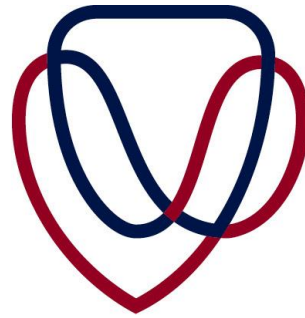


UNIVERSITY OF THE  
FREE STATE  
UNIVERSITEIT VAN DIE  
VRYSTAAT  
YUNIVESITHI YA  
FREISTATA



UFS  
UV

***IMMUNOGENICITY AND SEROLOGICAL  
APPLICATIONS OF FLAVIVIRUS ED III PROTEINS  
AND MULTIPLEX RT-PCR FOR DETECTING NOVEL  
SOUTHERN AFRICAN VIRUSES***

---

**Lehlohonolo Mathengtheng**

**Thesis submitted in fulfillment of the requirements for the degree Ph.D Virology in the Department of Medical Microbiology and Virology, Faculty of Health Sciences, University of the Free State, Bloemfontein**

**Promotor: Prof Felicity Burt, Department of Medical Microbiology and Virology, Faculty of Health Sciences, University of the Free State, Bloemfontein**

**January 2015**

# Table of contents

Table of contents .....	2
Declaration.....	i
Acknowledgements.....	ii
Financial Support .....	iii
Lehlohonolo Mathengtheng, An Obituary .....	v
Publications and presentations.....	vii
List of figures.....	ix
List of tables .....	xii
List of abbreviations.....	xiii
Abstract.....	xviii
LITERATURE REVIEW .....	1
1.1. Introduction and history .....	1
1.2. Classification and molecular characteristics of flaviviruses .....	1
1.3. Transmission and epidemiology of flaviviruses .....	3
1.3.1. Tick-borne flaviviruses .....	3
1.3.2. Mosquito-borne flaviviruses .....	7
1.4. West Nile virus and Wesselsbron virus in South Africa. ....	9
1.5. Other flaviviruses previously identified in South Africa.....	11
1.6. Prevention and treatment of <i>flavivirus</i> infections.....	14
1.7. The WNV-E protein as a diagnostic agent and candidate vaccine.....	15
1.8. Laboratory diagnosis of <i>flavivirus</i> infection. ....	17
1.9. Influence of vertebrate hosts on emerging viruses .....	18
1.9.1. Hantaviruses .....	19
1.9.2. Hantaviruses in Africa .....	19
Problem identification, aim and objectives of the study.....	23
CHAPTER 2 .....	26
THE PREPARATION OF RECOMBINANT <i>FLAVIVIRUS</i> ENVELOPE DOMAIN III (EDIII) PROTEINS USING A BACTERIAL EXPRESSION SYSTEM. ....	26
2.1 Introduction .....	26
2.2. Aim .....	27

2.3. Materials and methods .....	27
2.3.1. Selection of viruses .....	27
2.3.2. Sequence alignment and phylogenetic analyses .....	28
2.3.3. Extraction of RNA .....	28
2.3.4. Amplification of EDIII genes .....	28
2.3.5. Purification of PCR products .....	29
2.3.6. Cloning of amplicons and transformation of cells .....	29
2.3.7. Colony PCR assay for confirmation of positive transformants .....	30
2.3.8. Plasmid purification .....	31
2.3.9. Double digestions.....	31
2.3.10. Determination of DNA concentrations .....	31
2.3.11. DNA sequencing of positive constructs .....	31
2.3.12. Protein expression and SDS-PAGE analysis.....	32
2.3.13. Protein solubility analyses .....	33
2.3.14. Purification of over-expressed proteins and refolding of denatured proteins.....	34
2.3.15. Protein concentration determination.....	35
2.3.16. Characterization of proteins by Western blot analysis.....	35
2.4. RESULTS.....	35
2.4.1. Amino acid alignments and phylogenetic tree .....	35
2.4.2. One step RT-PCR for amplification of the genes encoding the EDIII protein of flaviviruses .....	36
2.4.3. T/A cloning of the EDIII amplicons of LGTV, WNV and WESSV into pGEM®-T- Easy vector .....	37
2.4.4. Cloning of the EDIII genes of LGTV, WNV and WESSV into pQE-80L and pCOLD-TF expression vectors.....	38
2.4.5. Sequence data to confirm the identity of inserts .....	41
2.4.6. SDS-PAGE analyses for expressed proteins .....	42
2.4.7. Protein purification and determination of protein concentration .....	49
2.4.8. Western blot analysis of recombinant proteins .....	51
2.5. SUMMARY .....	52
CHAPTER 3 .....	53
DEVELOPMENT AND USE OF SEROLOGICAL ASSAYS TO DETERMINE THE PRESENCE OF FLAVIVIRUSES IN THE FREE STATE PROVINCE, SOUTH AFRICA.....	53
3.1. Introduction .....	53
3.2. Aim .....	54
3.3. Materials and methods .....	54
3.3.1. Preparation of KUNV cell lysate antigen.....	54

3.3.2. Collection of serum samples.....	55
3.3.3. Preparation of indirect IgG ELISA.....	56
3.3.4. Validation of indirect IgG ELISA.....	56
3.3.5. ELISA Specificity testing .....	56
3.3.6. Analysis of field samples .....	57
3.4. RESULTS.....	57
3.4.1. Validation of ELISA .....	57
3.4.2. Specificity testing .....	57
3.4.3. Screening of field samples .....	60
3.4.4. Detection profiles of field samples by indirect IgG ELISA. ....	61
3.4.5. Geographical location of positive reactors. ....	62
3.5. Summary .....	63
CHAPTER 4 .....	65
MOLECULAR ASSAYS FOR DETECTION OF TICK-BORNE AND RODENT-BORNE VIRUSES.....	65
4.1. Introduction .....	65
4.2. Aim .....	68
4.3. Materials and methods .....	68
4.3.1. RNA controls .....	68
4.3.2. Human and rodent samples for preliminary screening .....	68
4.3.3. RNA extraction .....	69
4.3.3.1. Human serum samples .....	69
4.3.3.2. Rodent organs.....	69
4.3.4. Primers and probes.....	69
4.3.4.1. Flavi-CCHFV nested multiplex PCR.....	70
4.3.4.2. Flavi-Hantavirus TaqMan® multiplex PCR assay .....	70
4.3.5. PCR cycling conditions for the Flavi-CCHFV nested multiplex PCR.....	71
4.3.6. Validation of the Flavi-Hantavirus TaqMan® multiplex PCR assay .....	72
4.3.6.1. Synthesis of RNA controls .....	72
4.3.6.2. Preparation of DNA standards.....	73
4.3.6.3. Synthesis of cDNA .....	73
4.3.6.4. Cycling conditions for Flavi-Hantavirus TaqMan® multiplex PCR assay .....	74
4.3.6.5. Intravariability and intervariability of the real-time assay .....	75
4.3.6.6. Sensitivity of the real-time assay .....	75
4.3.6.7. Sequencing of amplicons and analyses of nucleotide sequences .....	75
4.3.6.8. Screening of samples using hantavirus commercial serological assays.....	75

4.4. RESULTS.....	76
4.4.1. Flavi-CCHFV nested multiplex PCR.....	76
4.4.2. Flavi-Hantavirus TaqMan® multiplex PCR assay .....	78
4.4.2.1. Preparation of DNA standards .....	80
4.4.2.2. Detection of DNA controls and generation of standard curves.....	80
4.4.2.3. Sensitivity of the real-time assay .....	88
4.4.2.4. PCR results for human and rodent samples.....	89
4.4.2.5. Results for the commercial hantavirus IgG assay. ....	91
4.5. Summary .....	91
CHAPTER 5 .....	94
IMMUNOGENICITY OF WEST NILE AND LANGAT EDIII PROTEINS. ....	94
5.1 Introduction .....	94
5.2. Aim .....	95
5.3. Materials and methods .....	95
5.3.1. Animal inoculations.....	95
5.3.2. Antibody responses.....	97
5.4. Results.....	97
5.4.1. Immune responses elicited by recombinant EDIII proteins.....	97
5.4. Summary .....	99
Chapter 6.....	100
Discussion.....	100
References .....	107
Appendix A: Vector maps and multiple cloning sites for plasmids used.....	123
Appendix B Alignment of flavivirus NS5 sequence data .....	126
Appendix C. Alignment of hantavirus sequence data for design of primers and probes .....	128
Appendix D Sequence determined for CCHF from patient 24.....	129
Appendix E Raw data for hantavirus ELISA .....	130
Appendix F Ethics statement and approval .....	131

## Declaration

I, Felicity Jane Burt, certify that the thesis hereby submitted for the Doctor of Philosophy Virology degree at the University of the Free State is the independent effort of **Lehlohonolo Mathengtheng** and has not previously been submitted for a qualification at another university. I furthermore waive copyright of the dissertation in favour of the University of the Free State.

---

Felicity Burt

# Acknowledgements

I would like to thank God for his support and love towards Mathengtheng during his life.

On behalf of Mathengtheng I would like to extend appreciation and gratitude to the following people:

- The Department of Medical Microbiology and Virology, National Health Laboratory Service and University of the Free State for providing the facilities to complete the laboratory work. The staff at the UFS Animal Unit who assisted with the animal work.
- His mother, Belina. I know that you would have been at the top of his list.
- His brothers, Lerato and Lebogang.
- Family members, uncles, aunts and cousins who were so much a part of his life. Please understand that I have not mentioned each family member by name as Matheng would have done. You can be proud of his achievements.
- Mathengtheng had so many friends it is difficult to name them all and there are likely many I am unaware of and whom he would have wished to include in the list. Please understand if I have omitted you, it is not intentional. I would like to specifically mention his colleagues from the Vector-borne and Zoonotic Pathogens Research Group: Rudo Samudzi, Shannon Smouse, Azeeza Rangunwala, Kulsum Kondiah, Carina Combrinck, Manie Hanekom, Danelle Pieters, Natalie Viljoen, Armand Bester, Stephen Makatsa, Nteboheleng Bafazini, Tumelo Sekee.
- The staff in the Department of Medical Microbiology and Virology, including Prof AA Hoosen, Dr D Goedhals, Mrs A van der Spoel, Mrs M Peens and all the staff and medical technologists. The Christmas parties will never be the same without your infectious laughter, enthusiasm, joy and entertainment.
- On behalf of Matheng I would like to thank his many other friends and colleagues for their love, support, friendship and encouragement.

# **Financial Support**

We acknowledge the following sources of funding

- University of the Free State Grow Our Own Timber Fellowship
- Polio Research Foundation
- National Health Laboratory Service Research Trust
- School of Medicine, University of the Free State Postgraduate Bursary



## **Dedication**

On behalf of Lehlolonolo Mathengtheng I dedicate his thesis to his mother, Belina.

## **Remember me**

Do not shed tears when I have gone but smile because I have lived. Do not shut your eyes and pray to God that I'll come back but open your eyes and see all that I have left behind. I know your heart will be empty because you cannot see me but still I want you to be full of the love we shared. You can turn your back on tomorrow and live only for yesterday or you can be happy for tomorrow because of what happened between us yesterday. You can remember me and grieve that I have gone or you can cherish my memory and let it live on. You can cry and lose yourself, become distraught and turn your back on the world or you can do what I want-Smile, wipe away the tears, learn to love again and go on. (David Harkins 1980).

# Lehlohonolo Mathengtheng, An Obituary

By Felicity Burt

June 1985-Sept 2014



*Lehlohonolo Mathengtheng was born on the 10 June 1985 in Virginia in the Free State, South Africa. At the time of his death he lived in Bloemfontein but frequently visited his mother, whom he often spoke of with great love and pride, and his family in Odendaalsrus.*

*Mathengtheng, or as he was called by his colleagues and friends, Matheng, attended primary school from 1991 to 1997 (Grade 1- Grade 7) at Thusanong Primary School, Odendaalsrus. He completed his schooling from 1982 to 2002 (Grade 8- Grade 12) at Eldoret Secondary School, also in Odendaalsrus. He obtained his Matric with Merit in the six subjects including Afrikaans Second Language Higher Grade (HG), English Second Language HG, Sesotho First Language HG, Physical Science HG, Biology HG and Mathematics. During his secondary school years, Mathengtheng was awarded High School prestigious certificates for Sesotho, English, Afrikaans, Biology and Accounting. In addition to these certificates, he won the First National Bank award for the Best Matric Student in Languages and was proficient in seven languages.*

*Mathengtheng continued with his tertiary education at the University of the Free State (UFS) in Bloemfontein. In 2005 he graduated with a Bachelor of Science (BSc) degree in Microbiology. During his undergraduate years he won several awards. In 2004 he received the Free State Botanical Society's award for Best Student in Second Year Botany at the UFS, and the following year was the recipient of the award for Best Student in Third Year Botany. In 2006 he was invited to become a Golden Key member for being among the top 15% of the University's top academic achievers.*

*After receiving his undergraduate degree, he continued with postgraduate studies and was awarded a BSc Honours with Distinction in Microbiology in 2006 and shortly after he was awarded a Master of Science (MSc) in Microbiology. At the time of his death Mathengtheng was nearing completion of his Doctor of Philosophy (PhD) in Medical Virology.*

*From June 2007 to December 2009, Mathengtheng was appointed by the Grow Our Own Timber (GOOT) Fellowship program as a Research Fellow in the Department of Medical Microbiology and Virology in the School of Medicine. During this period he participated in activities determined by the GOOT Academic Group while contributing academically to the Department, assisting with managerial tasks and studying towards his PhD. His responsibilities included lecturing, training and assessment of biomedical science students. Specifically students in BMedSc 3<sup>rd</sup> year, BMedSc Honours, MBChB, technicians, technologists and Intern Medical Scientists in both Microbiology and Virology. At the time of his death he was employed as a Medical Scientist and Junior lecturer in Department of Medical Microbiology and Virology in a joint position with the National Health Laboratory Service (NHLS) Universitas, and the Faculty of Health Sciences, UFS.*

*His research output included presentations at national and international microbiology and infectious diseases conferences. He presented his research results annually at the Research Faculty Forum, Faculty of Health Sciences. Mathengtheng did not just present his work but he frequently, and well deservedly, was the recipient of prizes and awards. In 2008 he was a member of the team awarded the Best Team Exhibition for the National Innovation Competition attending the finals held in Cape Town. The award was presented by the Minister of Science and Technology. In 2010 he was the recipient of the TATA® prestigious award for Top 40 postgraduate candidates in South Africa 2010. He was the winner of the Best Junior Laboratory Paper in 2009 and 2012 at the Faculty of Health Sciences Research Forum and was the runner up in 2011 and 2014. At provincial level he was received the Free State Department of Health Research Day Best Oral Presentation award for Clinical and Laboratory Medicine Track. In 2011 he was the Runner-up in the “3-Minute-Thesis Competition” PhD category, at the UFS.*

*Matheng presented his work with such enthusiasm that he enthralled audiences and inspired younger students. He always had time to assist other members of the Research Group to prepare for their presentations giving them academic advice and the confidence to stand up in front of an audience.*

*More recently he attended and presented his research at three international conferences: the 14<sup>th</sup> International Congress on Infectious Diseases (ICID), held in Miami, Florida, USA in 2010, the 9<sup>th</sup> Young Scientist Association (YSA) PhD symposium held at the Medical University of Vienna, Austria in 2013 and the 16<sup>th</sup> International Congress on Infectious Diseases (ICID) held in Cape Town, South Africa in 2014. Not only was he able to take home awards from national conferences but when he attended the meeting symposium in Austria he was winner of the Young Scientists Association (YSA) PhD Symposium-Best Poster Presentation Award, from the Medical University of Vienna, Austria.*

*As part of his PhD research, he studied the serological presence of flaviviruses in the Free State province by preparation of recombinant antigens that were functional in immunoassays. His research also involved evaluation of candidate vaccines and the development of molecular assays for the investigation of zoonotic pathogens and arboviruses. He was first author of a recent publication in an international scientific journal and co-author of a review article on arboviruses in South Africa.*

*Matheng excelled and achieved not only academically but also in leadership roles, participating in Central RAG committee (2003-2004), Botanical Society of South Africa, Free State branch (2005-2007) and Executive Member of the Postgraduate Committee in 2012. He was a member of UFS Choir (2003-2005), actively involved in his church, frequently asked to be the master of ceremonies for various corporate and social events and enjoyed writing short stories. Sport was not high on his list of activities, although he did briefly mention that he thought he would try tennis when his PhD was complete.*

*The previous pages list his academic achievements through school and university. Matheng died within weeks of submitting his thesis for his PhD, however his achievements were far greater and more valuable. Matheng had a PhD in “Living Life”. He lived his life to the full every single day, his cup was never half empty but always half full, his optimism inspired many around him and he won the hearts of friends and colleagues. He touched an uncountable number of people of all ages during his lifetime. He was a cherished son, brother, cousin, nephew, friend. He will be remembered, his broad and cheerful smile will be sorely missed, we will laugh and shed tears when we reminisce and we will be grateful that we knew an outstanding scholar, a friend and a special person.*

*Rest in peace our dear friend Matheng.*

# Publications and presentations

## Publication in international peer-reviewed journal.

In print

Mathengtheng L, Burt FJ. Use of envelope domain III for detection and differentiation of flaviviruses in the Free State province, South Africa. *Vector-Borne and Zoonotic Diseases* 2014;14:261-271.

Burt FJ, Goedhals D, Mathengtheng L. Arboviruses in southern Africa: are we missing something? *Future Virology* 2014; 9: 993-1008.

In preparation

Mathengtheng L, Burt FJ. Evidence for mild infection of Crimean-Congo haemorrhagic fever virus in South Africa (In preparation).

Mathengtheng L, Burt FJ. Development of multiplex real time RT-PCT for rodent-borne hantaviruses and application in surveillance. (In preparation).

## Supplement in international peer-reviewed journal.

Mathengtheng L, Burt FJ. Development of a recombinant antigen and multiplex PCR for differentiation of tick-borne and mosquito-borne flaviviruses. *International Journal of Infectious Diseases* 01/2010; 14. DOI:10.1016/j.ijid.2010.02.1594

## Presentations International

Mathengtheng L & Burt FJ: Development of a recombinant antigen and multiplex PCR for differentiation of tick-borne and mosquito-borne flaviviruses. The 14<sup>th</sup> International Congress on Infectious Diseases (ICID), Miami, Florida, USA. 9-12 March 2010.

Mathengtheng L & Burt FJ. Use of enveloped domain III protein for detection and differentiation of flaviviruses in Free State, South Africa. 9<sup>th</sup> Young Scientist Association (YSA) PhD symposium. Medical University of Vienna, Austria. 19-20 June 2013.

Mathengtheng L & Burt FJ. Development of immunoassays for detection of flaviviruses in the Free State Province, South Africa. 16<sup>th</sup> International Congress on Infectious Diseases (ICID). Cape Town, South Africa, 2-5 April 2014.

## Presentations National

Mathengtheng L & Burt FJ. The development and evaluation of recombinant antigens to differentiate between the members of flaviviruses. Virology Africa Congress. UCT Graduate School of Business and the Breakwater Lodge, Cape Town, South Africa. 29 November-2 December 2011.

Mathengtheng L & Burt FJ. Use of recombinant domain III protein for detection and differentiation of flaviviruses in the Free State province. South African Society of Microbiology (SASM). Bela Bela, Limpopo 24-27 November 2013.

## Awards

**2011:** Runner-up, Faculty of Health Sciences Forum 2011- Best Junior Laboratory Paper. Mathengtheng L & Burt FJ. A chaperone-based expression system expressed a serologically functional protein for West Nile virus. Faculty of Health Sciences Forum 2011, University of the Free State 25-26 August 2011.

**2011:** Runner-up: 3-Minute-Thesis Competition-Ph.D. category. University of the Free State.

**2012:** Winner: Faculty of Health Sciences Forum-Best Junior Laboratory Paper. Mathengtheng L & Burt FJ. Serological survey for evidence of flaviviruses in the Free State province. Faculty of Health Sciences Forum University of the Free State 23-24 August 2012.

**2014:** Runner-up, Faculty of Health Sciences Forum 2011- Best Junior Laboratory Paper. Mathengtheng L & Burt FJ.

**Provincial award**

**2013:** Winner: Free State Department of Health Research day-Best presentation award: Clinical and Laboratory Medicine Track

**2010:** Recipient of the TATA® prestigious award for Top 40 postgraduate candidates in South Africa

**International award**

**2013:** Winner: Young Scientists Association (YSA) PhD Symposium-Best poster presentation award, at the Medical University of Vienna, Austria.

## List of figures

Figure 1: The genome organization of the polyprotein of flaviviruses depicting structural and non-structural proteins.....	2
Figure 2: Schematic representation of a mature and an immature virion of tick-borne encephalitis virus. ....	3
Figure 3: Schematic drawing of the transmission cycle of tbev. The virus is transmitted in nature by hard ticks of the ixodidae family. Small rodents of the genera myodes and apodemus are natural hosts of the virus although rodentia and eulipotyphla may contribute in viral transmission (Pfeffer and Dobler, 2010). The publisher grants permission for use and redistribution of the image as outlined in the license ( <a href="http://creativecommons.org/licenses/by/2.0/">http://creativecommons.org/licenses/by/2.0/</a> ) .....	6
Figure 4: Schematic representation of the transmission of WNV in nature. The virus is maintained between mosquitoes and birds and spillover to incidental hosts is achieved through the bite of an infected mosquito. ....	8
Figure 5: Hantavirus phylogenetic tree illustrating high divergence of hantaviruses detected in Africa. ....	22
Figure 6: Phylogenetic comparison of the EDIII protein of various flaviviruses. The neighbour-joining tree was constructed by using MEGA version 4 software (Katoh & Standley, 2013) and 1000 bootstrap replicates by using the maximum composite likelihood algorithm. ....	36
Figure 7: Agarose gel electrophoresis analysis of EDIII amplicons from the one step RT-PCR using genomic viral RNA as template. Amplicons were loaded on the gel after purification using the Wizard® SV gel and PCR clean-up system (Promega). ....	37
Figure 8: Agarose gel electrophoresis analysis of pGEM®-T easy vector (Promega) from transformed bacterial cells double digested with <i>Bam</i> HI and <i>Hind</i> III to confirm the presence of EDIII genes of LGTV, WNV and WESSV. ....	38
Figure 9: Agarose gel electrophoresis analysis of pQE-80L plasmid double-digested with <i>Bam</i> HI and <i>Hind</i> III for screening of the presence of LGTVEDIII insert. ....	39
Figure 10: Agarose gel electrophoresis analysis of pQE-80L plasmid double-digested with <i>Bam</i> HI and <i>Hind</i> III for screening of the presence of WNVEDIII insert. ....	40
Figure 11: Agarose gel electrophoresis analysis of amplicons obtained from a colony PCR assay used to screen for positive pQE80L-WESSVEDIII constructs. ....	40
Figure 12: Agarose gel electrophoresis analysis for screening of pCOLD-WNVEDIII constructs by double digestion with <i>Bam</i> HI and <i>Hind</i> III. ....	41
Figure 13: SDS-PAGE analysis of pQE80L-LGTVEDIII at intervals over a 24-hour period. ....	42
Figure 14: SDS-PAGE analysis of pQE80L-LGTVEDIII following a protein solubility study at 4 and 6 hours post-induction. Mixed fractions (MF) consisted of both the soluble fraction (SF) and insoluble fraction (IF), whereas the SF was harvested in the cytoplasm and the IF from inclusion bodies. ....	44
Figure 15: SDS-PAGE analysis of bacterial cell lysates containing pQE80L-WNVEDIII protein purified under native conditions. ....	45
Figure 16: SDS-PAGE analysis of pQE80L-WNVEDIII following a protein solubility study at 2, 3 and 4 hours post-induction. Mixed fractions (MF) consisted of both the soluble fraction (SF) and insoluble fraction (IF), whereas the SF was harvested in the cytoplasm and the IF from inclusion bodies. ....	45
Figure 17: SDS-PAGE analysis of pCOLDTF-WNVEDIII over a 24-hour period. The bacterial culture carrying the pCOLDTF plasmid without the EDIII gene was used as a control. ....	46

Figure 18: SDS-PAGE analysis of pCOLDTF-WNVEDIII following a protein solubility study over a 24-hour period. Mixed fractions (MF) consisted of both the soluble fraction (SF) and insoluble fraction (IF), whereas the SF was harvested from the cytoplasm and the IF from inclusion bodies. ....	47
Figure 19: SDS-PAGE analysis of pQE80L-WESSVEDIII at intervals over a 24-hour period. ....	48
Figure 20: SDS-PAGE analysis of pQE80L-WESSVEDIII following a protein solubility study at 2, 4 and 6 hours post-induction. Mixed fractions (MF) consisted of both the soluble fraction (SF) and insoluble fraction (IF), whereas the SF was harvested from the cytoplasm and the IF from inclusion bodies.....	49
Figure 21: (A) SDS-PAGE analysis of r-EDIII proteins expressed using pCOLD-TF and pQE-80L expression vectors after protein purification. Lane 1: Page Ruler™ Prestained Protein Ladder, Lane 2: LGTVEDIII expressed using pQE-80L vector, Lane 3: WESSVEDIII expressed using pQE-80L, Lane 4: WNVEDIII expressed using pCOLDTF vector, Lane 5-6: pCOLDTF and pQE-80L expression vectors respectively, expressed without a <i>flavivirus</i> gene. (B) Purification of r-WESSVEDIII with increased number of washes. Lane 1: Fermentas PageRuler™ Prestained Protein Ladder, Lanes 2-5: purified proteins eluted from Protino® columns. CL= total bacterial cell lysates. ....	50
Figure 22: Western blot analysis of the r-LGTVEDIII protein expressed using the pQE-80L plasmid. The dark band indicates the reactivity of the His-antibody with the recombinant protein. ....	51
Figure 23: Western blot analysis of the r-EDIII proteins of WNV and WESSV. The dark bands indicate the reactivity of the His-antibody with the recombinant protein. ....	51
Figure 24: Validation of KUNV cell lysate antigen for detection of anti- <i>Flavivirus</i> antibodies from various species in ELISA. The cut-off value of the assay was determined to be net OD <sub>405</sub> =0.23 as indicated by the horizontal line. ....	58
Figure 25: Evaluation of the LGTVEDIII recombinant protein for specificity against mosquito-borne flaviviruses in ELISA. The cut-off value of the assay was determined to be net OD <sub>405</sub> =0.28 as indicated by the horizontal line.....	58
Figure 26: Evaluation of the WNVEDIII recombinant protein for differentiation between antibodies raised against mosquito-borne and tick-borne flaviviruses in ELISA. The cut-off value of the assay was determined to be net OD <sub>405</sub> =0.226 as indicated by the horizontal line. ....	59
Figure 27: Evaluation of the WESSVEDIII recombinant protein for differentiation between antibodies raised against mosquito-borne and tick-borne flaviviruses in ELISA. The cut-off value of the assay was determined to be net OD <sub>405</sub> =0.23 as indicated by the horizontal line. ....	60
Figure 28: Profiles for the detection of samples positive on the KUNV assay by the r-EDIII proteins of LGTV, WNV and WESSV. ....	62
Figure 29: Correlation of the detection of anti- <i>flavivirus</i> IgG antibody results to the geographical distribution in 4 districts of the Free State province. ....	63
Figure 30: Agarose gel electrophoresis analysis of partial NS5 amplicons obtained in mono-reactions from the one step RT-PCR using genomic viral RNA as template at an annealing temperature of 45 °C. ....	76
Figure 31: Agarose gel electrophoresis analysis of partial NS5 and S segment amplicons of flaviviruses and CCHFV, respectively. ....	77
Figure 32: Agarose gel electrophoresis analysis of partial NS5 and S segment amplicons of flaviviruses and CCHFV, respectively. ....	77
Figure 33: Agarose gel electrophoresis analysis of amplicons derived from partial S segment genes of hantaviruses using the T7 forward primer and gene-specific reverse primers in a GoTaq® DNA polymerase (Promega) reaction. ....	78

Figure 34: Agarose gel electrophoresis analysis of amplicons derived from the RNA of the partial S segment genes of hantaviruses using gene-specific primers in a Titan® One Tube RT-PCR System (Roche) reaction. ....	79
Figure 35: Agarose gel electrophoresis analysis of amplicons derived from amplification of cDNA of partial S segment genes of hantaviruses using gene-specific primers in a GoTaq® DNA polymerase (Promega) reaction. Lane 1: Fermentas O'GeneRuler™ DNA ladder mix molecular weight marker (Thermo Scientific), lanes 2-5 amplicons derived from partial S segment genes of representative hantaviruses. ....	80
Figure 36: The detection of flavivirus controls using the FLAVI probe in a real-time touchdown PCR assay...	81
Figure 37: Amplification curves for LGTV DNA concentration standards of $10^0$ - $10^6$ using the FLAVI probe. ....	82
Figure 38: Standard curve generated using the log concentrations of LGTV DNA standards tested using FLAVI probe plotted against corresponding cycle threshold values. ....	82
Figure 39: Amplification curves for WNV DNA concentration standards using the FLAVI probe. ....	83
Figure 40: Standard curve generated using the log concentrations of WNV DNA standards tested using FLAVI probe plotted against corresponding cycle threshold values. ....	83
Figure 41: Amplification curves for HNSL DNA concentration standards of $10^0$ - $10^6$ using the HNSL probe. ....	84
Figure 42: Standard curve generated using the log concentrations of HNTV DNA standards tested using HNSL probe plotted against corresponding cycle threshold values. ....	84
Figure 43: Amplification curves for PUUV DNA concentration standards of $10^0$ - $10^6$ using the ASPBR probe. ....	85
Figure 44: Standard curve generated using the log concentrations of PUUV DNA standards tested using ASPBR probe plotted against corresponding cycle threshold values. ....	85
Figure 45: Amplification curves for SANGV DNA concentration standards of $10^0$ - $10^6$ using the SANGV probe. ....	86
Figure 46: Standard curve generated using the log concentrations of SANGV DNA standards tested using SANGV probe plotted against corresponding cycle threshold values. The efficiency of the assay must be as close to 2 as possible and the error approximately 0. ....	86
Figure 47: Results of the real-time multiplex assay for determination of the limit of detection of DNA controls by probes. ....	89
Figure 48: Agarose gel electrophoresis analysis of partial NS5 and S segment amplicons of flaviviruses and CCHFV. ....	90



## List of tables

Table 1: Summary of the classification of members of the family <i>Flaviviridae</i> . (Modified from Swanepoel and Burt, 2008).....	4
Table 2: <i>Flavivirus</i> diagnosis algorithms. (Modified from Domingo <i>et al.</i> , 2011) .....	18
Table 3: Summary of African hantaviruses identified to date. (Modified from Witkowski <i>et al.</i> , 2014) .....	20
Table 4: Primers targeting the EDIII genes of LGTV, WNV, WESSV. Restriction sites flanking the 5' and 3' positions are underlined. ....	28
Table 5: Reagent mixtures for the amplification of EDIII genes using Titan® one step reverse transcriptase PCR kit (Roche) .....	29
Table 6: Reaction mix for ligation of EDIII genes into PQE-80L and PCOLD-TF expression vectors.....	30
Table 7: Sequencing reactions prepared according to recommendations included in the BigDye® Terminator Kit .....	31
Table 8: Preparation of 8% resolving gel and 4 % stacking gel for analysis of proteins by SDS-PAGE.....	33
Table 9: Amino acid sequences of the EDIII proteins of various flaviviruses .....	36
Table 10: Amplicon concentration before and after purification.....	37
Table 11: Protein concentrations for recombinant antigens purified from a 100 culture and recombinant antigens pooled from multiple cultures. ....	49
Table 12: Determination of cut-off values for indirect IgG ELISA. ....	57
Table 13: Summary of samples tested on serological assays for presence of <i>flavivirus</i> antibodies .....	61
Table 14: Differentiation of field samples by r-EDIII proteins of WNV and WESSV. ....	62
Table 15: Nucleotide sequences and properties of primers and probes used in the development of multiplex PCR assays. ....	71
Table 16: Reaction setup for the second round of Flavi-CCHFV nested multiplex PCR using GoTaq® polymerase.....	72
Table 17: PCR cycling conditions for the Flavi-Hantavirus TaqMan® multiplex PCR assay .....	74
Table 18: Estimated DNA copy numbers for control DNA samples.....	80
Table 19: Average DNA concentrations for high and low controls extrapolated within a multiplex assay using the Lightcycler® software.....	87
Table 20: Average cycle threshold values for high and low controls obtained in a multiplex PCR assay using the Lightcycler® software.....	88
Table 21: Results for patient sera screened using the Euroimmun Eurasia and America hantavirus IgG ELISA kits. ....	91
Table 22: Immunization schedule for immunogenicity study. ....	96
Table 23: Immunization schedule for control mice .....	96
Table 24: Total IgG antibody responses in mice against KUNV or LGTV antigen and ratio of IgG1:IgG2a .....	98

## List of abbreviations

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ADE	antibody dependent enhancement
Ag	antigen
ALTV	Altai virus
AMV	Avian myeloblastosis virus
ANDV	Andes virus
ARRV	Ash River virus;
ARTV	Artybash virus;
ASAV	Asama virus;
ASIV	Asikkala virus;
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
AZGV	Azagny virus
BANV	Banzi virus
BBQ	BlackBerry® Quencher
BLAST	Basic Local Alignment Search Tool
Bla	beta lactamase
bp	base pairs
BOWV	Bowé virus
BSL	biosafety laboratory
C	capsid
°C	degrees Celcius
CBNV	Cao Bang virus
CCHFV	Crimean-Congo haemorrhagic fever virus
CDC	Centres for Disease Control and Prevention
cDNA	complementary DNA
cfu	colony forming units
CHOV	Choclo virus
CMV	cytomegalovirus
CSF	cerebrospinal fluid
CV	coefficient of variation
DENV	dengue virus
DTV	deer tick virus

DNA	deoxyribonucleic acid
dNTP	dinucleotide triphosphate
DOBV	Dobrava-Belgrade virus
dsDNA	double stranded DNA
D	domain
DTT	dithiothreitol
E	envelope
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EMEM	Eagle's Minimal Essential Medium
FRET	fluorescence resonance energy transfer
G	glycoprotein
h	hour
HCl	hydrochloric acid
HFRS	haemorrhagic fever with renal syndrome
HPS	hantavirus pulmonary syndrome
HCPS	hantavirus cardiopulmonary syndrome
HIS	histidine
HMAF	hyperimmune mouse ascitic fluid
HRPO	horseradish peroxidase
HTNV	Hantaan virus
HUPV	Huanqpi virus
Ig	immunoglobulin
IPTG	isopropyl $\beta$ -D-1-thiogalactopyranoside
IS	insoluble fraction
JEJV	Jeju virus
JEV	Japanese encephalitis virus
JMSV	Jemez Springs virus
KADV	Kadam virus
kb	kilobases
KKMV	Kenkeme virus
KFDV	Kyasanur Forest disease virus
KILV	Kilimanjaro virus
KUNV	Kunjin virus
L	large
LAMP	loop-mediated isothermal amplification

LB	Luria-Bertani
LEW	lysis-equilibrium-wash
LGTV	Langat virus
LHEV	Lianghe virus
LIV	Louping ill virus
LNA	locked-nucleic acid
LNV	Laguna Negra virus
LOD	limit of detection
LQUV	Longquan virus
M	membrane
MAPV	Maporal virus
MEGA	molecular evolutionary genetics analysis
MF	mixed fraction
MGBV	Magboi virus
MHAF	mouse hyperimmune ascitic fluid
min	minutes
MJNV	Imjin virus
mg	milligram
ml	millilitre
mM	millimolar
MOUV	Mouyassué virus
MVSV	Murray Valley encephalitis virus
ND	not done
ng	nanogram
NS	non-structural
NVAV	Nova virus
OD	optical density
OHFV	Omsk haemorrhagic fever virus
OXBV	Oxbow virus
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
POWV	Powassan virus
prM	pre-membrane
PUUV	Puumala virus
PVDF	polyvinylidene fluoride

QDLV	Qiandao Lake virus
r-EDIII	recombinant EDIII
RFV	Royal Farm virus
RIOMV	Rio Mamore virus
RKPV	Rockport virus
RPLV	Camp Ripley virus
rpm	rotations per minute
RNA	ribonucleic acid
RT-PCR	reverse-transcriptase polymerase chain reaction
RVFV	Rift Valley fever virus
S	small
SANGV	Sangassou virus
SEOV	Seoul virus
SERV	Serang virus
SNV	Sin Nombre virus
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SF	soluble fraction
SFV	Semliki Forest virus
SLEV	St Louis encephalitis virus
SPOV	Spondweni virus
SWSV	Seewis virus
TANGV	Tanganya virus
TBEV-Eu	tick-borne encephalitis virus - European
TBE-FE	Far Eastern tick-borne encephalitis
TBEV-Sib	Siberian tick-encephalitis virus
TDV	tetravalent dengue vaccine
TEMED	tetramethylethylenediamine
Th	T-helper
TIGV	Tigray virus
TMA	transcription-mediated amplification
Tris	trisaminomethane
UK	United Kingdom
ULUV	Uluguru
μ	microlitre
u	units

USA	United States of America
USUV	Usutu virus
WESSV	Wesselsbron virus
WHO	World Health Organisation
WNV	West Nile virus
$x g$	gravitational force
X-gal	5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside
YFV	yellow fever virus
ZIKV	Zika virus

## **Abstract**

West Nile virus (WNV) is endemic to southern Africa but the true burden of disease associated with WNV infection remains unknown in this region. The presence of the mosquito-borne Wesselsbron virus (WESSV) has also been established in southern Africa. Although not considered a serious human pathogen, WESSV has been associated with encephalitis in humans. No routine testing is performed for WESSV diagnosis in South African patients and hence, similar to WNV infections, the virus remains unreported and overlooked. The presence of tick-borne flaviviruses in southern Africa on the other hand, has not been established despite the presence of suitable vectors. A challenge associated with serological identification of flaviviruses is the high level of cross-reactivity between members of flaviviruses and the impracticality of using neutralization assays. Serological assays using reagents that can be handled in a biosafety level 2, or lower facility, were developed and evaluated for the detection and differentiation of tick- and mosquito-borne flaviviruses in the Free State province of South Africa. A total of 2393 serum samples from a variety of species including humans, cattle and sheep were tested using Kunjin virus (KUNV) cell lysate antigen for the detection of anti-flavivirus antibodies in an indirect IgG enzyme-linked immunosorbent assay (ELISA). To further differentiate positive reactors on KUNV assay for antibodies against tick- or mosquito-borne flaviviruses, recombinant envelope domain III (r-EDIII) proteins of Langat virus (LGTV), WNV and WESSV were expressed in a bacterial expression system and used in ELISA. A total of 722 samples were positive on the KUNV assay of which 71, 457 and 431 were positive on the r-LGTVEDIII, r-WNVEDIII and r-WESSVEDIII assays, respectively. A total of 70 samples were reactive on the KUNV assay but not on any of the other assays, suggesting that there are other flaviviruses circulating in the Free State province for which specific r-EDIII assays were not available. Collectively, the results suggest a strong presence of flaviviruses co-circulating in the Free State province with an abundance of mosquito-borne flaviviruses. There is evidence suggesting the presence of tick-borne flaviviruses but it has yet to be confirmed. The EDIII protein is a useful tool that can be utilized in the detection and differentiation of flaviviruses in resource-limited laboratories. Vertebrate hosts play a role in the maintenance and circulation of flaviviruses and, although not involved in the direct transmission of tick- and mosquito-borne flaviviruses, form a link for virus transmission between vectors. In addition to rodent involvement in maintenance of flaviviruses, rodents have also been implicated in the transmission of other medically significant viruses such as arenaviruses, lyssaviruses and hantaviruses. Arboviruses and viral haemorrhagic fevers are among the most pathogenic and devastating disease agents in many parts of the world. It is therefore important for surveillance of such pathogens to be conducted as they may result in considerable public health implications. Molecular assays were developed for the detection of a selected number of arboviruses and viral haemorrhagic fevers, specifically Crimean-Congo haemorrhagic fever virus (CCHFV), mosquito-borne and tick-borne flaviviruses, as well as hantaviruses. To date, the

presence of hantaviruses have not been confirmed in southern Africa despite their emergence in the western and eastern parts of Africa in recent years. In our study, serum samples of patients presenting with a tick-bite and febrile illness without diagnosis were screened for hantavirus IgG antibodies using commercial assays that represent the American and Eurasian hantavirus species. The overall seropositivity rate obtained was 10% and 6% for assays representing the Eurasia and America hantavirus species, respectively. The emergence of hantaviruses in Africa and their seroprevalence in the Cape region of South Africa as well as in our study warranted the development of a molecular assay to further investigate the presence of these viruses in southern Africa. In order to achieve this, a real-time RT-PCR was designed and optimized. The assay was designed by identifying in-house primers targeting the partial region of the S-segment of hantaviruses and hydrolysis probes targeting the inner region of the amplicon. The probes were based on nucleotide sequences targeting the *Murinae*-associated hantaviruses for the HNLS probe, *Sigmodontinae*- and *Arvicolinae*-associated hantaviruses for the ASPRB probe, as well as the SANGV probe for the African hantavirus Sangassou virus. The flavivirus RT-PCR targeted the NS5 region with a probe shown to successfully detect RNA samples that represent eight different flavivirus species. The hantavirus primers and probes were evaluated using RNA transcribed from synthetic genes representing the different hantaviral genotypes and subsequently reverse transcribed cDNA. The limit of detection was determined to range from ~160 to ~17 copies of DNA for the various hantaviral probes and flavivirus probe.

In addition, a conventional multiplex PCR assay aimed at detecting CCHFV and flavivirus RNA in samples collected from undiagnosed patients presenting with a tick-bite and febrile illness was developed by using nested primers targeting the partial region of the genome of the S-segment of CCHFV and hemi-nested primers targeting the partial region of the NS5 gene of flaviviruses. When clinical samples from patients with known tick-bites, mild disease and no diagnosis were screened, a patient was retrospectively diagnosed as having a CCHFV infection. This result highlights the need for awareness to arboviruses and viral hemorrhagic fevers in mild cases that may easily be overlooked but constitute a significant public health risk. Similarly, there needs to be an increase in awareness for travelers to South Africa at risk of returning to their country with an exotic viral haemorrhagic fever, highlighting the need for increased awareness and increased diagnostic capacity for arboviruses.

Finally the current lack of registered human vaccines warrants continued investigation of the immunogenicity of selected viral proteins. The recombinant antigens developed for serological purposes were further employed in this study to determine the immunogenicity of the envelope domain III proteins of WNV and LGTV in a mouse model. Small molecule antigens or weakly immunogenic antigens frequently require an adjuvant to stimulate a stronger immune response. In addition, adjuvants can shift an immune response towards a Th1 or Th2 response as required



based on immune correlates of protection. Groups of mice were immunized with purified r-WNVEDIII or r-LGTVEDIII protein alone, r-WNVEDIII or r-LGTVEDIII protein in combination with one of three adjuvants, including saponin, Titermax® gold and Alhydrogel® or one of the three adjuvants without a flavivirus protein. In the absence of any adjuvant the results from WNV protein alone were inconclusive whereas a strong IgG1 response was induced by LGTV EDIII. Briefly, protein alone or mixed with alum elicited a predominantly Th2 response whereas protein in combination with saponin or Titermax® gold induced a mixed Th1 and Th2 response. Mice immunized with r-WNVEDIII reacted against KUNV native antigen indicating that the protein was expressed in conformation exposing epitopes that are required to induce a detectable antibody response. The formulation of the WNV and LGTV proteins with different adjuvants produced similar results with a shift in response depending on the adjuvant. Despite an absence of being able to assess cell mediated responses using antigen stimulated splenocytes and profiling cytokine production as initially planned, the results do confirm that r-WNVEDIII and r-LGTVEDIII proteins are immunogenic in the absence of complete E protein, with ability to induce detectable antibody when formulated with adjuvant and that different adjuvants are able to have an immunomodulatory influence on the type of response induced.

.

---

## Opsomming

Wes-Nyl-virus (WNV) is endemies in suider-Afrika, maar die werklike siektelas wat met WNV-infeksie geassosieer word, is steeds onbekend in hierdie streek. Die teenwoordigheid van die muskiet-gedraagde Wesselsbron-virus (WESSV) is ook in suider-Afrika aangetoon. Alhoewel dit nie as 'n ernstige menspatogeen beskou word nie, word WESSV geassosieer met enkefalitis in mense. Geen roetinetoetsing word gedoen vir die diagnose van WESSV in Suid-Afrikaanse pasiënte nie en daarom, soortgelyk aan WNV-infeksies, bly die virus onaangemeld en oor die hoof gesien. Aan die anderkant is die teenwoordigheid van bosluis-gedraagde flavivirusse in suider-Afrika nog nie vasgestel nie, ten spyte van die teenwoordigheid van geskikte vektore. Die hoë vlak van kruisreaktiwiteit tussen flavivirusse en die gebruik van neutraliserende toetse wat onprakties is, is uitdagings geassosieer met die serologiese identifikasie van flavivirusse. Serologiese toetse wat reagens gebruik wat in 'n bioveiligheidsvlak 2 of 'n laer fasiliteit gebruik kan word, was ontwikkel en geëvalueer vir die waarneming en differensiasie van bosluis- en muskiet-gedraagde flavivirusse in die Vrystaat provinsie in Suid-Afrika. 'n Totaal van 2393 serummonsters van verskeie spesies, insluitend mense, beeste en skape, was getoets met behulp van die Kunjin-virus (KUNV) sel lisaat-antigeen vir die aantoning van anti-flavivirus teenliggame in 'n indirekte IgG ensiem-gekoppelde immunosorbent-toets (ELISA). Ten einde verdere positiewe reageerders op KUNV toetsing vir teenliggame teen bosluis- en muskiet-gedraagde flavivirusse te onderskei, was rekombinante omhulsel domein III (r-EDIII) proteïene van Langat-virus (LGTV), WNV en WESSV in 'n bakterieë uitgedruk en in die ELISA gebruik. 'n Totaal van 722 monsters het positief getoets met die KUNV toets, waarvan 71, 457 en 431 positief getoets het vir die r-LGTVEDIII, r-WNVEDIII en r-WESSVEDIII toetse, onderskeidelik. 'n Totaal van 70 monsters het reageer op die KUNV toets, maar nie met enige van die ander toetse nie, wat 'n aanduiding is dat daar ander flavivirusse waarvoor daar nie spesifieke r-EDIII toetse beskikbaar is nie, in die Vrystaat provinsie sirkuleer. Gesamentlik dui die resultate op 'n sterk teenwoordigheid van flavivirusse wat in die Vrystaat provinsie sirkuleer met verskeie muskiet-gedraagde flavivirusse wat ko-sirkuleer. Daar is bewyse wat op die teenwoordigheid van bosluis-gedraagde flavivirusse dui, alhoewel dit nog nie bevestig is nie. Die EDIII proteïen is 'n nuttige middel wat vir die waarneming en differensiasie van flavivirusse in hulpbron-beperkte laboratoriums gebruik kan word.

Gewerwede gashere speel 'n rol in die onderhoud en sirkulasie van flavivirusse en, hoewel nie betrokke by die direkte oordrag van bosluis- en muskiet-gedraagde flavivirusse nie, vorm 'n skakel vir virusoordrag tussen vektore. Bykomend tot knaagdierbetrokkenheid in die handhawing van flavivirusse, word knaagdiere ook geïmpliseer in die oordrag van ander medies-belangrike virusse soos arenavirusse, lyssavirusse en hantavirusse. Arbovirusse en virale hemorragiese koors virusse is van die mees patogeniese en vernietigende siekte-agente in baie dele van die wêreld. Dit is

daarom belangrik dat waarneming van sulke patogene uitgevoer word, omdat hierdie agent tot omvangryke openbare gesondheidsimplikasies kan lei. Molekulêre toetse was ontwikkel vir 'n geselekteerde aantal arbovirusse en virale hemorragiese koors agente, spesifiek Krimeaanse-Kongo hemorragiese koors virus (KKHKV), muskiet- en bosluis-gedraagde flavivirusse, asook hantavirusse. Huidiglik is die teenwoordigheid van hantavirusse in suidelike Afrika nog nie bevestig nie, ten spyte van hulle onlangse verskyning in die westelike en oorstelike dele van Afrika. In hierdie studie was die serummonsters van pasiënte wat met 'n bosluisbyt en koorsagtige siektebeeld sonder 'n diagnose presenteer het, getoets vir hantavirus IgG teenliggame met behulp van kommersiële toetse wat die Amerikaanse en Eurasiese hantavirus spesies verteenwoordig. Die algehele seropositiewe vlak wat gevind was, was 10% en 6% vir toetse wat die Amerikaanse en Eurasiese hantavirus spesies verteenwoordig, onderskeidelik. Die verskyning van hantavirusse in Afrika en die seroprevalensie van hierdie virusse in die Kaapse streek van Suid-Afrika en in ons studie, het die ontwikkeling van 'n molekulêre toets regverdig om die teenwoordigheid van hierdie virusse in suider-Afrika verder te ondersoek. Ten einde dit te bereik, was 'n reële tyd trutranskriptase polimerase ketting reaksie (RT-PCR) ontwerp en geöptimiseer. Die toets was ontwerp deur in-huis peilstukke 'n deel van die S-segment van hantavirusse teiken en hidrolise probes wat die binneste streek van die ampikon teiken, te identifiseer. Die probes is gebaseer op die nukleotiedopeenvolgings wat die *Murinae*-geassosieerde hantavirus teiken vir die HNLS probe, *Sigmodontinae*- en *Arvicolinae*-geassosieerde hantavirusse vir die ASPRB probe, asook die SANGV probe vir die Afrika hantavirus Sangassou-virus. Die flavivirus RT-PCR teiken die NS5 streek met 'n probe wat suksesvol RNS in monsters op spoor van agt verskillende flavivirus spesies. Die hantavirus peilstukke en probes is geëvalueer met behulp van getranskribeerde RNS van sintetiese gene wat verteenwoordigend is van die verskillende hantavirale genotipes en trugtranskribeerde kDNS. Die limiet van opsporing was gevind om van ~160 tot ~17 kopieë van DNS vir die verskillende hantavirus probes en die flavivirus probe te wees.

