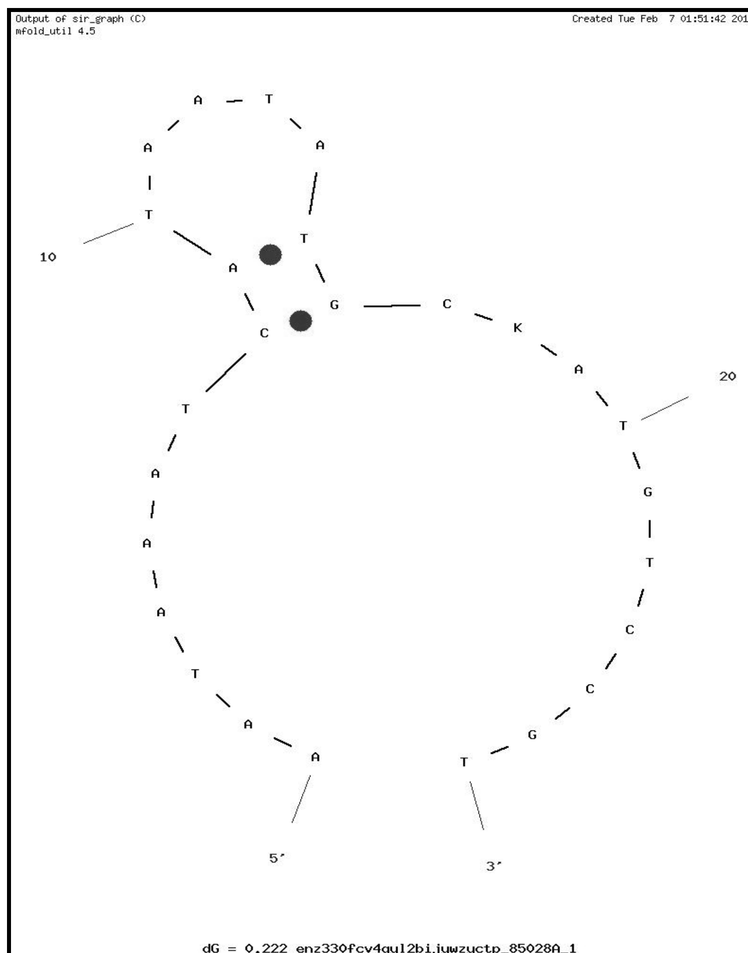
   Delta G -4.7 kcal/mol

   Base Pairs 4

```
5' AATAAATCATAATATGCKATGTCCGT
                 :::: ::         | | ||
3'       TGCCTGTAKCGTATAATACTAAATAA
```

2. Hairpin:

   Delta G 0.22 kcal/mol



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Secondary structures for AlprobeJC1

1. Self dimer:

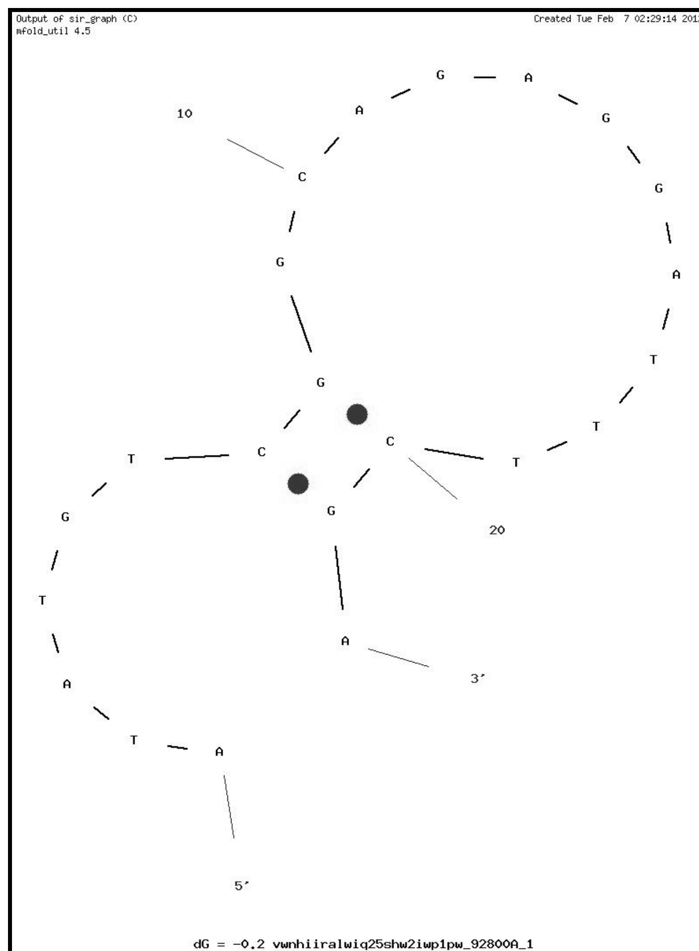
   Delta G -6.76 kcal/mol

   Base Pairs 4

```
5' ATATGTCGGCAGAGGATTTCGA
                                  | | | |
3'                                AGCTTTAGGAGACGGCTGTATA
```

2. Hairpin:

   Delta G -0.2 kcal/mol



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Secondary structures for AlprobeJC2

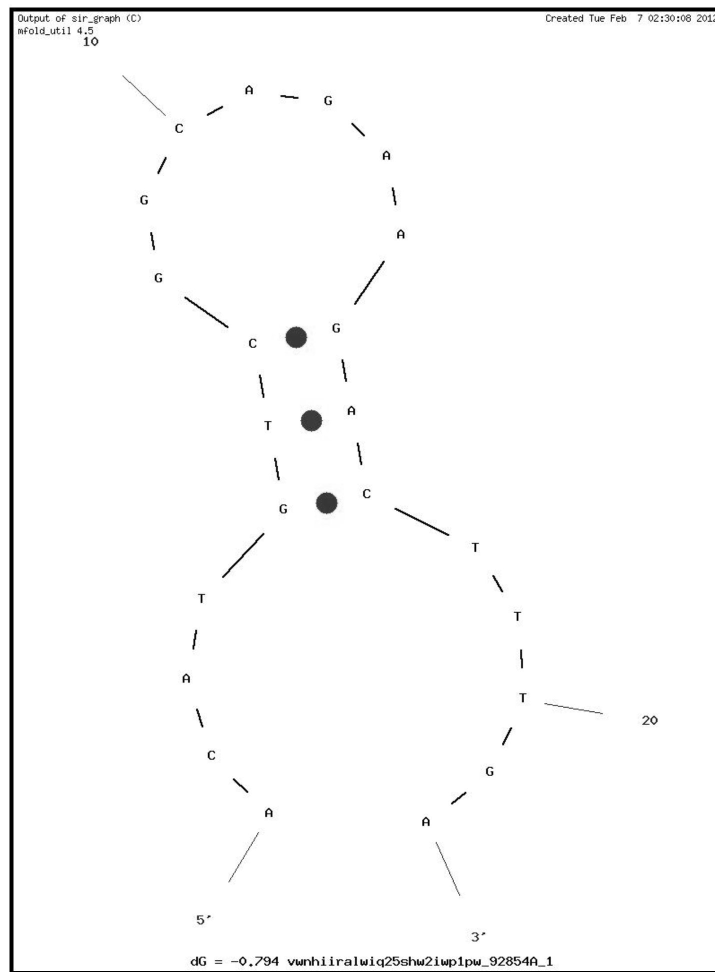
1. Self dimer:

   Delta G -8.07 kcal/mol

   Base Pairs 6

2. Hairpin:

   Delta G -0.79



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