'n Gewone multipleks PCR toets wat gerig was op die opsporing van KKHKV en flavivirus RNS in monsters versamel vanaf ongediagnoseerde pasiënte met 'n bosluisbyt en koorsagtige siektebeeld, is verder ontwikkel deur gebruik te maak van geneste peilstukke gerig teen 'n deel van die S-segment van KKHKV, en hemi-geneste peilstukke gerig teen 'n deel van die NS5 geen van flavivirusse. Wanneer kliniese monsters van pasiënte met bosluisbyte, 'n matige siektebeeld en geen diagnose ondersoek is, is 'n pasiënt retrospektief gediagnoseer met KKHKV. Die resultate beklemtoon die belang van die bewustheid van arbovirusse en virale hemorragiese koors agente in matige gevalle wat maklik oor die hoof gesien kan word, maar 'n beduidende openbare gesondheidsrisiko verteenwoordig. Soortgelyk behoort die bewustheid opgeskerp te word vir reisigers na Suid-Afrika wat die risiko loop om na hulle land terug te keer met 'n eksotiese virale

hemorragiese koors, wat verder die behoefte beklemtoon vir toenemende bewustheid en diagnostiese kapasiteit vir arbovirusse.

Laastens regverdig die huidige gebrek aan geregistreerde menslike entstowwe voortgesette ondersoek na die immunogenisiteit van geselekteerde virale proteïene. Die rekombinante antigene wat ontwikkel is vir serologiese doeleindes, was verder in hierdie studie aangewend om die immunogenisiteit van die omhulsel domein III proteïene van WNV en LGTV in 'n muismodel te bepaal. Klein-molekule antigene of swak immunogeniese antigene vereis dikwels 'n versterkingsmiddel om 'n sterker immuunrespons te stimuleer. Versterkingsmiddels kan verder 'n immuunrespons verskuif na 'n Th1 of Th2 respons soos nodig, gebaseer op die immuunkorrelate van beskerming. Groepe muise was geïmmuniseer met gesuiwerde r-WNVEDIII of r-LGTVEDIII proteïene alleenlik, r-WNVEDIII of r-LGTVEDIII proteïene in kombinasie met een van drie versterkingsmiddels, naamlik saponien, Titermax® gold en Alhydrogel®, of een van die drie versterkingsmiddels sonder 'n flavivirus proteïen. In die afwesigheid van enige versterkingsmiddel was die resultate van die WNV proteïen alleenlik twyfelagtig, terwyl 'n sterk IgG1 respons deur LGTV EDIII geïnduseer is. In kort, proteïen alleenlik of gemeng met alum het 'n oorwegend Th2 respons uitgelok, terwyl proteïen in kombinasie met saponien of Titermax® gold 'n gemengde Th1 en Th2 reaksie geïnduseer het. Muise wat met r-WNVEDIII geïmmuniseer was, het teen natuurlike KUNV antigeen gereageer, wat daarop dui dat die proteïen uitgedruk is in konformasie met blootstelling van die epitope wat vereis word om 'n waarneembare teenliggaamrespons te induseer. Die formulering van die WNV en LGTV proteïene met verskillende adjuvante het soortgelyke resultate opgelewer, met 'n verskuiwing in respons afhangend van die versterkingsmiddel. Ten spyte van die onvermoë om selbemiddelde reaksies te assesser met behulp van antigeen-gestimuleerde splenosiete en karakterisering van sitokienproduksie soos aanvanklik beplan, het die resultate bevestig dat r-WNVEDIII en r-LGTVEDIII proteïene immunogenies was in die afwesigheid van volledige E proteïen, met die vermoë om waarneembare teenliggame te induseer wanneer geformuleer met versterkingsmiddel, en dat verskillende versterkingsmiddels die vermoë het om 'n immuunmodulerende invloed op die tipe respons wat geïnduseer word, uit te oefen.

# CHAPTER1

## LITERATURE REVIEW

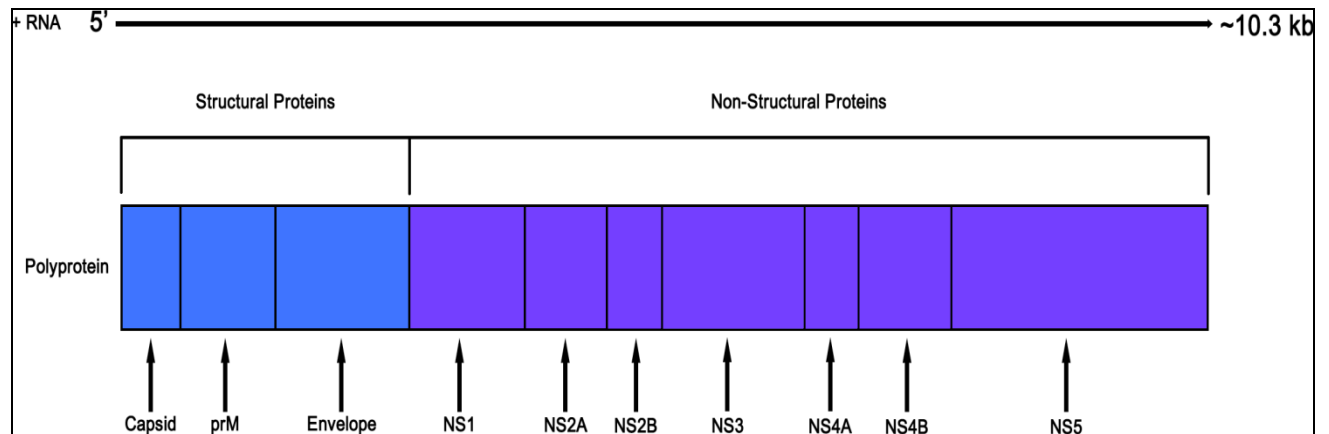
### 1.1. Introduction and history

The genus *Flavivirus* is a diverse group which comprises of mosquito-borne and tick-borne viruses, as well as viruses for which there are no known vectors (Porterfield, 1975). The prototype of the genus is the mosquito-borne yellow fever virus (YFV) that was first described in 1848 by Clark Nott and later in 1881 by a Cuban physician, Carlos Finlay, who undertook research that proved that mosquitoes serve as vectors for this virus. The vaccine strain of YFV designated 17D was successfully developed by a South African, Max Theiler in 1937 (Theiler and Smith, 1937). The complete genome of YFV-17D was later sequenced in 1985 (Rice *et al.*, 1985), making it the first complete genome of any flavivirus to be sequenced. This marked the advancement in the molecular understanding and typing of the genus. Many viral species have been described and subsequently included in the genus since, and the genus currently comprises of over 70 viruses (Karabatsos, 1985; Pybus *et al.*, 2002). While pathogenesis in humans has not been established for some of the members, other members belonging to this genus are significant medical pathogens causing disease including encephalitis, hemorrhagic fever and fatalities. Vaccine development targeted against the prevention of pathogenic flaviviruses in humans has public health implications.

### 1.2. Classification and molecular characteristics of flaviviruses

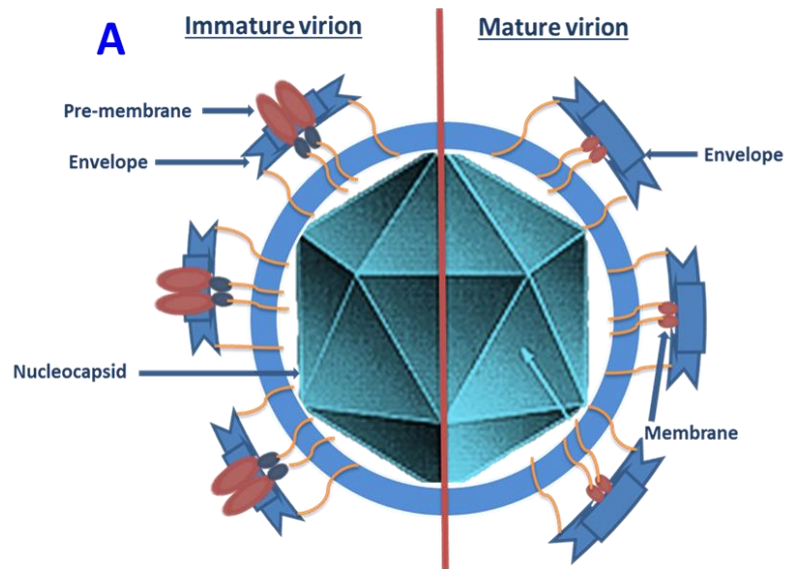
The genus *Flavivirus* is classified as one of the three genera of the family *Flaviviridae*. The other two genera are *Pestivirus* and *Hepacivirus* with bovine viral diarrhea virus 1 and Hepatitis C virus as type species, respectively (Swanepoel and Burt, 2008). Members of the *Flavivirus* genus are spherical, enveloped viruses with a diameter of approximately 50 nm and comprises of three structural and seven non-structural proteins (Calisher and Gould, 2003). The three structural proteins are envelope (E), pre-membrane (prM/M) and capsid (C) while the seven non-structural proteins are NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 (Rice *et al.*, 1985). All the proteins are encoded for by a single-stranded, positive sense RNA genome of approximately 11 kilobases (kb) in length. Due to its positive sense characteristic, the genome serves as RNA template for translation and encodes for a polyprotein. Following post-translational modifications, the polyprotein is then cleaved into these 10 proteins (Lindenbach and Rice, 2003). The organization of the proteins in the genome is illustrated in Figure 1. As observed in the figure, the NS5 protein (~103 kDa) is the largest protein followed by the NS3 (~70 kDa). The E protein is the largest of the structural proteins (~53 kDa) and consists of three domains designated domains 1 to 3 (DI, DII and DIII). Mandl and co-workers (1989) studied the structure of the envelope and reported that the three domains have

the following functions: DI forms a barrel-like structure, DII projects along the virus surface between the transmembrane regions of the homodimer subunit and DIII maintains an immunoglobulin constant. It was further elucidated that DIII serves as the receptor binding region (Crill and Roehrig, 2001). This domain is discussed in detail later in this review.



**Figure 1: The genome organization of the polyprotein of flaviviruses depicting structural and non-structural proteins**

The capsid protein is the smallest (~11 kDa) of all the proteins and reported to be a highly basic protein. The third structural protein is the membrane (M) protein. The prM is approximately 26 kDa in size and serves as the glycoprotein precursor of the M protein. Furthermore, the prM contributes to one of the two forms of the virion viz. the immature and the mature virion forms. The prM is found in the immature virions and becomes proteolytically cleaved into M during maturation resulting in a mature virion. During this process of maturation, the pr is secreted leaving the mature M fragment (Murray *et al.*, 1993). Figure 2 illustrates these two forms of virions and the differences between them. The structures of virions have been studied extensively for tick-borne encephalitis virus (TBEV).



**Figure 2: Schematic representation of a mature and an immature virion of tick-borne encephalitis virus.**

### **1.3. Transmission and epidemiology of flaviviruses**

Human infection with flavivirus can range from mild to severe disease. Severe disease may include symptoms ranging from encephalitis to hemorrhagic fever even death. The vectors of tick- and mosquito-borne flaviviruses are primarily *Ixodes* sp. and *Culex* sp., respectively as well as *Aedes* sp. for some mosquito-borne flaviviruses. However, it has been observed that most flaviviruses can infect and be transmitted by several different species of a vector. TBEV is regarded as the most important of all the mammalian tick-borne flaviviruses. Though ticks of the *Ixodes* sp. are considered to be the principal vectors of TBEV, the virus has previously been isolated from 18 different tick-species (Gould et al. 2003). Once infected with TBEV, *Ixodes* ticks play an important role in the viral transmission cycle and maintenance in nature.

#### **1.3.1. Tick-borne flaviviruses**

Studies focusing on the *Flavivirus* tick-borne group have indicated that TBEV is one of the most dangerous members of the group. TBEV continues to be the cause of viral neurological disease in Europe, the former Soviet Union, and Asia. Approximately 11, 000 people contract the disease annually, despite historical under-reporting of this disease (CDC, 2014).

Table 1: Summary of the classification of members of the family *Flaviviridae*. (Modified from Swanepoel and Burt, 2008)

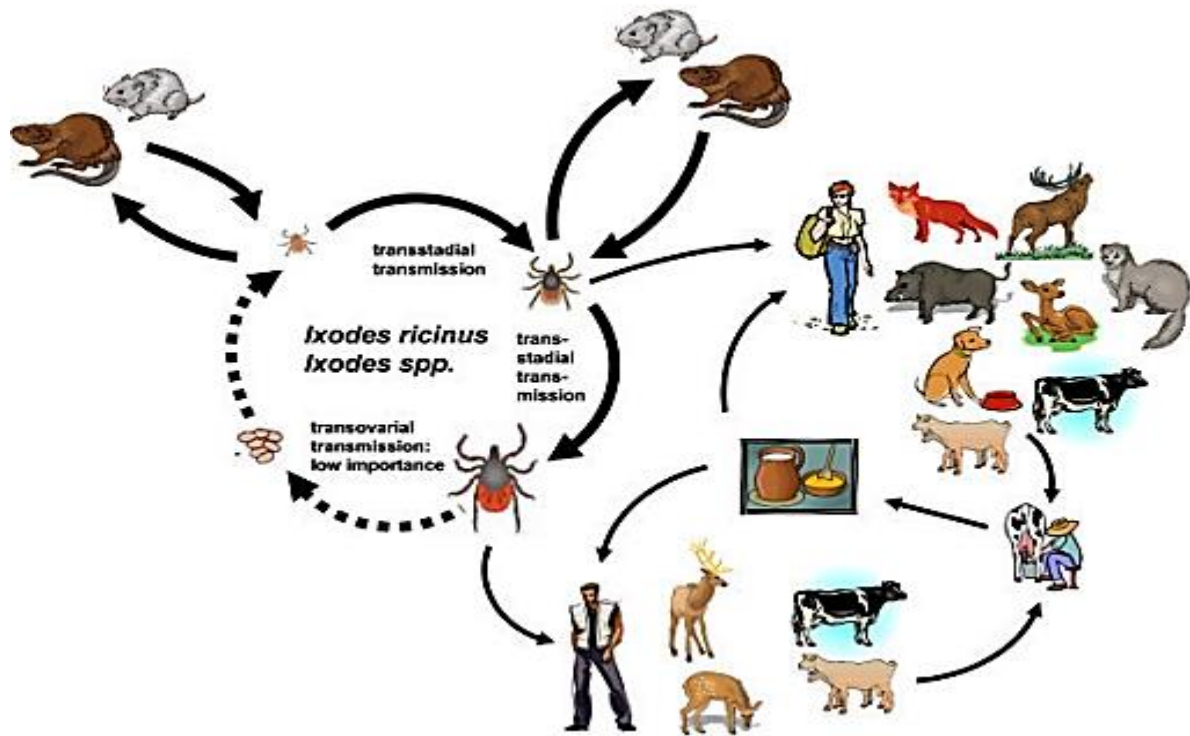
<b>Genus <i>Hepacivirus</i></b>
<b>Genus <i>Pestivirus</i></b>
<b>Genus <i>Flavivirus</i></b>
<b>Cell fusing agent group</b>
<b>Mosquito-borne groups</b>
<b>Aroa group</b>
<b>Dengue group</b>
<b>Japanese encephalitis group</b>
Japanese encephalitis virus (JEV)
Murray Valley encephalitis virus (MVEV)
St Louis encephalitis virus (SLEV)
Usutu virus (USUV)
West Nile virus (WNV)
Kunjin virus (KUNV)
<b>Kokobera group</b>
<b>Ntaya group</b>
<b>Spondweni group</b>
Spondweni virus (SPOV)
Zika virus (ZIKV)
<b>Yellow fever group</b>
Banji virus (BANV)
Wesselsbron virus (WESSV)
Yellow fever virus (YFV)
<b>Tick-borne groups</b>
<b>Seabird tick-borne group</b>
<b>Kadam tick-borne group</b>
Kadam virus (KADV)
<b>Mammalian tick-borne group</b>
Royal Farm virus (RFV)
Powassan virus (POWV)
Deer tick virus (DTV)
Kyasanur Forest disease virus (KFDV)
Langat virus (LGTV)
Omsk hemorrhagic fever virus (OHFV)
<b>Tick-borne encephalitis virus types and subtypes</b>
Louping ill virus (LIV)
Western tick-borne encephalitis virus - European (TBEV-Eu)
Far Eastern tick-borne encephalitis (TBEV-FE)
Siberian tick-encephalitis virus (TBEV-Sib)
<b>Viruses with no known arthropod vector</b>
<b>Entebbe bat group</b>
<b>Rio Brava group</b>
<b>Modoc group (rodent-associated)</b>
<b>Unclassified</b>



TBEV is further divided into three subtypes which include the far eastern subtype and the Siberian subtype which are primarily transmitted by the tick vector *Ixodes persulcatus*, as well as the European subtype which is primarily transmitted by *Ixodes ricinus* (Ecker *et al.*, 1999). Due to its relatively lengthy lifespan, the tick is the main reservoir of TBEV (Kozuch *et al.*, 1990). The virus becomes transmitted to a tick when ticks at different developmental stages (nymphs and larvae) co-feed on the same animal, typically rodents. During a feed on a viraemic vertebrate host the virus infects the salivary glands of the vector and becomes shed in the saliva. Humans and other mammals are incidental hosts and acquire infection through a bite of an adult tick (Figure 3). Due to the transmission of the virus being dependant on the tick species, the epidemiology of the virus strongly correlates to that of the vector.

Although ticks have been described as the primary mode of transmission of TBEV, this virus has also been reported to be transmitted through other unconventional routes. The virus has been reported to cause infection through consumption of dairy products derived from unpasteurized milk of infected livestock (Lindquist and Vapalahti, 2008). Recently, a small outbreak of TBEV associated with the consumption of raw goat milk was reported in Slovenia (Hudopisk *et al.*, 2013). In this outbreak, 3 individuals showed symptoms of acute TBEV infection 2 days after consumption of raw milk from the same goat. TBEV infection was confirmed in all patients by detection of anti-TBEV IgG and IgM antibodies as well as the presence of neutralizing antibodies. No TBEV RNA was detectable by RT-PCR. Interestingly, the fourth person who had also consumed the infected milk remained healthy. It was later discovered that this individual had received 3 boosters of the TBEV immunization between the years 1995 and 2010. Following laboratory testing, this individual was found to have no detectable anti-TBEV IgM antibodies but high levels of anti-TBEV IgG antibodies and neutralizing antibodies. The goat whose milk was consumed was found to have detectable TBEV RNA in both the serum and the milk. The virus has also been transmitted to humans through contact with infected tissue during slaughtering of viraemic goats and blood transfusions (Wahlberg *et al.*, 1989).

Other tick-borne flaviviruses implicated as the cause of encephalitis in humans include louping ill virus (LIV), Powassan virus (POWV) and deer tick virus (DTV) which is a subtype of POWV. LIV derives its name from the odd leaping behaviour of infected sheep. LIV is the only flavivirus that has been identified in the United Kingdom. The disease has been reported in Scotland, northern England, north Wales and southwest England (Gritsun *et al.*, 2003). The condition of vegetation found in these areas is moist and supports the long-term maintenance of the vector *I. ricinus* (Nuttall and Labuda, 2005).



**Figure 3: Schematic drawing of the transmission cycle of tbev. The virus is transmitted in nature by hard ticks of the ixodidae family. Small rodents of the genera myodes and apodemus are natural hosts of the virus although rodentia and eulipotyphla may contribute in viral transmission (Pfeffer and Dobler, 2010). The publisher grants permission for use and redistribution of the image as outlined in the license (<http://creativecommons.org/licenses/by/2.0/>)**

POWV was named after a town in northern Ontario, Canada, where the first fatal human case of the disease was identified. The patient was a 5 year-old boy who died in 1958 after developing encephalitis (McClellan and Donohue, 1959). The virus circulates in Canada as well as in the eastern and western regions of the United States and far eastern Russia. DTV was isolated in the northeastern region of the United States from a tick vector *I. scapularis*. Although encephalitis has been reported in young laboratory mice, Langat virus (LGTV) is avirulent in adult rodents in nature. Additionally, the virus has not been associated with human disease though serological prevalence has been reported in humans residing in endemic Malaysia where the virus was first isolated in 1956 (Smith, 1956). Historically, the virus was suggested to be phylogenetically (Calisher, 1988) and antigenically (Potterfield, 1975) distinct from other flaviviruses. The virus was later shown to be closely related to Skalica virus, a flavivirus that was isolated from a bank vole in 1976 (Guirakhoo *et al.*, 1991).

Two additional subgroups of the tick-borne viruses known as the seabird group and Kadam group have been identified. The Kadam group only includes one species known as Kadam virus (KADV) (Grard *et al.*, 2006). Other viruses within the tick-borne flavivirus group cluster according to the illness they cause. One such group is the “hemorrhagic tick-borne viruses”. As suggested by the

name, the group comprises of viruses that cause hemorrhagic fever viz. Omsk hemorrhagic fever virus (OHFV), Kyasanur forest disease virus (KFDV) and Alkhurma virus (ALKV).

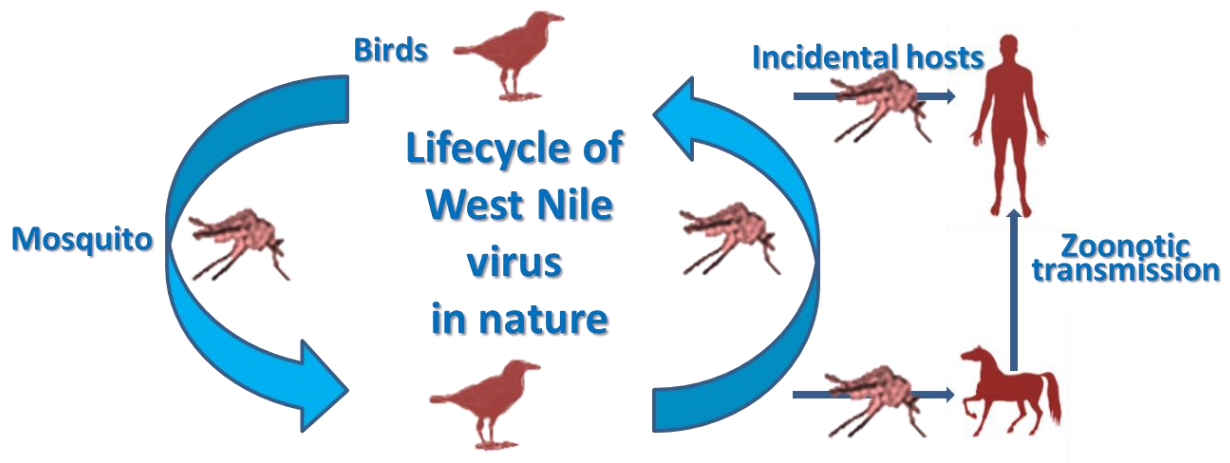
### **1.3.2. Mosquito-borne flaviviruses**

The mosquito-borne group is subdivided into seven groups, some of which have been implicated as major human pathogens. One such group, the Japanese encephalitis group, includes Japanese encephalitis virus (JEV), Kunjin virus (KUNV), St. Louis encephalitis virus (SLEV) and West Nile virus (WNV). Disease caused by JEV has been reported in humans, horses, swine and other domestic animals. Encephalitis cases suggestive of JEV have been reported from as early as 1871 in Japan (Halstead and Jacobson, 2003). The virus is currently endemic in many parts of Asia and is reportedly the leading cause of childhood encephalitis. SLEV obtained its name from a major outbreak in St. Louis, Missouri during 1933, where more than 1000 cases of encephalitis were observed. The virus was later isolated from deceased patients by inoculation into Rhesus monkeys. SLEV is currently found in southern Canada, Argentina and North America (Reisen, 2003).

Using complete genome sequences, Coia and co-investigators (1988) showed that WNV and KUNV viruses are 93 % homologous in the coding regions and the Committee for the taxonomy of viruses reported some strains of WNV falling in a unique lineage with isolates of KUNV. This high degree of similarity between the two viruses suggests that KUNV is a subtype or Australian variant of WNV (Hall, 2006). A more recent study used phylogenetic analyses to indicate that certain strains of WNV are more similar to KUN and form a separate lineage to the rest of WNV strains suggesting the addition of more subtypes in the WNV group (Hall *et al.*, 2006). Both viruses are associated with febrile illness with rash or mild encephalitis in humans (Scherret *et al.*, 2001) as well as neurological disease in horses (Badman *et al.*, 1984; Venter *et al.*, 2010), as illustrated in a schematic diagram that shows various species involved in the transmission of WNV in nature (Figure 4).

The two viruses were isolated years apart: WNV was first isolated from the blood of a febrile woman in the West Nile district of Uganda in 1937 (Smithburn *et al.*, 1940), while KUNV was isolated from *Culex annulirostris* mosquitoes that were captured in northern Australia in 1960 (Komar, 2003). Cases involving WNV outbreaks in humans, horses and birds have been reported in Africa, Asia, Europe (Karabatsos, 1985) and more recently in the USA (CDC., 1999). The earliest recorded epidemic of WNV occurred in Israel in 1950 at a military camp near Pardes Hanna, north of Tel Aviv where 500 cases of infection were treated in hospital during the peak of the epidemic. Outbreaks were reported annually in Israel from 1951 to 1954 and again in 1957. In 1962 WNV was identified as the cause of an outbreak in France and cases were reported in 1963 and 1964 with few reports subsequent until more recently. Due to the itinerant nature of migratory birds which act as reservoirs

for WNV, the virus has spread to other parts of the world including the Americas in 1999. The first WNV epidemic to ever occur in the Americas was localized around New York City and resulted in 62 confirmed human cases, seven of whom had a fatal outcome (Rappole et al 2000). Several thousand captive and wild birds, particularly American crows, died during this epidemic which was later attributed to the introduction of an Israeli strain of WNV by migratory birds into the Americas. The virus has since been associated with various human outbreaks in the Americas with over 39 000 cumulative cases to date and approximately 17 000 deaths (CDC, 2014). In Europe, the circulation of the virus had been confirmed for a number of decades in regions of Eastern and Central Europe. In the summer of 2010 areas previously considered to be non-endemic for WNV in Europe such as Greece (Papa et al., 2010), reported outbreaks of the virus and this resulted in the highest number of cases ever reported in this region (ECDC, 2013).



**Figure 4: Schematic representation of the transmission of WNV in nature. The virus is maintained between mosquitoes and birds and spillover to incidental hosts is achieved through the bite of an infected mosquito.**

Initial phylogenetic analyses of isolates from geographically distinct regions showed that there were two distinct lineages of WNV (Lanciotti *et al.*, 1999). Isolates from central and northern Africa, Europe, Israel and USA grouped in lineage 1 while isolates solely from central and southern Africa as well as Madagascar grouped in lineage 2. The lineages did not correlate with pathogenicity, source of infection or geographic distribution. In later years, more strains that were divergent from the two main lineages were isolated in various geographical areas and additional lineages 3 (Lvov *et al.*, 2004) and 4 (Bakonyi *et al.*, 2005) were suggested for inclusion in WNV classification. To date, a fifth lineage has been proposed for WNV following phylogenetic analyses of 13 strains isolated in India that formed a novel divergent clade (Bondre *et al.*, 2007). Detection of WNV RNA

from *C. pipiens* mosquito pool in Spain, may also suggested a putative new WNV lineage (Vazquez *et al.*, 2010).

Other medically important groups of mosquito-borne flaviviruses are the dengue and the yellow fever groups. The dengue group comprises of five viruses, four of which are serotypes (1-4) of the dengue virus (DENV); while the yellow fever group includes many flaviviruses including yellow fever virus (YFV) and Wesselsbron (WESSV) virus. According to the World Health Organization (WHO) *Aedes aegypti* and *A. albopictus*, commonly known as tiger mosquitoes, are the primary vectors of DENV responsible for transmitting the virus to humans. The virus occurs in central and south America, south and southeast Asia, sub-Saharan Africa, the Caribbean and pacific regions (Thomas *et al.*, 2003). The virus currently infects an estimated number of 50-100 million people per year and 500 000 of these cases require hospitalization (WHO, 2013). The virus has been reported to cause a more severe form of disease in children than in adults, with a collective fatality rate of 2.5%.

The epidemiology of DENV differs from that of YFV in that YFV is only found in the tropical regions of South America and sub-Saharan Africa and not in Asia. The number of *Aedes* species infecting humans in Africa is much greater than that of the species in South America. Virus activity is found in many parts of Africa, with the highest number of cases occurring in west Africa where epidemics have been known for many centuries (Gould and Solomon, 2008). The majority of the cases in South America occur in the Amazon, Orinoco and Araguaia river regions (Barrett and Monath, 2003).

WESSV, which is a member of the YFV group, is not as common in humans as YFV and is rather implicated as a causative agent of abortion and death of newborn offspring of cattle, sheep and goats (Blackburn and Swanepoel, 1980). Swanepoel and Coetzer (1994) reported on an unusual case of laboratory infection of the virus, where the subject developed symptoms suggestive of encephalitis after spraying the virus into their eyes. The virus was first isolated from infected sheep in 1955 in the northern district of the Free State in South Africa, in a small rural town of Wesselsbron (Weiss *et al.*, 1956). Since its identification, WESSV has also been isolated in other parts of Africa such as Nigeria and the sub-Saharan African country of Madagascar (Tomori *et al.* 1981; Morvan *et al.*, 1990). WESSV was also isolated outside Africa from mosquitoes trapped in Thailand, suggesting an expansion in the epidemiology of this virus (Swanepoel and Coetzer 1994).

#### **1.4. West Nile virus and Wesselsbron virus in South Africa.**

Although WNV outbreaks have been reported in many parts of the world, it is in southern Africa however, where the largest epidemic of WNV was recorded. Specifically, the epidemic occurred

within the Karoo and Northern Cape Province of South Africa in 1974 and involved tens of thousands of human cases over a 2 500-km<sup>2</sup> area (McIntosh and Jupp, 1976). Between 1983 and 1984, another epidemic was recorded concurrently with a Sindbis virus epidemic in the Witwatersrand-Pretoria region of South Africa. Prior to these epidemics, sporadic cases of WNV had been recorded in South Africa within the former Transvaal and Orange Free State regions and seroprevalence was recorded in cattle sera collected in Zimbabwe (Blackburn and Swanepoel, 1980). A study performed using cattle sera collected between 1983 and 1984 from 50 herds across South Africa reported a seroprevalence of 1.3% for WNV (Burt *et al.*, 1996). A serological survey of dogs from the highveld region of South Africa showed that 138 of 377 (37%) had neutralizing antibodies against WNV (Blackburn *et al.*, 1989). A recent study showed a seropositivity rate of 56% when a total of 2 393 serum samples obtained from humans, cattle, sheep and wild animals in the Free State region were screened using an in house ELISA (Mathengtheng and Burt, 2014). Variation in seroprevalence likely reflects the seasonal changes and differences in rainfall with high seroprevalence detected after periods of exceptionally high rainfall.

Until recently, all South African isolates of WNV belonged to lineage 2 which was presumed to comprise less virulent strains and this was supported by the lower number of cases reported in this region compared to those reported in the Northern hemisphere. However, phylogenetic comparisons (Burt *et al.*, 2002) and transmission of neuroinvasive West Nile fever (Venter *et al.*, 2010) showed that pathogenic and neuroinvasive strains do exist in lineage 2 and WNV may have been underestimated in South Africa. Moreover, a WNV isolate belonging to lineage 1 was recently detected in South Africa in the brain of a pregnant mare presenting with signs of neurological disease (Venter *et al.*, 2011). Subsequent to onset of illness, the animal aborted her foetus and died. Further evidence of neurovirulent strains belonging to lineage 2 of WNV was reported in a total of five horses that presented with unexplained fever and/or neurological signs (Venter *et al.*, 2009). WNV infection was confirmed by RT-PCR and WNV was isolated from one of the horses. Four of these horses were located in the Gauteng province and one in the Western Cape region of South Africa.

Strains of WNV belonging to lineage 2 have been associated with outbreaks in Europe since 2004, demonstrating the expansion in the geographical distribution of lineage 2 strains. The first emergence of a lineage 2 strain was in birds of prey in Hungary and by the year 2008 the strain had spread throughout Hungary and into eastern Austria (Bakonyi *et al.*, 2006). In the year 2010, the first outbreak of WNV was reported in Greece where a total of 191 people presented with clinical symptoms of neuroinvasiveness (Papa *et al.*, 2010). All the patients were elderly and WNV infection lead to a fatal outcome in 32 of them. Following the laboratory confirmation for WNV in these

patients, mosquitoes were trapped at the sites where infections had occurred and the presence of WNV RNA was determined by RT-PCR assay. The detected WNV RNA from mosquito pools provided evidence that the strain belonged to lineage 2 and was similar to that associated with outbreaks in Hungarian birds and South African horses (Papa *et al.*, 2011).

In contrast to WNV, WESSV has not been associated with a fatal outcome of disease and cases involving human infection with WESSV may be underreported. Following the initial identification of the viral agent in 1955, an outbreak of RVFV in 1956 in the Kroonstad district, northeast of Wesselsbron, led to the demonstration of WESSV antibody in affected lambs although no virus could be isolated at this stage. WESSV was only successfully isolated from one lamb within this cluster in 1958. Due to the intertwined nature of outbreaks involving RVFV and WESSV, live partially attenuated vaccines based on these viruses were prepared and sold together for dual use in South Africa and Zimbabwe. Due to their abortifacient outcome in pregnant sheep, these vaccines were not recommended for use in pregnant animals. The virus was subsequently isolated at intervals between 1955 and 1983 from a variety of vertebrates including cows, sheep as well as humans. In a serological survey of human sera performed in South Africa, WESSV was recorded in 32% of samples collected in the subtropical region of KwaZulu Natal province and 0.7% in the southern Cape (McIntosh 1980). Recently, WESSV was isolated from two human cases in which an initial diagnosis of Rift Valley fever virus (RVFV) infection was suspected due to an outbreak of the RVFV that occurred in South Africa, with human and livestock infections, between 2010 and 2011. These isolates were included in a phylogenetic analysis of collective WESSV isolates based on the sequence data of the NS5 gene (Weyer *et al.*, 2013). The study identified 2 clades of WESSV circulating in southern Africa. Clade I included isolates collected from South Africa and Zimbabwe, while clade II was comprised exclusively of isolates from the KwaZulu Natal province of South Africa.

The true prevalence of WNV and WESSV in South Africa is not known and this is largely due to limited facilities that conduct routine diagnosis for infection with these pathogens and the high serological cross-reactivity between flaviviruses. The cross-reactivity of flaviviruses has been linked to common group epitopes on the E protein and frequently complicates differentiation among species of this genus (Gubler, 1998).

### **1.5. Other flaviviruses previously identified in South Africa**

To date, a total of four flaviviruses were identified for the first time in South Africa. All of the four existing South African flaviviruses were identified in the 1950s, namely: WESSV and Spondweni (SPOV) were identified in 1955, followed by Banzi virus (BANV) in 1956 and Usutu virus (USUV) in 1959 (Gould and Solomon, 2008). Apart from WNV, no other non-indigenous flavivirus species has

established endemicity in this region. The earliest report on DENV infection in the Natal region of South Africa dates back to 1897 with another outbreak reported in 1901. However, the largest outbreak of DENV in southern Africa was documented in the Natal region between 1926 and 1927 (Edington, 1927). During this outbreak, 50000 cases were recorded with 60 fatalities along the coast of Durban (McIntosh, 1986). Heavy rainfall likely contributed to the outbreak after importation of the virus; nevertheless the virus failed to establish itself in local mosquito populations (Sang et al, 2006). Dengue activity was reported in northern Mozambique in 1984 but fewer reports were known to exist until 2014 when an outbreak involving DENV2 was reported in the northern province of Cabo Delgado. The presence of DENV in other parts of Africa was established around the time the first reports were recorded in southern Africa, such as in Zanzibar (1823, 1870), Burkina Faso (1925), Egypt (1887, 1927) as well as Senegal (1927-1928). Between 1960 and 2010, 20 laboratory-confirmed outbreaks had been reported in 15 African countries with the highest burden occurring in the eastern parts of Africa (Were, 2012). Although all four serotypes of DENV have been reported in parts of Africa, DENV2 has been observed to cause the highest number of epidemics. DENV is not known to be endemic in southern Africa but over the past 25 years sporadic cases of infection have been documented in travelers returning to South Africa, Namibia and Zambia (Were, 2012). A retrospective study conducted in South Africa reported that travelers returning from South-East Asia and Central-West Africa were the most affected by dengue fever in this country (Msimang et al., 2014). Although Zanzibar is not regarded as a dengue fever risk area, European travelers returning from Zanzibar were confirmed positive for dengue fever in 2014 (NICD, 2014).

SPOV was isolated from pools of *Mansonia uniformis* mosquitoes in the northern Natal region of South Africa but the principal vector for this virus has been described as *Aedes circumluteolus* (Gould and Solomon, 2008). The virus was later associated with human disease in the western parts of Africa where American natives residing in Burkina Faso, Gabon and Cameroon had also contracted the virus (Wolfe *et al.*, 1982). Symptoms associated with SPOV infection in human include fever, chills, myalgia, nausea and a rash. The virus is pathogenic for adult white Swiss mice when inoculated intracerebrally and was reported to exhibit exclusive tropism with histopathological changes in nervous tissue of that host. Though an immune response was successfully evoked in vervet monkeys, guinea-pigs and rabbits, no clinical signs were observed (Kokernot *et al.*, 1957).

BANV was isolated from a febrile 9 year-old boy near the Usutu river (now called Maputo River) in South Africa (Smithburn *et al.*, 1959). The virus was isolated from the patient serum by inoculation into infant mice which showed signs of infection 5 days post-inoculation. The patient was bled again after 34 days and the virus was strongly neutralized in the presence of the convalescent sera. Using neutralization assays, the virus was further evaluated for cross-reactivity with other arboviruses and



showed weak reactivity against antibodies derived from WESSV, YFV, ZIKV and DENV1. No reactivity was observed with antibodies derived from flaviviruses WNV, SPOV, DENV2 and other non-flaviviral arboviruses. Since its identification, BANV has been isolated from mosquitoes, cattle, rodents and hamsters in South Africa, Mozambique, Zimbabwe and Kenya (McIntosh et al., 1976, Metslaar *et al.*, 1974) and also associated with a case of febrile illness in Tanzania (Williams and Woodall, 1964). Based on the predicted amino acid sequence of the NS5 gene of BANV, the virus demonstrated close relation to mosquito-borne flaviviruses and in particular, YFV (Fulop *et al.*, 1995).

USUV was isolated in 1959 from *Culex neavei* mosquitoes in the Natal region of South Africa and belongs to the JEV serocomplex of flaviviruses (Gould and Solomon, 2008). Subsequent to its identification, USUV was detected from different bird and mosquito species in other parts of Africa (Williams and Woodall, 1964) with the first case of human infection reported in the Central African Republic (Adam and Digouette, 2005), contrary to historical classification of USUV as a non-pathogenic flavivirus species. Until recently, the virus was only known to be endemic in Africa and had never been observed in other parts of the world. In the year 2001, USUV was observed for the first time outside Africa when considerable fatalities of Eurasian blackbirds (*Turdus merula*), great gray owls (*Strix nebulosa*) and barn swallows (*Hirundo rustica*) took place in Vienna, Austria (Weissenböck *et al.*, 2002). Infections in dead birds were confirmed by histological analyses of livers and spleens, which revealed multifocal acute necroses, and virus was isolated in Vero cells from organ homogenates, followed by RT-PCR confirmation. Since then, the virus has been detected in other European countries such as Hungary, Italy, Spain and Switzerland from birds and mosquito species (Vazquez *et al.*, 2011). Based on nucleotide homology (>99%) between outbreaks in Europe and similar clinical outcomes, the virus is believed to have been introduced once from Africa to Vienna and subsequently spread to central Europe. A more recent study by Weissenböck and co-authors (2013) has however shown evidence for the presence of USUV in Europe prior to the outbreak in Vienna. The authors retrospectively analyzed archived tissue samples collected from bird deaths that occurred in 1996 in the Tuscany region of Italy. Using partial sequencing of the NS5 region, the USUV sequences identified from archived tissue samples were shown to be identical to the sequences from USUV in Austria and 95% identical to the South Africa strain identified in 1959. The clinical impact of the virus was further elucidated when two immunocompromised individuals presented with neurological disorders due to USUV infection in Italy. One of the patients had undergone hemicolectomy (Pecorari *et al.*, 2009) and the other had undergone orthotopic liver transplantation (Cavrini *et al.*, 2009). These patients were diagnosed concurrently as part of a surveillance programme and may have been infected directly through a mosquito bite or indirectly

through an infected donor. These patients represent the first human cases of USUV neuroinvasive illness ever described (Vazquez *et al.*, 2011).

## **1.6. Prevention and treatment of flavivirus infections**

There are no antiviral drug therapies for any of the flaviviruses and treatment for patients with acute illness is supportive. There is, therefore, a high demand for vaccine development to curb disease caused by medically significant flaviviruses. Medical researchers have been advancing vaccine development for flaviviruses, but to date human vaccines have been registered for only four species of flaviviruses, namely: YFV, JEV, TBEV and KFDV (Heinz and Stiasny, 2012).

Since its development in 1937, the YF-17D vaccine has been highly effective in reducing the burden of YF infection in endemic areas. However, in the last 10 years serious adverse events such as viscerotropic and neurotropic disease have been associated with administration of the YF vaccine, particularly in elderly and immunocompromised individuals (Barrett and Teuwen, 2009). Additionally, adverse events in some vaccinees are exacerbated by allergic reactions from chicken embryo proteins acquired from embryonated eggs during vaccine preparation. In order to circumvent these obstacles, a purified inactivated vaccine was produced in Vero cells and reported to be safe and immunogenic in human trials (Monath *et al.*, 2011). Similar to the initial preparation of YF-17D vaccine, the inactivated TBEV vaccine is also produced in embryonated eggs with efficacy rates of 98% (Heinz *et al.*, 2007).

Tauber and colleagues (2007) also reported on a newer preparation of inactivated JEV vaccine produced in Vero cells. The initial JEV vaccine was based on inactivated whole virus and live attenuated preparations derived from mouse brain and hamster kidney cells (Halstead and Thomas, 2010). The latest JEV vaccine purified from Vero cells is based on the attenuated SA-14-14-2 strain and is commercially available in China and other parts of the world.

Vaccine development against DENV has, over the years, been confronted with more particular challenges due to the four serologically distinct serotypes of this virus. The serotypes have been designated DENV1 to DENV4 and although their respective geographical origin is not known, evidence suggests that DENV originated in Africa and subsequently evolved into the four serotypes (Holmes and Twiddy, 2003). In addition to the lack of an animal model that reproduces the disease observed in humans (Durbin and Whitehead, 2013), vaccine development against DENV is defied by antibody-dependant enhancement (ADE) of disease that has been observed in DENV-infected humans. ADE occurs as a result of the limited period of heterologous protection by antibodies raised against one serotype of DENV, against subsequent infection with another serotype of DENV. Epidemiological observations have reported that previous infection with one serotype can

predispose humans to the severe forms of DENV during secondary infection with a heterologous serotype (Guzman and Vazquez, 2010). An effective solution to this phenomenon is a tetravalent DENV vaccine (TDV) that can induce simultaneous protection against all four serotypes of DENV upon administration. A recent phase II challenge study by Sun and co-authors (2013) demonstrated the significance of high levels of neutralizing antibodies in protection against challenge with underattenuated strains of DENV in humans that were previously immunized with the TDV vaccine. Additionally, no signs of viremia or enhanced disease were associated with virus challenge in the study, suggesting that the vaccine may have potential in averting the long-standing hurdle of ADE. The findings of this study were viewed as motivation and a point of reference to initiating challenge studies involving native DENV with humans as challenge models (Durbin and Whitehead, 2013)

### **1.7. The WNV-E protein as a diagnostic agent and candidate vaccine.**

The E protein is the major surface protein of flaviviruses and it is found on the viral surface in a heterodimer with the viral membrane (M) protein (Rey *et al.*, 1995). A number of studies have reported that the majority of antibodies elicited by infection with a flavivirus are directed to epitopes within the E and NS1 proteins of the virus (Schepp-Berglind *et al.*, 2007). Focus has been directed towards the E-protein due to its ability to induce neutralizing antibody responses. Besides the stimulation of B and T cell immune responses, the M and E viral proteins also play a role in host range, tissue tropism, replication as well as virion assembly (Dauphin and Zientara, 2006).

The E protein of flaviviruses comprises of three domains designated domain I to III. Of the three domains, the EDIII domain has been the most intensely investigated. This is due to the observation that this domain is highly antigenic with primary linear epitopes (Beasley and Barrett, 2002) based on X-ray crystallography (Rey *et al.*, 1995), and is probably involved in viral receptor binding (Crill and Roehrig, 2001). Martina *et al.* (2008) explained that the domain contains predominantly subcomplex and type-specific epitopes which mainly induce neutralizing antibodies. In addition, the EDIII can be expressed as a recombinant protein that has the ability to fold as a functional fragment capable of inducing neutralizing antibodies and may therefore have the potential for use as a subunit vaccine.

WNV remains a medically significant flavivirus for which no human vaccine has been licensed. Many researchers have evaluated in-house candidate vaccines for efficacy against WNV infection in various species including humans (Ng *et al.*, 2003; Ledgerwood *et al.*, 2011). Currently, three veterinary vaccines are commercially available in the USA for immunization against WNV in horses and two vaccines are licensed for use in geese in Israel (Posadas-Herrera *et al.*, 2010). Preparations for two of these vaccines were based on the inactivation of whole virus and co-administration with an adjuvant (Ng *et al.*, 2003; Minke *et al.*, 2004; Samina *et al.*, 2005). These

vaccines require boosters and provide 80-94% protection in vaccinated animals. Additionally, inactivated vaccines are much safer in vaccinees as they do not pose a risk of reversion to wild type compared to live attenuated vaccines when administered in immunocompromised individuals. A disadvantage commonly associated with inactivated vaccines however, is that protective immunity is frequently short lived. Recombinant proteins may present a solution to developing both safe and highly immunogenic vaccines. Two of the commercially available veterinary vaccines were developed using recombinant technology in the form of a canarypox virus which expresses WNV E antigens (RecombiTek) and a chimeric virus that uses the YFV genome as a backbone (ChimeriVax-WNV) (Monath *et al.*, 2006)

A review of literature on the functions and properties of WNV proteins illustrates the importance of the prM /E proteins in protective immunity and the role of NS1 proteins in protection against neuroinvasiveness, inducing a cell mediated immune response as well as playing a role in humoral immunity. It has been shown that antibodies directed against the E protein are able to neutralize the virus and that the dominant neutralizing epitopes have been mapped to ED III region. Advances in vaccine technology such as recombinant live virus, subunit and DNA vaccines, have been used to develop several novel vaccine candidates. Recombinant vaccines have been constructed using the prM/E gene or truncated forms of the E gene. Attempts to develop an efficacious vaccine against WNV has involved recombinant vaccines or modified live vaccines. For example, modified live vaccines using canary pox vector to express WNV prM and E (Karaca *et al.*, 2005) and a live chimera vaccine containing prM and E expressed using a YF-17D vector were administered to horses prior to lethal challenge and were both shown to prevent fatalities and reduce clinical signs and symptoms in the animals. (Seino *et al.*, 2007).

Other novel vaccine candidates include a DNA plasmid vaccine encoding prM and E proteins producing virus like particles (VLP) and can induce protective neutralizing antibody in mice. A truncated E protein protected mice against challenge and antisera from an immunized horse conferred protection in challenged mice (Ledizet *et al.*, 2005). Another truncated E and NS1 based vaccine produced in a *Drosophila* cell expression system induced neutralizing antibody and a cellular response in mice (Lieberman *et al.*, 2007). Although these vaccines have shown that a neutralizing antibody response can readily be induced against prM and E proteins, an ideal vaccine should also induce a strong cellular immune response that is important in clearance of natural infections. Schepp-Berglind *et al.*, (2007) describe a novel vaccine based on adenovirus mediated expression of West Nile C, prM, E and NS1 proteins which induced both humoral and cellular immune responses in mice.

There are certain obstacles that need to be considered when developing commercial human vaccines: (i) DNA vaccines, although providing good responses in animals, frequently have poor immunogenicity in humans (ii) subunit vaccines are difficult to produce in large quantities and (iii) live-vectored vaccines may be ineffective (Widman *et al.*, 2008). In some instances, a truncated E protein was administered with adjuvant to induce stronger immune responses. Martina *et al.* (2008) and Chu *et al.*, (2007) focused on the EDIII expressed protein and coupled the protein with oligodeoxynucleotides (CpG-DNA) as an adjuvant to generate a high titer of WNV-neutralizing Abs, which was also shown to neutralize a related *Flavivirus*, Japanese encephalitis virus. The authors also demonstrated that these vaccines could invoke a cellular immune response in the animal model. A phase I clinical trial using a DNA vaccine encoding the prM and the E glycoprotein of the NY99 strain of WNV under the transcriptional control of the CMV/R promoter was conducted in 30 healthy adults aged between 18 and 65 years old (Ledgerwood *et al.*, 2011). The 3-dose regimen showed characteristics of safety and tolerance, with induced T cell and antibody responses in the majority of vaccinees. The vaccine elicited responses of greater magnitude when compared to the earlier-generation construct of the vaccine that only utilized the CMV promoter.

### **1.8. Laboratory diagnosis of flavivirus infection.**

Serological diagnosis of flaviviruses is widely used in many laboratories but may be compromised by the high degree of antigenic cross-reactivity between species, and the time required for detection of antibodies during acute infections. Virus isolation is the “gold standard” for accurate diagnosis of flavivirus infection but it is a laborious technique that requires skilled personnel to keep the virus viable without contamination and requires a longer time to result. In this regard, molecular techniques have been the most rapid and accurate means of diagnosis prior to virus isolation. PCR-based techniques rely on the detection of viral nucleic acid and therefore present an added advantage for detection of both viable and non-viable infectious agents. The challenge observed with the use of molecular techniques however, is the inability to detect strains of a virus that circulate in different geographical areas (e.g.: YFV vaccinal, African or American strains). To subvert this challenge, molecular diagnostic tests must be updated continuously to include sequence data of newly isolates viruses (Domingo *et al.*, 2011).

As shown in Table 2, serum can be collected for both serological and molecular diagnosis of medically important flaviviruses. Additional clinical samples are acceptable for molecular diagnosis of flaviviruses, depending on the suspected viral pathogen. Importantly, the clinical history of the patient must be known for the correct test to be performed in order to achieve accurate diagnosis of infection.

**Table 2: *Flavivirus* diagnosis algorithms. (Modified from Domingo *et al.*, 2011)**

<b>Virus</b>	<b>Acute phase</b>	<b>Convalescent phase</b>	<b>Preferred sample (molecular tests)</b>
YFV	RT-PCR, IgM, virus isolation.	IgM, IgG.	Serum, plasma and tissue.
DENV	RT PCR, NS1 Ag, IgM, virus isolation.	IgM, IgG.	Serum, plasma, CSF and PBMCs.
WNV	RT-PCR, IgM, IgG.	IgM, IgG.	CSF and serum.
JEV	RT-PCR, IgM, IgG.	IgM, IgG.	CSF, serum, blood and PBMCs.
TBEV	RT-PCR, IgM, IgG.	IgM, IgG.	CSF and serum.

**Ag= Antigen, CSF=Cerebrospinal fluid, PBMC=Peripheral blood mononuclear cell, RT-PCR=Reverse transcriptase PCR. Serological tests may also apply.**

According to Domingo and co-authors (2011) following onset of illness, flaviviruses can be found in serum or plasma generally 2-7 days. The immune response arises between 5-7 days from onset with IgM antibodies peaking after day 15. The IgM antibodies can persist for months (DENV) or even years (WNV). Depending on the flavivirus concerned and the specific host responses, IgG antibodies occur between days 8 and 10 but tend to remain detectable throughout life.

### **1.9. Influence of vertebrate hosts on emerging viruses**

The role of vertebrate hosts in the maintenance and circulation of flaviviruses has serious public health implications (Calisher and Gould, 2003). Vertebrate hosts are not involved in the direct transmission of tick- and mosquito-borne flaviviruses but form a link for virus transmission between vectors. The hosts can vary from (but are not limited to) birds in WNV transmission, monkeys in YFV transmission and rodents in TBEV transmission (Ludwig and Iacono-Connors, 1993). In central Europe, the major role in TBEV circulation has been attributed to rodents, particularly the yellow-necked mouse *Apodemus flavicollis* and bank vole (*Myodes glareolus*) (Labuda *et al.*, 1997). Rodent hosts play a significant role in TBEV transmission, as they are able to promote both viremic and non-viremic types of transmission (Knap *et al.*, 2012). The resistance to flavivirus-induced disease in wild mouse populations has previously been reported, and was shown to extend to both mosquito- and tick-borne flaviviruses (Brinton and Perelygin, 2003). According to the authors, this resistance to infection ensures successful maintenance of flaviviruses in mice without leading to a lethal outcome. This phenomenon was attributed to a resistant allele of a single dominant autosomal *Flv* gene which is constitutively expressed without the induction of interferon in infected mice. These mice were observed to achieve this resistance by maintaining a low viral titer contrary to susceptible mice.

In addition to their involvement in maintenance of flaviviruses, rodents have also been implicated in the transmission of other medically significant viruses such as arenaviruses, lyssaviruses and

hantaviruses. These viruses continue to emerge in many parts of the world and cause serious human illnesses. Lujo virus, a relatively novel arenavirus, was described in South Africa in 2008 from a woman who had been infected in Zambia (Briese *et al.*, 2009). The virus was transmitted nosocomially from the index patient to four other patients and resulted in a case fatality rate of 80% (4/5). Characterization of the highly pathogenic Lujo virus emphasizes the need for increased virus surveillance efforts in Africa. Another important group of rodent-borne viruses, hantaviruses, was shown to emerge in Africa. Prior to 2006, no hantavirus species had been identified in Africa until hantavirus RNA was detected in a wood mouse trapped in Guinea, west Africa (Klempa *et al.*, 2006). This highlights the role of vertebrate hosts not only in the circulation, but also in the emergence of viruses. This relatively recent finding suggests that hantaviruses may be present in other parts of Africa. The following section will focus on hantaviruses due to their notably continuous emergence in Africa.

### **1.9.1. Hantaviruses**

Hantaviruses are rodent-borne viruses transmitted to humans via faecal and urine aerosols. Hantavirus species are associated with a range of nephrotic diseases in Asia and Europe known variously as haemorrhagic nephrosonephritis, Korean haemorrhagic fever, Songo fever, epidemic haemorrhagic fever and nephropathia epidemica (Settergren *et al.*, 1987) but use of the generic term haemorrhagic fever with renal syndrome (HFRS) is advocated. The first outbreak of the disease was first reported during the Korean war in the 1950s. The disease was later reported in the Americas in 1993 in the Four Corners region, where it was initially referred to as Four Corners disease (Jonsson *et al.*, 2010). The disease is now referred to as hantavirus pulmonary syndrome (HPS) or hantavirus cardiopulmonary syndrome (HCPS) in the Americas. The prototype of the hantavirus genus, Hantaan virus (HTNV), was first identified with its reservoir, the striped field mouse (*A. agarrarius*), by Lee and co-workers in 1983 (Lee *et al.*, 1983). Since this unprecedented finding in hantavirus research, increased surveillance efforts lead to the identification of pathogenic hantaviruses such as Dobrava virus (DOBV) and Seoul virus (SEOV) in Asia (Golovljova *et al.*, 2000), Puumala virus (PUUV) in Europe (Clement *et al.*, 2003) as well as Sin Nombre virus (SNV) in the Americas (Hjelle *et al.*, 1994). To date, over 21 species of pathogenic hantaviruses have been transmitted from the rodent host to humans resulting in infections ranging from proteinuria to pulmonary edema and hemorrhagic illnesses (Jonsson *et al.*, 2010).

### **1.9.2. Hantaviruses in Africa**

Hantaviruses have historically been known to be carried by rodents of the family *Muridae* in nature and were divided into three groups based on the rodent host they infected, namely: *Murinae*,

*Arvicolinae* and *Sigmodontinae* (Ulrich *et al.*, 2002). However, recent studies have reported on the isolation of novel hantaviruses in a variety of other species including shrews, moles and bats (Guo *et al.*, 2013; Kang *et al.*, 2011). Thottapalayam virus (TPMV) was isolated from an Asian house shrew (*Suncus murinus*) in 1964 and represents the first non-rodent hantavirus ever identified (Song *et al.*, 2007). Since the identification of the first African hantavirus, Sangassou virus (SANGV), an additional eight hantaviruses have been identified in the African continent by detection of genomic RNA. Unpredictably, seven of the nine current African hantaviruses were identified from non-rodent hosts, as shown in Table 3 and Figure 5. Tanganya virus (TANGV) was the first non-rodent hantavirus to be identified in Africa (Klempa *et al.*, 2007) and introduced the notion of insectivores, and not just rodents, as possible carriers for hantaviruses. Although this virus was phylogenetically divergent from rodent-borne hantaviruses as expected, it also exhibited divergence from TPMV which was the only shrew-borne hantavirus species at the time. Subsequent to this finding, Azagny virus (AZGV), Tigray virus (TIGV), Kilimanjaro virus (KILV), Uluguru (ULUV) as well as Bowe virus (BOWV) were all identified in shrews from West Africa (Witkowski *et al.*, 2014). In addition, a total of two hantaviruses designated Magboi virus (MGBV) and Mouyassue (MOUV) were identified in bats, further expanding the known host range for hantaviruses. The identification of hantaviruses in bats has public health implications due to the itinerant nature of bats as this may increase transmissibility of hantavirus species to humans across large geographical areas. Following the identification of MGBV from a hairy slit-faced bat in Sierra Leone, the reclassification of the initial three phylogenetic groups was proposed by Weiss and co-authors (2012).

**Table 3: Summary of African hantaviruses identified to date. (Modified from Witkowski *et al.*, 2014)**

Proposed virus name	Host animal	Geographical location	Reference
Sangassou (SANGV)	African wood mouse ( <i>Hylomyscus simus</i> )	Guinea	Klempa <i>et al.</i> , 2006
Tanganya (TANGV)	Therese' shrew ( <i>Crocidura theresae</i> )	Guinea	Klempa <i>et al.</i> , 2007
Azagny (AZGV)	West African pygmy shrew ( <i>Crocidura obscurior</i> )	Cote d'Ivoire	Kang <i>et al.</i> , 2011
Magboi (MGBV)	Hairy slit-faced bat ( <i>Nycteris hispida</i> )	Sierra Leone	Weiss <i>et al.</i> , 2012
Mouyassue (MGBV)	Banana pipistrelle ( <i>Neoromicia nanus</i> )	Cote d'Ivoire	Sumibcay <i>et al.</i> , 2012
Tigray (TIGV)	White-footed mouse ( <i>Stenocephalemys albipes</i> )	Ethiopia	Meheretu <i>et al.</i> , 2012
Kilimanjaro (KILV)	Kilimanjaro mouse shrew ( <i>Myosorex zinki</i> )	Tanzania	Kang <i>et al.</i> , GenBank entries
Uluguru (ULUV)	Geata mouse shrew ( <i>Myosorex geata</i> )	Tanzania	Kang <i>et al.</i> , GenBank entries
Bowe (BOWV)	Doucet shrew ( <i>Crocidura douceti</i> )	Guinea	Gu <i>et al.</i> , 2013



Although the African hantaviruses have still to be confirmed as medically significant, a serological study in West Africa revealed a 1.2% seroprevalence of hantaviruses in humans (8/649) with neutralizing antibodies detected in 6 of the 8 positive sera (Klempa *et al.*, 2010). In the course of this seroprevalence study, an important observation was made in an 8 year-old boy presenting with typical HFRS symptoms. A serum sample collected after the acute phase of the illness showed high titers of IgM against SANGV (1:1600) whereas a sample collected 1 year later showed no evidence of IgM antibodies. The second sample also demonstrated a reciprocal neutralizing antibody titer of 80 only against SANGV and none of the other hantaviruses. The results of this research strongly suggest a correlation between SANGV and the HFRS illness in the patient. In a South African study, the seroprevalence was determined as 1% in a cohort of 1442 patient sera (Witkowski *et al.*, 2014). Despite the low seroprevalence rate, the authors were of the opinion that most cases may remain undetected due to antigenic differences in novel hantaviruses to current serological tests. A comparison was also made to Germany which has an overall prevalence rate of 1% but nearly 3000 patients are recorded per population of 80 million for hantavirus disease. Detection of antibodies against hantaviruses is achieved by using assays based on the nucleoprotein since it is the most abundant viral protein which also induces a strong humoral response in both humans and rodents (Jonsson *et al.*, 2010)

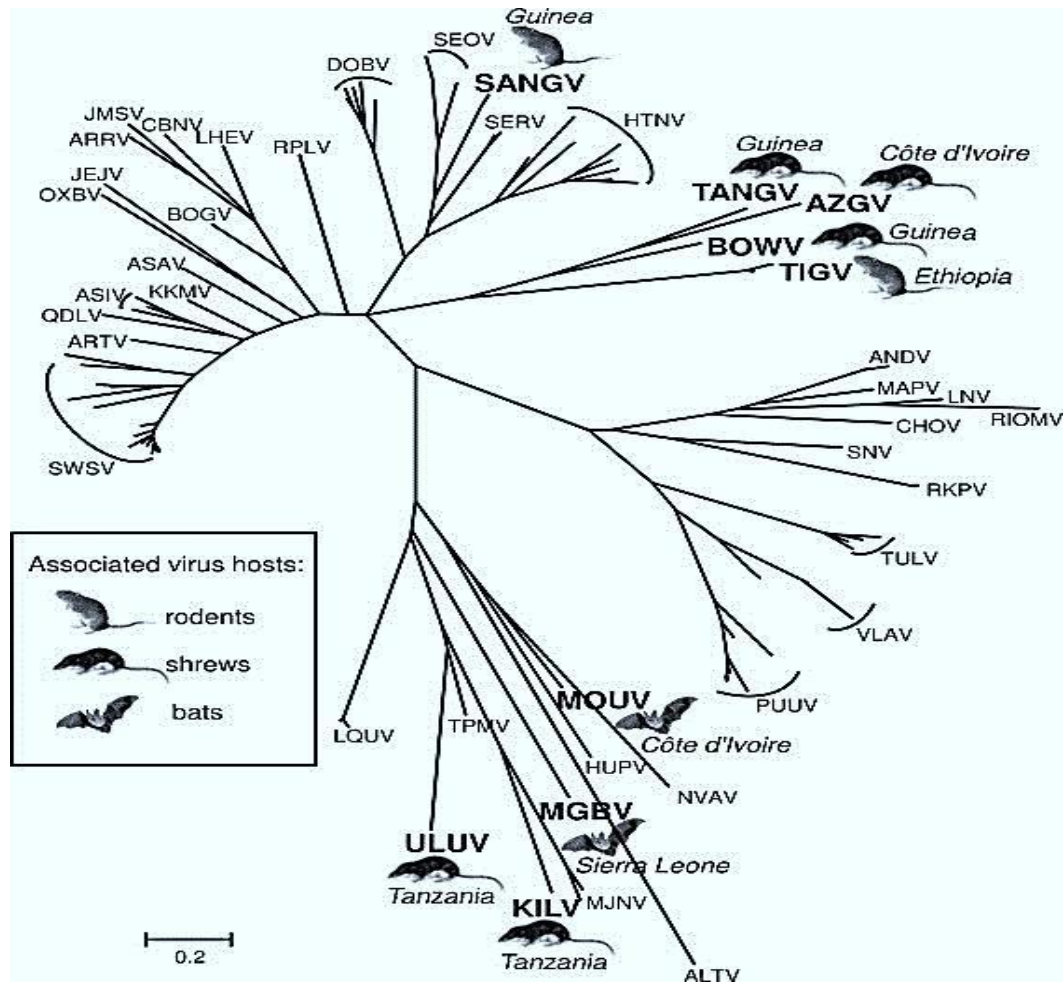


Figure 5: Hantavirus phylogenetic tree illustrating high divergence of hantaviruses detected in Africa.

Hantaviruses detected in Africa are marked by names in bold, the host symbol, and the country of origin. The phylogenetic tree was constructed on the basis of partial L segment sequences (306 nucleotides) in the MEGA5 program (Tamura *et al.*, 2011) by using the Maximum Likelihood (GTR+G evolutionary model). Scale bar indicates an evolutionary distance of 0.2 substitutions per position in the sequence. ALTV, Altai virus; ANDV, Andes virus; ARRV, Ash River virus; ARTV, Artybash virus; ASAV, Asama virus; ASIV, Asikkala virus; AZGV, Azagny virus; BOGV, Boginia virus; BOWV, Bowé virus; CBNV, Cao Bang virus; CHOV, Choclo virus; DOBV, Dobrava-Belgrade virus; HUPV, Huanqpi virus; HTNV, Hantaan virus; JEJV, Jeju virus; JMSV, Jemez Springs virus; KILV, Kilimanjaro virus; KKMV, Kenkeme virus; LHEV, Lianghe virus; LNV, Laguna Negra virus; LQUV, Longquan virus; MAPV, Maporal virus; MGBV, Magboi virus; MJNV, Imjin virus; MOUV, Mouyassué virus; NVAV, Nova virus; OXBV, Oxbow virus; PUUV, Puumala virus; QDLV, Qiandao Lake virus; RIOMV, Rio Mamore virus; RKPV; Rockport virus; RPLV, Camp Ripley virus; SANGV, Sangassou virus; SEOV, Seoul virus; SERV, Serang virus; SWSV, Seewis virus; SNV, Sin Nombre virus; TGNV, Tanganya virus; TIGV, Tigray virus; TPMV, Thottapalayam virus; TULV, Tula virus; ULUV, Uluguru virus; VLAV, Vladivostok virus. (Witkowski *et al.*, 2014). This image is used in accordance with the regulations outlined by the publisher (<http://www.elsevier.com/legal/elsevier-website-terms-and-conditions>) and permission was also granted by the corresponding author Prof. Detlev Kruger of the Charite-University Medicine, Berlin, Germany.

## **Problem identification, aim and objectives of the study.**

WNV is endemic to southern Africa but the true burden of disease associated with WNV infection remains unknown in this region. One of the largest epidemics of WNV ever recorded occurred in the Karoo and Northern Cape Province of South Africa in the year 1974 and involved tens of thousands of human cases over a 2,500-km<sup>2</sup> area (McIntosh and Jupp, 1976). Between 1983 and 1984, another epidemic was recorded in combination with a Sindbis virus epidemic in the Witwatersrand-Pretoria region of South Africa. Despite the occurrence of these epidemics, lineage 2 isolates of WNV (the lineage known to circulate in southern Africa) were regarded to be less virulent as they appear to be less frequently associated with fatalities and neurological complications compared to isolates in eastern Europe or the USA. However, phylogenetic comparisons (Burt *et al.*, 2002) and transmission of neuroinvasive West Nile fever (Venter *et al.*, 2010) demonstrated that pathogenic and neuroinvasive strains do exist in lineage 2 and WNV may have been underestimated in this region. Due to the atypical clinical picture of the mild form of disease, low awareness, as well as limited diagnostic capacity, cases involving WNV infection may remain underreported and overlooked.

The presence of the principal vector of WNV, mosquitoes belonging to the genus *Culex*, contributes to the maintenance, transmission and possible outbreaks of the virus especially during rainy seasons. These cases may be self-limiting and as such become eradicated without any form of medical intervention. It is however evident from a South African study by Zaayman and Venter (2012) that WNV infection in this region is not limited to the mild form of disease. This retrospective investigation was performed on samples obtained from hospitalized patients with febrile illness or neurological disease of unknown etiology in the Pretoria region of South Africa revealed the presence of WNV neutralizing antibodies in 36 out of 206 samples (Zaayman and Venter, 2012). Four of the positive samples were CSF collected from patients who were presenting with neurological signs and symptoms. One of the samples was positive for lineage 2 WNV RNA when subjected to RT-PCR. This particular patient further presented with a rash and was screened for measles and mumps at the time of illness, both of which were negative. The patient was not tested for WNV. Additionally, the increase in documented cases involving neuroinvasive WNV infection in horses (Venter *et al.*, 2009; Venter *et al.*, 2011) together with the associated risk of zoonotic transmission signify a need for prevention and control of WNV infection. Due to the fact that there is currently no WNV vaccine registered for use in humans, research for possible candidate vaccines is warranted. Current studies are moving focus from the use of live attenuated vaccines due to the risk they pose to certain individuals, such as immunocompromised and elderly individuals. An alternative solution is the evaluation of recombinant proteins as a means to identify immunogenic regions of the viral genome that may be useful in eliciting a protective immune response.

The presence of the mosquito-borne WESSV has also been established in southern Africa. Since it was first isolated in 1956 in the small town of Wesselsbron, in the Free State province, the virus has been known to cause disease in livestock leading to severe weight loss and abortion. Although not considered a serious human pathogen, WESSV has been associated with encephalitis in humans (Swanepoel and Coetzer, 1994). Currently, no routine testing is performed for WESSV diagnosis in South African patients. Similar to WNV infections, the virus remains unreported and overlooked. A recent study revealed evidence of WESSV circulation in southern Africa and transmission to humans when two isolates of this virus were obtained from human sera during a RVFV outbreak (Weyer *et al.*, 2013). Another challenge associated with proper identification of WESSV is the high level of cross-reactivity between members of flaviviruses. To circumvent the cross-reactivity between flaviviruses, the gold standard for flavivirus identification which is virus neutralization assay can be used. However, for biosafety level (BSL) 3 pathogens such as WESSV and WNV, virus neutralization assays cannot be performed in low-resource laboratories and it is also not practical for routine use. There is a very limited number of institutions that have facilities higher than a BSL 2 laboratory in developing countries. Therefore, there is a need for reagents that have the ability to differentiate between flaviviruses with the added advantage of being handled in a BSL 2 or lower facility.

The presence of tick-borne flaviviruses in southern Africa on the other hand, has not been established. Tick-borne flaviviruses are known to be prevalent in Europe where they cause serious human diseases and in some cases death. In a seroprevalence study that investigated the presence of other tick-borne viruses in suspected cases of CCHFV infection, serological evidence suggested the presence of tick-borne flaviviruses (Burt *et al.*, 1996). The finding is supported by the natural occurrence of the *Ixodes* tick species in various parts of southern Africa. Additionally, there are diagnostic services for tick-borne infections such as CCHFV and *Rickettsia* but these tests confirm infections in 10 % of the cases while the remainder of infections remains undiagnosed. This raises the question of whether the remaining undiagnosed causative agents could be flaviviruses, as infected patients present with signs of illness after a tick bite but remain undiagnosed. Similarities in clinical manifestations in patients infected with CCHFV and other viral hemorrhagic fevers also play a role in the impediment of proper diagnosis. More common diseases such as malaria, bacterial septicemias, leptospirosis, viral hepatitis and viral herpes need to be ruled out during the differential diagnosis. In Africa, particular consideration needs to be given to rickettsial infections caused by *R. africae* and *R. conorri*. Further consideration must be given to hantaviruses in Africa following an increase in detection of hantavirus RNA since 2006 when the first hantavirus was identified in this region. These viruses have been detected in species other than rodents such as shrews and bats. Moreover, serological detection of hantavirus antibodies in humans was reported in the 1980s

and recently in 2010 (Witkowski *et al.*, 2014). Although SANGV has been suggested to be the cause of HFRS in a 9 year-old boy (Klempa *et al.*, 2010), no African hantavirus has been confirmed to cause disease in humans. To date, a total of nine hantaviruses have been identified in the western and eastern parts of Africa from a mouse, shrew and a bat. While these viruses were all identified in the western regions of Africa, no hantaviruses have ever been identified in southern Africa. The identification of these novel hantaviruses in the western regions of Africa therefore suggests the possibility of other hantaviruses that may be circulating among susceptible hosts in southern Africa. Some members of hantaviruses play a significant role in human infections and as such may remain overlooked in southern Africa due to the non-specific clinical picture in infected patients and limited awareness in southern Africa.

For these reasons, the presence and epidemiology of flaviviruses and hantaviruses warrants investigation in southern Africa. In addition, the need for a vaccine and the potential application of domain III flavivirus envelope proteins as candidate vaccines warrants investigation of the immunogenicity of these proteins in an animal model.

Our study was aimed at achieving the following goals:

1. Determine the seroprevalence of flaviviruses in the Free State province of South Africa.
2. Develop molecular assays for differential diagnosis of flaviviruses, hantaviruses and CCHFV.
3. Characterize the immune response elicited by the envelope domain III protein of WNV.

The objectives of this study were to:

- i. Prepare recombinant EDIII proteins of LGTV, WNV and WESSV in a bacterial expression system for use in immunoassays.
  - ii. Develop multiplex PCR assays for the detection of flaviviruses, hantaviruses and CCHFV.
  - iii. Characterize the immune response elicited by the recombinant EDIII protein of WNV when administered in combination with saponin, alhydrogel and Titermax® gold as adjuvant.
-

## CHAPTER 2

### THE PREPARATION OF RECOMBINANT *FLAVIVIRUS* ENVELOPE DOMAIN III (EDIII) PROTEINS USING A BACTERIAL EXPRESSION SYSTEM.

#### 2.1 Introduction

Recombinant proteins have become important tools for the medical, biopharmaceutical, enzyme and agricultural industries. By means of genetic engineering, desired proteins are identified and generated to meet the enormous demand experienced in these industries. The first step in developing a recombinant protein is getting the target gene cloned into an expression vector and the desired protein will be subsequently expressed using an expression system. There is a wide variety of protein expression systems available and these include cell lines derived from bacteria, yeasts, molds, mammals, plants and insects. Factors to consider when an expression system is chosen include protein modifications, functionality, production speed, cost and yield (Demain and Vaishnav, 2009).

An article by Terpe (2006) described *Escherichia coli* (*E. coli*) as one of the earliest and most widely used hosts for the production of heterologous proteins. Advantages to using this system are based on the rapid nature of the expression, low cost, as well as the high yield of protein produced. The bacterium has the ability to alter the metabolic carbon flow, can accumulate recombinant proteins of up to 80% of its dry weight and survives a variety of environmental conditions. However, there are certain drawbacks that have been associated with this system. These include the production of unglycosylated proteins, proteins produced with endotoxins as well as proteins produced as inclusion bodies, which are inactive and require refolding (Jenkins and Curling, 1994). Codon bias and disulfide bond formation have also been identified as challenges when using bacterial expression systems (Sahdev *et al.*, 2008). Although these drawbacks may present a challenge with regards to using this system, it is still seen as the least expensive, easiest and quickest way to express proteins (Demain and Vaishnav, 2009).

In addition to the expression of a desired protein, consideration must be made concerning its purification. The successful purification of a recombinant protein will depend on the type of purification system used relative to the expression system that was utilized to obtain the protein. Affinity tags have become significant tools for purifying recombinant proteins from crude lysates and do not require prior removal of nucleic acid or other cellular material. A study that compared the efficiency of eight elutable affinity tags indicated major differences in purity, yield and cost among

the tags (Lichty *et al.*, 2005). It was observed in the study that histidine (HIS) tags provide good yield of tagged proteins using inexpensive methods, but only moderate purity from *E. coli* extracts and relatively poor purification from yeast, insect and mammalian extracts.

The recombinant EDIII (r-EDIII) protein of flaviviruses has been expressed using various strains of *E.coli* (Chavez *et al.*, 2013; Chu *et al.*, 2007; Bhardwaj *et al.*, 2001). The r-EDIII protein was biologically functional in immunoassays when it was expressed as a fusion protein to glutathione S-transferase (Bhardwaj *et al.*, 2001) and a thioredoxin tag (Chu *et al.*, 2007). The protein was further reported to be stable and useful as a tool for detection of homologous antibodies and differentiation between antisera raised against various species of flaviviruses (Holbrook *et al.*, 2004; Chavez *et al.*, 2013).

## **2.2. Aim**

The aim of the study was to prepare antigens for detection and differentiation of flaviviruses in immunoassays.

## **2.3. Materials and methods**

### **2.3.1. Selection of viruses**

LGTV strain TP21, classified as a class II pathogen that can be cultured within the confines of a BSL II facility was selected as a representative tick-borne flavivirus. The r-EDIII protein of Langat virus was selected for use in differentiating antibodies raised against tick-borne flaviviruses from those raised against mosquito-borne flaviviruses in serum. The r-EDIII proteins derived from tick-borne flaviviruses have previously been demonstrated to lack the ability to differentiate antisera raised against viruses within the tick-borne flavivirus serocomplex (Holbrook *et al.*, 2004) and this was the basis of selecting a tick-borne flavivirus species, LGTV, for detection of antisera raised against viruses belonging to this serocomplex. Members belonging to the tick-borne flavivirus serocomplex have also been shown to have highly conserved amino acid residues within the EDIII region (Bhardwaj *et al.*, 2001). The recombinant EDIII proteins of two mosquito-borne flaviviruses: WNV strain SA93/01 and WESSV strain SAH177 were prepared for detection and differentiation of mosquito-borne flaviviruses from tick-borne flaviviruses, as well as to differentiate antibodies raised against WNV from antibodies raised against WESSV. In their native form, these viruses can only be handled in a BSL 3 laboratory and this is a hindrance in laboratories that lack such resources. In many developing countries such as South Africa, the majority of laboratories do not have the privilege of facilities above a BSL II containment laboratory. This highlights the need for preparation of recombinant proteins which are safe to handle in resource-limited laboratories. If validated, the

use of recombinant proteins can allow such laboratories to perform research and diagnosis for endemic pathogens thus contributing towards improved public health.

### 2.3.2. Sequence alignment and phylogenetic analyses

In order to determine similarities in the EDIII proteins of flaviviruses, the amino acid sequences of the following viruses (Genbank accession numbers) were aligned using MAFFT version 7 program (Katoh & Standley. 2013): WNV (EF429198.1), JEV (AEN14607.1), LGTV (EU790644.1), LIV (NC001809.1), POWV (AAL32144.1), WESSV (DQ859058.1), YFV (AAD45531.1), KUNV (AF196505.1), TBEV (KC414090.1). The amino acid sequences were used to construct a neighbour-joining phylogenetic tree using MEGA version 4 software (Tamura *et al.* 2007).

### 2.3.3. Extraction of RNA

LGTV, WNV and WSLV were propagated by intracerebral inoculation of suckling mice or provided by Professor Janusz Paweska, and later harvested. RNA was extracted and purified from viruses infecting brain tissue using the Qiagen RNeasy mini kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Briefly, the tissue was disrupted and homogenized in buffer RLT and the lysate was added into a QIAshredder spin column. The lysate was further centrifuged for 3 mins at full speed and 70% ethanol added to the supernatant. The mixture was transferred to an RNeasy spin column and the column washed with buffer RW1 and buffer RPE. RNA was eluted from the column by addition of RNase-free water and stored at -70 °C until needed.

### 2.3.4. Amplification of EDIII genes

Primers targeting the complete open reading frame of EDIII genes of LGTV, WNV and WESSV were identified using sequence data retrieved from GenBank and flanked with sites for *Bam*HI and *Hind*III restriction endonucleases (Promega, WI, USA) on the 5' and 3' positions, respectively as shown in Table 4. A total of three additional nucleotides (TTT) was added upstream of the restriction sites to increase the efficiency of the restriction reaction.

**Table 4: Primers targeting the EDIII genes of LGTV, WNV, WESSV. Restriction sites flanking the 5' and 3' positions are underlined.**

Primer name	Virus strain used	Primer positions	Primer sequence	T <sub>m</sub> (°C)
LGTV3F	LGTV strain	nt.1928-1954	TTTGGATCCAAAGGATTGACATACACAGTCTGTGA	55
LGTV3R	TP21	nt. 2155-2131	TTTAAGCTTCTTCTGAAACCACTGATGGTTCAGG	58
WNV3F	WNV strain	nt. 1858-1880	TTTGGATCCAAAGGGAACAACATATGGAGTATG	52
WNV3R	SA93/01	nt. 2166-2144	TTTAAGCTTAGATTTGTGCCAGTGGTGGTTT	53
WESSV3F	WESSV strain	nt. 1826-1846	TTTGGATCCAAAGGTTCAACCTACTCAATG	49
WESSV3R	SAH177	nt. 2125-2106	TTTAAGCTTTGATCTTTGCCAGTGAT	46

An RT-PCR assay was performed using Titan® One Tube RT-PCR System (Roche, Basel, Switzerland) according to the manufacturer's instructions. The kit reverse transcribes RNA into



cDNA and amplifies the cDNA with a thermal cycling reaction in one singular tube. This is achieved by the enzymes AMV reverse transcriptase and Expand high fidelity, which form part of the Titan® enzyme mix. Reactions for RT-PCR were prepared as shown in Table 5 below.

**Table 5: Reagent mixtures for the amplification of EDIII genes using Titan® one step reverse transcriptase PCR kit (Roche)**

Reagent	Volume (µl)
Primers (20 pmol/µl)	0.75 each
RNAse Inhibitor (40 Units/µl)	0.25
RT-PCR Buffer (5x)	10
Dithiothreitol (100 mM)	2.5
dNTP (10mM each)	1
Enzyme mix (5 u/µl)	1
RNA template (1-5 ng)	1
Water to a final volume of 50µl	
<b>Total</b>	<b>50</b>

Briefly, the reactions were incubated in a Perkin Elmer thermocycler Gene Amp PCR system 2400 (Applied Biosystems, CA) using the following conditions: 50 °C for 30 min followed by 30 cycles of denaturation at 94 °C for 10s, annealing at 45 °C for 30s, elongation at 68 °C for 45s and one final elongation cycle at 68 °C for 7 min. Amplicons were stored at -20 °C until needed. Agarose gel electrophoresis was performed for confirmation of results and the DNA of the predicted size was excised from the gel and purified.

### 2.3.5. Purification of PCR products

Amplicons were purified from excised gel slices using the Wizard® SV gel and PCR clean-up system (Promega) according to manufacturer's instructions. Briefly, the membrane binding solution was added to the gel slice and incubated at 50-65 °C until the gel slice was completely dissolved. The mixture was then transferred to the minicolumn and incubated for 1 min at room temperature. The column was centrifuged at 16000 x g for 1 min and the column was washed with 700 µl and 500 µl membrane wash solution, respectively. DNA was eluted with nuclease-free water by centrifugation of the minicolumn at 16000 g for 1 min.

### 2.3.6. Cloning of amplicons and transformation of cells

Amplicons were ligated into pGem-T-easy® cloning vector (Promega) using T/A cloning according to parameters suggested by the manufacturer. The ligation reactions were set up by adding 5 µl of 2X Rapid Ligation Buffer (400mM Tris-HCL, 100mM MgCl<sub>2</sub>, 100mM DTT, 5mM ATP, pH 7.8), 1 µl

pGEM®-T Easy Vector (50 ng/μl), 1 μl T4 DNA Ligase enzyme (100U) and 3 μl of PCR product. The reactions were mixed by pipetting and incubated overnight at 4 °C. Ligation mixtures were used to transform high-efficiency JM109 competent cells ( $\geq 1 \times 10^8$  cfu/μg DNA) (Promega). Cells were plated out on Luria Bertani (LB) agar plates containing 100 μg/ml ampicillin, supplemented with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and 80 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), and plates were incubated overnight at 37 °C. Positive transformants were selected using blue/white selection and confirmed by digestion of plasmids using restriction endonucleases (*Bam*HI and *Hind*III) or by performing PCR on cell lysates, as described in sections 2.3.10 and 2.3.11. Following restriction enzyme digestion of plasmids from positive transformants, genes derived from LGTV, WNV and WESSV were subcloned into pQE-80L expression vector (Qiagen) and the gene derived from WNV was further subcloned into pCold-TF expression vector (Takara Bio, Paris, France), which allows the co-expression of the target protein with a chaperone that assists the protein with solubility and better folding. Both pQE-80L and pCOLD-TF vectors comprise of a 6X HIS-tag that facilitates purification of over-expressed proteins. Vector maps and multiple cloning sites for all the plasmids that were used are illustrated in Appendix A. Ligation mixtures were prepared as follows (Table 6):

**Table 6: Reaction mix for ligation of EDIII genes into PQE-80L and PCOLD-TF expression vectors**

Reagent	pQE-80L system	pCOLD-TF system
Expression vector double digested with <i>Bam</i> HI and <i>Hind</i> III	(pQE-80L; 50 ng) 2μl	(pCOLD-TF; 50 ng) 2 μl
10 XT4 ligase buffer (400mM Tris-HCL, 100mM MgCl <sub>2</sub> , 100mM DTT, 5mM ATP, pH 7.8)	1 μl	1 μl
T4 ligase enzyme (1U/μl)	1 μl	1 μl
Purified EDIII gene double digested with <i>Bam</i> HI and <i>Hind</i> III	6 μl	6 μl
Total	10 μl	10 μl

### 2.3.7. Colony PCR assay for confirmation of positive transformants

Cell lysates were obtained by selecting a colony of cells and resuspending it in 30 μl of nuclease-free water. The mixture was heated for 5 mins at 95 °C to lyse cells and 25 μl of the supernatant was used as template in a PCR assay. The forward primer targeting the plasmid and a reverse primer targeting the insert were used to confirm the integration and orientation of the insert in the plasmid. The forward primers for each plasmid were as follows: pGEM®-T easy, T7 universal primer, 5'-TAATACGACTCACTATAGGG-3', pQE promoter primer 5'-CCCAGAAAGTGCCACCTG-3' and pCOLD-TF-F1 primer 5'-CCACTTTCAACGAGCTGATG-3'. The PCR reaction was performed to obtain results more rapidly before starter cultures could be prepared for downstream reactions.

### 2.3.8. Plasmid purification

Plasmids were purified from *E.coli* cells by using the QIAprep Spin Miniprep Kit (Qiagen) and all the reagents were provided in the kit. Briefly, the pelleted bacterial cells were resuspended in 250 µl buffer P1 and an equal volume of buffer P2 was added, followed by addition of 350 µl buffer N3. The mixture was centrifuged at maximum speed and the supernatant was loaded onto the QIAprep spin column. The column was washed with 500 µl buffer PB and 750 µl buffer PE. The DNA was eluted by adding 50 µl of buffer EB and stored at -20 °C until needed.

### 2.3.9. Double digestions

In order to ensure compatibility, the same restriction endonucleases were used to digest the insert and the vector. *Bam*HI and *Hind*III (Promega) were selected as the enzymes of choice as restriction sites were present in the multiple cloning sites on all the vectors used and restriction sites were absent in all the genes of interest. Buffer E (Promega) was used for all reactions due to its 100 % compatibility for both *Bam*HI and *Hind*III (information from supplier). Double digestions for the vectors PQE-80L and PCOLD-TF were prepared as follows: 10X Buffer E 2.5 µl, Vector 1 µl, *Bam*HI 1 µl, *Hind*III 1 µl and distilled water to a total volume of 25 µl.

### 2.3.10. Determination of DNA concentrations

DNA concentration for amplicons was determined by using the Quant-IT™ dsDNA BR assay (Invitrogen™, OR, USA) according to the manufacturer's instructions and using reagents included in the kit.

### 2.3.11. DNA sequencing of positive constructs

The sequencing of positive transformants designated pQE-80L-LGTVDIII, pQE-80L-WNVDIII, pCOLD-TF-WNVDIII and pQE-80L-WESSVDIII was performed using the BigDye® Terminator Sequencing Kit (Applied Biosystems). Reactions were prepared as shown in Table 7 below.

**Table 7: Sequencing reactions prepared according to recommendations included in the BigDye® Terminator Kit**

Reaction component	Reaction volume
Terminator Ready Reaction	2µl
Sequencing primers: forward/reverse (0.8 picomolar/primer)	4µl
1 × Sequencing buffer	2µl
DNA template (~300bp, range: 3-10 ng)	2µl
Total	10µl

The following thermal cycling conditions were used for each reaction: 96 °C for 1 min, followed by 30 cycles of 96 °C for 10 sec, 50 °C for 5 sec and 60 °C for 4 min. Post-reaction cleanup was performed using the ethylenediaminetetraacetic acid (EDTA)/ethanol precipitation protocol. Briefly, 5

$\mu$ l of 125 mM EDTA and 60  $\mu$ l absolute ethanol was added to the sequencing reaction. Following precipitation at room temperature for 15 mins, the mixture was centrifuged at 4 °C for 20 mins at 14000 g, pellets washed with 500  $\mu$ l of 70 % ethanol and centrifuged at 4 °C for 10 mins at 14000 g. Pellets were dried in a 37 °C incubator until completely dry and submitted to The Department of Microbiology, Biochemistry and Food Biotechnology, Faculty of Natural and Agricultural Sciences, University of the Free State (Bloemfontein, South Africa) for gel electrophoresis. The nucleotide sequence data of the genes encoding the EDIII proteins of LGTV, WNV and WESSV were edited using ChromasPro Version 1.34 software. A nucleotide analysis was performed on the edited sequence by using the Basic Local Alignment Search Tool (BLAST) software accessed from the website [www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov) to confirm the identity of viruses. Sequencing results were also used to confirm the correct orientation of sequences and that there were no mutations in the sequence. The presence of restriction sites for *Bam*H1 and *Hind*III1, start and stop codons as well as the His-tag were also confirmed.

### **2.3.12. Protein expression and SDS-PAGE analysis**

The resulting constructs designated pQE-80L-LGTVDIIDIII and pQE-80L-WNVDIIDIII were used to transform JM109 competent cells ( $\geq 1 \times 10^8$  cfu/ $\mu$ g DNA) whereas pCOLD-TF-WNVDIIDIII and pQE-80L-WESSVDIIDIII constructs were used to transform *E. coli* OverExpress C43 (DE3) competent cells ( $\geq 1 \times 10^6$  cfu/ $\mu$ g DNA) (Lucigen, WI, USA). Mock antigens were prepared using cells transformed with pCOLD-TF and pQE-80L plasmids lacking any of the *Flavivirus* DIIDIII genes. The *E. coli* transformants were propagated in 5 ml LB broth containing 100  $\mu$ g/ml ampicillin and incubated overnight with shaking at 200 rpm. A 2 ml aliquot of the overnight culture was inoculated into 40 ml of LB broth containing ampicillin and incubated at 37 °C with shaking at 200 rpm. Cells transformed with the pQE-80L vector system were grown at 37 °C until an optical density (OD) reading of between 0.6 and 0.8 at 600nm was reached while cells transformed with the pCOLD-TF system were grown at 37 °C to an OD reading of between 0.4 and 0.5 at 600nm. Bacterial cultures were induced with 1mM IPTG and allowed to grow for 6 hours at 37 °C and 24 hours at 16 °C for the pQE-80L and pCOLD-TF systems, respectively. A total of 2 samples were collected from the cultures at various intervals. The first sample was used to determine OD readings and the second sample was loaded onto an SDS-PAGE gel (comprised of 8 % resolving gel and 4 % stacking gel as shown in Table 8) subsequent to re-suspension in PBS. The amount of PBS used to resuspend bacterial pellets was calculated using this formula: Volume of PBS =  $A_{600nm} / 0.5 \times 150 \mu$ l. Fractions of 80  $\mu$ l of all re-suspended samples were collected and boiled at 95 °C for 5 mins in 20  $\mu$ l 5X SDS-PAGE sample buffer, (Lane Marker Reducing Sample Buffer; Thermo Scientific, Illinois, USA) and incubation at 95°C for 5 minutes. of which 25  $\mu$ l was loaded onto an SDS-PAGE gel. The gel was run at 150 V for 1 hour 45 mins and stained overnight with Coomassie brilliant blue stain (containing 45% methanol, 10% glacial acetic acid, 0.2% Coomassie Brilliant Blue) with gentle shaking. The stain

was discarded and the gels were destained with approximately 600ml destaining solution (containing 45% methanol and 7% glacial acetic acid). The gels were then dried on a gel dryer for approximately 2 hours at 70 °C.

**Table 8: Preparation of 8% resolving gel and 4 % stacking gel for analysis of proteins by SDS-PAGE.**

<b>Reaction component (8 % resolving gel)</b>	<b>Reaction volume</b>
30% acrylamide solution/0.8% bisacrylamide stock solution (Merck, USA)	21.4 ml
1M Tris-HCl pH 8.8 stock solution	30 ml
10% SDS	0.8 ml
1.5% ammonium persulfate (freshly prepared)	4 ml
TEMED	0.02 ml
Distilled H <sub>2</sub> O	Up to final volume
Total	80 ml
<b>Reaction component (4 % stacking gel)</b>	<b>Reaction volume</b>
30% acrylamide solution/0.8% bisacrylamide stock solution	2 ml
1M Tris-HCl pH 6.8 stock solution	1.9 ml
10% SDS	0.15 ml
Glycerol	1 ml
1.5% ammonium persulphate	0.7 ml
TEMED	0.02 ml
Distilled H <sub>2</sub> O	up to final volume
Total	15 ml

### **2.3.13. Protein solubility analyses**

Samples collected at various intervals during protein induction were spun down and supernatant discarded. Pellets were re-suspended in 500 µl cold PBS. *N*-Lauroylsarcosine sodium salt (Sigma-Aldrich), a mild detergent that releases membrane-bound proteins, was added at a final concentration of 7.5 % and cells were sonicated in a sonicator bath (Branson). A total of 80 µl of the sample (soluble and insoluble) was prepared as described above and loaded onto an 8 % SDS-PAGE gel. The remaining re-suspended sample (~ 420 µl) was centrifuged for a further 20 mins at 13000 g and 80 µl of the supernatant was collected (soluble fraction), prepared as described above and loaded onto an 8 % SDS-PAGE gel. Following removal of the entire supernatant fraction, the pellet was re-suspended in 500 µl cold PBS and 80 µl was collected (insoluble fraction), prepared as described above and loaded onto an 8 % SDS-PAGE gel.

### **2.3.14. Purification of over-expressed proteins and refolding of denatured proteins**

The bacterial cultures expressing r-LGTVEDIII, r-WNVEDIII and r-WESSVEDIII proteins were collected, clarified and lysed by resuspending the pellet in 5 ml BugBuster Protein Extraction Reagent (Novagen, WI, USA) per gram of pelleted cells. Lysozyme (Novagen), an enzyme that facilitates lysis of bacterial cells, was added to a final concentration of 1mg/ml. In order to degrade any nucleic acid that may have been released from bacterial cells, benzonase (Novagen) was added to final concentration of 0.25 units/ml. Bacterial cells were stirred for 30 mins at room temperature and then sonicated on ice using 10 × 15 sec bursts, with a 15 sec cooling period on ice between each burst. After sonication the cells were centrifuged at 10000 g for 30 min at 4°C. At this point, supernatants were used for protein purification under native conditions using the Protino® Ni-TED packed columns (Macherey-Nagel, Duren, Germany) and pellets were discarded; or pellets were further purified if protein purification was performed from the insoluble fraction (under denaturing conditions). To prepare crude lysates for purification under denaturing conditions, bacterial cell pellets were resuspended in 2.5 ml LEW buffer (supplied in kit) and centrifuged at 10000 g for 30 mins at 4°C. Pellets were then resuspended in 500 µl LEW supplemented with 8 M urea (now referred to as DS buffer). The cells were sonicated on ice as described above and stirred on ice for a further 60 mins. Crude lysates for protein purification were obtained when the mixture was centrifuged at room temperature for 30 mins at 10000 g. Recombinant EDIII proteins were purified from the crude lysates by using the Protino® Ni-TED packed columns (Macherey-Nagel) under native (pQE-80L-LGTVDIII, pQE-80L-WNVDIII, pCOLD-TF-WNVDIII, pQE-80L-WESSVDIII) or denaturing (pQE-80L-WNVDIII, pCOLD-TF-WNVDIII, pQE-80L-WESSVDIII) conditions using buffers supplied in the kit. Columns were equilibrated with 1X LEW buffer and the lysates were eluted through the column, allowing HIS-tagged proteins to bind to the Nickel-charged resin in the column. The columns were then washed with 1X LEW buffer to get rid of unbound proteins. HIS-tagged proteins were eluted with 1X elution buffer which contains 250 mM imidazole. Imidazole is instrumental in breaking the bond formed between the HIS-tag and the nickel-charged resin, allowing the HIS-tag protein to be released and eluted through the column. For purification of proteins under denaturing conditions, the use of LEW buffer was substituted with DS buffer and 8M urea was added to the elution buffer. Proteins purified under denaturing conditions were renatured by the removal of urea in the solution using Millipore filters with a 10 kDa or 30 kDa molecular weight cut off (Millipore Corporation, USA), depending on the size of the eluted protein. All eluted proteins were collected and stored at -80 °C until needed.

### **2.3.15. Protein concentration determination**

Concentrations for r-EDIII proteins were determined by using the Quant-IT™ protein assay (Invitrogen™) according to the manufacturer's instructions and using reagents included in the kit.

### **2.3.16. Characterization of proteins by Western blot analysis**

Recombinant proteins were characterized by Western Blot analyses using the Pierce® Fast Western Blot Kit (Thermo Scientific, MA, USA) according to the manufacturer's instructions. Briefly, 8% SDS-PAGE gels used to separate recombinant proteins were soaked in transfer buffer (25mM Tris, 192mM glycine, 20% methanol, pH 7.4 for 15min. PVDF nitrocellulose membrane (Roche) was moistened with methanol for 1-3 seconds, incubated in distilled water for 1-2 minutes and then soaked in transfer buffer for 2-3 minutes. Proteins were transferred to a nitrocellulose membrane using a Biorad® Trans-Blot® SD semi-dry transfer cell (Biorad, CA, USA) at 20 V for 1 hour. The membrane was washed in 1X Wash Buffer and incubated in antibody diluent containing 100µg/ml mouse anti-His<sub>6</sub>. The membrane was further incubated in a 10-fold dilution of optimized anti-mouse horseradish peroxidase (HRPO) reagent for 5 minutes at room temperature. Subsequent to washing for 5 mins, the membrane was transferred to SuperSignal® West Pico working solution (supplied in kit) and incubated for up to 5 mins at room temperature. The membrane was exposed to an X-ray film, which was incubated in fixer and developer.

## **2.4. RESULTS**

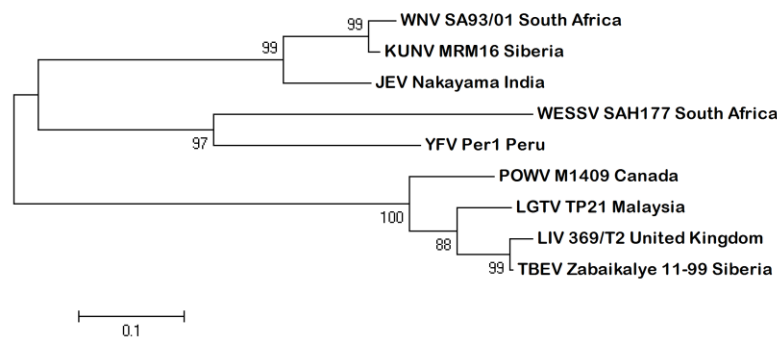
### **2.4.1. Amino acid alignments and phylogenetic tree**

In order to determine relatedness in EDIII proteins of flaviviruses, amino acid sequences of the EDIII region of flaviviruses were aligned as shown in Table 9 and subsequently used to construct a phylogenetic tree. As seen in Figure 6, members belonging to the tick-borne serocomplex clustered together. Similarly, mosquito-borne representatives also clustered together and branches were in agreement with the various serocomplexes that mosquito-borne flaviviruses belong to. The closely related YFV and WESSV formed a separate clade, similarly with WNV and KUNV.

**Table 9: Amino acid sequences of the EDIII proteins of various flaviviruses**

VIRUS	AMINO ACID SEQUENCES
WNV	KGTTYGVCSKAFKFAAGTADTGHGTVVLELQYTGTDGPKVPISSVASLNDLTPVGRLV 59
KUNV	KGTTYGVCSK-AFRFLGTPADTGHGTVVLELQYTGTDGPKIPISSVASLNDLTPVGRLV 59
JEV	KGTTYGMCTE-KFSFAKNPADTGHGTVVIELSYSGSDGPKIPIVSVASLNDMTPVGRLV 59
LIV	KGLTYTMDKSKFAWKRTPTDSGHDTVVMVETVFSGS-KPCRIPVRAVAHGSPDVNVAMLI 59
TBEV	KGLTYTMDKTKFAWKRTPTDSGHDTVVMVETVFSGT-KPCRIPVRAVAHGSPDVNVAMLI 59
LGTV	KGLTYTVC DKTKFTWKRAPTDSGHDTVW-EVGFSGT-RPCRIPVRAVAHGVPVNVVA-LI 57
POWV	KGTTYSMCDKAKFKWKRVPVDSGHDTVVMVETVFSGDKPCRIPVRAVAHGVPVNVVAMLI 60
WESSV	KGSTYS-CK--RGSFAKQPVEDHGTAV-QIKVTTG-APCRIPVIAADS-AGTENRGSVI 54
YFV	KGTSYKMTD-KMSFVKNPDTTGHGTAVMQVKVPKG-APCRIPVMVADDLTASVNVKGI 58
	** :* * : * : : * . * . * : : . * : : * : . : :
WNV	TVNPFVSVATANSKVLIELEPPFGDSYIVVGRGEQQINHHW--- 100
KUNV	TVNPFVSVATANSKVLIELEPPFGDSYIVVGRGEQQINHHW--- 100
JEV	TVNPFVATSSANSKVLVEMEPFGDSYIVVGRGDKQINHHWHKA 103
LIV	TPNPTI---ENDGGGFIEMLPPGDNIIVVG---ELSHQWFQT 96
TBEV	TPNPTI---ENNGGGFIEMLPPGDNIIVVG---ELSHQWFQK 96
LGTV	TPNPT---ENNGGGFIE-QLPPGDNIIVVG---DLNHQWFQK 92
POWV	TPNPTI---ETNGGGFIEMLPPGDNIIVVG---DLSQQWFQK 97
WESSV	TTNPIAA--SNNDEVLVEISPPFGESYIIVGNGDDKLTYHWQRS 96
YFV	TVNPIAS--TNEDEVLIEVNPPFGDSYIIVGTGDSRLTYQWHKE 100
	* ** :. : : * . * * :. * ** :. : *

Symbols have been used to denote the homology between amino acids. \* identical, : conserved substitutions, . semi-conserved substitutions, space denotes variable substitutions. Genbank accession numbers WNV: EF429198.1, JEV: AEN14607.1, LGTV: EU790644.1, LIV: NC001809.1, POWV: AAL32144.1, WESSV: DQ859058.1, YFV: AAD45531.1, KUNV: AF196505.1, TBEV: KC414090.1.



**Figure 6: Phylogenetic comparison of the EDIII protein of various flaviviruses.** The neighbour-joining tree was constructed by using MEGA version 4 software (Kato & Standley. 2013) and 1000 bootstrap replicates by using the maximum composite likelihood algorithm. Scale-bar indicates nucleotide substitutions. Genbank accession numbers WNV: EF429198.1, JEV: AEN14607.1, LGTV: EU790644.1, LIV: NC001809.1, POWV: AAL32144.1, WESSV: DQ859058.1, YFV: AAD45531.1, KUNV: AF196505.1, TBEV: KC414090.1.

### 2.4.2. One step RT-PCR for amplification of the genes encoding the EDIII protein of flaviviruses

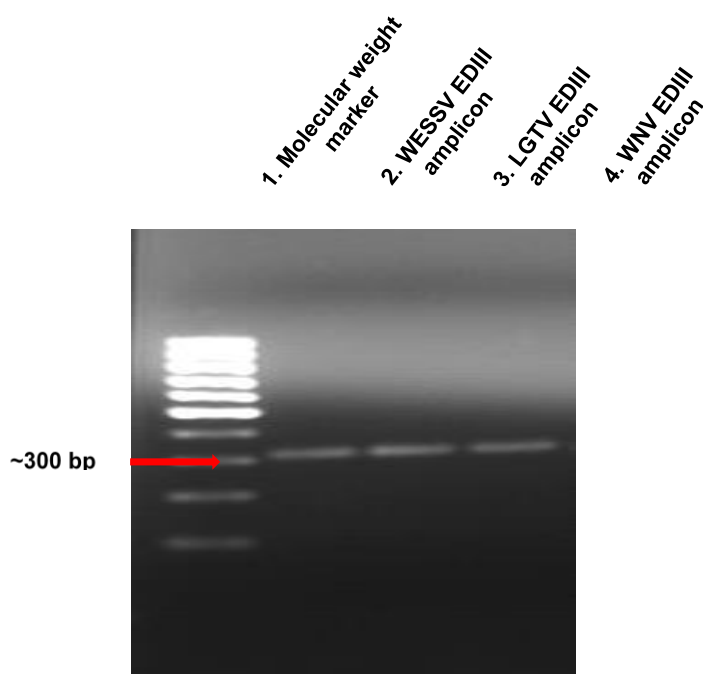
The genes encoding the EDIII of LGTV, WNV and WESSV were successfully amplified using the Titan® One Tube RT-PCR system (Roche). All amplicons were purified using the Wizard® SV gel and PCR clean-up system (Promega). A total of 5 µl of each amplicon was loaded onto a 1 %



agarose gel and analyzed by electrophoresis as shown in Figure 7. The DNA concentrations of amplicons after purification are shown in Table 10.

**Table 10: Amplicon concentration before and after purification**

Gene targeted in PCR assay	DNA concentration of amplicon after purification (ng/ $\mu$ l)
LGTV-EDIII	98.2
WNV-EDIII	88.8
WESSVEDIII	100.4



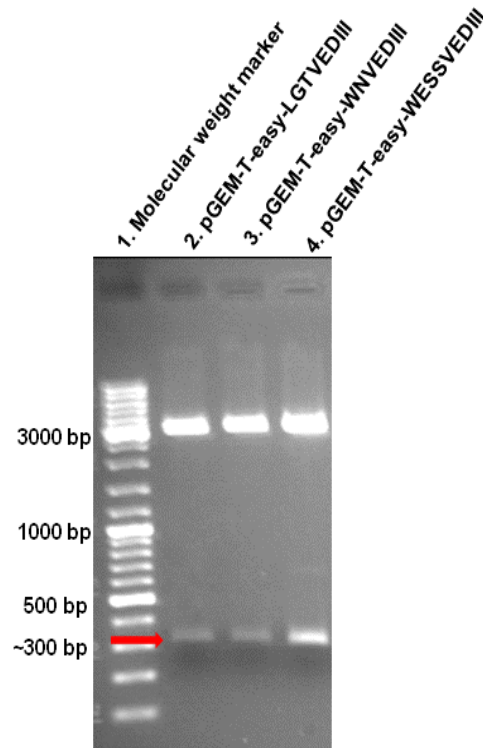
**Figure 7: Agarose gel electrophoresis analysis of EDIII amplicons from the one step RT-PCR using genomic viral RNA as template. Amplicons were loaded on the gel after purification using the Wizard® SV gel and PCR clean-up system (Promega).**

Lane 1 – Fermentas O’GeneRuler™ 100bp DNA ladder molecular weight marker (Thermo Scientific) comprising DNA fragments from 100 to 1000 bp fragments, Lane 2 - 5 $\mu$ l PCR product of amplified WESSV EDIII gene; Lane 3 - 5 $\mu$ l PCR product of amplified LGTV EDIII gene, Lane 4 - 5 $\mu$ l PCR product of amplified WNV EDIII gene.

### 2.4.3. T/A cloning of the EDIII amplicons of LGTV, WNV and WESSV into pGEM®-T-Easy vector

As described in MATERIALS AND METHODS 2.3.8, EDIII amplicons were ligated into pGEM®-T easy vector and the ligation mixtures were used to transform *E.coli* cells. Following purification of plasmids from bacterial cells, the presence of inserts were confirmed by restriction endonucleases

*Bam*HI and *Hind*III. Double digestion reactions were loaded onto a 1 % agarose gel and visualized under UV light as shown in Figure 8.



**Figure 8: Agarose gel electrophoresis analysis of pGEM®-T easy vector (Promega) from transformed bacterial cells double digested with *Bam*HI and *Hind*III to confirm the presence of EDIII genes of LGTV, WNV and WESSV.**

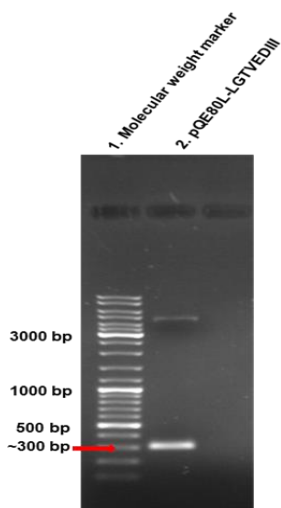
Lane 1 – Fermentas O’GeneRuler™ DNA ladder mix molecular weight marker (Thermo Scientific) comprising DNA fragments from 100 to 10000 bp fragments, Lane 2 - 5µl of double digested pGEM-T-easy-LGTVEDIII, Lane 3 - 5µl of double digested pGEM-T-easy-WNVEDIII, Lane 4 - 5µl of double digested pGEM-T-easy-WESSVEDIII.

#### **2.4.4. Cloning of the EDIII genes of LGTV, WNV and WESSV into pQE-80L and pCOLD-TF expression vectors.**

In order to facilitate ligation of EDIII genes of LGTV, WNV and WESSV; supercoiled pQE-80L and pCOLDTF expression vectors were double digested with *Bam*HI and *Hind*III to create sticky ends. Digested plasmids were gel purified. Following plasmid purification the DNA concentration of pQE-80L and pCOLDTF were determined to be 53.1 ng/ µl and 78.4 ng/ µl.

EDIII genes were rescued from pGEM®-T easy vector and sub-cloned into pQE-80L vector and the EDIII of WNV was further subcloned into pCOLD-TF vector. The ligation mixtures were used to transform *E. coli* cells which were subsequently plated on LB/AMP agar plates containing ampicillin. Positive transformants were screened by double digestions, or by colony PCR using bacterial lysates as template. Double digestion reactions and products obtained from the colony PCR were loaded on 1 % agarose gels and visualized. The positive pQE80L-LGTVEDIII construct was

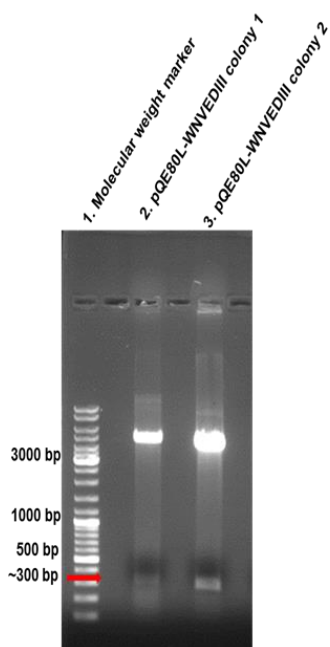
confirmed as shown in Figure 9 and glycerol stocks of the bacterial cell colony carrying the positive constructs were prepared for downstream reactions and stored at  $-80^{\circ}\text{C}$  until needed.



**Figure 9: Agarose gel electrophoresis analysis of pQE-80L plasmid double-digested with *Bam*HI and *Hind*III for screening of the presence of LGTVEDIII insert.**

**Lane 1 – Fermentas O’GeneRuler™ DNA ladder mix molecular weight marker (Thermo Scientific) comprising DNA fragments from 100 to 10000 bp fragments, Lane 2 - 5 $\mu$ l of double-digested pQE-80L plasmid from colony 1 containing LGTVEDIII.**

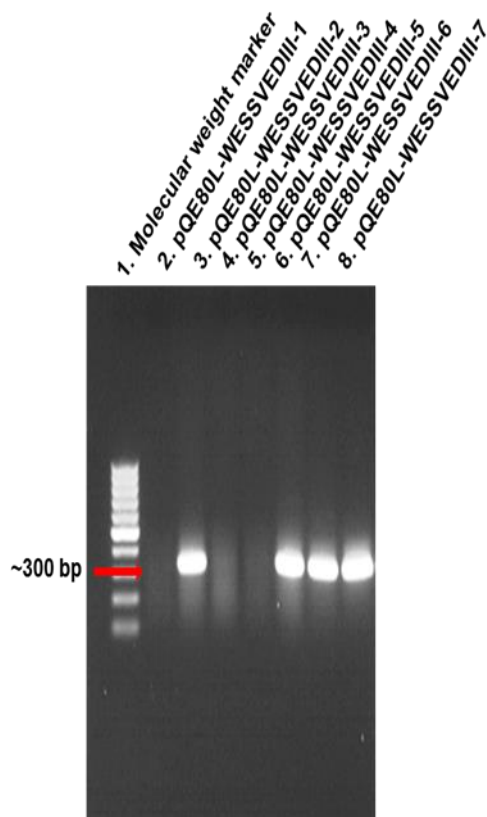
Two bacterial colonies (designated colony 1 and 2) were screened for pQE80L-WNVEDIII construct by double digestions with *Bam*HI and *Hind*III as shown in Figure 10. Colony 2 was selected for downstream reactions and the corresponding bacterial colony was preserved by preparation of glycerol stocks and storage at  $-80^{\circ}\text{C}$ .



**Figure 10: Agarose gel electrophoresis analysis of pQE-80L plasmid double-digested with *Bam*HI and *Hind*III for screening of the presence of WNVEDIII insert.**

Lane 1 – Fermentas O’GeneRuler™ DNA ladder mix molecular weight marker (Thermo Scientific) comprising DNA fragments from 100 to 10000 bp fragments, Lane 2 - 5µl of double-digested pQE-80L plasmid from colony 1 containing WNVEDIII, Lane 3 - double-digested pQE-80L plasmid from colony 1 containing WNVEDIII.

In order to confirm whether there was a positive pQE80L-WESSVEDIII construct, a total of seven bacterial colonies (designated colony 1-7) were selected from the LB/AMP agar plate and used as template for PCR. As illustrated in Figure 11, only four colonies out of seven were positive. Colony 2 was selected for downstream reactions and a glycerol stock was prepared to facilitate the storage of the corresponding bacterial colony at -80 °C.



**Figure 11: Agarose gel electrophoresis analysis of amplicons obtained from a colony PCR assay used to screen for positive pQE80L-WESSVEDIII constructs.**

Lane 1 – Fermentas O’GeneRuler™ DNA ladder mix molecular weight marker (Thermo Scientific) comprising DNA fragments from 100 to 10000 bp fragments, Lanes 2 to 8: 5µl of amplicons obtained from a colony PCR assay performed on pQE80L-WESSVEDIII colonies 1 to 7.

The EDIII gene of WNV was further sub-cloned into pCOLDTF expression vector. A total of six bacterial colonies (designated colony 1-6) were selected from LB/AMP plates and screened for the presence of positive pCOLDTF-WNVEDIII constructs by double digestions using *Bam*HI and *Hind*III, as illustrated in Figure 12.

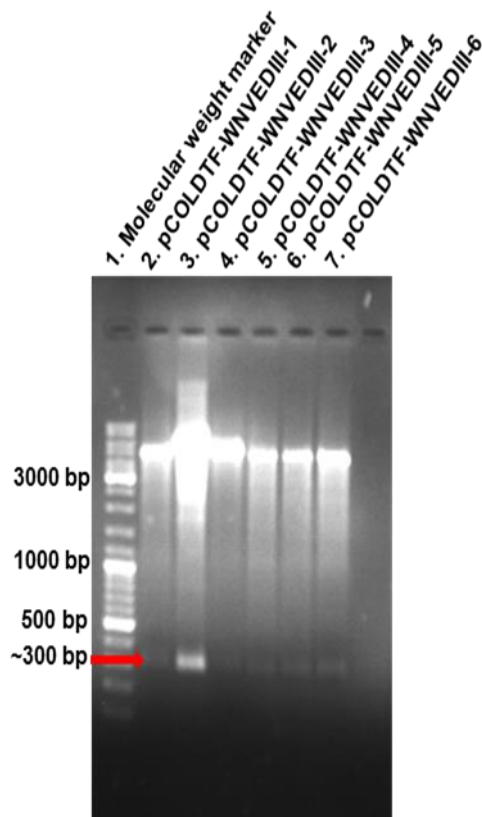


Figure 12: Agarose gel electrophoresis analysis for screening of pCOLD-WNVEDIII constructs by double digestion with *Bam*HI and *Hind*III.

Lane 1 – O’GeneRuler™ DNA ladder mix molecular weight marker (Thermo Scientific) comprising DNA fragments from 100 to 10000 bp fragments, Lanes 2 to 7: 5µl of double digestion reactions for screening of pCOLD-WNVEDIII colonies 1 to 6.

All colonies were positive for pCOLD-WNVEDIII. Colony 2 was selected for downstream reactions, preserved in 15% glycerol and stored at -80 °C until needed.

#### 2.4.5. Sequence data to confirm the identity of inserts

Sequence data was used to establish the fidelity of the EDIII genes ligated into expression vectors as well as to determine whether the genes were in the correct frame relative to the start and stop codons of the vector. The sequence data for all the four constructs (pQE80L-LGTVEDIII, pQE80L-WNVEDIII, pQE-80L-WESSVEDIII and pCOLDTF-WNVEDIII) confirmed the correct frame.

Additionally, no base changes were observed when the genes were aligned with reference strains from GenBank. The correct frame of the insert and the fidelity of the gene are among important factors that need to be considered for the subsequent expression and functionality of the target proteins.

#### 2.4.6. SDS-PAGE analyses for expressed proteins

Cell lysates were prepared and loaded onto 8 % SDS-PAGE gels for visualization. Proteins were separated under denaturing conditions to confirm the presence of overexpressed target genes. The 13 kDa r-EDIII protein of LGTV was expressed in a bacterial expression system as shown in Figure 13. The protein yield increased over time and this suggested that the protein was not toxic towards the cells. Following the successful expression of the r-LGTVEDIII protein, a solubility analysis was performed to ascertain whether the protein was soluble in the cell matrix or rather embedded inside inclusion bodies. This was performed at various growth intervals (not shown) and it was observed that the protein was saturated in the soluble form for up to six hours. The protein would then be transitioned to inclusion bodies if cells were grown beyond this point. An illustration of the solubility study at four and six hours post-induction is shown in Figure 13. Based on this observation, the protein was then harvested at 6 hours and purified from the soluble fraction as shown in Figure 14.

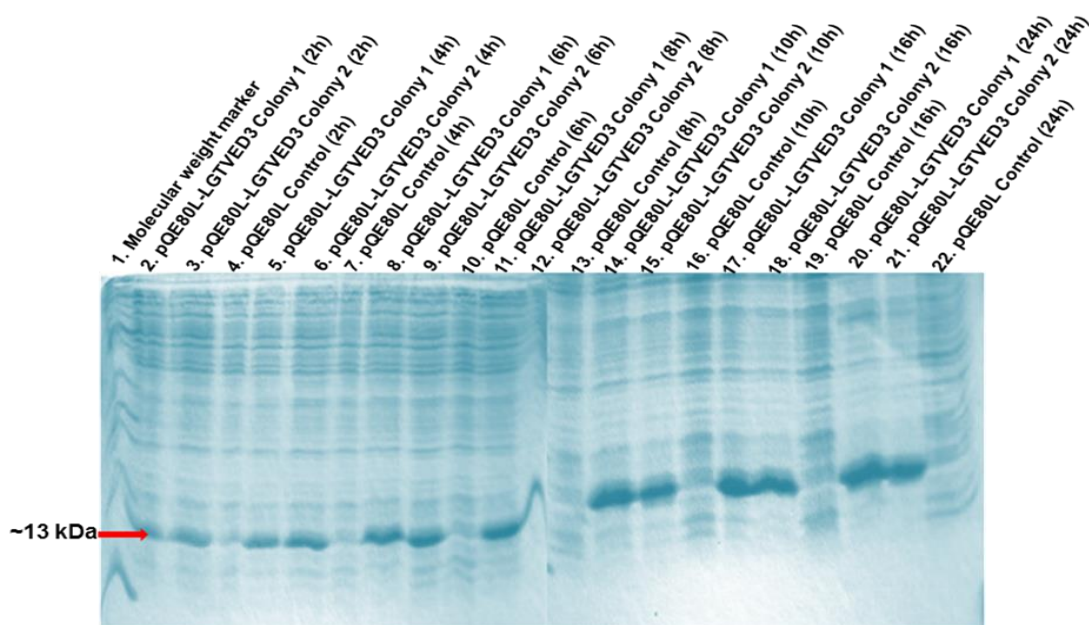
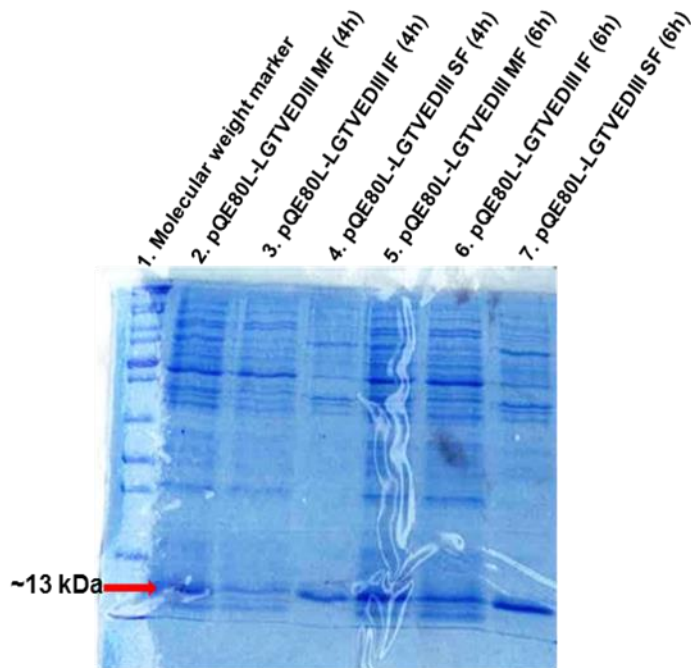


Figure 13: SDS-PAGE analysis of pQE80L-LGTVEDIII at intervals over a 24-hour period.

The bacterial culture was grown in duplicate and designated colony 1 and colony 2. The bacterial culture carrying the pQE-80L plasmid without the EDIII gene was used as a control.

Lane 1: Fermentas PageRuler™ Unstained protein molecular marker (Thermo Scientific), Lanes 2-22: cell lysates harvested at 2h, 4h, 6h, 8h, 10h, 16h and 24h from bacterial cultures.



**Figure 14: SDS-PAGE analysis of pQE80L-LGTVEDIII following a protein solubility study at 4 and 6 hours post-induction. Mixed fractions (MF) consisted of both the soluble fraction (SF) and insoluble fraction (IF), whereas the SF was harvested in the cytoplasm and the IF from inclusion bodies.**

**Lane 1: Fermentas PageRuler™ Unstained protein molecular marker (Thermo Scientific), Lane 2: total bacterial cell lysate at 4h, Lane 3: bacterial cell lysate from the insoluble fraction at 4h, Lane 4: bacterial cell lysate from the soluble fraction at 4h, Lane 5: total bacterial cell lysate at 6h, Lane 6: bacterial cell lysate from the insoluble fraction at 6h, Lane 7: bacterial cell lysate from the soluble fraction at 6h.**

The expression of pQE80L-WNVEDIII was confirmed using SDS-PAGE analysis as shown in Figure 15. As seen in Figure 16, the protein remained in the soluble phase up to 2 hours post-induction and transitioned to the insoluble phase in subsequent samples. Additionally, a considerable amount of the protein was washed off the Protino® protein columns during wash steps and this affected the yield that could be obtained from a bacterial culture (Figure 15). This suggested that the protein was folding incorrectly and thus the HIS-tag may have remained hidden, preventing binding of the protein to the Nickel-charged column. There was no binding even when the amount of protein loaded onto the column was decreased considerably, suggesting that the columns were not overloaded with protein, which would also decrease their binding capacity. Decreasing the number of wash steps resulted in a highly impure protein, whereas increasing the number of wash steps resulted in significantly low protein concentration in the final eluate. To address this shortcoming, the protein was purified under denaturing conditions (not shown) but was later abandoned as it repeatedly showed no reactivity to anti-WNV antibodies in immunoassays even after re-folding (by removal of urea). This protein was also not detectable by Western blot analysis. The lack of



reactivity in immunoassays suggested that the protein folded incorrectly after the urea was removed from the solution, concealing epitopes that are important for recognition by homologous antibodies.

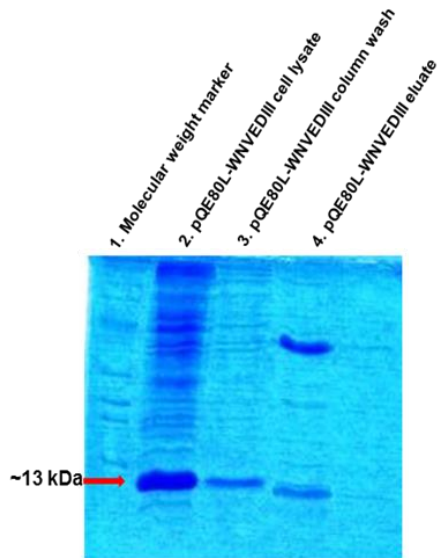


Figure 15: SDS-PAGE analysis of bacterial cell lysates containing pQE80L-WNVEDIII protein purified under native conditions.

Lane 1: Fermentas PageRuler™ Unstained protein molecular marker (Thermo Scientific), Lane 2: total bacterial cell lysate of cells expressing WNVEDIII protein derived from pQE-80L vector, Lane 3: eluate collected during the column wash step, demonstrating the loss of WNVEDIII protein, Lane 3: eluate collected from the column after the final elution step.

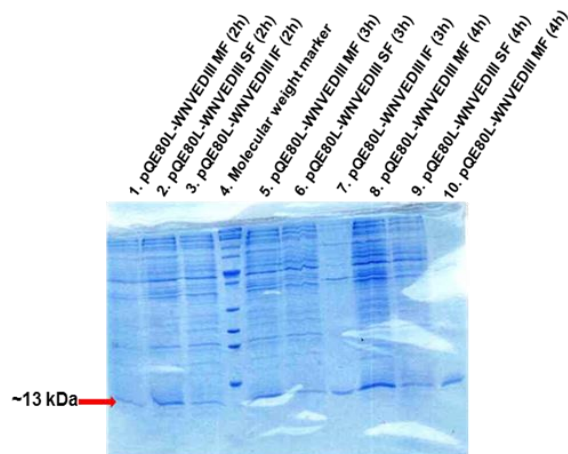
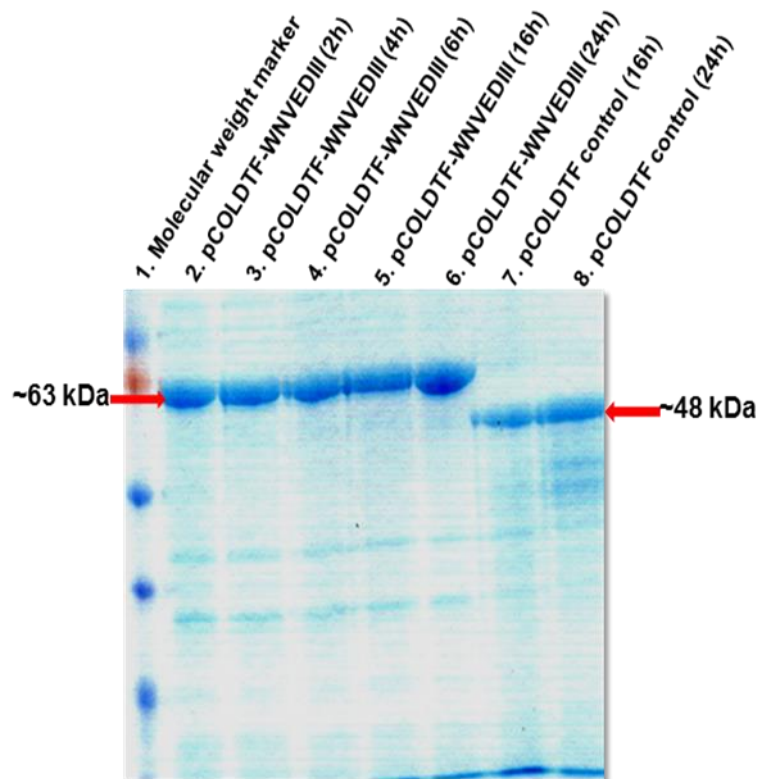


Figure 16: SDS-PAGE analysis of pQE80L-WNVEDIII following a protein solubility study at 2, 3 and 4 hours post-induction. Mixed fractions (MF) consisted of both the soluble fraction (SF) and insoluble fraction (IF), whereas the SF was harvested in the cytoplasm and the IF from inclusion bodies.

Lane 1: total bacterial cell lysate at 2h, Lane 2: bacterial cell lysate from the soluble fraction at 2h, Lane 3: bacterial cell lysate from the insoluble fraction at 2h, Lane 4: Fermentas PageRuler™ Unstained protein molecular marker (Thermo Scientific), Lane 5: total bacterial cell lysate at 3h, Lane 6: bacterial cell lysate from the soluble fraction at 3h, Lane 7: bacterial cell lysate from the insoluble fraction at 3h, Lane 8: total bacterial cell lysate at 4h, Lane 9: bacterial cell lysate from the soluble fraction at 4h, Lane 10: bacterial cell lysate from the insoluble fraction at 4h.



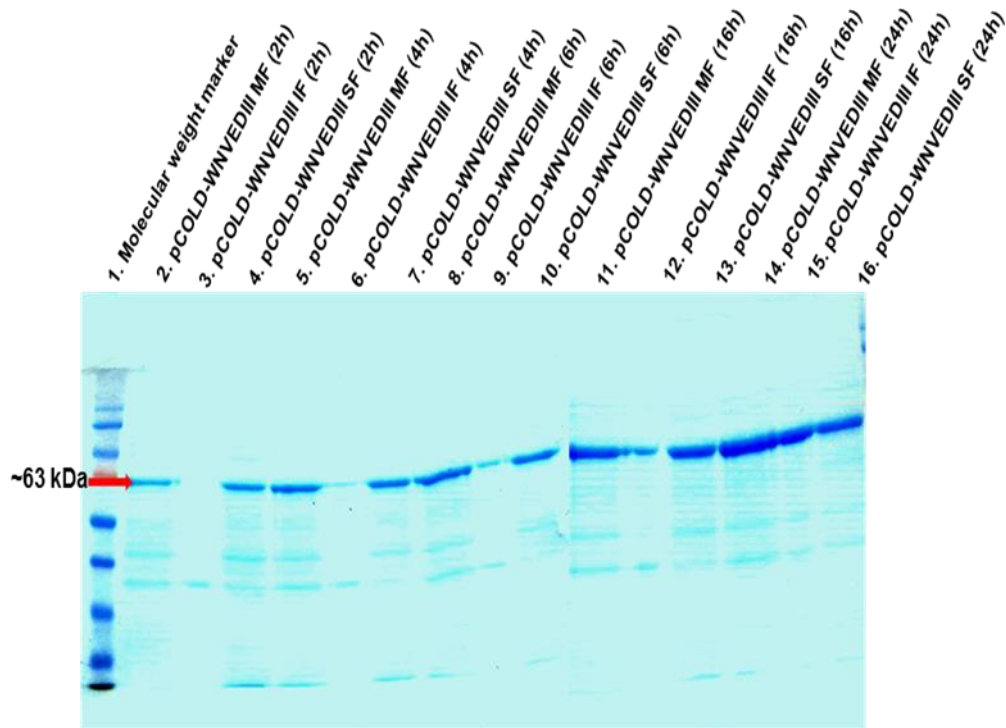
Due to the limitations encountered with the protein derived from the pQE80L-WNVEDIII construct, the EDIII gene of WNV was further subcloned into a pCOLDTF plasmid (Takara). According to the manufacturer, the vector promotes the folding and solubility of proteins due to the trigger factor (TF), an *E.coli* chaperone. The system uses cold shock expression and this is achieved optimally at 16 °C. TF is a 48 kDa protein that is associated with ribosomes, and is considered to be coupled with protein translation to promote folding of nascent polypeptides (Quing *et al.*, 2004). Additionally, the vector has a His-tag sequence on the N-terminal region, allowing improved recovery and purification of the fusion protein. Bacterial cells transformed with the pCOLDTF-WNVEDIII were cultured and harvested at various intervals to confirm protein expression (Figure 17). When compared to the control protein on the gel, there was overexpression from the bacterial cells containing the pCOLDTF-WNVEDIII construct, indicating the successful expression of the target protein. The size of the protein obtained from bacterial cells expressing pCOLDTF plasmid was ~48 kDa in size whereas the test protein had a size of ~ 63 kDa, which indicates co-expression of the WNVEDIII protein with the TF.



**Figure 17: SDS-PAGE analysis of pCOLDTF-WNVEDIII over a 24-hour period. The bacterial culture carrying the pCOLDTF plasmid without the EDIII gene was used as a control.**

Lane 1: Fermentas PageRuler™ Prestained protein ladder (Thermo Scientific), Lanes 2-6: bacterial cell lysates expressing WNVEDIII harvested at 2h, 4h, 6h, 16h and 24h. Lanes 7-8: bacterial cell lysates expressing pCOLDTF at 16h and 24h.

Following the successful expression of the r-WNVEDIII using the pCOLDTF system, protein solubility analyses were performed over a 24h period to ascertain whether the protein was in the soluble or insoluble phase (Figure 18).



**Figure 18: SDS-PAGE analysis of pCOLDTF-WNVEDIII following a protein solubility study over a 24-hour period. Mixed fractions (MF) consisted of both the soluble fraction (SF) and insoluble fraction (IF), whereas the SF was harvested from the cytoplasm and the IF from inclusion bodies.**

**Lane 1: Fermentas PageRuler™ Prestained protein molecular marker (Thermo Scientific), Lanes 2, 5, 8, 11 and 14: total bacterial cell lysate at 2h, 4h, 6h, 16h and 24h, respectively. Lanes 3, 6, 9, 12 and 15: bacterial cell lysate from the insoluble fraction at 2h, 4h, 6h, 16h and 24h, respectively. Lanes 4, 7, 10, 13, 16: bacterial cell lysate from the soluble fraction at 2h, 4h, 6h, 16h and 24h, respectively.**

It was observed that the protein was present in the soluble phase throughout the 24-hour period, a significant change in property compared to the r-WNVEDIII protein expressed using the pQE system which remained in the soluble phase for less than 4 hours post-induction.

In contrast to the r-EDIII protein of WNV, the r-WESSVEDIII was successfully expressed using the pQE-80L vector, purified and used in immunoassays (Chapter 3). Figure 19 demonstrates the expression of r-WESSVEDIII over a 24-hour period, though there is a notable decrease in protein load at 24 hours. This was observed repeatedly with this protein and suggests that the increased accumulation of the pQE-derived r-WESSVEDIII may be toxic towards bacterial cells.

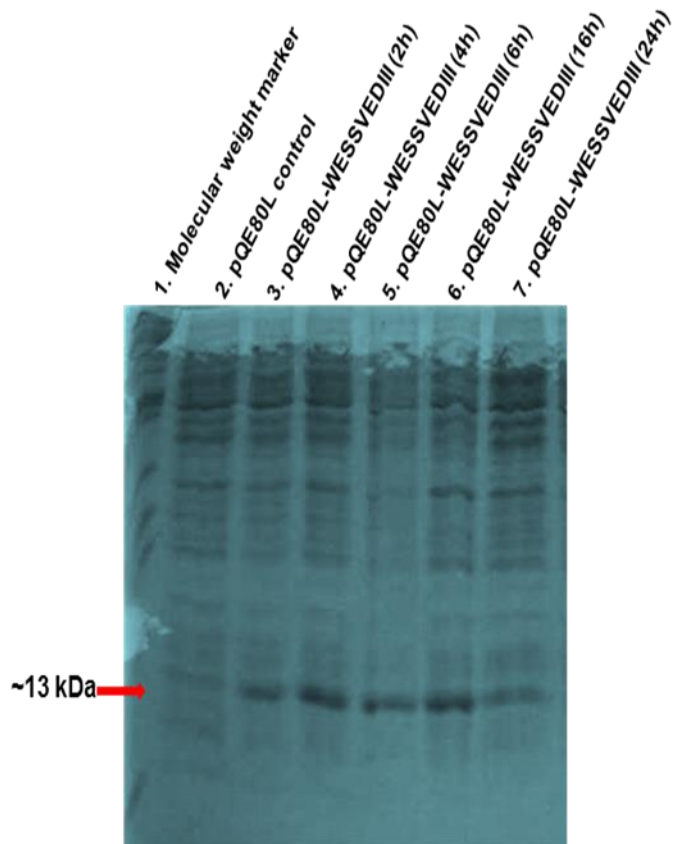
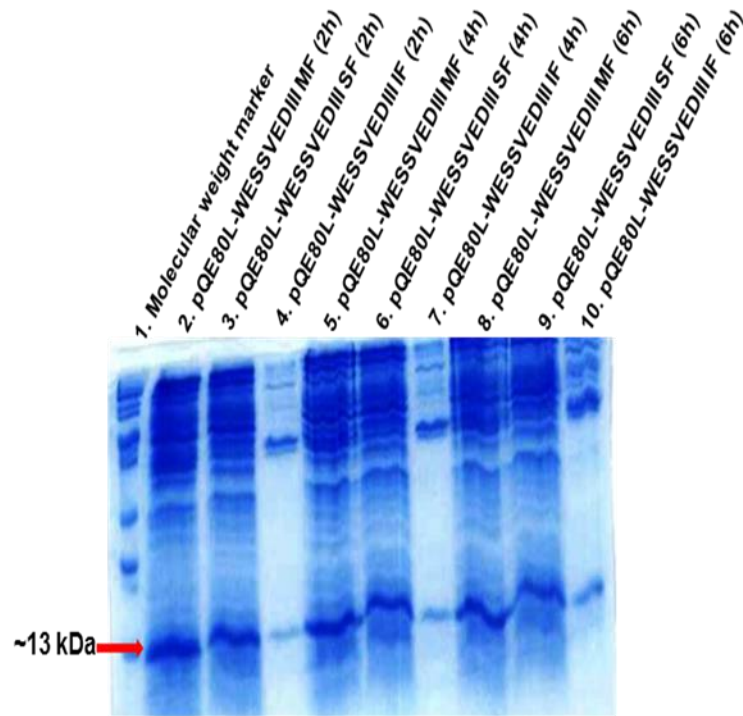


Figure 19: SDS-PAGE analysis of pQE80L-WESSVEDIII at intervals over a 24-hour period.

Lane 1: Fermentas PageRuler™ Unstained protein molecular marker (Thermo Scientific), Lanes 2-7: bacterial cell lysates harvested at 2h, 4h, 6h, 8h, 16h and 24h.

Solubility analyses were performed on the r-WESSVEDIII to ascertain the solubility profile for this protein as well as to facilitate purification. Based on the decline in the amount of r-WESSVEDIII protein over time, the solubility study was restricted to 6 hours, which also proved to be the threshold for the most accumulated soluble protein (Figure 20). The protein was therefore harvested at 6 hours post-induction for downstream procedures.



**Figure 20: SDS-PAGE analysis of pQE80L-WESSVEDIII following a protein solubility study at 2, 4 and 6 hours post-induction. Mixed fractions (MF) consisted of both the soluble fraction (SF) and insoluble fraction (IF), whereas the SF was harvested from the cytoplasm and the IF from inclusion bodies.**

Lane 1: Fermentas PageRuler™ Unstained protein molecular marker (Thermo Scientific), , Lanes 2, 4 and 6: total bacterial cell lysate at 2h, 4h and 6h, respectively. Lanes 3, 6, and 9: bacterial cell lysate from the soluble fraction at 2h, 4h and 6h, respectively. Lanes 4, 7 and 10: bacterial cell lysate from the insoluble fraction at 2h, 4h and 6h, respectively.

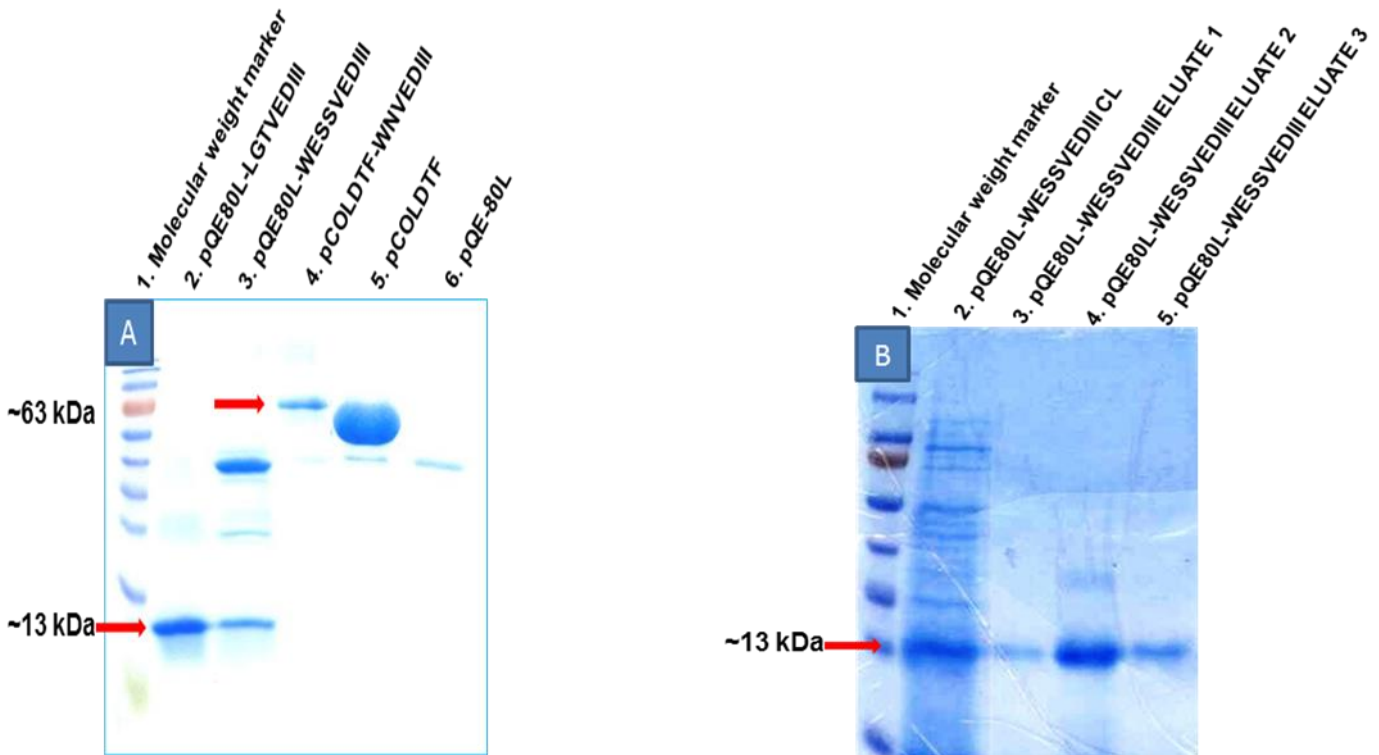
#### 2.4.7. Protein purification and determination of protein concentration

After the expression of the r-EDIII proteins of LGTV, WNV and WESSV were confirmed, they were purified in order to remove non-specific proteins. A pure protein is required for immunoassays as it will reduce background and non-specific binding in the assays. Expressed proteins were purified according to the solubility profiles obtained. Protein concentrations determined using the Qubit™ system for each r-EDIII are shown in Table 11. Recombinant WNVEDIII derived from PQE is not included in the table due to its non-functionality in immunoassays and difficulty in purification.

**Table 11: Protein concentrations for recombinant antigens purified from a 100 culture and recombinant antigens pooled from multiple cultures.**

Recombinant protein	Protein concentration per 100 ml bacterial culture (µg/ml)	Final protein concentrations (µg/ml) pooled to 10 ml
LGTVEDIII (pQE system)	549	700
WNVEDIII (pCOLD system)	900	1460
WESSVEDIII (pQE system)	547	828

The r-LGTVEDIII protein was harvested at 6 hours post-induction and purified under native conditions as shown in Figure 21. Similarly, the r-WESSVEDIII was also harvested at 6 hours post-induction and purified under native conditions. However, as seen in Figure 21.A, the r-WESSVEDIII prep had non-specific bands after purification. This was corrected by increasing the number of washes through the column and no additional bands were observed (Figure 21.B). The r-WNVEDIII derived from the pCOLDTF vector was initially purified under denaturing conditions at 24 hours but later proved to be non-functional in immunoassays, even after the removal of urea. This protein was then purified under native conditions at 24 hours and proved to be useful in immunoassays (Chapter 3). This can likely be attributed to the TF that allowed the protein to remain soluble even at 24 hours (Figure 18), which was not observed with any of the other recombinant proteins expressed without a chaperone. Bacterial cell lysates from cells transformed with pQE-80L and pCOLDTF lacking a *Flavivirus* gene were also purified and showed no indication of expression of any flavivirus protein (Figure 21).



**Figure 21: (A) SDS-PAGE analysis of r-EDIII proteins expressed using pCOLD-TF and pQE-80L expression vectors after protein purification. Lane 1: Page Ruler™ Prestained Protein Ladder, Lane 2: LGTVEDIII expressed using pQE-80L vector, Lane 3: WESSVEDIII expressed using pQE-80L, Lane 4: WNVEDIII expressed using pCOLDTF vector, Lane 5-6: pCOLDTF and pQE-80L expression vectors respectively, expressed without a *Flavivirus* gene. (B) Purification of r-WESSVEDIII with increased number of washes. Lane 1: Fermentas PageRuler™ Prestained Protein Ladder, Lanes 2-5: purified proteins eluted from Protino® columns. CL= total bacterial cell lysates.**

### 2.4.8. Western blot analysis of recombinant proteins

Following protein purification, recombinant proteins were loaded onto an 8 % SDS-PAGE gel and then transferred to a nitrocellulose membrane. Proteins were then reacted with an anti-His antibody for characterization. The r-LGTVEDIII was used for validation of the method and there was reactivity that was observed, as shown by the appearance of a dark band of the expect size on a film (Figure 22). Subsequent to this, the r-EDIII of WNV and WESSV were also characterized by Western blot analysis as shown in Figure 23.

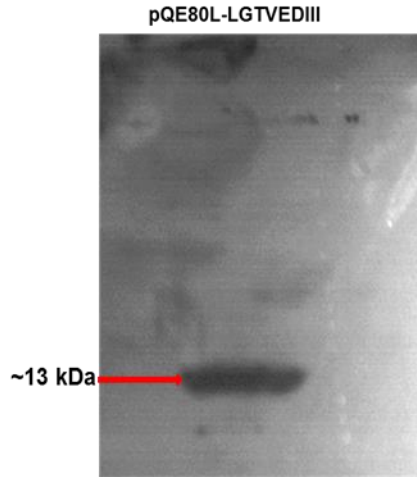


Figure 22: Western blot analysis of the r-LGTVEDIII protein expressed using the pQE-80L plasmid. The dark band indicates the reactivity of the His-antibody with the recombinant protein.

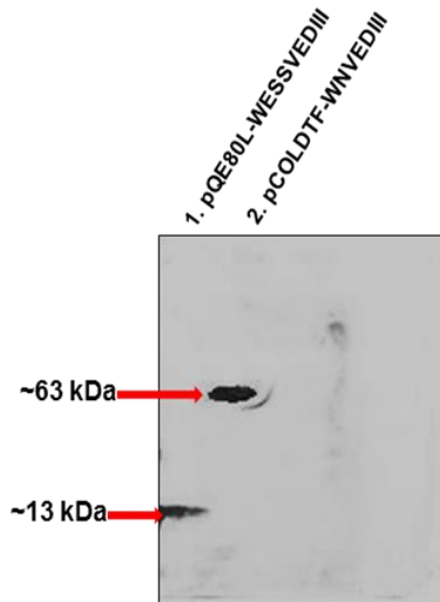


Figure 23: Western blot analysis of the r-EDIII proteins of WNV and WESSV. The dark bands indicate the reactivity of the His-antibody with the recombinant protein.

## 2.5. SUMMARY

The genes encoding the EDIII proteins of LGTV, WNV and WESSV were each amplified in a reverse transcription PCR assay. The genes were cloned into PGEM® T-easy vector using T/A cloning and the ligation mixtures were used to transform competent *E.coli* JM109 cells. The presence of the genes was confirmed by double digestion of the PGEM® T-easy using *Bam*HI and *Hind*III and the genes were further subcloned into pQE-80L vector. The gene encoding the EDIII gene of WNV was additionally subcloned into the pCOLDTF vector. The presence of the genes were confirmed by double digestion of the plasmids using *Bam*HI and *Hind*III except for the gene encoding the EDIII protein of WESSV, which was confirmed by 'colony' PCR, using the forward primer that targets the plasmid and a gene-specific reverse primer to amplify DNA template from cell lysates. Positive constructs were further sequenced to confirm the correct frame of the gene and also that no base changes had occurred in the gene. Positive transformants were used for protein expression by induction with IPTG. Following induction of protein expression, bacterial cell lysates were evaluated using SDS-PAGE analysis. The SDS-PAGE analyses confirmed the presence of r-EDIII protein of LGTV, WNV and WESSV. The r-EDIII proteins of LGTV and WESSVEDIII that were derived from the pQE-80L plasmid were successfully expressed and purified. The purification of the r-EDIII of WNV derived from pQE-80L was however more complex as there was no binding between the His-tag and the resin in the column. The protein was rather washed off the column during initial wash steps, in the absence of imidazole, suggesting that no strong bond was formed between the protein and the Nickel in the column. Additionally, this protein was not detectable by Western blot analysis and did not show functionality in immunoassays such as ELISA. The focus was shifted to using pCOLDTF which is a chaperone-coupled vector that utilizes cold shock technology for protein expression. The 48 kDa chaperone is reported to enhance solubility and the correct folding of target proteins. Using this system, the r-WNVEDIII was shown to remain soluble for 24 hours, compared to 4 hours with the pQE-80L vector. The r-WNVEDIII protein was therefore successfully prepared and purified. The three recombinant proteins (excluding the r-WNVEDIII derived from pQE-80L) were purified under native conditions based on the solubility profiling. Characterization of the proteins was achieved by using Western blot analysis which was based on reacting the immobilized recombinant proteins with the anti-His antibody. All antigens were prepared for use in immunoassays.

---



## CHAPTER 3

### DEVELOPMENT AND USE OF SEROLOGICAL ASSAYS TO DETERMINE THE PRESENCE OF FLAVIVIRUSES IN THE FREE STATE PROVINCE, SOUTH AFRICA.

#### 3.1. Introduction

Serological assays continue to play a pivotal role in the detection and diagnosis of flaviviruses in many parts of the world. Development of these assays has previously been based on the use of native viruses but more recently, recombinant proteins derived from flaviviruses have been the focus of serological assay development. Cargnelutti and co-workers (2011) evaluated the ability of the recombinant envelope glycoproteins preM/E expressed in baby hamster kidney cells in a fluorescent antibody assay for detection of WNV antibodies. Sera collected from horses, mice and chickens immunized with an inactivated WNV vaccine as well as sera from horses acutely infected with WNV were shown to be detectable using this assay. The assay substantiated the value of serological techniques in anti-*Flavivirus* antibody detection from a variety of species. Additionally, an epitope-blocking ELISA was also developed for the universal detection of antibodies to West Nile virus (Sotelo *et al.*, 2011). The principle of the assay was based on the binding of a monoclonal antibody to whole virus particles thus neutralizing infection *in vitro* by recognizing a neutralizing epitope within the envelope glycoprotein. The monoclonal antibody was used to compete with WNV-specific serum antibodies for virus-binding *in vitro* and the assay showed specificity of 79.5-96.5% and sensitivity of 100%. This method was reported to have potential in diagnosis and disease surveillance particularly for small birds. In another study, a total of 54 domestic birds and 391 wild birds were evaluated for seroprevalence against WNV using a plaque-reduction neutralization microtest with Vero cells (Hubalek *et al.*, 2008). It was observed in that study that none of the domestic birds were positive but a proportion of wild birds (5.9%) was positive, with one bird having antibodies against USUV.

Although there is limited data on serosurveillance of flaviviruses in southern Africa, Burt and co-authors (1996) evaluated a total of 2116 cattle sera for the seroprevalence of tick-borne pathogens using ELISA. Two tick-borne flaviviruses, louping ill virus and Kadam virus were among viruses that were detected in the cohort with prevalences of 1.5% and 0.5%, respectively. The authors further tested samples for reactivity against the mosquito-borne WNV and reported that 1.3% of samples had antibodies specific for this virus. This study was instrumental in suggesting that tick-borne flaviviruses may be prevalent in southern Africa. Low awareness for flaviviruses in this region may be attributed to mild infections that are self-limiting, high burden of other diseases such as



tuberculosis and HIV infections, serological cross-reactivity that leads to other flaviviral species being overlooked and limited facilities for diagnosis. Recombinant technology holds promise for laboratories with limited facilities as antigens that do not require high containment laboratories can be produced and used for detection and diagnosis. Such antigens however, have to be validated before they can be implemented for routine testing.

### **3.2. Aim**

The aim of this study was to prepare a KUNV cell lysate antigen and use it with r-EDIII proteins of LGTV, WNV and WESSV to screen serum samples collected from a variety of species in the Free State province for antibodies against flaviviruses.

### **3.3. Materials and methods**

#### **3.3.1. Preparation of KUNV cell lysate antigen**

KUNV strain MRM16, which is a subtype of WNV, was selected as a representative native *Flavivirus* antigen for detection of antibodies raised against both tick- and mosquito-borne flaviviruses in immunoassays. This conclusion was based on the ability of a native *Flavivirus* antigen to detect heterologous *Flavivirus* antibodies due to the high degree of cross-reactivity between members of this genus, but this was first confirmed using an in-house specificity test. More importantly, KUNV does not require a high containment facility and can be handled in a BSL 2 laboratory which is the highest facility that our laboratory has access to. Briefly, Vero cells [American Tissue Culture Collection (ATCC) no. CCL-81™] were cultured in a total of nine Corning® 150 cm<sup>2</sup> tissue culture flasks (Corning, USA) containing BioWhittaker® Minimum Essential Medium Eagle (EMEM). The media was enriched with 10% fetal bovine sera (Delta products, Johannesburg, South Africa) with the addition of 1X BioWhittaker® non-essential amino acids, 2 mM L-Glutamine (Lonza, IL, USA) and 100 U/mL Penicillin-Streptomycin (Sigma-Aldrich®, MO, USA). Culture flasks were incubated at 37 °C for 48 hours and upon a confluency of over 90%, the media was discarded. A 1ml preparation of the MRM16 strain of KUNV was diluted 10-fold and aliquots of 1ml of the diluted virus were each added to the flasks and allowed to adsorb to cells for one hour at 37 °C. EMEM growth media with 2% fetal bovine serum was added to the cultures and flasks were incubated. Cells were scraped from the flasks before cytopathic effects (CPE) using a rubber policeman and decanted into BD Falcon™ centrifuge bottles (BD Biosciences, NJ, USA). Following centrifugation for 10 mins at 8000 g, supernatants were discarded and pellets resuspended in borate buffered saline (pH 9). Cells were further pelleted by centrifugation at a speed of 8000 g for 10 mins at a temperature of 4 °C.

Pellets were resuspended in borate saline (pH 9), 1 % Triton X100, 0.1 % sodium dodecyl sulfate (SDS) and sonicated in a sonicator bath (Branson, CT, USA) over 10 intervals of 15s bursts and 15s cooling periods until all the particulates were disrupted and centrifuged at 4000 g for 10 mins. Supernatants were collected in polypropylene tubes, aliquoted and stored at -70 °C until needed. Mock antigen was prepared as outlined above, using uninfected cells.

### **3.3.2. Collection of serum samples**

Human sera with antibodies positive for WNV, YFV and LIV confirmed using hemagglutination inhibition test by an external laboratory were obtained with informed consent from volunteers with a history of infection. Mouse hyperimmune ascitic fluid (MHAF) (polyclonal) against tick-borne encephalitis virus (TBEV) was obtained from Dr. Robert Tesh from the Arbovirus Reference Center in Texas, USA, and MHAF against WNV, WESSV, Zika virus (ZIKV), Dengue virus serotype (DENV-1), Spondweni virus (SPOV), YFV, LIV, WNV, Kadam virus (KADV), and Banzi virus (BANV) were collectively obtained from Professor Janusz Paweska from the Center for Emerging and Zoonotic Diseases at the National Institute for Communicable Diseases in South Africa. Serum samples collected between 2008 and 2011 from 196 human patients in the Free State province presenting with a tick-bite associated with acute illness and no diagnosis were obtained anonymously from the routine serology laboratory of the Medical Microbiology department at the University of the Free State. A total of 2052 serum samples collected between 2007 and 2012 from cattle submitted for routine screening for brucellosis in four districts within the Free State province and serum samples from 88 sheep, 4 horses, 11 rats (*Aethomys namaquensis*), 5 mice (*Mastomys coucha* and *Rhabdomys pumilo*), 2 shrews (*Elephantulus myurus*), 2 bats (species unidentified), 9 rooihartbees (*Alcelaphus buselaphus*), 9 eland (*Tragelaphus oryx*), 5 warthogs (*Phacochoerus africanus*), 3 buffalos (*Syncerus caffer*), 2 gemsbuck (*Oryx gazella*), 2 giraffes (*Giraffa camelopardalis*) and 2 zebras (*Equus quagga*) were obtained from the Free State Veterinary Services Laboratory in Bloemfontein, South Africa or collected at various sites in the Free State. The four districts from which samples were obtained include Motheo district (name recently changed to Mangaung Metropolitan Municipality district) which covers the central region of the province, Lejweleputswa district which forms part of the northern Free State, Xhariep district which covers the southern parts of the province and Thabo Mofutsanyane district which constitutes the south-eastern region of the Free State. No samples were collected from the remaining district, Fezile Dabi.

### **3.3.3. Preparation of indirect IgG ELISA**

Following the preparation of the KUNV cell lysate antigen and r-EDIII proteins of WNV, LGTV and WESSV; indirect IgG ELISA assays were developed for the detection of anti-*Flavivirus* antibodies in serum samples from the species mentioned above. For each assay, the antigen was diluted in 1X phosphate buffered saline (PBS) in an optimal dilution determined by performing checkerboard titrations, used to coat 96-well PolySorp immuno-plates (Nunc™, Roskilde County, Denmark) and incubated overnight at 4 °C. Wells were washed with 1% Tween/PBS between incubations of 1 hour at 37 °C. After blocking wells with 200 µl of 10% skimmed milk in PBS, serum samples diluted 100-fold in 2% skimmed milk/PBS were added to each well. Anti-species secondary antibodies conjugated to horseradish peroxidase (Zymed Inc, California, USA and KPL, District of Columbia, USA) were optimally diluted 4000-fold and added to the wells except for serum samples collected from wildlife and rodents, where Pierce® recombinant protein A/G conjugate horseradish peroxidase (Thermo Scientific, Massachusetts, USA) was used at a 10000-fold dilution. The reactions were visualized by addition of 2, 2'-azino-di (3-ethylbenzthiazoline-6-sulfonate) (ABTS®) peroxidase substrate (KPL) and OD readings were read at 405 nm using a Sunrise absorbance reader (Tecan, North Carolina, USA).

### **3.3.4. Validation of indirect IgG ELISA**

Cut-off values for each assay were determined by calculating the mean net OD (sample OD on test antigen - sample OD on mock antigen) + 2 (KUNV, r-WNVEDIII, r-WESSVEDIII) or 3 (r-LGTVEDIII) standard deviations depending on the intensity of the background. A panel of 10 known negative sera, confirmed by hemagglutination assay in an external laboratory, was utilized and reacted with the test antigen as well as the mock antigen in ELISA. Each assay was performed in triplicate over a number of days to ensure repeatability of results. All the results obtained were pooled and utilized in determining each of the cut-off values.

### **3.3.5. ELISA Specificity testing**

The specificity of immunoassays was evaluated by cross-reacting KUNV antigen and recombinant proteins with samples positive for antibodies raised against flaviviruses that are known to be endemic in southern Africa (WNV, WESSV), those that have previously been reported in southern Africa (SPOV, BANV) as well as non-endemic flaviviruses that are known to be significant human pathogens (DENV, YFV, TBEV), together with other tick- and mosquito-borne flaviviruses representing additional serogroups (KADV, ZIKV).

### 3.3.6. Analysis of field samples

Following testing of serum samples by ELISA, samples were correlated to geographic distribution to ascertain areas that suggest high endemicity, and possible co-infections (positive reactors for more than one assay) were discerned.

## 3.4. RESULTS

### 3.4.1. Validation of ELISA

Cut-off values for each ELISA were determined from reacting panels of negative serum samples as outlined in Table 12 below. Mean net OD values and the corresponding standard deviations were calculated for all the assays. The mean net OD was added to 2 or 3 standard deviations based on the intensity of the background in the wells of the reaction plate.

**Table 12: Determination of cut-off values for indirect IgG ELISA.**

Antigen	Net OD value	Standard deviation	Cut-off value
KUNV cell lysate	0.05473	0.08723 X2	0.23
r-LGTVEDIII	0.1834	0.032295 X3	0.28
r-WNVEDIII	0.1006	0.0629 X2	0.226
r-WESSVEDIII	0.105	0.12494 X2	0.23

### 3.4.2. Specificity testing

The ability of KUNV cell lysate antigen to detect antibodies against tick- and mosquito-borne flaviviruses is illustrated in Figure 24. All samples collected from species that were confirmed to be positive for flaviviruses using hemagglutination inhibition assay performed at an external laboratory were detected using this assay and it was discriminatory against sera that were negative for flaviviral antibodies. These samples represented various *Flavivirus* serogroups as previously shown in Table 1.

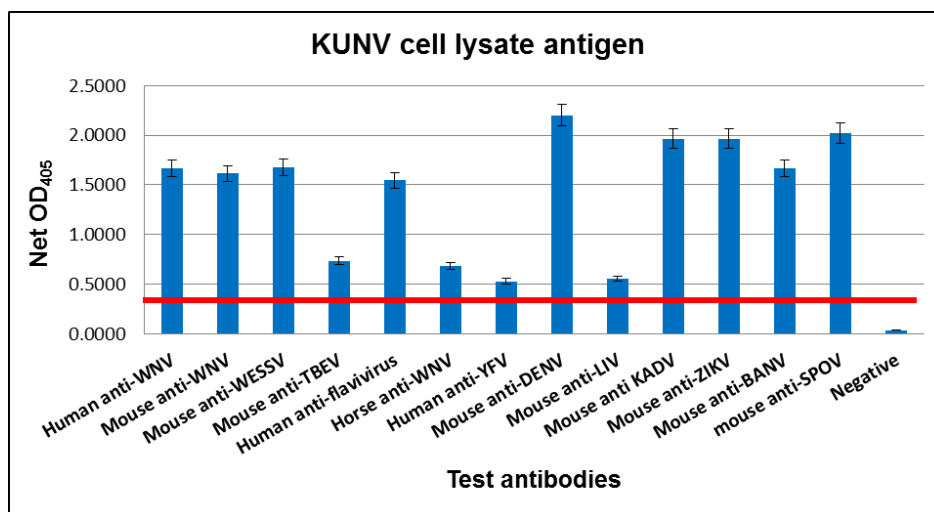


Figure 24: Validation of KUNV cell lysate antigen for detection of anti-Flavivirus antibodies from various species in ELISA. The cut-off value of the assay was determined to be net OD<sub>405</sub>=0.23 as indicated by the horizontal line.

To ascertain if the r-LGTVEDIII protein would differentiate between tick-borne and mosquito-borne flaviviruses, the protein was reacted with sera positive for antibodies against tick-borne TBEV, LIV, KADV and 7 mosquito-borne flaviviruses: WNV, YFV, WESSV, BANV, SPOV, DENV and ZIKV as shown in Figure 25. The assay was able to differentiate antibodies directed against the three tick-borne flaviviruses from those against the mosquito-borne and flavivirus-negative sera.

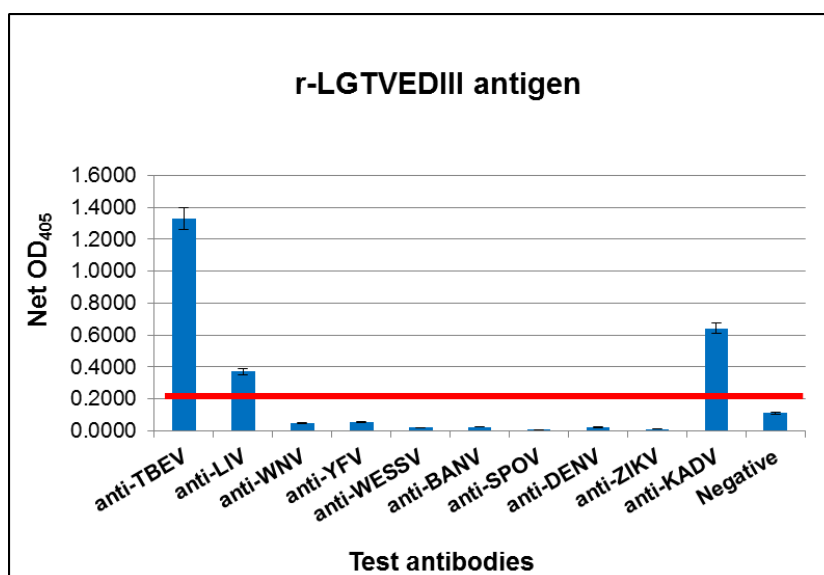
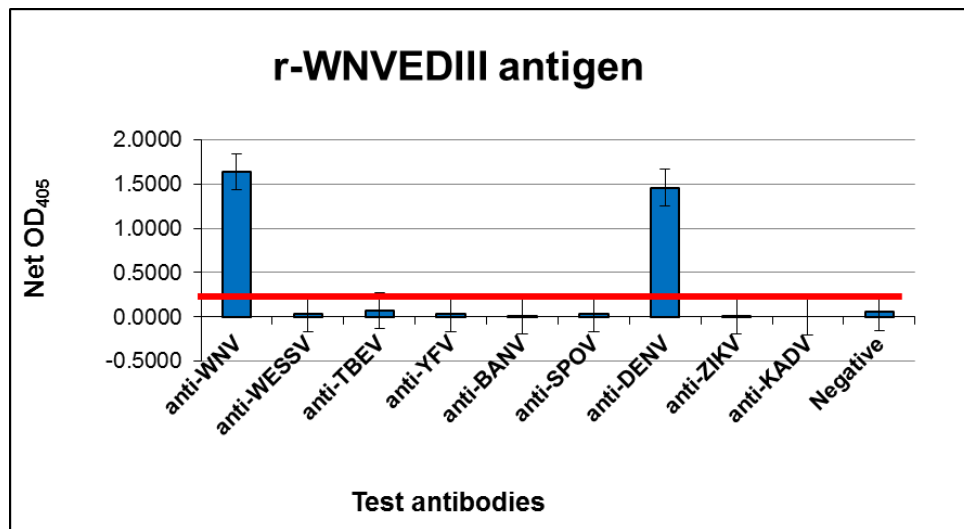


Figure 25: Evaluation of the LGTVEDIII recombinant protein for specificity against mosquito-borne flaviviruses in ELISA. The cut-off value of the assay was determined to be net OD<sub>405</sub>=0.28 as indicated by the horizontal line.

Similarly, the r-WNVEDIII protein was able to differentiate antibodies raised against tick-borne flaviviruses from those raised against WNV as well as sera with no anti-flavivirus antibodies (Figure 26). Though the WNVEDIII immunoassay was able to differentiate between anti-WESSV and anti-WNV antibodies demonstrating a differentiation potential to species level, the assay was not able to differentiate antibodies raised against DENV. Though this may pose a major limitation in areas where both these viruses are endemic, we proceeded with the study as DENV is not known to be endemic to southern Africa. For all the other samples containing antibodies raised against flaviviruses, the assay showed a high discriminatory potential as no equivocal OD readings were obtained.



**Figure 26: Evaluation of the WNVEDIII recombinant protein for differentiation between antibodies raised against mosquito-borne and tick-borne flaviviruses in ELISA. The cut-off value of the assay was determined to be net OD<sub>405</sub>=0.226 as indicated by the horizontal line.**

The r-WESSVEDIII antigen was able to differentiate between anti-WESSV and anti-WNV antibodies as well as sera containing antibodies raised against a tick-borne flavivirus. This is an important attribute in regions that are endemic for both these viruses, such as southern Africa. Samples that are negative for flavivirus antibody were also not detected. Antibodies raised against YFV could however, not be differentiated (Figure 27). This was not a major limitation as YFV is not endemic in southern Africa.

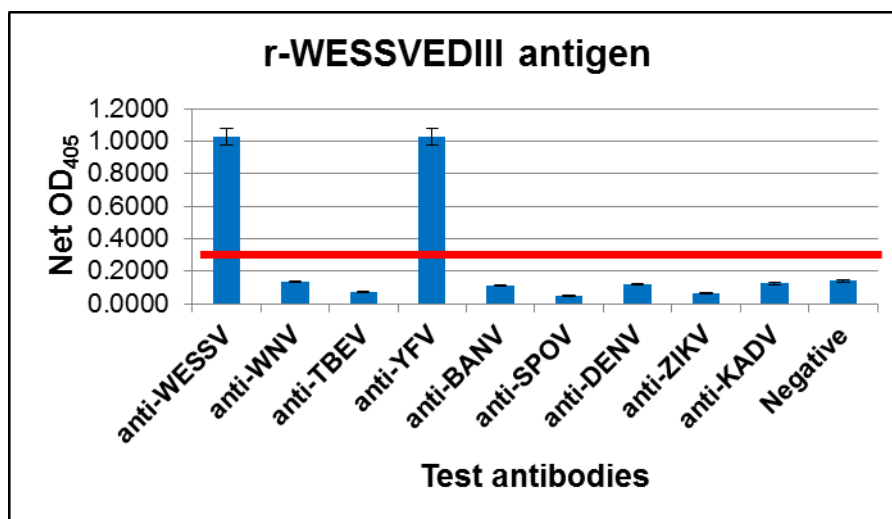


Figure 27: Evaluation of the WESSVEDIII recombinant protein for differentiation between antibodies raised against mosquito-borne and tick-borne flaviviruses in ELISA. The cut-off value of the assay was determined to be net OD<sub>405</sub>=0.23 as indicated by the horizontal line.

### 3.4.3. Screening of field samples

Subsequent to their validation, the four assays were further used to detect anti-flavivirus antibodies in sera collected from a variety of species including humans, cattle and sheep. The initial screening for anti-flavivirus antibodies was performed using the KUNV immunoassay and samples that were confirmed as positive based on the cut-off value were further tested using the more specific recombinant LGTV, WNV and WESSV assays. As shown in Table 13, a total of 722 samples were positive on the KUNV assay and were further tested using the r-EDIII assays. Raw data for each ELISA run was not included as it is too extensive, but it is available on request.

Of the 722 samples that reacted with the KUNV antigen, the majority of samples were positive on the r-WNVEDIII (n=457) and r-WESSVEDIII (n=431). This was in agreement with the endemicity of both these viruses in this region. A total of 71 samples reacted with the r-LGTVEDIII protein, which is a representative of tick-borne flaviviruses. This suggests, but does not confirm the circulation of flaviviruses that belong to the tick-borne serocomplex in this region. Approximately a third of clinical samples sent to the Microbiology and Virology department of the University of the Free State collected from humans with a tick bite, also reacted with the KUNV antigen (n=67). A large portion of these samples reacted with the r-WESSVEDIII antigen (n=54), followed by reactivity on the r-WNVEDIII (n=47). The r-LGTVEDIII antigen reacted with the least number of human samples (n=18).

**Table 13: Summary of samples tested on serological assays for presence of *Flavivirus* antibodies**

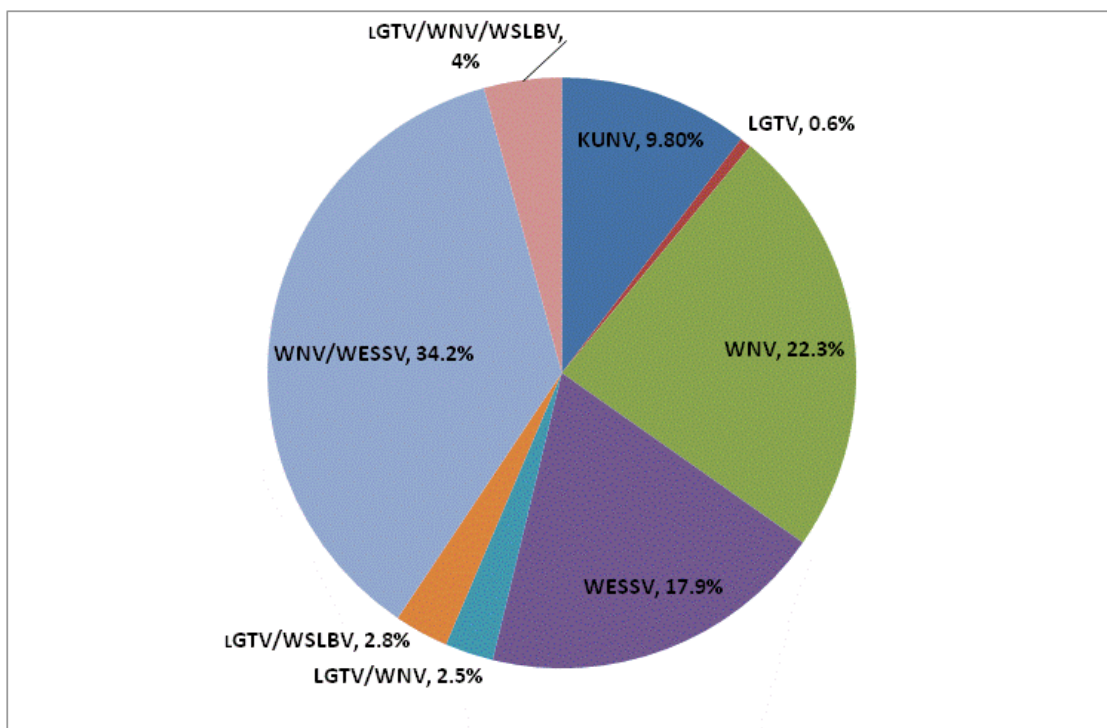
Species tested	Total number of samples	KUNV assay	r-LGTVEDIII assay	r-WNVEDIII assay	r-WESSVEDIII assay
Human	196	67	18	47	54
Cattle	2052	595	39	372	376
Sheep	88	31	14	26	1
Horses	5	3	0	3	0
Rats	11	0	ND	ND	ND
Mice	5	0	ND	ND	ND
Shrews	2	0	ND	ND	ND
Bats	2	0	ND	ND	ND
Rooihaartbees	9	8	0	1	0
Eland	9	8	0	1	0
Warthog	5	5	0	4	0
Buffalo	3	2	0	1	0
Gemsbuck	2	2	0	2	0
Giraffe	2	0	ND	ND	ND
Zebra	2	1	0	0	0
TOTAL	2393	722	71	457	431

ND: No further tests done as samples were negative for antibody against KUNV.

#### **3.4.4. Detection profiles of field samples by indirect IgG ELISA.**

While a large proportion (40.8%) of the samples was detected using only one of the r-EDIII protein assays, the rest of the samples were either detected by multiple r-EDIII assays (43.5%) or the KUNV assay only (9.8%), as illustrated in Figure 28. A total of 34.2% of the samples reacted against both r-WNVEDIII and r-WESSVEDIII antigens, with mono-detections of the r-WNVEDIII and r-WESSVEDIII assays resulting in 22.5% and 17.9%, respectively. Mono-detection of r-WNVEDIII and r-WESSVEDIII further proves the potential of the r-EDIII protein to differentiate between antibodies raised against the serologically-cross reactive but antigenically distinct WNV and WESSV.





**Figure 28: Profiles for the detection of samples positive on the KUNV assay by the r-EDIII proteins of LGTV, WNV and WESSV.**

Table 14 outlines the number of samples that were differentiated by the r-EDIII proteins of WNV and WESSV. Samples that were detected on both the r-LGTVEDIII and r-WNVEDIII assays accounted for 2.5% whereas those detected on r-LGTVEDIII and r-WESSVEDIII accounted for 2.8%. A small proportion of samples (4%) were detected on all three r-EDIII assays and the mono-detection of r-LGTVEDIII assay was the lowest at 0.6%. The remaining 5.6% of samples not illustrated in Figure 30 could not be tested further due to insufficient sample.

**Table 14: Differentiation of field samples by r-EDIII proteins of WNV and WESSV.**

Assay result	WESSV-positive	WESSV-negative
WNVEDIII-positive	246	207
WNVEDIII-negative	178	91

### 3.4.5. Geographical location of positive reactors.

To further understand the putative prevalence of flaviviruses in the Free State province, samples detected using the immunoassays developed in this study were correlated to their site of collection and the results were analyzed per district (Figure 29). All districts that were surveyed showed similar distribution for samples that were detected using the KUNV assay only, and they ranged between 8-12% in each district. Samples that were detected using the r-WNVEDIII were abundant in all districts (35-50%), with the highest number in the Xhariep and Lejweleputswa regions. Similarly, there was an ample representation of the

samples detected using the r-WESSVEDIII assay in all the districts (28-55%) with dominance in the Motheo (47%) and Thabo Mofutsanyane (54%) districts. Detection of samples observed using the r-LGTVEDIII was the lowest in all regions (average 5%) and no detection was observed in the Thabo Mofutsanyane district. The Xhariep district however, showed a high presence of samples detected using the r-LGTVEDIII antigen (12%).

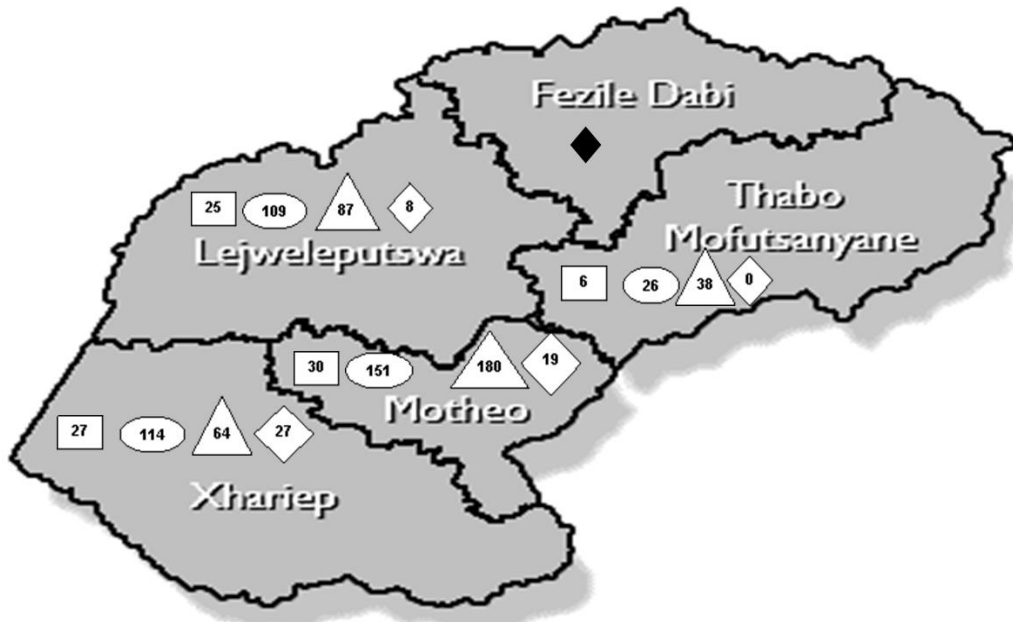


Figure 29: Correlation of the detection of anti-flavivirus IgG antibody results to the geographical distribution in 4 districts of the Free State province.

Various symbols have been utilized to represent the number of samples detected on each of the assays developed in this study. □ = KUNV, ○ = WNVEDIII, △ = WESSVEDIII, ◇ = LGTVEDIII, ◆ = no samples were collected in this district.

### 3.5. Summary

The presence of mosquito-borne flaviviruses in southern Africa is known but their prevalence has not been fully established. In addition, cases of flavivirus disease may have been underreported in this region due to limited routine diagnostic services. An assay that can detect antibodies against flaviviruses could have a role in diagnosis and in serological surveillance of various species. Due to the high cross-reactivity between members of this genus, it is essential to have an assay that can differentiate between them. Most flaviviruses that are medically significant pathogens such as WNV, DENV, YFV, TBEV and JEV require BSL 3 containment facilities for culturing and handling. This is challenging for laboratories that have limited resources and limits diagnosis or surveillance for circulation of such pathogens. Recombinant antigens are safe alternative reagents that may be handled within BSL2 or lower facilities. The r-EDIII protein of flaviviruses is a useful tool that has

been demonstrated to have potential in differentiating between the highly cross-reactive flaviviruses (Holbrook et al. 2004). We aimed to express and subsequently use this protein derived from LGTV, WNV and WESSV to assess its efficacy as a reliable reagent. KUNV cell lysate antigen was used as the initial screening assay for antibodies raised against flaviviruses. The antigen was evaluated for specificity against antibodies raised against both tick-borne and mosquito-borne flaviviruses representing various serogroups. The antigen was useful in detecting all samples in the panel and excluded samples that were not positive for flaviviruses. Due to the fact that a native flavivirus antigen was used, there was potential to detect flaviviral antibodies owing to the cross-reactivity between members of this genus. This was validated for flaviviruses that are known to circulate in southern Africa (WNV, WESSV), those previously recorded in this region (ZIKV, SPOV, BANV) as well as non-endemic flaviviruses representing additional serogroups (TBEV, KADV, DENV, YFV, LIV). Recombinant EDIII proteins of LGTV, WNV and WESSV were also evaluated for specificity between *Flavivirus* species. r-LGTVEDIII was in agreement with previous reports that it can discriminate tick-borne flaviviruses from mosquito-borne flaviviruses but is unable to differentiate between members of the tick-borne serocomplex. KADV, TBEV and LIV were detected using this assay and none of the samples positive for mosquito-borne flaviviruses were detected. The ability for differentiation between r-WNVEDIII and r-WESSVEDIII was also evaluated. This was important as both these viruses are known to circulate in the Free State province. The two assays were able to discriminate antibodies raised against WNV and WESSV and reacted only to homologous antibodies. Both these assays were however not mono-specific for heterologous antibodies when the panel of positive sera was tested. In addition to detecting antibodies raised against WNV, the r-WNVEDIII antigen also detected antibodies raised against DENV. Similarly, the r-WESSVEDIII also reacted with antibodies raised against YFV. These limitations did not deter the study from proceeding as both DENV and YFV are known to be non-endemic in southern Africa. Using KUNV antigen for screening of samples indicated a 30% (722/2393) positivity rate in the samples collected. The majority of the samples (~35%) were positive on the recombinant antigens derived from mosquito-borne flaviviruses WNV and WESSV. A smaller proportion of samples (~4%) reacted with the r-LGTVEDIII protein suggesting the possible circulation of tick-borne flaviviruses in this region. Samples that were detected on more than one assay accounted for ~43%, suggesting that multiple flaviviruses may be circulating in the same regions within the Free State province. Additionally, some samples that reacted with KUNV antigen did not react with any of the recombinant antigens, suggesting that additional flaviviruses for which recombinant proteins were not available may be circulating in the Free State province. Though these results account for a preliminary report on flavivirus seroprevalence in the Free State, a confirmatory report will require virus neutralization assay which is the gold standard for flavivirus identification.

---

## CHAPTER 4

### MOLECULAR ASSAYS FOR DETECTION OF TICK-BORNE AND RODENT-BORNE VIRUSES.

#### 4.1. Introduction

Tick-borne viruses are currently found in six major viral families which include *Bunyaviridae* and *Flaviviridae* (Karabatsos, 1985). When transmitted to humans, virus species belonging to *Bunyaviridae* and *Flaviviridae* may cause infections ranging from a mild to a more serious clinical presentation that may result in a fatal outcome. In addition to serological assays, molecular assays continue to be useful and reliable tools for the detection of tick-borne viruses in a variety of vertebrate species. Molecular techniques are especially significant in confirming infections during the acute stage in humans and animals, and may also be applied for detection of viral nucleic acid from arthropod vectors and viral reservoirs when conducting epidemiological and surveillance studies. Virus species that belong to flaviviruses have been detected using a variety of multiplex PCR formulations including the use of SYBR® Green, heminested reverse-transcriptase (RT)-PCR and TaqMan®-based assays (Lanciotti 2003, Bae *et al.*, 2003; Nunes *et al.*, 2011).

Whilst most of these assays focus on the detection of flaviviruses confined to a specific geographical area (Domingo *et al.*, 2011; Wodak *et al.*, 2010); a one-step quantitative RT-PCR aimed at detecting nucleic acid extracted from geographically and phylogenetically distinct flaviviruses has been developed (Patel *et al.*, 2013). The latter was initially based on the use of 17 forward and 19 reverse primers and 22 probes representing the various RNA targets, but resulted in a suboptimal reaction assay probably due to primer/probe competition (Patel *et al.*, 2013). When a set of three pairs of degenerate primers was designed and three locked-nucleic acid (LNA) probes were utilized, the specificity and sensitivity of the assay was significantly increased. The assay was able to detect RNA from 30 different flaviviruses, verifying the broad spectrum of specificity achieved. In South Africa, a nested real-time RT-PCR that distinguishes WNV lineage 1 and 2 using fluorescent resonance energy transfer (FRET) probes was designed and has been used for diagnosis and surveillance applications (Zaayman *et al.*, 2009; Venter *et al.*, 2009). Additional molecular techniques recently developed for detection of flaviviruses include nucleic acid sequence based amplification (NASBA), loop-mediated isothermal amplification (LAMP) and transcription-mediated amplification (TMA) (Domingo *et al.*, 2011). Due to their highly conserved nucleotide

sequences among flavivirus species, the NS3 and NS5 genes of the positive single-stranded RNA genome of flaviviruses are commonly used as targets for flavivirus multiplex assays (Scaramozzino *et al.*, 2001; Sekaran and Artsob 2007; Johnson *et al.*, 2010).

Species of the *Bunyaviridae* family consist of a tripartite negative sense single-stranded RNA genome. The *Bunyaviridae* family comprises of five genera: *Hantavirus*, *Nairovirus*, *Orthobunyavirus*, *Phlebovirus* and *Tospovirus*. CCHFV is a type species of the genus *Nairovirus* and constitutes the S, M and L segments which encode for the nucleocapsid, two envelope glycoproteins G<sub>n</sub> and G<sub>c</sub>, as well as a RNA dependant RNA polymerase, respectively (Clerx *et al.*, 1981). The S-segment encoded nucleoprotein has been described as the most abundant and immunodominant viral protein inducing high levels of specific humoral antibodies in CCHFV-infected patients. Transmission of CCHFV occurs by tick-bite and contact with infected people, livestock and tissue. The virus is widely distributed in Africa, Asia and eastern Europe with a case-fatality rate of between 10 and 50%. Ticks belonging to the *Hyalomma* genus serve as principal vectors for the virus and their distribution correlates to that of the virus (Whitehouse 2004). For diagnosis in the acute stages of disease, traditional virus isolation methods for CCHFV either in cell culture or mice is performed, but the assay must be carried out in a maximum containment facility (BSL4), which presents a challenge for most laboratories in resource-limited countries. In addition to using recombinant antigens for demonstration of IgM antibodies as alternative means for diagnosis (Saijo *et al.*, 2005), RT-PCR serves as an ideal substitute that can be utilized to detect the viral RNA with accurate and reliable results. The use of specific probes for detection of CCHFV has also been demonstrated to have the ability to discriminate between reassorted and non-reassorted strains of the virus (Kondiah *et al.*, 2010). Moreover, Ke and co-authors (2011) were able to use padlock probes for detection of CCHFV without the need for a PCR assay.

Another genus belonging to *Bunyaviridae*, namely *Hantavirus*, is rather associated with rodents and not ticks. Hantaviruses are known to have a strict correlation with their rodent hosts and thus share a common geographical distribution with the rodent host. Some hantavirus species are significant human pathogens that have been associated with large outbreaks of disease. Hantaviruses are associated with a range of nephrotic diseases in Asia and Europe with varying degrees of severity. In the Americas, these viruses are associated with severe respiratory disease and have a fatality rate that can exceed 50%. Hantaviruses have historically been known to be carried by rodents of the family *Muridae* in nature and have been divided into three groups based on the rodent host they infect, namely, *Murinae*, *Arvicolinae* and *Sigmodontinae* (Ulrich *et al.*, 2002). However, recent studies have reported on the isolation of novel hantaviruses in a variety of other species including shrews, moles and bats (Guo *et al.*, 2013; Kang *et al.*, 2011). Since 2006, hantavirus RNA has also

been detected in rodents and insectivores in West Africa, Tanzania and Ethiopia. (Klempa *et al.*, 2007; Kang *et al.*, 2011; Weiss *et al.*, 2012, Witkowski *et al.*, 2014). Though African hantaviruses have not been associated with human disease except for a suspected case of HFRS in a nine-year old boy, a serological survey demonstrated anti-hantavirus antibodies in humans, suggesting previous exposure to hantaviruses (Klempa *et al.*, 2010). Unlike humans, rodents are able to maintain hantaviruses in nature without any progression to either a clinical or a fatal outcome. Transmission to humans occurs by aerosolized excreta from carrier rodents as a result of their continuous shedding of the virus. In the Americas, patients infected with a hantavirus can evolve from acute febrile illness to severe pneumonia with respiratory failure and cardiac shock within 12 to 24 hours (Jonsson *et al.*, 2010). This highlights the need for rapid diagnosis of hantaviruses in suspected cases of infection. RT-PCR has been used to detect hantavirus genomes in clinical samples such as blood, serum and tissue fragments as early as one day after onset of illness (Padula *et al.*, 2007). Due to the possibility of low levels of viral RNA in human and rodent samples, a nested RT-PCR has previously been used to increase the sensitivity of the assay (Hjelle *et al.*, 1994).

The increase in host range and geographical distribution of hantaviruses demonstrates the potential of their emergence in parts of the world that were previously regarded to be non-endemic for hantaviruses. Prior to 2006, no hantavirus had been identified in Africa. However, within the past eight years, hantavirus RNA has been detected in rodents, insectivores and bats, resulting in nine new species being described in Africa (Witkowski *et al.*, 2014). In all instances where hantaviruses were detected in non-rodent hosts, it was strongly suggested that the viruses had not spilled over to, but were rather closely associated with their non-rodent hosts. Although a one percent seroprevalence rate has been suggested for hantaviruses in the Cape region of South Africa (Witkowski *et al.*, 2014), no hantavirus has been identified in any of the southern regions of Africa to date, despite the presence of suitable hosts. Due to the atypical clinical picture that patients present with following hantavirus infection such as acute febrile illness, renal failure and cardiac shock, cases involving hantavirus infection may easily be missed in a routine diagnostic settings. Diagnostic services for *Rickettsia* and CCHFV exist in southern Africa for patients presenting with a tick-bite and febrile illness but these only confirm infection in 10% of cases and the rest remain undiagnosed. Similarities in clinical manifestations observed in patients infected with CCHFV and other viral hemorrhagic fevers also hamper proper diagnosis. More common diseases such as malaria, bacterial septicemias, leptospirosis, viral hepatitis and viral herpes need to be ruled out during the differential diagnosis. The lack of diagnosis in 90% of patients with tick-bite and/or rural residents or visitors to rural areas with possible exposure to arboviruses and rodent-borne viruses, suggests that development of multiplex assays is warranted. Hence this chapter was aimed at

developing molecular assays for the detection of tick-borne and rodent-borne viruses not previously identified and for which routine diagnostic assays are not available in South Africa.

## **4.2. Aim**

The aim of this study was to develop multiplex RT-PCR assays for detection of mosquito- and tick-borne viruses and multiplex RT-PCR for detection of hantaviruses in rodent samples.

## **4.3. Materials and methods**

### **4.3.1. RNA controls**

#### **Flaviviruses**

A total of eight RNA controls representing *Flavivirus* species were available for use in the laboratory. RNA from WNV strain SA93/01, LIV strain Negishi 3248/49/P10 and WESSV strain SAH177 were kindly provided by Professor Janusz Paweska, National Institute for Communicable Diseases (NICD) in Johannesburg, South Africa. Ntaya virus (NTAV) original strain VR-78, LGTV strain TP21, YFV strain 17D, ZIKV strain MR766 and KUNV strain MRM16 were propagated by intracerebral inoculation of suckling mice. Brain tissue was harvested from mice that succumbed and RNA was extracted using the Qiagen RNeasy mini kit (Qiagen) according to manufacturer's instructions and as previously mentioned (Chapter 2).

#### **CCHFV**

RNA of CCHFV strain SPU 203/88 was supplied to us by Professor Janusz Paweska, National Institute for Communicable Diseases (NICD) in Johannesburg, South Africa and used as a control.

#### **Hantaviruses**

Genes representing the partial region of the S-segment of HTNV, SNV, PUUV and SANGV were synthesized and supplied by GenScript (NJ, USA). All genes were supplied in a pUC 57 vector and were the basis of the RNA transcripts that were subsequently prepared.

### **4.3.2. Human and rodent samples for preliminary screening**

Serum samples collected between 2008 and 2011 from 196 human patients in the Free State province presenting with a tick-bite associated with acute illness and no diagnosis were obtained anonymously from the routine serology laboratory of the Medical Microbiology department at the University of the Free State. A total of 33 rodents and seven shrews collected around the

Bloemfontein area in the Free State province, South Africa, were also included in the study. Specifically, these included 13 Namaqua rock rats (*Aethamys namaquensis*), nine southern multimammate mice (*Mastomys coucha*), nine four-striped grass mice (*Rhabdomys pumilio*), two southern African vlei rats (*Otomys irroratus*) and seven eastern rock elephant shrew (*Elephantulus myurus*).

### **4.3.3. RNA extraction**

#### **4.3.3.1. Human serum samples**

RNA was extracted from human serum samples using the QIAamp® Viral RNA mini kit (Qiagen) according to manufacturer's instructions and using buffers provided in the kit. In brief, 140 µl of serum was added to buffer AVL-carrier RNA and mixed by vortexing. Following incubation at room temperature for 10 mins, ethanol was added to the sample mixture. The sample mixture was then applied to a QiAamp® mini column and centrifuged. Spin columns were washed with buffer AW1 followed by the addition of buffer AW2. After centrifugation, RNA was eluted by addition of buffer AVE or nuclease-free water.

#### **4.3.3.2. Rodent organs**

Total RNA was extracted from pooled rodent organs (liver, spleen, kidneys) using Trizol® reagent as recommended by the supplier (Life Technologies, CA, USA). In brief, 1 ml of Trizol reagent was added to organs and homogenized using a needleless syringe. After chloroform was added, the homogenate was mixed vigorously and centrifuged at 4 °C. The aqueous phase of the mixture was removed and 500 µl of 100% isopropanol was added. Following incubation at room temperature for 10 mins, the mixture was centrifuged and RNA was washed using ethanol. The resulting RNA pellet was air-dried for 5 mins. The pellet was resuspended in nuclease-free water, incubated at 60 °C for 15 mins and stored at -70 °C until needed.

#### **4.3.4. Primers and probes**

This section outlines oligos that were utilized in developing the two multiplex PCR assays. The first assay, referred to as the Flavi-CCHFV nested multiplex PCR assay was aimed at detecting flaviviruses and CCHFV from human samples using nested (CCHFV) and hemi-nested (flaviviruses) primers. The second assay, designated Flavi-Hantavirus TaqMan® multiplex PCR assay, was designed for the detection of flaviviruses and hantaviruses in rodent samples using specific primers and hydrolysis probes in a real-time RT-PCR assay.



#### **4.3.4.1. Flavi-CCHFV nested multiplex PCR**

As outlined in Table 15, two sets of CCHFV primers designated F2, R3 and F3, R2 as previously described by Rodriguez *et al.*, (1997) were used. The primer sets target a ~536 bp region (F2, R3) and an inner ~260 bp region (F3, R2) of the S-segment in a two-round nested RT-PCR assay. For detection of flaviviruses, in-house degenerate primers previously identified using a multiple alignment of the NS5 region of flaviviruses were used (Samudzi R, 2008). The primers were designated FlaviF1 and FlaviR1 and target a ~414 bp region of the NS5 gene. In this study, two additional reverse primers targeting the inner region of the ~414 bp amplicon were designed and designated FlaviR2 and FlaviR3. Two reverse primers were necessary due to genetic variability between the flaviviruses as one primer would need to have an excessive number of degenerate nucleotides. The primers, FlaviR2 and FlaviR3, were designed to target a ~353 bp inner region and a ~349 bp inner region of the NS5 gene respectively, when used in combination with FlaviF1 in a hemi-nested PCR assay. Multiple sequence alignments used for identification of *Flavivirus* primers are included in Appendix B.

#### **4.3.4.2. Flavi-Hantavirus TaqMan® multiplex PCR assay**

In-house *Flavivirus* primers FlaviF1 and FlaviR1 targeting a ~414 bp region of the NS5 gene were used as described above. For detection of hantaviruses, two sets of primers were designed based on sequence alignment of the S-segment of hantaviruses representative of the three classical lineages of the genus as well as SANGV, the first African hantavirus species identified and the only African hantavirus that has been isolated in the laboratory to date. (Appendix C). Specifically, nucleotide alignments were based on *Murinae*-associated hantaviruses (HTNV, SEOV), *Sigmodontinae*-associated hantaviruses (SNV, ANDV), *Arvicolinae*-associated hantavirus (PUUV) and SANGV. Based on the multiple sequence alignments a degenerate primer referred to as Hanta F was designed from a conserved region of the genome of representatives of three hantavirus lineages. An additional forward primer, SANGF, was designed to detect a partial region of the S-segment of SANGV virus genome. Two reverse primers were designed and designated ASPrev and HSrev. ASPrev was designed to amplify the partial S-segment of the genome of representative *Sigmodontinae*- and *Arvicolinae*-associated hantaviruses, while HSrev was designed to amplify the partial S-segment of the genome of *Murinae*-associated hantaviruses and SANGV. Hydrolysis probes were designed based on the homology of the viral nucleotides within the region of the designed primers. A total of three probes were designed for the detection of the hantavirus genome, namely the HNSL probe (*Murinae*-associated hantaviruses), ASPRB probe (*Sigmodontinae*- and *Arvicolinae*-associated hantaviruses) as well as the SANGV probe (SANGV). A *Flavivirus* probe (FLAVI) was designed based on the inner region of the NS5 gene that is flanked by FlaviF1 and FlaviR1 binding regions. All probes were labeled with a 6-carboxyfluorescein (6FAM) dye and

coupled to a BlackBerry® Quencher (BBQ). Oligonucleotide sequences are shown below in Table 15. Due to their limitations in length being consequential to low melting temperatures and stability, probes designated Flavi and ASPR were modified to include locked nucleic acids (LNAs) which are made of nucleic acid analogues in which the ribose ring is locked by a methylene bridge connecting the 2'-O atom and the 4'-C atom, making it inaccessible for base-pairing.

**Table 15: Nucleotide sequences and properties of primers and probes used in the development of multiplex PCR assays.**

Primer name	Genomic positions*	Oligonucleotide sequence	Tm (°C)
<b>Flaviviruses</b>			
FlaviF1	8847-8863	5'-ATGGCHATGACWGACAC-3'	45
FlaviR1	9259-9242	5'-CCACATGWACCADATGGC-3'	48
FlaviR2	9199-9180	5'-CYXTTYCCCATCATGTTXTA -3'	44
FlaviR3	9195-9178	5'-TSCCCATCATGWTGTAYACRCA-3'	51
<b>CCHFV</b>			
F2	135-153	5'-TGGACACCTTCACAACTC-3'	49
R3	670-653	5'-GACAAATTCCTGCACCA-3'	48
F3	290-309	5'-GAATGTGCATGGGTTAGCTC-3'	52
R2	549-530	5'-GACATCACAATTCACCAGG-3'	50
<b>Hantaviruses</b>			
HantaF	1-26	5'-TAGTAGTAGACTCCTTGAYAAGCTAC-3'	55
SANGF	1-23	5'-TAGTAGTAGACTCCCTAAYGAGC-3'	53
ASPrev	162-144	5'-GTTAACRTCATCSGGGTC-3'	48
Hsrev	135-114	5'-CTCATACTGYTTTTCTGCATCC-3'	51
<b>Probes</b>			
HNSL	37-60	6FAM-ATGGCAACTATGGAGGAAMTSCAG-BBQ	57
SANGV	41-63	6FAM -AAAAAATGGCAACTAGAGGAG-BBQ	55
ASPRB+LNA	37-46	6FAM-CTGGA+A+T+G+A+G-BBQ	55
FLAVI+LNA	9042-9055	6FAM -TACAWCATGA+T+G+GG-BBQ	61

\*Flavivirus primers and probe relative to the WNV strain Madagascar-AnMg798 (GenBank accession: DQ176636); CCHFV primers relative to the S segment of CCHFV strain SPU415/85 (GenBank accession: DQ211648) and as previously described (Rodriguez *et al.*, 1997); HantaF primer, HSrev primer and HNSL probe relative to the S segment of Hantaan virus strain HV004 (GenBank accession: JQ083395); SANGF primer and SANGV probe relative to the S segment of SANGV strain SA14 N (GenBank accession: JQ082300), ASPrev relative to the S segment of PUUV strain Udmurtia/338Cg/92 (GenBank accession: Z30708).

#### 4.3.5. PCR cycling conditions for the Flavi-CCHFV nested multiplex PCR

In order to determine the ability of the FlaviF1 and FlaviR1 primers to detect individual flavivirus RNA controls, an RT-PCR assay was performed using Titan® One Tube RT-PCR System kit (Roche) according to the manufacturer's instructions and using cycling conditions as previously described for this system (Table 4, Chapter 2) and the annealing temperature was set at 45 °C. However, the annealing temperature was lowered to 43 °C when the assay was assembled as a multiplex PCR for the simultaneous detection of flaviviruses and CCHFV. For the first round of the

multiplex assay, a total of 10 µl of RNA extracted from human serum samples was used, whereas 1 µl was added for each of the control RNA samples for validation of the assay. For the second round of the assay, 1 µl of the amplicon was used as template DNA and amplified using the GoTaq® DNA polymerase (Promega). The reaction setup for the second round assay was assembled as shown in Table 16 below. Cycling conditions were commenced with an initial denaturation step at 95 °C for 2 mins, followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 42 °C for 1 min and extension at 72 °C for 30 sec. Final extension was set at 72 °C for 5 mins. DNA products were visualized using 1% agarose gel electrophoresis.

**Table 16: Reaction setup for the second round of Flavi-CCHFV nested multiplex PCR using GoTaq® polymerase**

Reagent	Volume (µl)
5X GoTaq® reaction buffer	10
dNTP mix, 10 Mm each	1
Primers each, 20 pmol	0.75
GoTaq® DNA polymerase (5U/µl)	0.25
Template DNA	1
Nuclease-free water to	50

#### 4.3.6. Validation of the Flavi-Hantavirus TaqMan® multiplex PCR assay

In order to utilize this assay as a tool for viral nucleic acid detection, procedures were undertaken to validate its reliability as outlined below.

##### 4.3.6.1. Synthesis of RNA controls

Synthetic genes representing the partial region of the S-segment of HTNV, SNV, PUUV and SANGV were received in a pUC57 plasmid and amplified with gene-specific primers using the GoTaq® DNA polymerase (same reaction setup as shown in Table 16). The resulting amplicons were ligated into a pGEM-T-easy® vector using T/A cloning. Ligation mixtures were used to transform *E. coli* XL-10 Gold competent cells (Agilent Technologies, CA, USA). Positive transformants were subcultured and subsequently used for plasmid extraction and purification. The pGEM-T-easy® vector has a T7 promoter and this was used to confirm the orientation of the ligated genes using a T7 primer, as well as to facilitate RNA synthesis using the MEGAscript® kit (Life Technologies) according to manufacturer's instructions. Initially, the genes were amplified with the T7 primer and a gene-specific reverse primer using GoTaq® DNA polymerase. Amplicons obtained were used as templates for RNA transcription. For RNA transcription, the transcription reaction mixture was assembled at room temperature. The reaction mixture consisted of 8 µl of 10 mM NTP

solution, 2 µl of 10X MEGAscript® reaction buffer, 2 µl of MEGAscript® enzyme mix, 1 µl of template DNA (0.1-1 µg), and nuclease-free water to a total volume of 20 µl. The reaction mixture was mixed by gentle pipetting and incubated at 37 °C for 16 hours. RNA was isolated using the SV Total RNA isolation system (Promega) using reagents included in the kit. In summary, the transcription reaction mixture was added to RNA lysis buffer. Subsequent to the addition of RNA dilution buffer, the reaction was mixed and centrifuged for 10 mins. Ethanol was added to the cleared lysate and the mixture transferred to the spin basket assembly. RNA was washed and the DNase mix added to the membrane of the column and incubated at room temperature for 15 mins. DNase stop solution was added to the membrane and centrifuged. The membrane was washed twice with RNA wash solution before the RNA was eluted using 40 µl nuclease-free water and stored at -70 °C until required. For RNA controls of flaviviruses, represented by LGTV and WNV, viral genomic RNA was used.

#### **4.3.6.2. Preparation of DNA standards**

Subsequent to RNA transcription, RNA controls for HTNV, PUUV, SANGV and LGTV were used as templates for the synthesis of DNA standards using Titan® One Tube RT-PCR System (Roche). The RNA controls were selected as they represented positive controls for each of the probes designed. The resultant amplicons were excised out of an agarose gel and purified using the Wizard® SV gel and PCR clean-up system (Promega) as shown previously. The DNA concentration was determined by using a NanoDrop spectrophotometer (Thermo Scientific) and DNA copy numbers were calculated as  $\text{DNA copies} = \frac{\text{amount dsDNA (ng)} \times 6.022 \times 10^{23}}{(\text{length of dsDNA (bp)} \times 10^9 \times 650 \text{ (molar mass per base pair)})}$ . Based on the calculated copy number, amplicons were serially diluted 10-fold,  $10^6$ - $10^0$ . Thus, a standard curve was constructed for each of the probes using 10-fold diluted DNA standards. Using the software for the LightCycler® 2.0 instrument (Roche), the standard curve was constructed by plotting the logarithmic values of the concentration of each DNA standard against the corresponding cycle threshold. Using the software, the resultant standard curve was also used to estimate the efficiency of the PCR assay, which is most optimal when a value of 2 is achieved with an error value of 0.

#### **4.3.6.3. Synthesis of cDNA**

RNA extracted from rodent organs using Trizol® reagent was reverse-transcribed into cDNA using Super Script® first-strand synthesis system for RT-PCR (Life Technologies) according to manufacturer's instructions. In short, 5 µl of rodent-derived RNA (10 pg-5 µg) was mixed with 1 µl of a 10 mM dNTP mix and 1 µl of each primer at a concentration of 2 pmol (FlaviF1, FlaviR1, HantaF, SANGF, ASPrev, Hsrev). Nuclease-free water was added up to a volume of 13 µl and the reaction

mixture was incubated at 65 °C for 5 mins. The reaction was incubated on ice and 4 µl First-strand buffer (5X), 1 µl DTT (0.1 M), 1 µl Rnase OUT™ (40 U/µl) and 1 µl SuperScript™ III RT (200 U/µl) were added. The reaction mixture was incubated at 55 °C for 60 mins and then inactivated by incubation at 70 °C for 15 mins. The synthesized cDNA was used as template for the Flavi-Hantavirus TaqMan® multiplex PCR assay. The reliability of this method was also confirmed using known RNA controls.

#### 4.3.6.4. Cycling conditions for Flavi-Hantavirus TaqMan® multiplex PCR assay

Mono-reactions using gene-specific primers and probes as well as multiplex reactions were performed using the LightCycler® TaqMan® Master kit (Roche) according to parameters recommended by the supplier. In brief, the reaction setup consisted of 0.5 µl of each 20 pmol primer, 2 µl of each 2 pmol hydrolysis probe, 4 µl of the LightCycler® TaqMan® 5X master mix, 2 µl of DNA template and PCR-grade water to a total volume of 20 µl. A total of 1 µl of DNA template was used for controls. The PCR assay was performed in a LightCycler® 2.0 instrument (Roche) which is a carousel-based system. Due to the complex nature of the assay, owing to numerous primer sets and probes, the assay was designed as a “Touchdown PCR assay”. The PCR assay was based on three programs: pre-incubation, amplification and cooling as demonstrated in Table 17. Fluorescence as a measure of DNA amplification was detected after each extension step. After temperature gradient assays of between 55-60 °C were performed, the primary annealing temperature was finally set at 55 °C, which was decreased by 0.5 °C per cycle until an annealing temperature of 50 °C was reached after 10 cycles. The gradual decrease in annealing temperature is the basis of touchdown PCR.

**Table 17: PCR cycling conditions for the Flavi-Hantavirus TaqMan® multiplex PCR assay**

Analysis mode	Cycles	Segment	Target temperature	Hold time	Acquisition mode
<b>Pre-Incubation</b>					
None	1		95 °C	10 min	None
<b>Amplification</b>					
Quantification	45	Denaturation	95 °C	10 sec	None
		Annealing	55 °C	40 sec	None
		Secondary target (Step size =0.5 °C, Step delay =1 cycle)	50 °C	40 sec	None
		Extension	72 °C	1 sec	Single
<b>Cooling</b>					
None	1		40 °C	30 sec	None

#### **4.3.6.5. Intravariability and intervariability of the real-time assay**

Intra- and inter-assay reproducibility were evaluated for the multiplex real-time assay using a high ( $10^6$ ) and a low ( $10^2$ ) concentration standard of DNA for HTNV, PUUV, SANGV and LGTV; which are representative targets for each of the TaqMan® probes. The high and low concentration standards were evaluated in triplicate within individual PCR runs and also in triplicate between independent runs. The intravariability and intervariability were expressed as a percentage coefficient of variation (% CV) based on the equation: (standard deviation/the mean cycle threshold) x 100.

#### **4.3.6.6. Sensitivity of the real-time assay**

Initially, 10-fold serial dilutions of the DNA standards ( $10^0$ - $10^6$ ) of HTNV, PUUV, SANGV and LGTV were tested in mono-reactions using gene-specific primers and probes (Table 17) and the data obtained was used to generate standard curves. Two standards with the lowest copy number ( $10^0$ ,  $10^1$ ) were used to determine the limit of detection (LOD) for each of the assays.

#### **4.3.6.7. Sequencing of amplicons and analyses of nucleotide sequences**

Total RNA obtained from human and rodent samples was screened for viral RNA using the multiplex PCR assays and amplicons were sequenced bidirectionally using the BigDye® sequencing kit (Applied Biosystems) following manufacturer's instructions and as previously explained. The nucleotide sequence was edited using ChromasPro Version 1.34. A nucleotide analysis was performed on the edited sequence by using the BLAST software to confirm the identity of viruses.

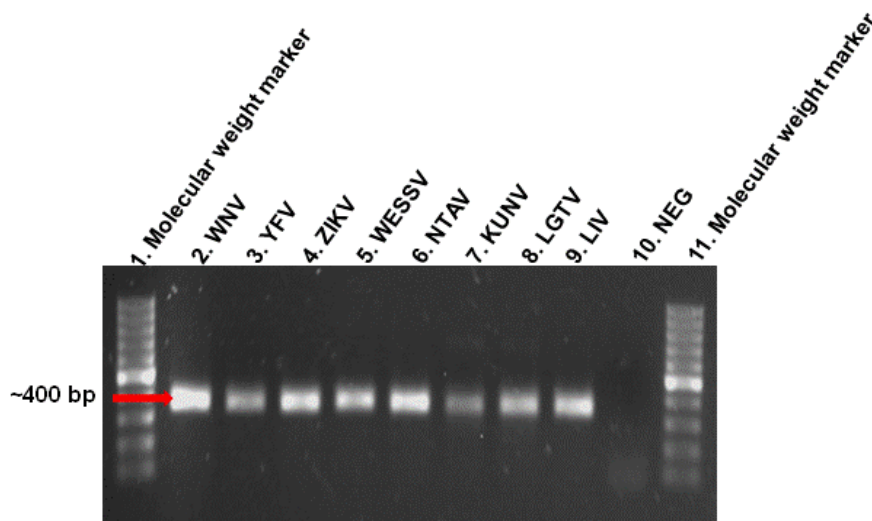
#### **4.3.6.8. Screening of samples using hantavirus commercial serological assays**

A total of 176 of the 196 humans serum samples included in this study were available for testing and were screened using the Anti-Hanta virus pool 1 "Eurasia" ELISA (IgG) and the Anti-Hantavirus pool 2 "America" ELISA (IgG) (Euroimmun, Germany). The two ELISA kits provide a quantitative analysis for IgG antibodies against HNTV, DOBV and PUUV for the Eurasia assay; as well as SNV and ANDV for the America assay. Briefly, calibrators, controls and diluted patient sera were transferred to antigen-coated wells and incubated for 1 hr at 37 °C. Subsequently, a peroxidase-labelled anti-human IgG solution was added, followed by ABTS substrate and finally the reaction was stopped and photometric measurement determined 30 mins after the reaction was stopped. Results were evaluated by calculating a ratio of the extinction value of the control or patient sample over the extinction value of the calibrator 2 (low positive), and reported as negative (ratio <0.8), borderline (ratio  $\geq$ 0.8 to <1.1) or positive (ratio  $\geq$ 1.1).

## 4.4. RESULTS

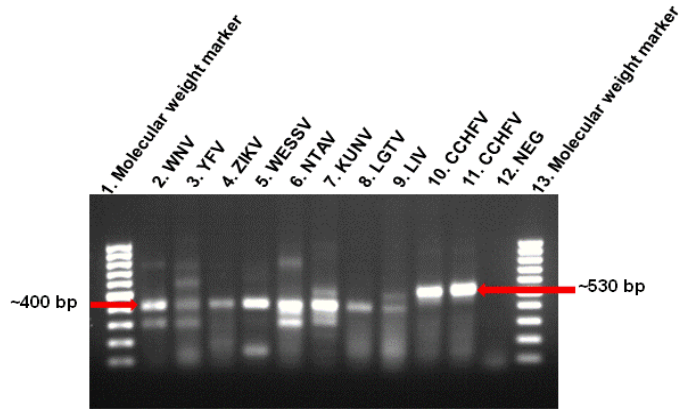
### 4.4.1. Flavi-CCHFV nested multiplex PCR

RNA controls for flaviviruses and CCHFV were amplified using Titan® One Tube RT-PCR System kit (Roche). PCR products were separated by electrophoresis using a 1% agarose gel and visualized under UV light. Initially, the reliability of in-house FlaviF1 and FlaviR1 primers was determined in individual reactions. All the eight controls available in our laboratory were detected using this assay as shown in Figure 30. Following testing of primers, the Flavi-CCHFV nested multiplex PCR assay was evaluated for its ability to detect RNA extracted from eight flaviviruses and two CCHFV isolates. The first-round assay was performed using the Titan® One Tube RT-PCR System kit (Roche) and the image of the agarose gel that was used for visualization is shown in Figure 31. It is evident from these images that there was a better optimization of the assay in mono-reactions than in the first round of the multiplex reaction. This can be deduced from the amount of non-specific bands observed in Figure 31, which represent amplicons of the multiplex PCR assay.



**Figure 30: Agarose gel electrophoresis analysis of partial NS5 amplicons obtained in mono-reactions from the one step RT-PCR using genomic viral RNA as template at an annealing temperature of 45 °C.**

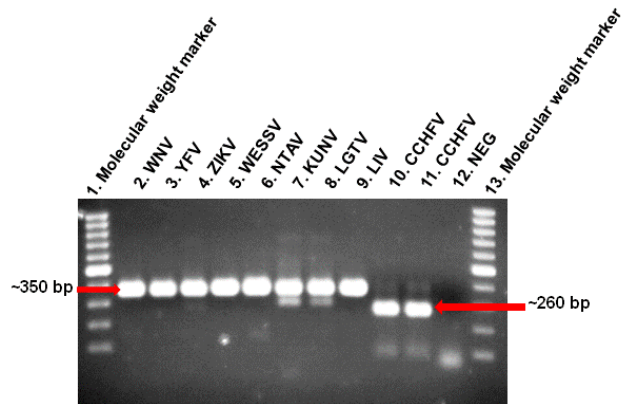
**Lanes 1 and 11: Fermentas O'GeneRuler™ 100bp DNA ladder molecular weight marker (Thermo Scientific) comprising DNA fragments from 100 to 1000 bp fragments, Lanes 2 – 9: 5µl PCR product of flavivirus controls used in the study. Lane 10: Negative control reaction without any flavivirus RNA.**



**Figure 31: Agarose gel electrophoresis analysis of partial NS5 and S segment amplicons of flaviviruses and CCHFV, respectively.**

Amplicons were obtained following a multiplex reaction from the one step RT-PCR using genomic viral RNA as template at an annealing temperature of 43 °C. Lanes 1 and 11: Fermentas O'GeneRuler™ 100bp DNA ladder molecular weight marker (Thermo Scientific) comprising DNA fragments from 100 to 1000 bp fragments, Lanes 2 – 9: 5µl PCR product of flavivirus controls used in the study, Lanes 10 and 11: CCHFV, Lane 12: Negative control reaction without any flavivirus or CCHFV RNA.

The second round of the assay was performed using GoTaq® DNA polymerase (Promega). For this assay, products of the first round multiplex assay (Figure 31) were used as templates for amplification. Results were analyzed by agarose gel electrophoresis as shown in Figure 32. The optimization of the second round PCR is visibly improved due to the reduction in non-specific bands as well as primer dimers.



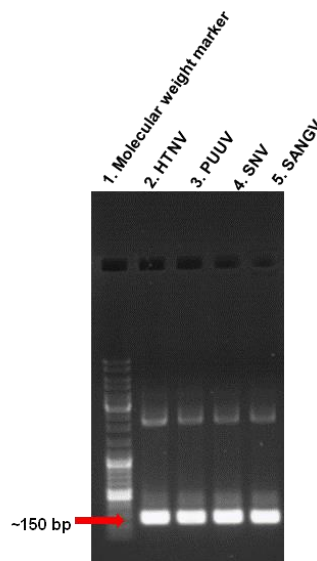
**Figure 32: Agarose gel electrophoresis analysis of partial NS5 and S segment amplicons of flaviviruses and CCHFV, respectively.**

Amplicons were obtained following a second round of a multiplex reaction using GoTaq® DNA polymerase (Promega) and products of the first-round assay as template DNA at an annealing temperature of 42 °C. Lanes 1 and 11: Fermentas O'GeneRuler™ 100bp DNA ladder molecular weight marker (Thermo Scientific) comprising DNA fragments from 100 to 1000 bp fragments, Lanes 2 – 9: 5µl PCR product of flavivirus controls used in the study, Lanes 10 and 11: CCHFV, Lane 12: Negative control reaction without any flavivirus or CCHFV RNA.



#### 4.4.2. Flavi-Hantavirus TaqMan® multiplex PCR assay

This assay was designed to detect viral nucleic acid in real-time using hydrolysis probes. Although flavivirus controls were available in the laboratory as depicted above, hantavirus controls were not available for use. To circumvent this shortfall, genes representing partial sequences of the S segment genes of HTNV, SNV, PUUV and SANGV were synthesized (GenScript). All synthetic genes were received in a pUC57 plasmid and amplified with gene-specific primers using the GoTaq® DNA polymerase to create the 'A' overhangs. The resulting amplicons were ligated into a pGEM-T-easy® vector using T/A cloning. Ligation mixtures were used to transform *E. coli* XL-10 Gold competent cells (Agilent Technologies) and positive transformants were subcultured and used for plasmid extraction and purification. Due to the presence of the T7 promoter in the pGEM-T-easy® vector, a T7 forward primer was used together with gene-specific reverse primers to confirm the orientation of the ligated primers as shown in Figure 33 below.

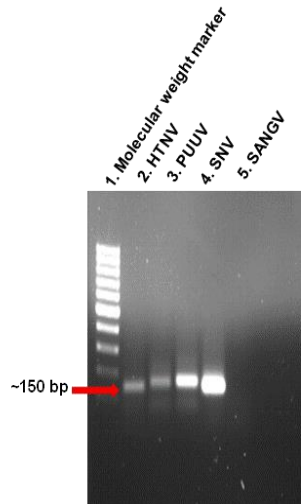


**Figure 33: Agarose gel electrophoresis analysis of amplicons derived from partial S segment genes of hantaviruses using the T7 forward primer and gene-specific reverse primers in a GoTaq® DNA polymerase (Promega) reaction.**

**Lane 1: Fermentas O'GeneRuler™ DNA ladder mix molecular weight marker (Thermo Scientific), lanes 2-5 amplicons derived from partial S segment genes of representative hantaviruses.**

Following confirmation of the orientation of the representative genes, the T7 promoter region was also utilized to facilitate RNA synthesis for these genes using the MEGAscript® kit (Life Technologies) according to manufacturer's instructions. To confirm the fidelity and correct sense of the RNA, Titan® One Tube RT-PCR System (Roche) was used for amplification of genes with gene-specific forward and reverse primers as shown in Figure 34. The HantaF primer was able to detect

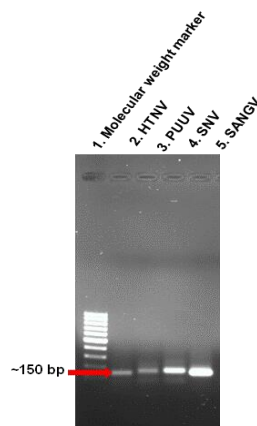
all the RNA controls except for SANGV, the African hantavirus. For this purpose, the SANGF primer was utilized in detecting the SANGV nucleic acid and similarly, this primer was also unable to detect any other hantavirus nucleic acid among the controls. For reverse primers, the ASPrev was able to detect nucleic acid representing SNV and PUUV. The primer HSrev was able to detect nucleic acid representing HTNV and SANGV.



**Figure 34: Agarose gel electrophoresis analysis of amplicons derived from the RNA of the partial S segment genes of hantaviruses using gene-specific primers in a Titan® One Tube RT-PCR System (Roche) reaction.**

**Lane 1: Fermentas O'GeneRuler™ DNA ladder mix molecular weight marker (Thermo Scientific), lanes 2-5 amplicons derived from partial S segment genes of representative hantaviruses.**

Part of the optimization of the assay included the synthesis of cDNA. Organ samples from rodents that were collected from the field were used for the isolation of total RNA using Trizol® reagent (Life Technologies). RNA was reverse-transcribed into cDNA using Super Script® first-strand synthesis system for RT-PCR (Life Technologies) with gene-specific primers and according to manufacturer's instructions. In addition to the reverse transcription of RNA derived from rodent samples, hantavirus RNA controls were reverse-transcribed. In order to confirm the dependability of the method, the presence of cDNA was confirmed by using GoTaq® DNA polymerase (Promega) to amplify the genes as shown in Figure 35.



**Figure 35: Agarose gel electrophoresis analysis of amplicons derived from amplification of cDNA of partial S segment genes of hantaviruses using gene-specific primers in a GoTaq® DNA polymerase (Promega) reaction. Lane 1: Fermentas O’GeneRuler™ DNA ladder mix molecular weight marker (Thermo Scientific), lanes 2-5 amplicons derived from partial S segment genes of representative hantaviruses.**

#### 4.4.2.1. Preparation of DNA standards

DNA concentrations of amplicons derived from flaviviruses (LGTV, WNV) and hantaviruses (PUUV, SANGV) were quantified using a NanoDrop spectrophotometer (Thermo Scientific). DNA standards were not prepared for SNV as only one standard curve was necessary for each probe thus PUUV was used for the generation of a standard curve for ASPRB probe as it could detect both these controls. DNA concentrations were further used to estimate the number of DNA copies for each control sample using the calculation provided above. Estimated DNA copy numbers are tabulated below:

**Table 18: Estimated DNA copy numbers for control DNA samples**

DNA control	Estimated copy numbers
LGTV	$1.6 \times 10^{11}$
WNV	$1.8 \times 10^{11}$
HNTV	$1.8 \times 10^{11}$
PUUV	$1.7 \times 10^{11}$
SANGV	$2.9 \times 10^{11}$

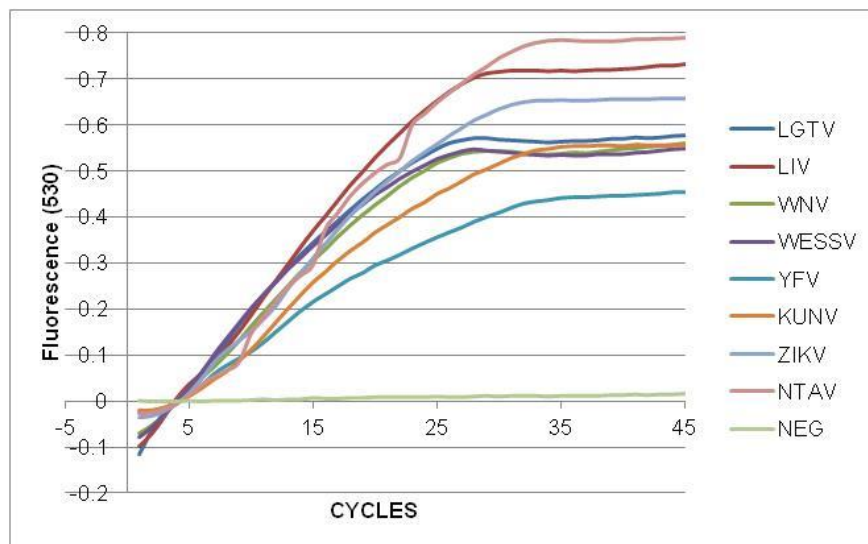
#### 4.4.2.2. Detection of DNA controls and generation of standard curves

Prior to setting up the touchdown multiplex PCR assay, probes were tested in singleplex reactions to confirm their ability to detect the RNA controls available in the laboratory. The probes included the FLAVI probe which was designed to detect flaviviruses available in our laboratory as well as

three probes that were designed to detect representatives of the three hantavirus lineages. All probes were labelled with a 6FAM dye and coupled to a BBQ.

### FLAVI probe

The FLAVI probe is a 14-mer oligo was modified to include LNAs. This probe was initially evaluated for its ability to detect nucleic acid extracted from the eight representative flaviviruses available in our laboratory. The cohort of flavivirus species included WESSV and WNV, which are known to be endemic in southern Africa; other mosquito-borne flaviviruses such as YFV, ZIKV, KUNV, NTAV and tick-borne flaviviruses LGTV and LIV. As demonstrated in Figure 36, the probe successfully detected all the controls tested as authenticated by the increase in fluorescence for all controls. There was no significant increase in fluorescence for the negative control and therefore no cycle threshold value determined. The highest level of fluorescence was obtained when NTA and LIV were tested, followed by ZIKV. The least level of fluorescence detected was with YFV nucleic acid. The level of fluorescence was similar for the remaining five controls.



**Figure 36: The detection of flavivirus controls using the FLAVI probe in a real-time touchdown PCR assay.**

**The probe was used to detect LGTV, LIV, WNV, WESSV, YFV, KUNV, ZIKV and NTAV. Neg denotes the negative control used.**

Following the detection of flavivirus controls using the FLAVI primer, LGTV and WNV were selected for generating representative standard curves for the tick-borne and mosquito-borne flaviviruses, respectively, to demonstrate the application of the probe. DNA standards of concentration  $10^0$  to  $10^6$  were tested using the probe as shown in Figures 37 and 38. Cycle threshold values were in

agreement with DNA standard concentrations, thus samples with higher concentrations of DNA had lower cycle threshold values.

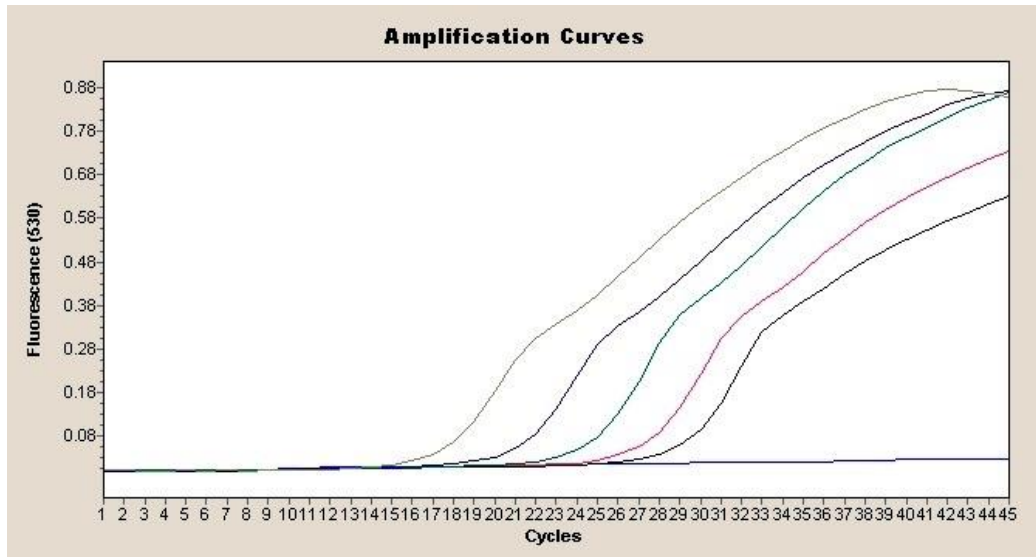


Figure 37: Amplification curves for LGTV DNA concentration standards of  $10^0$ - $10^6$  using the FLAVI probe.

The amplification curves were utilized to generate a standard curve for the assay which was also used to indicate the efficiency of the PCR assay, where the number 2 indicates an optimal reaction. The efficiency of the assay was determined to be 1.997 which indicates a highly optimal assay, with minimal error.

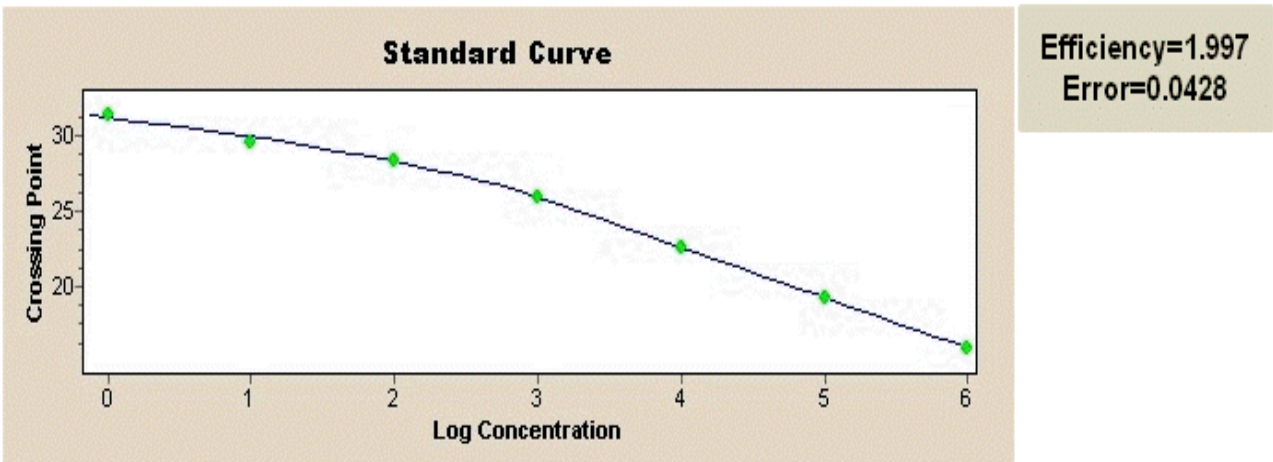


Figure 38: Standard curve generated using the log concentrations of LGTV DNA standards tested using FLAVI probe plotted against corresponding cycle threshold values.

The efficiency of the assay must be as close to 2 as possible and the error approximately 0.

Similarly, nucleic acid representing WNV was also tested to demonstrate the versatility of the FLAVI probe (Figure 39).

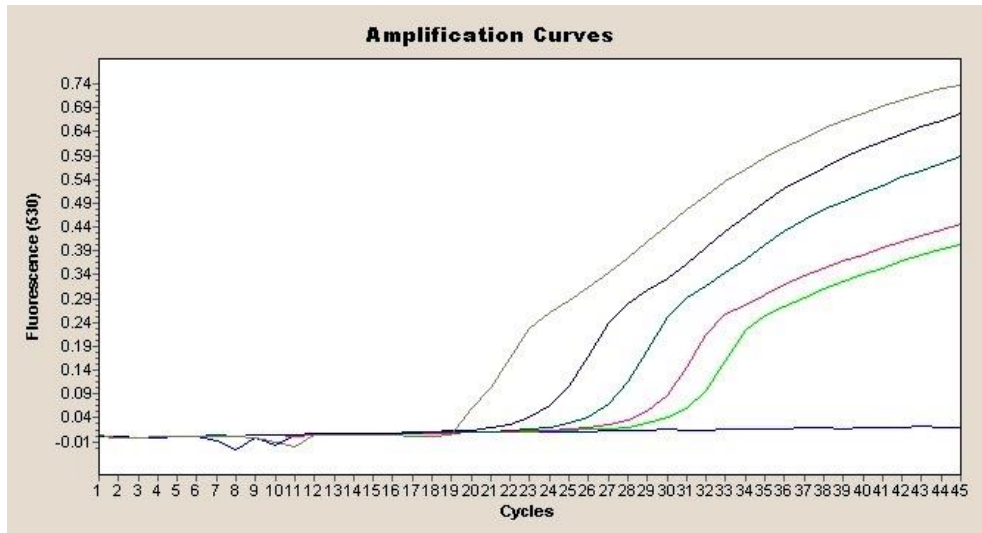


Figure 39: Amplification curves for WNV DNA concentration standards using the FLAVI probe.

The corresponding WNV standard curve resulting from the FLAVI probe indicated that the assay was reliable. The efficiency of the assay was determined to be 1.886 with an error of approximately 0 (Figure 40).

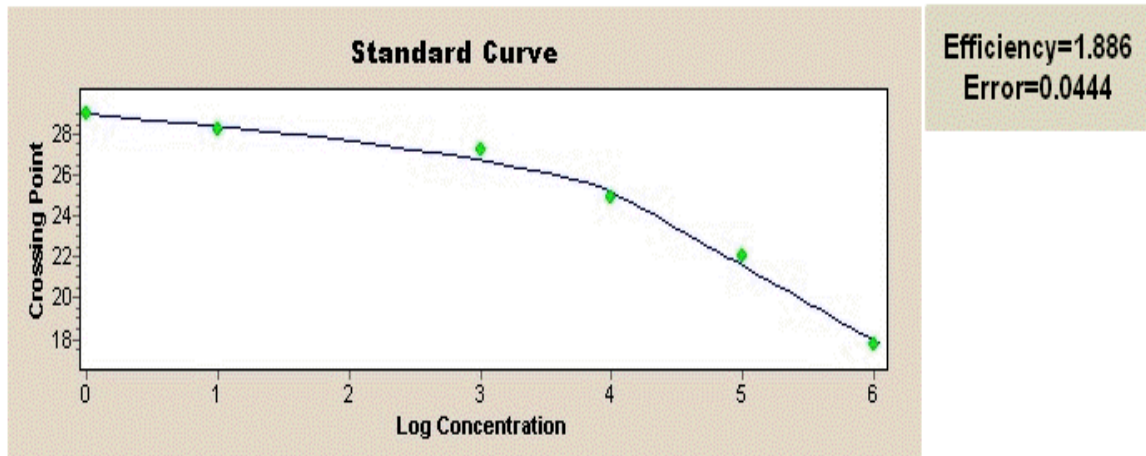


Figure 40: Standard curve generated using the log concentrations of WNV DNA standards tested using FLAVI probe plotted against corresponding cycle threshold values.

The efficiency of the assay must be as close to 2 as possible and the error approximately 0.



## HNSL probe

The HNSL probe is a 24-mer oligo that was designed to detect *Murinae*-associated hantaviruses such as HNTV and SEOV. The probe was used to detect serially diluted standards derived from the synthetic HNTV nucleic acid.

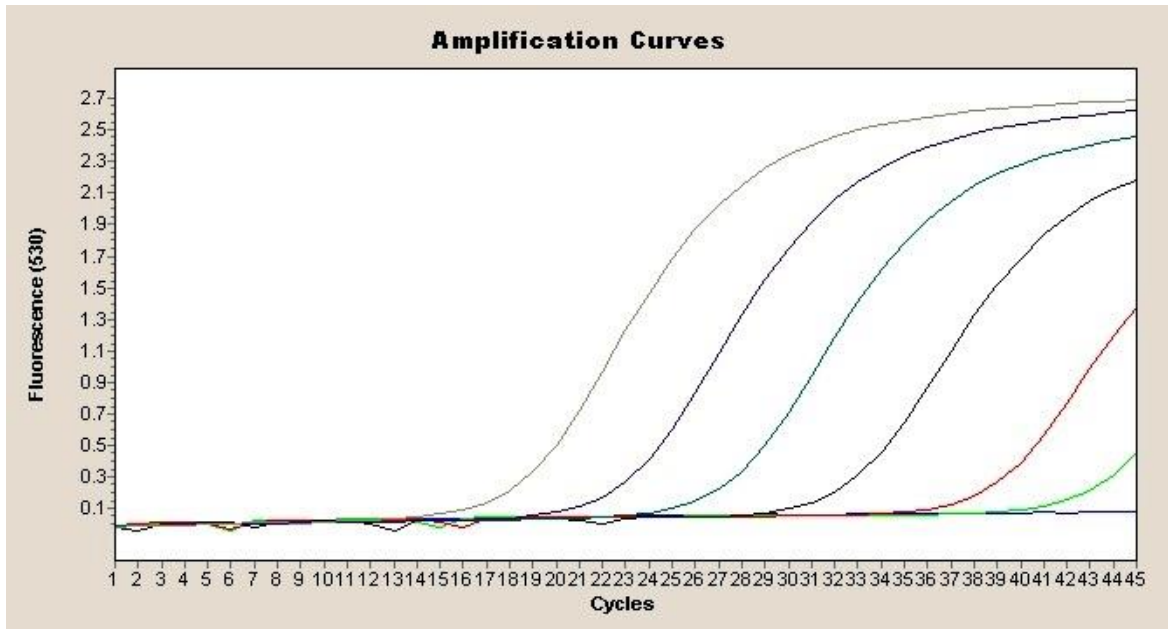


Figure 41: Amplification curves for HNSL DNA concentration standards of  $10^0$ - $10^6$  using the HNSL probe.

Figure 41 demonstrates the ability for HNSL probe to detected serially diluted DNA standards resulting in a two-fold increase in fluorescence for most of the samples. This data was used to generate a standard curve and also calculate the efficiency of the assay as illustrated in Figure 42.

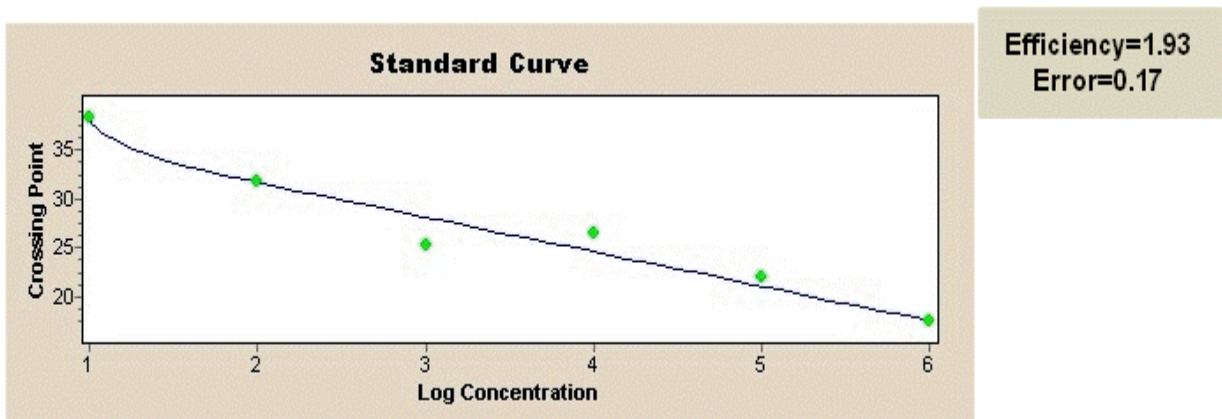


Figure 42: Standard curve generated using the log concentrations of HNTV DNA standards tested using HNSL probe plotted against corresponding cycle threshold values.

The efficiency of the assay must be as close to 2 as possible and the error approximately 0.

## ASPRB probe

The ASPBR probe is the shortest of all probes designed for this assay being only 10 nucleotides long. Due to its short length, the probe was modified to include LNAs for an increased melting temperature and stability. Based on nucleic acid alignments, this probe was designed to detect *Sigmodontinae* and *Arvicolinae*-associated hantaviruses. The probe was used to detect serially diluted standards derived from the synthetic PUUV nucleic acid as shown in Figure 43.

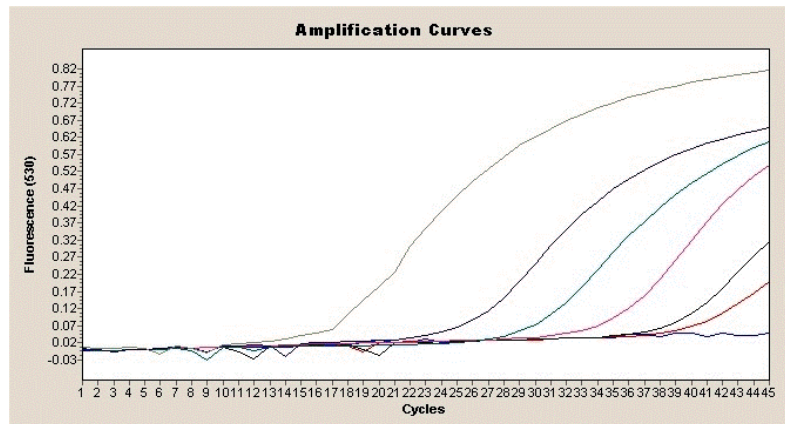


Figure 43: Amplification curves for PUUV DNA concentration standards of  $10^0$ - $10^6$  using the ASPBR probe.

Following the detection of serially diluted standards by the ASPBR probe, the data obtained was used for the subsequent preparation of a standard curve as shown in Figure 44. The standard curve demonstrated a lower efficiency at 1.4 however, the calculated error was much lower than 0. All the points were also accurately plotted on the linear curve.

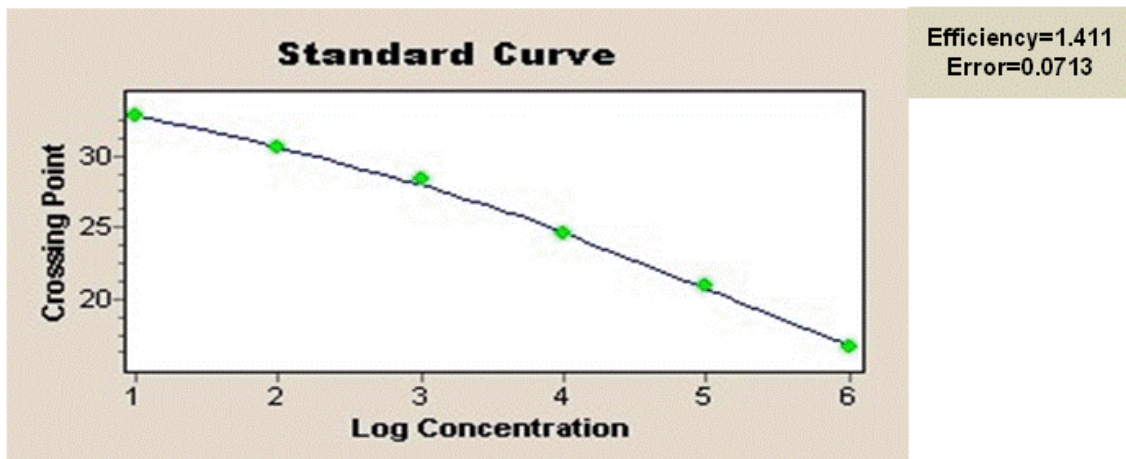


Figure 44: Standard curve generated using the log concentrations of PUUV DNA standards tested using ASPBR probe plotted against corresponding cycle threshold values.

The efficiency of the assay must be as close to 2 as possible and the error approximately 0.



## SANGV probe

The SANGV probe was based on the sequence derived from SANGV S segment. Due to differences exhibited by the sequence of the S segment of SANGV to other hantaviruses, the SANGV probe was exclusively based on the SANGV nucleotide sequence. SANGV was the first hantavirus to be detected in Africa and was found to be divergent from the classical hantavirus lineages. The probe was used to detect serially diluted standards derived from the synthetic SANGV nucleic acid as shown in Figure 45.

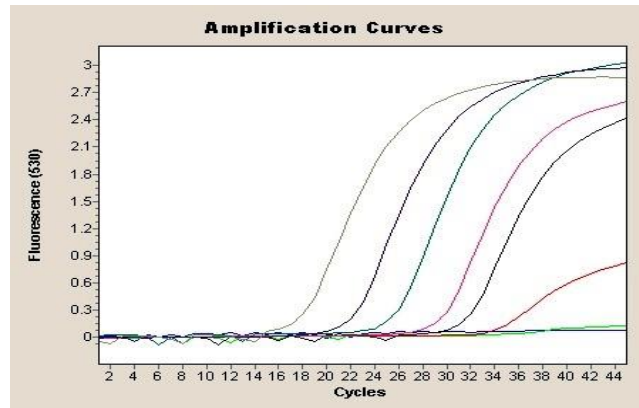


Figure 45: Amplification curves for SANGV DNA concentration standards of  $10^0$ - $10^6$  using the SANGV probe.

It is evident from Figure 46 that detected samples had up to three-fold increase in fluorescence when tested with the SANGV probe. The standard curve that was generated (Figure 46) was used to determine the efficiency of the PCR assay for this probe and this was 1.801 with an error rate less than 0.

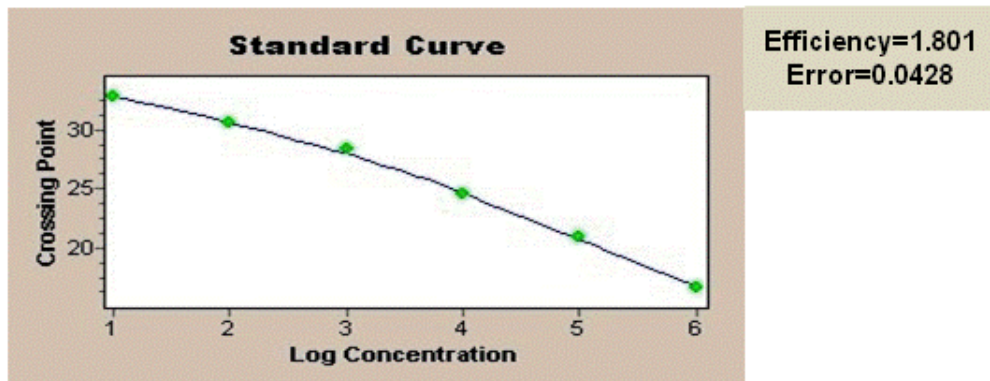


Figure 46: Standard curve generated using the log concentrations of SANGV DNA standards tested using SANGV probe plotted against corresponding cycle threshold values. The efficiency of the assay must be as close to 2 as possible and the error approximately 0.

### 4.4.2.3. Intravariability and intervariability of the real-time assay

Using the high ( $10^6$ ) and the low ( $10^2$ ) positive controls, the assay was evaluated for its repeatability in triplicate both within and between runs. Specifically, the high and the low controls were tested in triplicate within an assay, and the assay was repeated over three intervals thus each samples was tested a total of 9 times. This was performed using the low and the high positive controls for each of the four probes. The Lightcycler® software was used to extrapolate the DNA concentration of each sample within a run and an average was calculated for the three controls as shown in Table 19. The average DNA concentration obtained from each of the three PCR runs have been outlined and a mean calculated. As shown in the table, the overall mean for each control sample is in agreement with the standard concentrations of  $10^2$  and  $10^6$  in the low and high controls, respectively. Although there are variations between runs, they were not large enough to alter the calculated mean value from the theoretical dilution values of  $10^2$  and  $10^6$ .

**Table 19: Average DNA concentrations for high and low controls extrapolated within a multiplex assay using the Lightcycler® software.**

DNA control	LGTV	HNTV	PUUV	SANGV
<b>Low control (<math>10^2</math>)</b>		<b>Calculated concentrations</b>		
RUN1	9.49E+01	1.12E+02	4.23E+02	1.29E+02
RUN2	1.05E+02	1.61E+02	8.42E+01	9.56E+01
RUN3	1.01E+02	1.13E+02	1.06E+02	1.03E+02
<b>MEAN</b>	<b>1.00E+02</b>	<b>1.29E+02</b>	<b>2.04E+02</b>	<b>1.09E+02</b>
<b>High control (<math>10^6</math>)</b>				
RUN1	1.21E+06	1.24E+06	1.04E+06	1.06E+06
RUN2	1.01E+06	1.05E+06	1.03E+06	9.97E+05
RUN3	1.05E+06	9.63E+05	1.13E+06	9.45E+05
<b>MEAN</b>	<b>1.09E+06</b>	<b>1.08E+06</b>	<b>1.07E+06</b>	<b>1.00E+06</b>

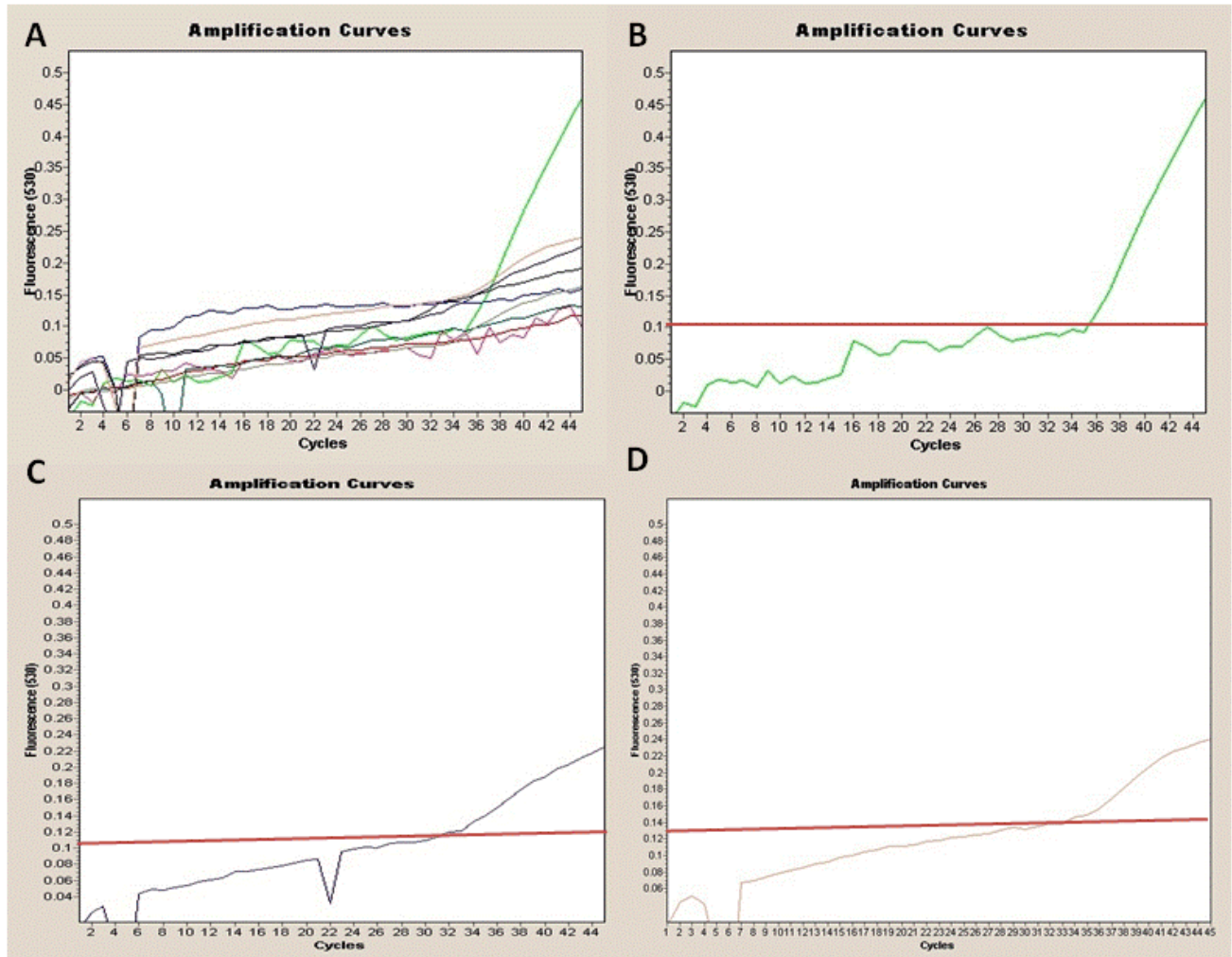
Similarly, cycle threshold values were determined using the Lightcycler® software for each of the samples within a run. Mean cycle threshold values were subsequently calculated and recorded for each of the three runs and the standard deviation was determined as shown in Table 20. The overall differences in the individual runs were expressed as %CV. Most samples were detected at similar cycle threshold values differing only by one cycle or less. The most difference was observed among low and high controls of PUUV, with a two-cycle difference and a CV of 3% for the high control. The overall CV for each probe were generally less than 3%

**Table 20: Average cycle threshold values for high and low controls obtained in a multiplex PCR assay using the Lightcyler® software.**

DNA control	LGTV	HNTV	PUUV	SANGV
<b>Low control (10<sup>2</sup>)</b>				
	<b>Cycle threshold values</b>			
RUN1	27.84	31.99	32.92	35.26
RUN2	27.29	31.40	34.21	36.15
RUN3	27.61	31.29	33.44	36.99
<b>MEAN</b>	<b>27.58</b>	<b>31.56</b>	<b>33.52</b>	<b>36.13</b>
<b>St deviation</b>	<b>0.28</b>	<b>0.38</b>	<b>0.65</b>	<b>0.87</b>
<b>%CV</b>	<b>1.0</b>	<b>1.2</b>	<b>1.9</b>	<b>2.4</b>
<b>High control (10<sup>6</sup>)</b>				
RUN1	17.49	18.52	23.1	17.22
RUN2	16.97	18.87	22.95	17.69
RUN3	17.41	19.60	24.24	18.16
<b>MEAN</b>	<b>17.29</b>	<b>19.00</b>	<b>23.43</b>	<b>17.69</b>
<b>St deviation</b>	<b>0.28</b>	<b>0.55</b>	<b>0.71</b>	<b>0.47</b>
<b>%CV</b>	<b>1.6</b>	<b>2.9</b>	<b>3.0</b>	<b>2.7</b>

#### 4.4.2.3. Sensitivity of the real-time assay

Table 20 demonstrates the ability of each probe to detect the low DNA control at a concentration of 10<sup>2</sup> DNA copies. To determine the limit of detection for each probe within the multiplex assay, controls ranging from 10<sup>0</sup> to 10<sup>1</sup> estimated DNA copies were tested. Figure 47A demonstrates all samples tested and the results obtained. Of all the samples tested, one sample corresponding to the 10<sup>1</sup> dilution of HNTV was detectable (Figure 47B) with a sharp increase in fluorescence at cycle 35. This sample was detectable using the HNSL probe and had a theoretical concentration of 18 copies of DNA which indicates the LOD for this probe. Some samples were ruled to be 'equivocal' as there was a slight increase in fluorescence with a cycle threshold value of less than 40. Cycle threshold values of more than 40 were not determined by the LightCycler®, but rather reported as (>40) and as such, those samples were ruled out as negative. Figure 48C demonstrates the slight increase in fluorescence at cycle 35 when a PUUV control with an estimated 17 copies of DNA was tested. In addition, a slight increase in fluorescence was also observed at cycle 38 when a SANGV control with an estimated 29 copies of DNA was tested. The lowest detectable control using the FLAVI probe was estimated at 160 copies of DNA. There was neither an increase in fluorescence observed nor a cycle threshold obtained for any of the negative controls



**Figure 47: Results of the real-time multiplex assay for determination of the limit of detection of DNA controls by probes.**

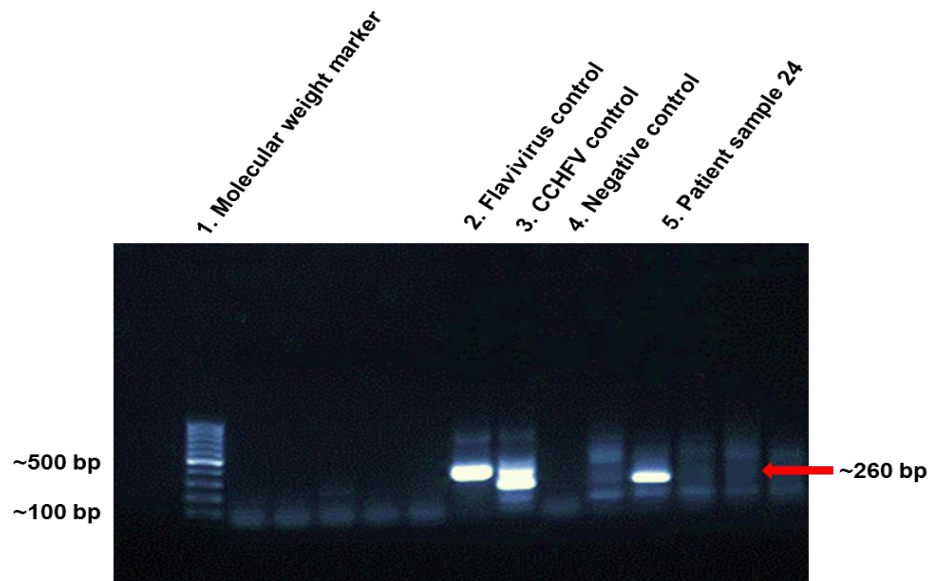
**A: Positive controls at  $10^1$  and  $10^0$  DNA copy numbers, B: sharp increase in fluorescence for HNTV control at 18 copies of DNA, C-D: results indicating a slight increase in fluorescence and cycle threshold values less than 40. C: ~17 copies of PUUV DNA, D: ~29 copies of SANGV DNA.**

#### **4.4.2.4. PCR results for human and rodent samples.**

RNA was extracted from a cohort of human and rodent samples for the purpose of preliminary screening using the in-house PCR assays designed in this study. This was performed to demonstrate the potential for application of these methods as significant tools for diagnosis and surveillance. When RNA obtained from organs harvested from mice was screened using the real-time PCR assay, no significant emission of fluorescence was detected indicating that samples were negative. These results were further confirmed by separation and subsequent visualization of

samples on agarose gels which were also in agreement with the results obtained using the LightCycler® instrument.

RNA obtained from human serum samples was also screened and one sample, designated Sample 24, had a band of the predicted size for CCHFV in the second round of the conventional multiplex assay as shown in Figure 48.



**Figure 48: Agarose gel electrophoresis analysis of partial NS5 and S segment amplicons of flaviviruses and CCHFV.**

Amplicons were obtained following a second round of a multiplex reaction using GoTaq® DNA polymerase (Promega) and products of the first-round assay as template DNA at an annealing temperature of 42 °C. Lane 1: Fermentas O'GeneRuler™ 100bp DNA ladder molecular weight marker (Thermo Scientific) comprising DNA fragments from 100 to 1000 bp fragments, Lane 2: 5µl PCR product of flavivirus control used, Lanes 3: CCHFV, Lane 4: Negative control reaction without any flavivirus or CCHFV RNA, Lane 5: Reaction with RNA obtained from the serum of a patient sample designated Sample 24, with a band indicating a positive reaction. The rest of the unlabeled lanes represent additional patient samples with no band of a predicted size.

The DNA amplicon obtained from amplification of the RNA representing sample 24 was excised from the agarose gel, purified using the Wizard® SV gel and PCR clean-up system (Promega), and subsequently sequenced bidirectionally with a pair of primers using the BigDye® sequencing kit according to manufacturer's instructions. The resultant sequences were edited using ChromasPro Version 1.34 software and analysed using the BLAST software for identification. Following the BLAST analysis, the organism was determined to be a CCHFV isolate that has a 91% homology to a previously identified South African strain, SPU 497/88, with a GenBank accession number of KJ682820.1. (Appendix D). Following the detection of CCHFV RNA in this patient sample, clinical

history of the patient submitted to the laboratory was perused. The treating physician submitted the patient blood sample with “tendonitis” as the only indicated symptom followed by a question mark. The clinician had requested that the sample be tested for evidence of tick-bite fever, rubella, yersinia and chlamydia infection.

#### 4.4.2.5. Results for the commercial hantavirus IgG assay.

A total of 176 serum samples collected from humans were tested for hantavirus antibodies using the Euroimmun assay. Raw data based on the ELISA is shown in Appendix E and results are summarized in Table 21. Using the Eurasia assay 17/176 (10%) of samples were positive, whereas 11/176 (6%) reacted with the America-based antigens. An additional 11 samples were regarded as borderline for the Eurasia assay, suggesting that there could be more positive samples within this cohort. A total of eight samples were detectable on both assays. Eight samples had a ratio greater than 2 suggesting a high antibody titer to the hantavirus antigens. One of these samples had an OD reading greater than that of both the positive control and the high calibrator.

**Table 21: Results for patient sera screened using the Euroimmun Eurasia and America hantavirus IgG ELISA kits.**

<b>Assay result</b>	<b>AMERICA-positive</b>	<b>AMERICA-negative</b>	<b>AMERICA-borderline</b>
<b>EURASIA-positive</b>	8	9	0
<b>EURASIA-negative</b>	3	144	0
<b>EURASIA-borderline</b>	0	11	1

#### 4.5. Summary

Factors such as urbanization, globalization, climate change, agricultural intensification and encroachment on wildlife habitat contribute towards the emergence and re-emergence of pathogens threatening biodiversity, water and food, as well as human and animal health. Pathogens may be introduced into the environment when suitable hosts and vectors transmit them to susceptible organisms. Arboviruses and viral hemorrhagic fevers are among the most pathogenic and devastating disease agents in many parts of the world. It is therefore important for surveillance of such pathogens to be conducted as they may result in considerable public health implications. Our study was aimed at developing molecular assays for the detection of a selected number of arboviruses and viral hemorrhagic fevers, specifically CCHFV, mosquito-borne and tick-borne

flaviviruses, as well as hantaviruses. To date, the presence of hantaviruses have not been confirmed in southern Africa despite their emergence in the western and eastern parts of Africa in the past eight years. SANGV, the first hantavirus to be reported, was tentatively associated with illness in an eight-year old boy within the Sangassou village in Guinea (Klempa *et al.*, 2010). Additionally, a serological survey using commercial assays in the Cape region of South Africa revealed a 1% seropositivity rate in human patients presenting with hantavirus-like disease (Witkowski *et al.*, 2014). In our study, serum samples of patients presenting with a tick-bite and febrile illness without diagnosis were screened for hantavirus IgG antibodies using commercial assays that represent the American and Eurasian hantavirus species. The overall seropositivity rate obtained was 10% and 6% for assays representing the Eurasia and America hantavirus species, respectively. The emergence of hantaviruses in Africa and their seroprevalence in the Cape region of South Africa as well as in our study warranted the development of a molecular assay to further investigate the presence of these viruses in southern Africa. In order to achieve this, a real-time RT-PCR was designed and optimized.

The assay was designed by identifying in-house primers targeting the partial region of the S-segment of hantaviruses and hydrolysis probes targeting the inner region of the amplicon were also identified. Identification of probes was based on the multiple sequence alignments of the partial S-segment genomes of various hantaviruses. A total of three probes were identified and designated HNLS, ASPRB and SANGV. The probes were based on nucleotide sequences targeting the *Murinae*-associated hantaviruses for the HNLS probe, *Sigmodontinae*- and *Arvicolinae*-associated hantaviruses for the ASPRB probe, as well as the SANGV probe for the African hantavirus Sangassou virus. In addition to the hantavirus-specific probes, an additional probe was designed for the detection of flaviviruses. The probe was identified from multiple nucleotide sequence alignments representing the partial region of the NS5 gene of mosquito-borne and tick-borne flaviviruses and designated FLAVI probe. This probe was shown to successfully detect RNA samples that represent eight different flavivirus species that were available for testing at our facility. The two pairs of primers and three probes targeting hantavirus nucleic acid, together with a pair of primers and a probe targeting flavivirus nucleic acid, were collectively used to develop and optimize a real-time multiplex PCR assay. In order to develop the assay, synthetic genes representing HNTV, SNV, PUUV and SANGV were obtained for the hantavirus controls as viral RNA was not available for this purpose and a BSL 3 facility is required for culturing these viruses. Synthetic genes were obtained in pUC57 vectors from the supplier and PCR was performed to generate 'A-overhangs' to flank amplicons. T/A cloning was subsequently used to ligate amplicons into pGEM-T easy vector and the T7 promoter site was used to transcribe RNA. The resultant RNA was confirmed by RT-PCR and the amplicons were sequenced. Genomic RNA was available for flaviviruses and served as controls



for the FLAVI probe. The assay was optimized as a two-step PCR assay and cDNA was synthesized using gene-specific primers. In order to determine the LOD for each probe, DNA standards ranging from  $\sim 10^6$  to  $\sim 10^0$  copies of DNA were prepared from amplicons obtained after performing RT-PCR. The LOD was determined to be  $\sim 160$ ,  $\sim 18$ ,  $\sim 17$ ,  $\sim 29$  copies of DNA for the probes designated FLAVI, HNSL, ASPBR and SANGV, respectively. The inter- and intra-variability of the assay for each of the probes had a coefficient of variation value less than or equal to three percent between all the runs. This assay is currently applied in screening rodent samples for hantavirus RNA and will be used to screen samples from patients presenting with a hantavirus-like illness resulting from an unknown cause.

A conventional multiplex PCR assay aimed at detecting CCHFV and flavivirus RNA in samples collected from undiagnosed patients presenting with a tick-bite and febrile illness was developed by using nested primers targeting the partial region of the genome of the S-segment of CCHFV and hemi-nested primers targeting the partial region of the NS5 gene of flaviviruses. The assay was nested to increase sensitivity and this also showed a significant decrease in non-specific bands during the optimization process. When clinical samples were screened, a serum sample representing patient 24 was observed to have a band of the predicted size when amplicons from the second round of the assay were visualized on an agarose gel. The band was excised and sequenced and BLAST analysis revealed that the isolate was a CCHFV strain with 91% nucleotide homology to a South African strain, designated SPU 497/88, which had been used as a positive control. These results highlight the need for awareness to arboviruses and viral hemorrhagic fevers in mild cases that may easily be overlooked. A more comprehensive reporting of patient history and description of acute illness will assist medical and laboratory personnel in accomplishing accurate differential diagnosis.

---



## CHAPTER 5

### IMMUNOGENICITY OF WEST NILE AND LANGAT EDIII PROTEINS.

#### 5.1 Introduction

The E protein is the major surface protein of flaviviruses and it is found on the viral surface in a heterodimer with the viral membrane (M) protein (Rey *et al.*, 1995). A number of studies have reported that the majority of antibodies elicited by infection with a flavivirus are directed to epitopes within the E and NS1 proteins of the virus (Schepp-Berglind *et al.*, 2007). Focus has been directed towards the E-protein due to its ability to induce neutralizing antibody responses (Mandl *et al.*, 2001). As described previously, the E protein of flaviviruses comprises of three domains designated domain I to III. Of the three domains, the EDIII domain is highly antigenic with primary linear epitopes (Beasley and Barrett, 2002) and is probably involved in viral receptor binding (Crill and Roehrig, 2001) and contains predominantly subcomplex and type-specific epitopes which mainly induce neutralizing antibodies. In addition, the EDIII can be expressed as a recombinant protein that has the ability to fold as a functional fragment capable of inducing neutralizing antibodies and may therefore have the potential for use as a subunit vaccine.

Various investigations of vaccine candidates have been reported. In some instances, a truncated E protein was administered with adjuvant to induce stronger immune responses. Martina *et al.* (2008) and Chu *et al.*, (2007) focused on the EDIII expressed protein and coupled the protein with oligodeoxynucleotides (CpG-DNA) as an adjuvant to generate a high titer of WNV-neutralizing Abs, which was also shown to neutralize a related *flavivirus*, Japanese encephalitis virus. The authors also demonstrated that these vaccines could invoke a cellular immune response in the animal model. Adjuvants can modulate the characteristics of the immune response with regard to various factors including the antibody titer, duration of response, isotype and subclass of antibody as well as properties of the cell mediated response. Adjuvants have the ability to elicit stronger immune responses and to shift the immune response towards a Th1 or Th2 response. Currently the only adjuvant that is approved for human use is alum. Aluminium compounds release antigen slowly increasing duration of antibody response however they have been shown to be weak adjuvants for eliciting strong humoral responses. Aluminium compounds are usually associated with aTh2 response. In contrast emulsion based adjuvants including Titermax® gold and saponin, are more frequently associated with mixed Th1 and Th2 responses. Titermax® gold was developed as an alternative to complete Freund's adjuvant but is safer to use. It is comprised of copolymers, CRL-8941, that bind the antigen and activate macrophages, a metabolizable oil and a stabilizer. Saponins are natural glycosides of steroid or triterpene that stimulate both humoral and cellular

immune responses but can have some toxic side effects in high concentrations. The immunogenicity of recombinant EDIII proteins was determined in combination with various adjuvants and alone.

## **5.2. Aim**

The aim of this chapter was to characterize the immune response elicited by the WNV and LGTV recombinant EDIII proteins when administered in combination with saponin, Alhydrogel® or Titermax® gold as adjuvant. (Note: The laboratory work was incomplete at the time of Mr Mathengtheng's death. The study was initially designed to also include investigation of cellular responses by cytokine profiling after stimulation of splenocytes harvested from immunized mice.)

## **5.3. Materials and methods**

### **5.3.1. Animal inoculations**

Animal experiments were approved by the University of the Free State Animal Ethics Committee (see Appendix F). For each protein, groups of mice were immunized according to the following protocol. Four groups of 6 week old female NIH mice, Groups A-D, with 6 mice per group were immunized as described in Table 22. The experiment described in Table 22 was repeated for r-WNV EDIII and r-LGTV EDIII proteins. Control mice were immunized as described in Table 23 with four groups of mice, Groups E-H, and 6 mice per group.

Animals were anaesthetized using inhalant halothane and exsanguinated at the end of the experiment. Anaesthetizing and bleeding animals was performed by the staff at the UFS Animal Unit. Serum obtained from the blood samples was tested for reactivity against homologous and heterologous flaviruses and for IgG isotype. Splenocytes were aseptically removed and cells harvested and stored for analysis of T cell proliferation and cytokine profiling\* in response to stimulations with homologous antigens.

(\*The cytokine profiling and T cell proliferation was incomplete at the time of Mr Mathengtheng's death and therefore has been omitted).

**Table 22: Immunization schedule for immunogenicity study.**

**Experimental groups**

Group	Number of mice	Inocula	Dose	Route	Schedule	End of experiment
A	6	Recombinant envelope protein plus adjuvant 1*	70µg protein plus 40µl of adjuvant in total volume of 100 µl	SC	Prime day 0 Boost day 14	Week 6
B	6	Recombinant envelope protein plus adjuvant 2*	70µg protein plus 1 mg of adjuvant in total volume of 100 µl	SC	Prime day 0 Boost day 14	Week 6
C	6	Recombinant envelope protein plus adjuvant 3*	70µg protein plus 60 µg of adjuvant in total volume of 100 µl	SC	Prime day 0 Boost day 14	Week 6
D	6	Recombinant envelope protein no adjuvant	70µg protein in total volume of 100 µl	SC	Prime day 0 Boost day 14	Week 6

\*Adjuvant 1: Titermax® gold, 2: Alhydrogel®, 3: saponin. Adjuvant preparation as per manufacturers' instructions, described in 5.3.1.

**Table 23: Immunization schedule for control mice**

**Control Group**

Group	Number of mice	Inocula	Dose	Route	Schedule	End of experiment
E	6	adjuvant 1*	40µl of adjuvant in total volume of 100 µl	SC	Prime day 0 Boost day 14	Week 6
F	6	adjuvant 2*	1 mg of adjuvant in total volume of 100 µl	SC	Prime day 0 Boost day 14	Week 6
G	6	adjuvant 3*	60 µg of adjuvant in total volume of 100 µl	SC	Prime day 0 Boost day 14	Week 6
H	6	Phosphate buffered saline	100 µl	SC	Prime day 0 Boost day 14	Week 6

\*Adjuvant 1: Titermax® gold, 2: Alhydrogel® 3: saponin . Adjuvant preparation as recommended by manufacturer.

Titermax® gold (Sigma-Aldrich, St Louis, USA) was prepared according to manufacturer's instructions. Briefly, a 50:50 water in oil emulsion was prepared using 100µl protein containing approximately 70-80µg protein/ml. Similarly an emulsion of protein in alhydrogel® (Sigma-Aldrich, St Louis, USA) was prepared using 50:50 mix of protein and emulsion. Saponin from Quillaja Bark (Sigma-Aldrich, St Louis, USA) was mixed with protein to give a final concentration of 70-80 µg protein in 0.05% saponin solution.

### **5.3.2. Antibody responses**

Antibody responses were determined using ELISA as described previously in Section 3.3.3. Serum samples from animal immunized using r-WNVEDIII were tested for antibody using Kunjin cell lysate antigen. Serum samples from mice immunized with r-LGTVEDIII were tested for antibody using recombinant LGTV antigen. In addition to detection of total antibody responses using anti mouse IgG HRPO, the ELISA was modified to identify IgG isotypes. Isotyping was performed using goat anti mouse IgG1 HRPO (Life Technology, Thermo Fisher Scientific, Wilmington, USA) and goat anti-mouse IgG2a HRPO (Life Technology, Thermo Fisher Scientific, Wilmington, USA) as detection antibodies as per manufacturer's instructions.

## **5.4. Results**

### **5.4.1. Immune responses elicited by recombinant EDIII proteins**

Groups of mice were immunized with purified r-WNVEDIII or r-LGTVEDIII protein alone, r-WNVEDIII or r-LGTVEDIII protein in combination with one of three adjuvants, including saponin, Titermax® gold and Alhydrogel® or one of the three adjuvants without a flavivirus protein. The mice received an initial prime followed by a boost two weeks later. At six weeks after onset of the experiment the mice were euthanased. Serum samples were tested for antibody using ELISA with KUNV cell lysate antigen or r-LGTVEDIII antigen. Cut off values determined in Section 3.4.1. to distinguish positive from negative samples were 0.23 and 0.28 for the KUNV ELISA and the LGTV ELISA, respectively. None of the control mice, that were immunized with an adjuvant alone and no protein, had detectable antibody responses against. Only 1/6 mice immunized with r-WNVEDIII alone had detectable antibody. In contrast all mice immunized with r-WNVEDIII plus an adjuvant had a detectable antibody responses as shown in Table 24. All mice immunized with r-WNVEDIII in combination with alum had detectable IgG1 antibody responses whereas no mice had a detectable IgG2a antibody response. Mice receiving r-WNV antigen in combination with saponin and Titermax® gold had both IgG1 and IgG2a antibody responses. Mice immunized with r-LGTVEDIII

alone had antibody against LGTV antigen using ELISA with higher IgG1 antibody responses compared with IgG2a (Table 24.)

**Table 24: Total IgG antibody responses in mice against KUNV or LGTV antigen and ratio of IgG1:IgG2a**

Immunogen	No of mice positive total IgG/total immunized	Mean net OD <sub>405</sub> ratios <sup>1</sup> IgG1:IgG2a	Mean net OD <sub>405</sub> IgG1 (Range)	No of positive mice IgG1	Mean net OD <sub>405</sub> IgG2a (Range)	No of positive mice IgG2a
r-WNVEDIII alone	1/6	1.68 <sup>1</sup>	0.13 (0.045-0.42)	1/6	0.083 (0.03-0.29)	1/6
r-WNVEDIII with Alhydrogel®	6/6	ND <sup>2</sup>	1.08 (0.66-1.80)	6/6	0.10 (0.05-0.18)	0/6
r-WNVEDIII with saponin	6/6	1.64	1.69 (1.54-1.91)	6/6	1.03 (0.75-1.34)	6/6
r-WNVEDIII with Titermax® gold	6/6	1.58	1.52 (1.14-1.82)	6/6	0.96 (0.37-1.56)	6/6
r-LGTVEDIII alone	6/6	17.8 <sup>1</sup>	1.21 (0.56-1.67)	6/6	0.16 (0.08-0.30)	2/6
r-LGTVEDIII with Alhydrogel®	6/6	ND	2.12 (1.93-2.26)	6/6	0.12 (0.068-0.23)	0/6
r-LGTVEDIII with saponin	6/6	2.1*	2.09 (1.94-2.26)	6/6	0.87 (0.22-1.42)	5/5 <sup>3</sup>
r-LGTVEDIII with Titermax® gold	6/6	3.82*	1.49 (0.86-2.2.4)	6/6	0.20 (0.06-0.78)	2/6

<sup>1</sup>Ratio calculated for mice with both detectable IgG1 and IgG2a

<sup>2</sup>ND not determined as no detectable IgG2a antibody

<sup>3</sup>one death 24 hours after inoculation.

Protein alone or mixed with alum elicited a predominantly Th2 response whereas protein in combination with saponin or Titermax® gold induced a mixed Th1 and Th2 response.

Mice immunized with r-WNVEDIII reacted against KUNV antigen indicating that the protein was expressed with conformation exposing epitopes that are required to induce a detectable antibody response. A native antigen representing the tick borne flaviviruses was not available to test serum samples from mice immunized with r-LGTVEDIII.

## 5.4. Summary

Small molecule antigens or weakly immunogenic antigens frequently require an adjuvant to stimulate a stronger immune response. In addition, adjuvants can shift an immune response towards a Th1 or Th2 response as required based on immune correlates of protection. The aim of this study was to characterize the response elicited using different adjuvants to stimulate both a strong humoral and cellular immune response based on antibody detection, isotyping antibody response and profiling cytokine secretion using stimulated splenocytes from immunized animals. In the absence of cytokine profiling, characterization of immune response was based on isotyping of antibody response. Briefly, Th1 responses are characterized by the production of IgG2a and IgG2b regulated by secretion of interferon gamma and interleukin-2 cytokines. In contrast, a Th2 response is characterized by production of IgG1 antibody and regulated by secretion of interleukin-4 and interleukin-10 secretion. The differentiation between IgG subclasses in the response was used as an indicator of the type of response induced. A ratio of IgG2a>IgG1 suggests a strong Th1 response and a IgG1>IgG2a ratio suggests a stronger Th2 response.

Mice receiving r-WNVEDIII alone had detectable antibody in only one mouse suggesting that the recombinant protein was weakly immunogenic. A significant increase in antibody (based on OD results and number of positive mice) was observed in animals receiving the protein in combination with an adjuvant. All mice receiving r-LGTVEDIII alone had detectable antibody however in retrospect this may have been dose related. Although the protein concentrations were the same, the WNVEDIII was expressed with a large TF chaperone protein, hence the final concentration of EDIII was less than that of LGTVEDIII protein. This may account for the differences in response. The combination of protein and alum (Alhydrogel®) produced a predictable result with detectable IgG1 in all mice and no detectable IgG2a in any of the animals. Although similar results were obtained from proteins in combination with saponin or Titer Max® gold which elicited a mixed response, overall the r-WNVEDIII induced antibody in all mice immunized whereas some mice receiving r-LGTVEDIII and Titer Max® gold were negative for IgG2a antibody. It is possible that the TF chaperone protein expressed with the WNVEDIII may have contributed to the response. With most viral infections cellular responses play an important role. Challenge studies and tests for neutralizing antibody will be required to determine if the EDIII region in combination with an emulsion adjuvant is sufficient to protect against infection.

## Chapter 6

### Discussion

The presence of mosquito-borne flaviviruses in southern Africa is known but their prevalence has not been fully established. In addition, cases of *flavivirus* disease may have been underreported in this region due to limited routine diagnostic services. An assay that can detect antibodies against flaviviruses could have a role in diagnosis and in serological surveillance of various species. Due to the high cross-reactivity between members of this genus, it is essential to have an assay that can differentiate between them. The r-EDIII protein of flaviviruses is a useful tool that has been demonstrated to have potential in differentiating between flaviviruses (Holbrook et al. 2004). This domain has been reported to comprise predominantly subcomplex and type-specific epitopes which mainly induce neutralizing antibodies (Oliphant et al. 2007) and has also been observed to be highly antigenic with primary linear epitopes (Beasley and Barrett, 2002) based on X-ray crystallography (Rey et al. 1995). The EDIII is probably involved in viral receptor binding (Crill and Roehrig, 2001) and can be expressed as a recombinant protein that has the ability to fold as a functional fragment (Martina et al. 2008). While this protein has the ability to differentiate between antibodies directed against tick- and mosquito-borne flaviviruses, our study has suggested that it has potential to differentiate between WESSV and WNV which are the only viruses known to occur in southern Africa to date. The potential of WESSVEDIII and WNVEDIII assays to differentiate between antibodies, as well as the high proportions of discrimination between field samples detected by these assays further shows the potential of the EDIII protein as a tool for surveillance. The gold standard for differentiation between flaviviruses is the neutralization assay but this could not be performed as our laboratory is limited to a biosafety level 2 facility and both WNV and WESSV are biosafety level 3 pathogens. The r-EDIII protein is therefore a valuable tool that may be beneficial for surveillance in low-resource laboratories that cannot perform neutralization assays due to viruses classified above level 2.

The amino acid sequences alignments of EDIII proteins of WNV and WESSV showed a homology of 36 % (Table 1). Additionally, the two viruses belong to different serogroups within the genus and this is also consistent with the phylogenetic tree that was constructed based on the amino acid sequences of various flaviviruses (Figure 1). The phylogenetic comparison between the EDIII protein of WNV and that of WESSV positions these viruses in distinct lineages of their respective serogroups, namely JEV and YFV. The r-WNVEDIII has previously been shown to have potential in differentiating homologous antibodies from those raised against viruses in the same serogroups, namely JEV, St. Louis encephalitis virus and Murray Valley encephalitis virus (Beasley et al. 2004). This further substantiates the discriminatory potential of the EDIII protein in immunoassays.

The EDIII proteins of KUNV and WNV grouped together on the phylogenetic tree similarly to a study that used phylogenetic analyses to indicate that certain strains of WNV are more similar to KUN and form a separate lineage to other WNV strains suggesting the addition of more subtypes in the WNV group (Hall et al. 2006). Both viruses are associated with febrile illness with rash or mild encephalitis (Scherret et al. 2001) as well as neurological disease in horses (Badman et al. 1984; Venter et al. 2010). The limitation that was observed with the r-WNVEDIII was the detection of antibodies raised against DENV. Though this may be a major problem in regions where DENV is endemic, it was not believed to play a major role in the detection of field samples as DENV is not endemic in southern Africa.

In contrast to WNV, WESSV has not been associated with a fatal outcome of disease and cases involving human infection with WESSV may be underreported. In a serological survey of human sera performed in South Africa, WESSV was recorded in 32% of samples collected in the subtropical region of KwaZulu Natal province and 0.7% in the southern Cape (McIntosh 1980). Recently, WESSV was isolated from two human cases in which an initial diagnosis of Rift Valley fever virus (RVFV) infection was suspected due to an outbreak of the RVFV that occurred in South Africa, with human and livestock infections, between 2010 and 2011. These isolates were included in a phylogenetic analysis of collective WESSV isolates based on the sequence data of the NS5 gene (Weyer et al. 2013). The study identified 2 clades of WESSV circulating in southern Africa. Clade I included isolates collected from South Africa and Zimbabwe, while clade II was comprised exclusively of isolates from the KwaZulu Natal province of South Africa. Our study also shows a high serodetection of human samples on the r-WESSV assay (54/196). Based on the specificity testing performed on the r-WESSVEDIII in ELISA none of the antibodies raised against other flaviviruses tested were detectable by this antigen except for the YFV antibodies, which is not endemic in southern Africa. Moreover, WNV is known to be the only other endemic flavivirus in this region and could be distinguished from WESSV. Additionally, humans from which samples were collected did not have a history of YFV vaccination which rules out YFV as a cause of seropositivity. The limited diagnostic service for WESSV disease can account for mild, self-limiting cases of infection being undiagnosed. The isolation of WESSV from samples collected during the recent RVFV outbreak indicates that there was circulation of other mosquito-borne viruses during this period (Weyer et al. 2013). This is not uncommon as the outbreak occurred during a period of high rainfall. In our study we observed that a considerable amount of the bovine serum samples that were positive on the KUNV anti-flavivirus antibody assay was collected after the 2010 outbreak of RVFV (NICD bulletin, 2011). A total of 537 samples were collected in this period of which 281 were positive on the assay. This increases the serodetection for flaviviruses to 52% compared to 21% (314/1515) that was observed in samples that were collected prior to the outbreak. Similarly, the ovine samples that were collected after the outbreak showed a more positive trend than those



collected prior. Of the 88 ovine samples tested, 40 were collected after the outbreak with a seropositivity of 68% (27/40) which is much higher than the 8% (4/48) collected before outbreak. Ovine samples that were positive for the r-LGTV samples were all collected after the outbreak with a seropositivity of 35% (14/40) compared to 0% (0/48) before the outbreak. This suggests that tick-borne flaviviruses were also circulating during the outbreak particularly in sheep than in cattle. While seropositivity of ovine samples seem to have increased after the outbreak on the r-WESSV and r-WNV assays, the seropositivity of bovine sera remained constant regardless of the outbreak. All human serum samples were collected prior to the 2010 RVFV outbreak.

Based on specificity testing, the r-LGTVEDIII protein was less stringent compared to the other recombinant proteins as it was able to react with antibodies raised against heterologous tick-borne flaviviruses and only excluded those raised against mosquito-borne flaviviruses. Our in-house specificity testing for r-LGTVEDIII against 7 samples positive for mosquito-borne flaviviruses belonging to various serotypes within the group and 3 samples positive for tick-borne flaviviruses provided repeatable data that excluded tick-borne flaviviruses from mosquito-borne flaviviruses. This observation positions the LGTVEDIII assay as a reliable tool for the detection of anti-tick-borne flavivirus antibodies and is consistent with the grouping of tick-borne flaviviruses in the phylogenetic tree. Holbrook and co-workers (2004) also reported that members of the tick-borne serocomplex of flaviviruses are highly cross-reactive and cannot be differentiated on the EDIII protein.

The r-EDIII proteins prepared in this study suggest the circulation of WNV, WESSV and tick-borne flaviviruses in the Free State province which has not been previously established. However, accurate identification of these samples requires confirmation by virus neutralization assay. Flaviviruses seem to be widely distributed in this province and their co-circulation has been suggested in various farms where samples would be collectively positive for WNV, WESSV and tick-borne flaviviruses. Antibodies raised against mosquito-borne flaviviruses were most abundant and this was not alarming as the presence of WNV and WESSV has been established in southern Africa and their transmission by the same mosquito vector would explain the high degree of co-circulation of WNV and WESSV in all districts. Though neutralization assays are needed for confirmation, the detection of antibodies by the r-LGTVEDIII suggests that tick-borne flaviviruses may be circulating in southern Africa. The detection of these antibodies was not only made in samples from livestock, but also in human patients that had had a tick bite and no diagnosis, suggesting but not confirming that tick-borne flaviviruses may have played a role in their illness. Future studies will focus on developing molecular techniques to detect isolates of novel mosquito-borne flaviviruses and confirm the presence of tick-borne flaviviruses.

The r-EDIII protein of flaviviruses is a safe, cost-effective and significant tool for the detection of flaviviruses, especially in low-resource laboratories. The protein can be used for differentiation between the serologically cross-reactive WNV and WESSV as well as tick-borne flaviviruses.

Though neglected, the putative prevalence of the WESSV in the Free State is suggested. Together with WNV and WESSV, tick-borne flaviviruses may also be circulating on the farms located in the Free State. The presence of other flaviviruses is also suggested but this data will have to be confirmed using virus neutralization test.

Rodents are known to be reservoir hosts of hantaviruses and other viral haemorrhagic fevers including arenaviruses and they are considered as transient hosts of CCHFV. Serological and molecular surveillance studies using wild caught rodents are important for investigating both the presence of these viruses and the role of rodents in their natural cycle. Currently pathogenic arenaviruses and hantaviruses are not known to be endemic in South Africa although Lujo has been identified from neighbouring Zambia and serological surveys have suggested that there exists a low hantavirus antibody prevalence in the country. However previous investigations have yet to produce conclusive evidence by means of hantavirus isolation or detection of hantaviral nucleic acid to confirm an indigenous South African hantavirus. Nevertheless the recent emergence or identification of hantaviruses in western and eastern Africa warrants continued surveillance in our country. Factors such as urbanization, globalization, climate change, agricultural intensification and encroachment on wildlife habitat contribute towards the emergence and re-emergence of pathogens threatening biodiversity, water and food, as well as human and animal health. Arboviruses and viral haemorrhagic fevers are among the most pathogenic and devastating disease agents in many parts of the world. It is therefore important for surveillance of such pathogens to be conducted as they may result in considerable public health implications.

Laboratory diagnosis of hantaviruses is usually based on antibody detection with the majority of patients presenting with detectable IgG and or IgM. Most commonly ELISA are applied for diagnostic and surveillance purposes. Previous and current serological surveys were based on American and European hantaviral antigens. In our study positive serology was detected in acute phase patients and the wide spectrum of disease presentation by American and European hantaviruses suggests that identification on a clinical basis will be difficult. A low seropositive rate was previously identified in the Cape in patients with presenting with hantavirus-like disease (Witkowski et al., 2014) but this approach may be limiting in the absence of known clinical symptoms associated with an African hantavirus. Serological cross reactivity must be taken into consideration. There is some suggestion that there is more cross reactivity during the acute stages compared with convalescent stages (Jonsson et al., 2010). Hence our results based on non African hantaviruses may have some validity but that for surveillance studies it may be important to widen the scope using African hantavirus antigens. With the limited number of African hantaviruses identified to date, it is difficult to determine the exact antigenic cross reactivity between African,

European and American hantaviruses. Future studies will include the preparation and application of recombinant NP based on African hantaviral gene sequences for surveillance.

Similarly the molecular assay, which should have application for all known hantaviruses, will be used in our laboratory to investigate larger numbers of wild caught rodents. The validation has suggested the assay has suitable sensitivity and specificity for detection of all known hantaviruses. The inclusion of representatives of all known hantavirus types may also allow differentiation of hantaviruses. It is possible that in shipping ports, rodents could be introduced from other continents with potential for establishment of exotic hantaviruses in local rodent populations.

In summary the identification of hantaviruses is expanding both geographically and by host range and specific assays targeting African hantaviruses but incorporating known hantaviruses are warranted. Since the first hantavirus was identified in Africa, eight more have been confirmed with one report suggesting febrile illness associated with African hantavirus infection. Continued monitoring of potential reservoir hosts should contribute to identifying novel viruses.

A conventional multiplex PCR assay aimed at detecting CCHFV and flavivirus RNA in samples collected from undiagnosed patients presenting with a tick-bite and febrile illness was developed by using nested primers targeting the partial region of the genome of the S-segment of CCHFV and hemi-nested primers targeting the partial region of the NS5 gene of flaviviruses. Flaviviruses and CCHFV were combined in a multiplex RT-PCR to target patients with a known tick-bite and no confirmed diagnosis. The idea was to incorporate an assay that could amplify tick-borne flaviviruses as a differential diagnosis for non CCHF cases. The detection of a patient with CCHFV nucleic acid and mild disease that was not submitted for testing for suspected viral haemorrhagic fever certainly raises alarms that increased awareness of viral haemorrhagic fever causing milder disease is required. CCHFV has the potential to cause nosocomial infections and what causes the variability in severity of disease has not been determined. A mild case therefore has potential to cause nosocomial infections with a more severe disease and possibly fatal outcome among health care and laboratory workers. These results highlight the need for awareness to arboviruses and viral hemorrhagic fevers in mild cases that may easily be overlooked. A more comprehensive reporting of patient history and description of acute illness will assist medical and laboratory personnel in accomplishing accurate differential diagnosis and awareness and diagnostic capacity will have important public health implications. The confirmation of a case of CCHFV indicates how cases may be missed, suggesting inefficient reporting of clinical history, improper differential diagnosis as a result of lack of awareness of mild cases.

There is still a lack of awareness regarding arboviruses that are circulating with potential to cause diseases and more work needs to be focused in this area to offer a wider range of assays and defining diseases associated specific arboviruses. For example there is little awareness of the chronic arthralgia associated with alphaviruses despite sporadic cases of Sindbis that occur annually. In addition patients that travel to South Africa and return to other countries with a known tick-bite and febrile illness and frequently identified as having a Rickettsial infection but it is unlikely that CCHFV will be included in the differential diagnosis when the patient presents with mild disease. South Africa is a popular tourist destination with many travelers visiting game reserves and participating in recreational activities such as hiking, camping and hunting which are likely to expose them to tick bites. Again this opens a potential health risk to laboratory and health care workers.

To summarize, although tick-borne flaviviruses and hantaviral diseases have not been confirmed as medically significant pathogens circulating in South Africa, both the vectors and reservoir hosts are present. Increase in diagnostic capacity, development of safe laboratory assays that will allow surveillance studies to be performed will all contribute to detection of novel pathogens of significance in our country.

Investigating immune responses elicited by viral proteins will contribute to vaccine development. Simultaneous expression of E protein and prM protein forming virus-like particles have been shown to protect against challenge. Similarly, the E protein of West Nile virus alone was shown to be protective in mice, inducing both humoral and cellular responses, with an enhancement of the immune responses obtained by boosting with recombinant domain III protein (Schneeweiss et al., 2011). Recombinant envelope domain III protein alone for dengue has been shown to be highly immunogenic in mice (Babu et al., 2008), hence in this study the aim was to determine the responses elicited by recombinant WNV and LGTV EDIII alone and with selected adjuvants.

Various subclasses of mouse immunoglobulin, referred to as isotypes IgG1, IgG2a, IgG2b and IgG3, are involved in immune responses to infection and the class that predominates during infection shift in response to the type of cytokines secreted. A Th1 response is characterized by production of IFN gamma and IL-2 cytokines with enhanced production of isotype IgG2a and inhibition of IgG1 production. In contrast a Th2 type response is associated with production of IL-4 which switches activated B cells to the IgG1 isotype. The presence of both IgG2a and IgG1 in some animals suggest that the vaccine can induce a mixed Th1 and Th2 response which should promote both cellular immunity and antibody production. In the absence of any adjuvant the results from WNV protein alone were inconclusive whereas a strong IgG1 response was induced by LGTV EDIII. The confirmation of recombinant proteins can influence the ability of the protein to induce a

protective neutralizing response and the absence of neutralizing antibody tests is a limitation of this study. However antibody was detected in sera from WNV inoculated mice using Kunjin antigen indicating the presence of epitopes on the r-WNVEDIII antigen that could induce an antibody that was recognized by a native flavivirus antigen. The formulation of the WNV and LGTV proteins with different adjuvants produced similar results with a shift in response depending on the adjuvant. Despite an absence of being able to assess cell mediated responses using antigen stimulated splenocytes and profiling cytokine production as initially planned, the results do confirm that r-WNVEDIII and r-LGTVEDIII proteins are immunogenic in the absence of complete E protein, with ability to induce detectable antibody when formulated with adjuvant and that different adjuvants are able to have an immunomodulatory influence on the type of response induced.

## References

Adam F, Digouette JP. Virus d'Afrique (base de données). [Viruses of Africa (database)]. Dakar: World Health Organization Collaborating Reference and Research Centre for arboviruses and haemorrhagic fever viruses (CRORA), Institut Pasteur de Dakar; Jul 2005. Available from <http://www.pasteur.fr/recherche/banques/CRORA>

Babu JP, Pattnaik P, Gupta N, Shrivastave A, Khan M, Rao L. Immunogenicity of a recombinant envelope domain III protein of dengue virus type-4 with various adjuvants in mice. *Vaccine* 2008; 26:4655-4663.

Badman RT, Campbell J, Aldred J. Arbovirus infection in horses-Victoria. *Commun Dis Intell* 1984; 17:5-6.

Bae JL, Lee JG, Kang TJ, Jang HS, et al. Induction of antigen specific systemic and mucosal immune responses by feeding animals transgenic plants expressing the antigen. *Vaccine* 2003; 21:4052-4058.

Bakonyi T, Hubálek Z, Rudolf I, Nowotny N. Novel flavivirus or new lineage of West Nile virus, central Europe. *Emerg Infect Dis* 2005; 11:225-231.

Bakonyi T, Ivanics E, Erdélyi K, Ursu K, et al. Lineage 1 and 2 strains of encephalitic West Nile virus, central Europe. *Emerg Infect Dis* 2006; 12:618-623.

Barrett AD, Monath TP. Epidemiology and ecology of yellow fever virus. *Adv Virus Res* 2003; 61:291-315.

Barrett AD, Teuwen DE. Yellow fever vaccine - how does it work and why do rare cases of serious adverse events take place? *Curr Opin Immunol* 2009; 21:308-313.

Bhardwaj S, Holbrook M, Shope RE, Barrett ADT, Watowich SJ. Biophysical characterization and vector-specific antagonist activity of domain III of the tick-borne flavivirus envelope protein. *J Virol* 2001; 75:4002-4007.

Beasley DWC, Barrett ADT. Identification of neutralizing epitopes within structural domain III of the West Nile virus envelope protein. *J Virol* 2002; 76:13097-13100.

Beasley DW, Holbrook M, Travassos Da Rosa A, et al. Use of recombinant envelope protein subunit antigen for specific serological diagnosis of West Nile Infection. *J Clin Microbiol* 2004; 42:2759-2765.

Blackburn NK, Swanepoel R. An investigation of flavivirus infections of cattle in Zimbabwe Rhodesia with particular reference to Wesselsbron virus. *J Hyg (Lond)* 1980; 85:1-33.

Blackburn NK, Reyers F, Berry WL, Shepherd AJ. Susceptibility of dogs to West Nile virus: A survey and pathogenicity trial. *J Comp Pathol* 1989; 100:59-66.

Bondre VP, Jadi RS, Mishra AC, Yergolkar PN, Arankalle VA. West Nile virus isolates from India: evidence for a distinct genetic lineage. *J Gen Virol* 2007; 88:875-884.

Briese T, Paweska JT, McMullan LK, Hutchison SK, et al. Genetic detection and characterization of Lujo virus, a new hemorrhagic fever-associated arenavirus from southern Africa. *PLoS Pathog* 2009; 5:e1000455.

Briese T, Jia XY, Huang C, Grady LJ, Lipkin WI. Identification of Kunjin/West Nile-like flavivirus in brains of patients with New York encephalitis. *Lancet* 1999; 354:1261-1262.

Brinton MA, Perelygrin AA. Genetic resistance to flaviviruses. *Adv Virus Res* 2003; 60:43-85.

Burt FJ, Spencer DC, Leman PA, Patterson B, Swanepoel R. Investigation of tick-borne viruses as pathogens of humans in South Africa and evidence of Dugbe infection in a patient with prolonged thrombocytopenia. *Epidemiol Infect* 1996; 116:353-361.

Burt FJ, Grobbelaar AA, Leman PA, Anthony FS, Gibson GVF, Swanepoel R. Phylogenetic relationships of southern African West Nile virus isolates. *Emerg Infect Dis* 2002; 8:820-826.

Calisher CH. Antigenic classification and taxonomy of flaviviruses (family *Flaviviridae*) emphasizing a universal system for the taxonomy of viruses causing tick-borne encephalitis. *Acta Virol.* 1988; 32:469-478.

Calisher CH, Gould EA. Taxonomy of the virus family *Flaviviridae*. *Adv Virus Res* 2003; 59:1-19.

Calisher CH, Karabatsos N, Dalrymple JM, Shope RE, et al. Antigenic relationships between flaviviruses as determined by cross-neutralization tests with polyclonal antisera. *J Gen Virol* 1989; 70:37-43.

Cargnelutti JF, Brum MCS, Weiblen R, Flores EF. Stable expression and potential use of West Nile virus envelope glycoproteins preM/E as antigen in diagnostic tests. *Braz J Microbiol* 2011; 42:1161-1166.

Cavrini F, Gaibani P, Longo G, Pierro AM, et al. Usutu virus infection in a patient who underwent orthotropic liver transplantation, Italy, August-September 2009. *Euro Surveill* 2009; 14:19448.

CDC. Outbreak of West Nile-like viral encephalitis--New York, 1999. *MMWR* 1999; 48:845-849.

CDC 2014. West Nile Virus activity by state – United States, 2014. Available from <http://www.cdc.gov/westnile/statsMaps/preliminaryMapsData/activitystatedate.html>.

Ceașu E, Erșcoiu S, Calistru P, Ispas D, et al. Clinical manifestations in the West Nile virus outbreak. *Rom J Virol* 1997; 48:3-11.

Chávez JH, Reis VP, Silva JR, Laure HJ, et al. Production and diagnostic application of recombinant domain III of West Nile envelope protein in Brazil. *Rev Soc Bras Med Trop* 2013; 46:97-99.

Chu JH, Chiang CC, Ng ML. Immunization of flavivirus West Nile recombinant envelope domain III protein induced specific immune response and protection against West Nile virus infection. *J Immunol* 2007; 178:2699-2705.

Clement J, Lameire N, Keyaerts E, Maes P, Van Ranst M. Hantavirus infections in Europe. *Lancet Infect Dis* 2003; 3:752-753.

Clerx JP, Casals J, Bishop DH. Structural characteristics of nairoviruses (genus *Nairovirus*, *Bunyaviridae*). *J Gen Virol* 1981; 55:165-178.

Coia G, Parker MD, Speight G, Byrne ME, Westaway EG. Nucleotide and complete amino acid sequences of Kunjin virus: definitive gene order and characteristics of the virus-specified proteins. *J Gen Virol* 1988; 69:1-21.



Crill WD, Roehrig JT. Monoclonal antibodies that bind to domain III of dengue virus E glycoprotein are the most efficient blockers of virus adsorption to Vero cells. *J Virol* 2001; 75:7769-7773.

Dauphin G, Zientara. West Nile virus: recent trends in diagnosis and vaccine development. *Vaccine* 2007; 25:5563-5576.

Demain AL, Vaishnav P. Production of recombinant proteins by microbes and higher organisms. *Biotechnol Adv.* 2009; 27:297-306.

Domingo C, Patel P, Linke S, Achazi K, Niedrig M. Molecular diagnosis of flaviviruses. *Future Virol* 2001; 6:1059-1074.

Durbin AP, Whitehead SS. The dengue human challenge model: has the time come to accept this challenge? *J Infect Dis* 2013; 207:697-699.

ECDC 2013. European Center for Disease Control and Prevention (ECDC). Epidemiological update: end of West Nile virus transmission season in Europe; November 2013. Available from [http://www.ecdc.europa.eu/en/press/news/\\_layouts/forms/News\\_DispatchForm.aspx?List=8db7286c-fe2d-476c-9133-18ff4cb1b568&ID=897](http://www.ecdc.europa.eu/en/press/news/_layouts/forms/News_DispatchForm.aspx?List=8db7286c-fe2d-476c-9133-18ff4cb1b568&ID=897)

Ecker M, Allison SL, Meixner T, Heinz FX. Sequence analysis and genetic classification of tick-borne encephalitis viruses from Europe and Asia. *J Gen Virol.* 1999; 80:179-185

Edington AD. "Dengue" as seen in the recent epidemic in Durban. *J Med Assoc South Africa* 1927; 1:446-448.

Fulop LD, Barrett AD, Titball RW. Nucleotide sequence of the NS5 gene of Banzi virus: comparison with other flaviviruses. *J Gen Virol* 1995; 76:2317-2321.

Gaunt MW, Sall AA, de Lamballerie X, Falconar AK, et al. Phylogenetic relationships of flaviviruses correlate with their epidemiology, disease association and biogeography. *J Gen Virol* 2001; 82:1867-1876.

Golovljova I, Vasilenko V, Prück T, Brus Sjölander K, Plyusnin A, Lundkvist A. Puumala and Dobrava hantaviruses causing hemorrhagic fever with renal syndrome in Estonia. *Eur J Clin Microbiol Infect Dis* 2000; 19:968-969.

Gould EA, de Lamballerie X, Zanotto PM, Holmes EC. Origins, evolution, and vector/host coadaptations within the genus *Flavivirus*. *Adv Virus Res* 2003; 59:227-314.

Gould EA, Solomon T. Pathogenic flaviviruses. *Lancet*. 2008; 371:500-509.

Grant-Klein RJ, Baldwin CD, Turell MJ, Rossi CA, et al. Rapid identification of vector-borne flaviviruses by mass spectrometry. *Mol Cell Probes* 2010; 24:219-228.

Grard G, Moureau G, Charrel RN, Lemasson JJ, et al. Genetic characterization of tick-borne flaviviruses: new insights into evolution, pathogenetic determinants and taxonomy. *Virology*. 2007; 361:80-92.

Gritsun TS, Nuttall PA, Gould EA. Tick-borne flaviviruses. *Adv Virus Res* 2003; 61:317-371.

Gubler DJ. The global pandemic of dengue/dengue haemorrhagic fever: current status and prospects for the future. *Ann Acad Med Singapore* 1998; 27:227-234.

Guirakhoo F, Heinz FX, Mandl CW, Holzmann H, Kunz C, Gresíková M. The relationship between the flaviviruses Skalica and Langat as revealed by monoclonal antibodies, peptide mapping and RNA sequence analysis. *J Gen Virol* 1991; 72:333-338.

Guo WP, Lin XD, Wang W, Tian JH, et al. Phylogeny and origins of hantaviruses harbored by bats, insectivores, and rodents. *PLoS Pathog*. 2013; 9:e1003159.

Guzman MG, Vazquez S. The complexity of antibody-dependent enhancement of dengue virus infection. *Viruses* 2010; 2:2649-2662.

Hall RA, Scherret JH, Mackenzie JS. Kunjin virus: An Australian variant of West Nile? *Ann N Y Acad Sci* 2006; 1:153-160.

Halstead SB, Jacobson J. Japanese encephalitis. *Adv Virus Res* 2003; 61:103-138.

Halstead SB, Thomas SJ. New vaccines for Japanese encephalitis. *Curr Infect Dis Rep* 2010; 12:174-180.

Heinz FX, Holzmann H, Essl A, Kundi M. Field effectiveness of vaccination against tick-borne encephalitis. *Vaccine* 2007; 25:7559-7567.

Heinz FX, Stiasny K. Flaviviruses and flavivirus vaccines. *Vaccine* 2012; 30:4301-4306.

Hjelle B, Jenison S, Mertz G, Koster F, Foucar K. Emergence of hantaviral disease in the southwestern United States. *West J Med* 1994; 161:467-473.

Holbrook MR, Shope RE, Barret ADT. Use of recombinant E protein domain III-based enzyme-linked immunosorbent assays for differentiation of tick-borne encephalitis serocomplex flaviviruses from mosquito-borne flaviviruses. *J Clin Microbiol* 2004; 42:4101-4110.

Holmes EC, Twiddy SS. The origin, emergence and evolutionary genetics of dengue virus. *Infect Genet Evol* 2003; 3:19-28.

Hubálek Z, Halouzka J, Juricová Z, Sikutová S, et al. Serologic survey of birds for West Nile flavivirus in Southern Moravia (Czech Republic). *Vector Borne Zoonotic Dis* 2008; 8:659-666.

Hudopisk N, Korva M, Janet E, Simetinger M, et al. Tick-borne encephalitis associated with consumption of raw goat milk, Slovenia, 2012. *Emerg Infect Dis* 2013; 19:806-808.

Jenkins N, Curling EM. Glycosylation of recombinant proteins: problems and prospects. *Enzyme Microb Technol* 1994; 16:354-364.

Johnson N, Wakeley PR, Mansfield KL, McCracken F, et al. Assessment of a novel real-time pan-flavivirus RT-polymerase chain reaction. *Vector Borne Zoonotic Dis* 2010; 10:665-671.

Jonsson CB, Figueiredo LT, Vapalahti O. A global perspective on hantavirus ecology, epidemiology, and disease. *Clin Microbiol Rev* 2010; 23:412-441.

Kang HJ, Kadjo B, Dubey S, Jacquet F, Yanagihara R. Molecular evolution of Azagny virus, a newfound hantavirus harboured by the West African pygmy shrew (*Crocidura obscurior*) on Côte d'Ivoire. *Virology* 2011; 28:373.

Karabatsos N (ed). International catalogue of arboviruses including certain other viruses of vertebrates (4th edition). San Antonio: American Society of Tropical Medicine and Hygiene, 1985.

Katoh K, Standley DM. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol Biol Evol* 2013; 30:772-780.

Karaca K, Bowen R, Austgen LE, Teehee M, et al. Recombinant canarypox vectored West Nile virus (WNV) vaccine protects dogs and cats against a mosquito WNV challenge. *Vaccine* 2005; 23:3808-3813.

Klempa B, Fichet-Calvert E, Lecompte E, Auste B, et al. Hantavirus in African wood mouse, Guinea. *Emerg Infect Dis* 2006; 12:838-840.

Klempa B, Fichet-Calvert E, Lecompte E, Auste B, et al. Novel hantavirus sequences in shrew, Guinea. *Emerg Infect Dis* 2007; 13:520-522.

Klempa B, Koivogui L, Sylla O, Koulemou K, et al. Serological evidence of human hantavirus infections in Guinea, West Africa. *J Infect Dis* 2010; 201:1031-1034.

Knap N, Korva M, Dolinšek V, Sekirnik M, et al. Patterns of tick-borne encephalitis virus infection in rodents in Slovenia. *Vector Borne Zoonotic Dis* 2012; 12:236-242.

Kokernot, RH, de Meillon B, Paterson HE, Heymann CS, Smithburn KC. Middelburg virus; a hitherto unknown agent isolated from *Aedes* mosquitoes during an epizootic in sheep in the eastern Cape Province. *S Afr J Med Sci* 1957; 22:145–153.

Kondiah K, Swanepoel R, Paweska JT, Burt FJ. A simple-probe real-time PCR assay for genotyping reassorted and non-reassorted isolates of Crimean-Congo hemorrhagic fever virus in southern Africa. *J Virol Methods* 2010; 169:34-38.

Komar N. West Nile virus: epidemiology and ecology in North America. *Adv Virus Res* 2003; 61:185-234.

Kozuch O, Labuda M, Lysý J, Weismann P, Krippel E. Longitudinal study of natural foci of Central European encephalitis virus in west Slovakia. *Acta Virol* 1990; 34:537-544.

Labuda M, Alves MJ, Elecková E, Kozuch O, Filipe AR. Transmission of tick-borne bunyaviruses by cofeeding ixodid ticks. *Acta Virol* 1997; 41:325-328.

Lanciotti RS. Molecular amplification assays for the detection of flaviviruses. *Adv Virus Res* 2003; 61:67-99.

Lanciotti RS, Roehrig JT, Deubel V, Smith J, et al. Origin of the West Nile virus responsible for an outbreak of encephalitis in the northeastern United States. *Science* 1999; 286:2333-2337.

Ledgerwood JE, Pierson TC, Hubka SA, Desai N, et al. A West Nile virus DNA vaccine utilizing a modified promoter induces neutralizing antibody in younger and older healthy adults in a phase I clinical trial. *J Infect Dis* 2011; 203:1396-1404.

Ledizet M, Kar K, Foellmer HG, Wang T, et al. A recombinant envelope protein vaccine against West Nile virus. *Vaccine* 2005; 23:3915-3924.

Lee HW, Baek LJ, Johnson KM. Isolation of Hantaan virus, the etiologic agent of Korean hemorrhagic fever, from wild urban rats. *J Infect Dis* 1983; 146:638-644.

Lichty JJ, Malecki JL, Agnew HD, Michelson-Horowitz DJ, Tan S. Comparison of affinity tags for protein purification. *Protein Expr Purif* 2005; 41:98-105.

Lieberman MM, Clements DE, Ogata S, Wang G, et al. Preparation and immunogenic properties of a recombinant West Nile subunit vaccine. *Vaccine* 2007; 25:414-423.

Lindenbach BD, Rice CM. Molecular biology of flaviviruses. *Adv Virus Res* 2003; 59:23-61.

Lindquist L, Vapalahti O. Tick-borne encephalitis. *Lancet* 2008; 371:1861-1871.

Ludwig GV, Iacono-Connors LC. Insect-transmitted vertebrate viruses: *Flaviviridae*. *In Vitro Cell Dev Biol Anim* 1993; 29A:296-309.

Lvov DK, Butenko AM, Gromashevsky VL, Kovtunov AI, et al. West Nile virus and other zoonotic viruses in Russia: examples of emerging-reemerging situations. *Arch Virol Suppl* 2004; 18:85-96.

Mandl CW, Guirakhoo F, Holzmann H, Heinz FX, Kunz C. Antigenic structure of the flavivirus envelope protein E at the molecular level, using tick-borne encephalitis as a model. *J Virol* 1989; 63:564-571.

Martina BE, Koraka P, van den Doel P, van Amerongen G, Rimmelzwaan GF, Osterhaus AD. Immunization with West Nile virus envelope domain III protects mice against lethal infection with homologous and heterologous virus. *Vaccine* 2008; 26:153-157.

Mathengtheng L, Burt FJ. Use of envelope domain III protein for detection and differentiation of flaviviruses in the Free State Province, South Africa. *Vector Borne Zoonotic Dis* 2014; 14:261-271.

McIntosh BM. The epidemiology of arthropod-borne viruses in southern Africa. DSc dissertation, University of Pretoria, Pretoria 1980; 144-159.

McIntosh BM. Mosquito-borne virus diseases of man in southern Africa. *S Afr Med J* 1986; Suppl:69-72.

McIntosh BM, Jupp PG, Dos Santos I, Meenehan GM. Epidemics of West Nile and Sindbis viruses in South Africa with *Culex (Culex) univittatus* Theobald as vector. *S Afr J Sci* 1976; 72:295-300.

McClellan DM, Donohue WL. Powassan virus: isolation of virus from a fatal case of encephalitis. *Can Med Assoc J* 1959; 80:708-711.

Metselaar D, Henderson BE, Kirya GB, Tukei PM, de Geus A. Isolation of arboviruses in Kenya, 1966-1971. *Trans R Soc Trop Med Hyg.* 1974; 68:114-123.

Minke JM, Siger L, Karaca K, Austgen L, et al. Recombinant canarypox vaccine carrying the prM/E genes of West Nile virus protects horses against a West Nile virus-mosquito challenge. *Arch Virol Suppl* 2004; 18:221-230.

Monath TP, Liu J, Kanesa-Thanan N, Myers GA, et al. A live, attenuated recombinant West Nile virus vaccine. *Proc Natl Acad Sci USA* 2006; 103:6694-6699.

Monath TP, Fowler E, Johnson CT, Balser J, et al. An inactivated cell-culture vaccine against yellow fever. *N Engl J Med* 2011; 364:1326-1333.

Morvan J, Fontenille D, Digoutte JP, Coulanges P. The Wesselsbron virus, a new arbovirus for Madagascar. *Arch Institut Pasteur Madagascar* 1990; 57:183-192.

Msimang V, Weyer J, le Roux C, Leman P, Kemp A, Paweska J. Update: Crimean-Congo haemorrhagic fever in South Africa. NICD. Communicable Diseases Surveillance Bulletin 2014; 11:58-62.

Murray JM, Aaskov JG, Wright PJ. Processing of the dengue virus type 2 proteins prM and C-prM. J Gen Virol 1993; 74:175-182.

Ng T, Hathaway D, Jennings N, Champ D, Chiang YW, Chu HJ. Equine vaccine for West Nile virus. Dev Biol (Basel) 2003; 114:221-227.

Nunes MR, Palacois G, Nunes KN, Casseb SM, et al. Evaluation of two molecular methods for the detection of yellow fever virus genome. J Virol Methods 2011; 174:29-34.

Nuttall PA, Labuda M. Tick-borne encephalitis. In: Tick-borne diseases of humans. Goodman JL, Dennis DT, Sonenshine DE (eds). Washington DC: ASM Press 2005; pp.150-163.

Ogden NH, Hailes RS, Nuttall PA. Interstadial variation in the attachment sites of *Ixodes ricinus* ticks on sheep. Exp Appl Acarol 1998; 22:227-232.

Oliphant T, Nybakken GE, Austin SK, Xu Q, et al. Induction of epitope-specific neutralizing antibodies against West Nile virus. J Virol 2007; 81:11828-11839.

Padula P, Martinez, Bellomo C, Maidana S, et al. Pathogenic hantaviruses, northeastern Argentina and eastern Paraguay. Emerg Infect Dis 2007; 13:1211-1214.

Papa A, Bakonyi T, Xanthopoulou K, Vázquez A, et al. Genetic characterization of West Nile virus lineage 2, Greece, 2010. Emerg Infect Dis 2011; 17:920-922.

Papa A, Danis K, Baka A, Bakas A, et al. Ongoing outbreak of West Nile virus infections in humans in Greece, July-August 2010. Euro Surveill 2010; 15:19644.

Patel P, Landt O, Kaiser M, Faye O, et al. Development of one-step quantitative reverse transcription PCR for the rapid detection of flaviviruses. Virol J 2013; 14:10:58.

Pecorari M, Longo G, Gennari W, Grottole A, et al. First human case of Usutu virus neuroinvasive infection, Italy, August-September 2009. *Euro Surveill* 2009; 14:19446.

Pfeffer M, Dobler G. Emergence of zoonotic arboviruses by animal trade and migration. *Parasit Vectors* 2010; 3:35.

Porterfield JS. The basis of arbovirus classification. *Med Biol* 1975; 53:400-405.

Porterfield JS. Antigenic characteristics and classification of Togaviridae. In: *The Togaviruses*. Schlesinger RW (ed). New York: Academic Press 1980; pp. 13-46.

Posadas-Herrera G, Inoue S, Fuke I, Muraki Y, et al. Development and evaluation of a formalin-inactivated West Nile virus vaccine (WN-VAX) for a human vaccine candidate. *Vaccine* 2010; 28:7939-7946.

Pulmanausahakul R, Khakpoor A, Smith DR. The development of flavivirus vaccines. *African J Biotechnol* 2010; 9:409-415.

Pybus OG, Rambaut A, Holmes EC, Harvey PH. New inferences from tree shape: numbers of missing taxa and population growth rates. *Syst Biol* 2002; 51:881-888.

Raboni SM, Levis S, Rosa ES, Bisordi I, et al. Hantavirus infection in Brazil: development and evaluation of an enzyme immunoassay and immunoblotting based on N recombinant protein. *Diagn Microbiol Infect Dis* 2007; 58:89-97.

Rappole JH, Derrickson SR, Hubálek Z. Migratory birds and spread of West Nile virus in the Western Hemisphere. *Emerg Infect Dis* 2000; 6:319-328.

Reisen WK. Epidemiology of St. Louis encephalitis virus. *Adv Virus Res* 2003; 61:139-183.

Rey FA, Heinz FX, Mandl C, Kunz C, Harrison SC. The envelope glycoprotein from tick-borne encephalitis virus at 2 Å resolution. *Nature* 1995; 375:291-298.

Rice CM, Lenches EM, Eddy SR, Shin SJ, Sheets RL, Strauss JH. Nucleotide sequence of yellow fever virus: implications for flavivirus gene expression and evolution *Science* 1985; 229:726-733.



Rift Valley fever outbreak: Interim report on the 2011 Rift Valley fever (RVF) outbreak in South Africa. 2011. Last updated on 20 May 2011. National Institute for Communicable Diseases (NICD) of the National Health Laboratory Services (NHLS). Accessed on 15 April 2013. Available from: [http://www.nicd.ac.za/?page=rift\\_valley\\_fever\\_outbreak&id=94](http://www.nicd.ac.za/?page=rift_valley_fever_outbreak&id=94)

Rodriguez LL, Maupin GO, Ksiazek TG *et al.*: Molecular investigation of a multisource outbreak of Crimean-Congo hemorrhagic fever in the United Arab Emirates 1997 *Am J Trop Med Hyg.* 57; 512-518.

Sahdev S, Khattar SK, Saini KS. Production of active eukaryotic proteins through bacterial expression systems: a review of the existing biotechnology strategies. *Mol Cell Biochem* 2008; 307:249–264.

Saijo M, Tang Q, Shimayi B, Han L, et al. Recombinant nucleoprotein-based serological diagnosis of Crimean-Congo hemorrhagic fever virus infections. *J Med Virol* 2005; 75:295-299.

Samina I, Khinich Y, Simanov M, Malkinson M. An inactivated West Nile virus vaccine for domestic geese-efficacy study and a summary of 4 years of field application. *Vaccine* 2005; 23:4955-4958.

Samudzi RR. 2008. Investigation to determine the presence of Kadam, a tick-borne virus, in southern Africa. Submitted in partial fulfillment BMedSc (Hons), 2012. University of the Free State.

Samudzi RR, Leman PA, Paweska JT, Swanepoel R, Burt FJ. Bacterial expression of Crimean-Congo hemorrhagic fever virus nucleoprotein and its evaluation as a diagnostic reagent in an indirect ELISA. *J Virol Methods* 2012; 179:70-76.

Sang R, Onyango C, Gachoya J, Mabinda E, et al. Tickborne arbovirus surveillance in market livestock, Nairobi, Kenya. *Emerg Infect Dis* 2006; 12:1074-1080.

Scaramozzino N, Crance JM, Jouan A, DeBriel DA, Stoll F, Garin D. Comparison of flavivirus universal primers pairs and development of a rapid, highly sensitive heminested reverse transcription-PCR assay for detection of flaviviruses targeted to a conserved region of the NS5 gene sequences. *J Clin Microbiol* 2001; 39:1922-1927.

Schepp-Berglind J, Luo M, Wang D, Wicker JA, et al. Complex adenovirus-mediated expression of West Nile virus C, PreM, E, and NS1 proteins induces both humoral and cellular immune responses. *Clin Vaccine Immunol* 2007; 14:1117-1126.

Scherret JH, Poidinger M, Mackenzie JS, Broom AK, et al. The relationships between West Nile and Kunjin viruses. *Emerg Infect Dis* 2001; 7:697-705.

Schneeweiss A, Chabierski S, Salomo et al., A DNA vaccine encoding the E protein of West Nile virus is protective and can be boosted by recombinant domain DIII. *Vaccine* 2011; 29: 6352-6357.

Seino KK, Long MT, Gibbs EP, Bowen RA, et al. Comparative efficacies of three commercially available vaccines against West Nile virus (WNV) in a short-duration challenge trial involving an equine WNV encephalitis model. *Clin Vaccine Immunol* 2007; 14:1465-1471.

Sekaran SD, Artsob H. Molecular diagnostics for the detection of human flavivirus infections. *Expert Opin Med Diagn* 2007; 1:521-530.

Settergren B, Juto P, Wadell G. Detection of specific serum immunoglobulin M in nephropathia epidemica (Scandinavian epidemic nephropathy) by a biotin-avidin-amplified immunofluorescence method. *J Clin Microbiol* 1987; 25:1134-1136.

Smith CE. A virus resembling Russian spring-summer encephalitis virus from an ixodid tick in Malaya. *Nature* 1956; 178:581-582.

Smithburn KC, Hughes TP, Burke AW, Paul JH. A neurotropic virus isolate from the blood of a native of Uganda. *Am J Trop Med Hyg* 1940; 20:471-492.

Smithburn KC, Paterson HE, Heymann CS, Winter PA. An agent related to Uganda S virus from man and mosquitoes in South Africa. *S Afr Med J* 1959; 33:959-962.

Song JW, Baek LJ, Schmaljohn, Yanagihara R. Thottapalyam virus, a prototype shrewborne hantavirus. *Emerg Infect Dis* 2007; 13:980-985.

Sotelo E, Llorente F, Rebollo B, Camuñas A, et al. Development and evaluation of a new epitope-blocking ELISA for universal detection of antibodies to West Nile virus. *J Virol Methods* 2011; 174:35-41.

Spohn G, Jennings GT, Martina BE, Keller I, et al. A VLP-based vaccine targeting domain III of the West Nile virus E protein protects from lethal infection in mice. *Virology* 2010;7:146.

Sun W, Eckels KH, Putnak JR, Lyons AG, et al. Experimental dengue virus challenge of human subjects previously vaccinated with live attenuated tetravalent dengue vaccines. *J Infect Dis* 2013; 207:700-708.

Swanepoel R. Wesselsbron virus disease. In: *The Arboviruses: epidemiology and ecology*. Monath, TP (ed). Boca Raton: CRC Press Inc 1989; pp. 32-57.

Swanepoel R, Burt FJ. Flaviviruses of veterinary importance. In: *Encyclopedia of Virology* (3rd edition). Mahy BWJ, van Regenmortel MHV (eds). Academic Press 2008; pp. 234-241.

Swanepoel R, Coetzer JAW. Rift Valley fever. In: *Infectious diseases of livestock with special reference to southern Africa*. Coetzer JAW, Thomson GR, Tustin RC (eds). Cape Town: Oxford University Press 1994; pp. 688-717.

Tamura K, Dudley J, Nei M, Kumar S. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* 2007; 24:1596-1599.

Tauber E, Kollaritsch H, Korinek M, Rendi-Wagner P, et al. Safety and immunogenicity of a Vero-cell-derived, inactivated Japanese encephalitis vaccine: a non-inferiority, phase III, randomised controlled trial. *Lancet* 2007; 370:1847-1853.

Theiler M, Smith HH. The use of yellow fever virus modified by *in vitro* cultivation for human immunization. *J Exp Med* 1937; 65:787-800.

Thomas SJ, Strickman D, Vaughn DW. Dengue epidemiology: virus epidemiology, ecology, and emergence. *Adv Virus Res* 2003; 61:235-289.

Tomori O, Monath TP, O'Connor EH, Lee VH, et al. Arbovirus infections among laboratory personnel in Ibadan, Nigeria. *Am J Trop Med Hyg* 1981; 30:855-861.

Ulrich R, Hjelle B, Pitra C, Krüger DH. Emerging viruses: the case 'hantavirus.' *Intervirology* 2002; 45:318-327.

Vázquez A, Sánchez-Seco MP, Ruiz S, Molero F, et al. Putative new lineage of West Nile virus, Spain. *Emerg Infect Dis* 2010; 16:549-552.

Vázquez A, Jimenez-Clavero MA, Franco L, Donoso-Mantke O, et al. Usutu virus: potential risk of human disease in Europe. *Euro Surveill* 2011; 16:19935.

Venter M, Human S, van Niekerk S, William J, et al. Fatal neurological disease and abortion in mare infected with lineage 1 West Nile virus, South Africa. *Emerg Infect Dis* 2011; 17:1534-1536.

Venter M, Human S, Zaayman D, Gerdes GH, et al. Lineage 2 West Nile virus as cause of fatal neurologic disease in horses, South Africa. *Emerg Infect Dis* 2009; 15:877-884.

Venter M, Steyl J, Human S, Weyer J, et al. Transmission of West Nile virus during horse autopsy. *Emerg Infect Dis* 2010; 16:573-575.

Virus Taxonomy online. <http://wildpro.twycrosszoo.org/s/00ref/websitescontents/w0752.htm>

Wahlberg JM, Boere WA, Garoff H. The heterodimeric association between the membrane proteins of Semliki forest virus changes its sensitivity to low pH during virus maturation. *J Virol* 1989; 63:4991-4997.

Weiss KE, Haig DA, Alexander RA. Wesselsbron virus-a virus not previously described associated with abortion in domestic animals. *Onderstepoort J Vet Res* 1956; 27:183-195.

Weiss S, Witkowski PT, Auste B, Nowak K, et al. Hantavirus in bat, Sierra Leone. *Emerg Infect Dis* 2012; 18:159-161.

Weissenböck H, Bakonyi T, Rossi G, Mani P, Nowotny N. Usutu virus, Italy, 1996. *Emerg Infect Dis* 2013; 19:274-277.

Weissenböck H, Kolodziejek J, Fragner K, Kuhn R, Pfeffer M, Nowotny N. Usutu virus activity in Austria, 2001-2002. *Microbes Infect* 2003; 5:1132-1136.

Weissenböck H, Kolodziejek J, Url A, Lussy H, Rebel-Bauder B, Nowotny N. Emergence of Usutu virus, an African mosquito-borne flavivirus of the Japanese encephalitis virus group, central Europe. *Emerg Infect Dis* 2002; 8:652-656.

Were F. The dengue situation in Africa. *Paediatr Int Child Health Suppl* 2012; 1:18-21.

Weyer J, Thomas J, Leman PA, Grobbelaar AA, Kemp A, Paweska JT. Human cases of Wesselsbron disease, South Africa 2010-2011. *Vector Borne Zoonotic Dis* 2013, 13:330-336.

Widman DG, Ishikawa T, Fayzulin R, Bourne N, Mason PW. Construction and characterization of a second-generation pseudoinfectious West Nile virus vaccine propagated using a new cultivation system. *Vaccine* 2008; 26:2762-2771.

Williams MC, Woodall JP. An epidemic of an illness resembling dengue in the Morogoro district of Tanganyika. *East Afr Med J* 1964; 41:271-275.

Whitehouse CA. Crimean-Congo hemorrhagic fever. *Antiviral Res* 2004; 63:146-160.

Witkowski PT, Klempa B, Ithete NL, Auste B, et al.. Hantaviruses in Africa. *Virus Res* 2014; 187:34-42.

Wodak E, Richter S, Bagó Z, Revilla-Fernández S, et al. Detection and molecular analysis of West Nile virus infections in birds of prey in the eastern part of Austria in 2008 and 2009. *Vet Microbiol* 2011; 149:358-366.

Wolfe MS, Calisher CH, McGuire K. Spondweni virus infection in a foreign resident of Upper Volta. *Lancet* 1982.; 2:1306-1308.

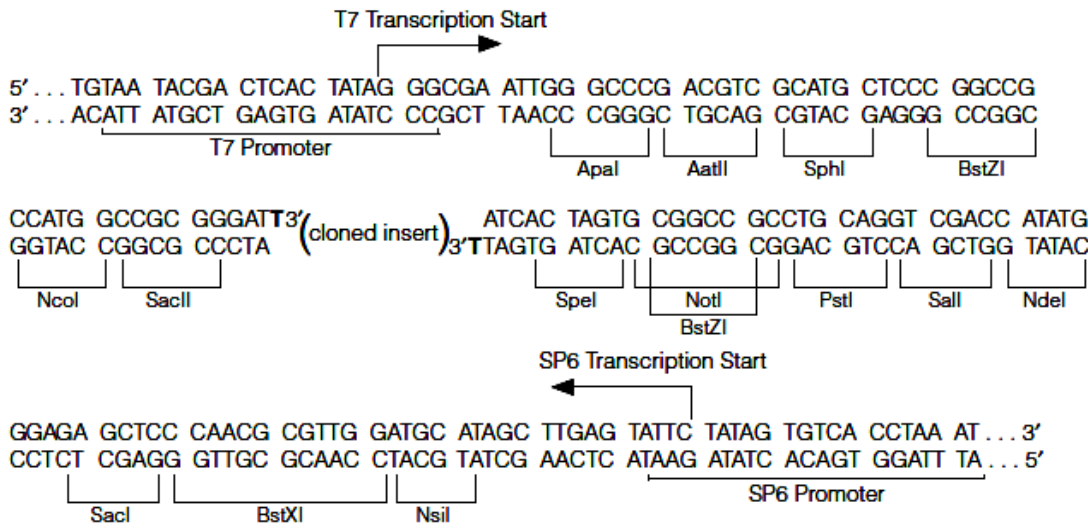
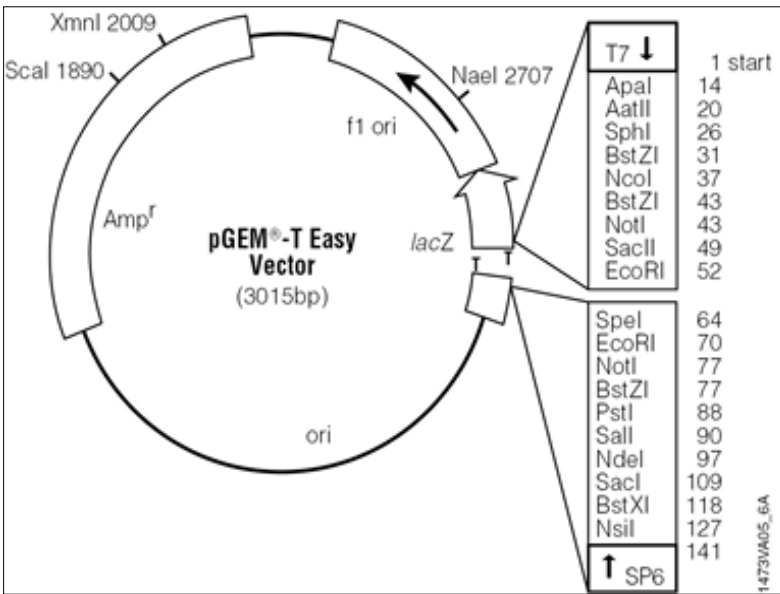
Zaayman D, Human S, Venter M. A highly sensitive method for the detection and genotyping of West Nile virus by real-time PCR. *J Virol Methods* 2009; 157:155-160.

Zaayman D, Venter M. West Nile neurologic disease in humans, South Africa, September 2008-May 2009. *Emerg Infect Dis* 2012; 18:2051-2054.

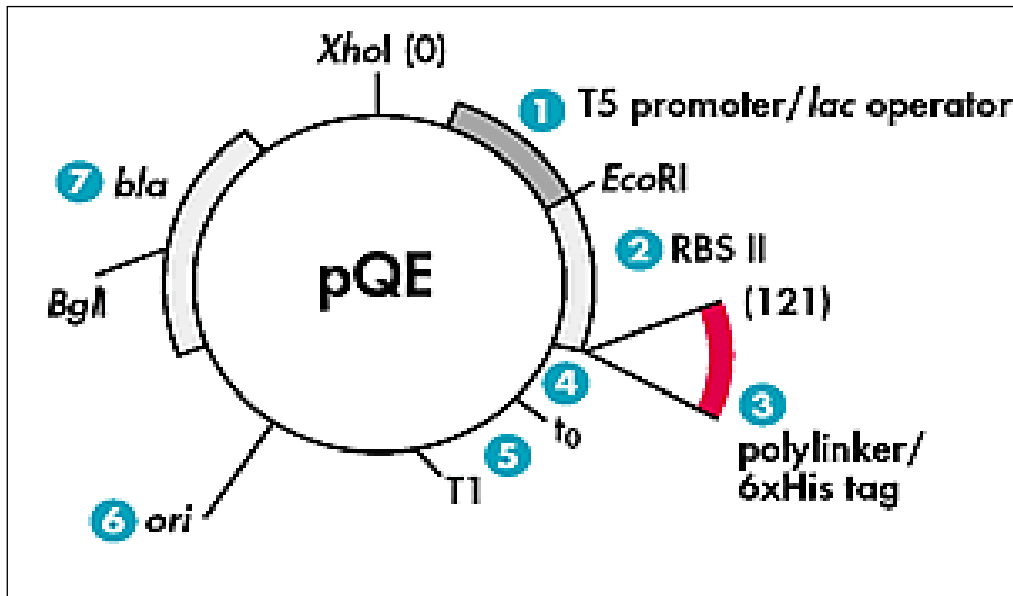
# Appendices

## Appendix A: Vector maps and multiple cloning sites for plasmids used.

### A1. Vector map and multiple cloning sites of pGEM®-T- Easy cloning vector (Promega).

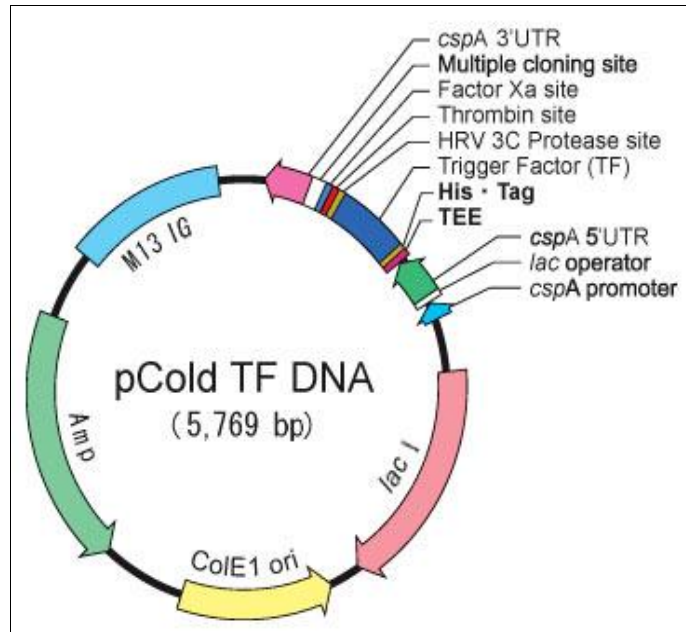


A2. Vector map and multiple cloning sites of pQE-80L expression vector (Qiagen).  
Description of the components of the vector are outlined below the vector.



Key	Description
1 Optimized promoter/operator element	Consists of the phage T5 promoter and two lacoperator sequences, which increase the probability of lac repressor binding and ensure efficient repression of the powerful T5 promoter
2 Synthetic ribosomal binding site RBSII	For efficient translation
3 6xHis-tag coding sequence	Either 5' or 3' to the polylinker cloning region
4 Translational stop codons	In all reading frames for convenient preparation of expression constructs
5 Two strong transcriptional terminators	t0 from phage lambda, and T1 from the rrnB operon of E. coli, to prevent read-through transcription and ensure stability of the expression construct
6 ColE1 origin of replication	From pBR322
7 beta-lactamase gene (bla)	Confers ampicillin resistance

A3. Vector map and multiple cloning sites of pCOLD-TF expression vector (Takara).



5' TAACGCTTCAAATCTGTAAAGCACGCCATATCGCCGAAAG

TFE -
His Tag

GCACACTTAATTATTAAGAGGTAATACACCATGAATCACAAAGTGCATCATCATCATCAC  
 SD Met Asn His Lys Val His His His His His His

pCold®-TF-F2 Primer →
pCold®-TF-F1 Primer →

ATG...Trigger Factor (1296 bp).... GCGAAAGTGACTGAAAAAGAAACCACTTCAACGAGCTGATGAACCAGCAGGCG  
 Met...Trigger Factor (432 aa)..... Ala Lys Val Thr Glu Lys Glu Thr Thr Phe Asn Glu Leu Met Asn Gln Gln Ala

HRV 3C Protease
Thrombin
Factor Xa

TCCGCGGGTCTGGAAGTTCTGTTCCAGGGGCCCTCCGCGGGTCTGGTGCCACGCGGTAGTGGTGGTATCGAAGGTAGG  
 Ser Ala Gly Leu Glu Val Leu Phe Gln Gly Pro Ser Ala Gly Leu Val Pro Arg Gly Ser Gly Gly Ile Glu Gly Arg

Nde I
Sac I
Kpn I
Xho I
BamHI
EcoRI
Hind III
Sal I
Pst I
Xba I

CATATG GAGCTC GGTACC CTCGAG GGATCC GAATTC AAGCTT GTCGAC CTGCAG TCTAGA TAGGTAATCTCTGCT  
 His Met Glu Leu Gly Thr Leu Glu Gly Ser Glu Phe Lys Leu Val Asp Leu Gln Ser Arg End

← pCold®-R Primer

TAAAAGCACAGAATCTAAGATCCCTGCCATTGGCGGGGATTTTTTTATTTGTTTTTCAGGAAATAAATAATCGAT 3'

transcription terminator



## Appendix B Alignment of flavivirus NS5 sequence data

```

LIV          ATGGCCATGACTGACACGACAGCCTTTGGGCAGCAGAGAGTGTTTAAAGAGAAAAGTTGAC 60
TBEV        ATGGCTATGACTGACACAACCGCTTTTCGGACAGCAGAGAGTGTTCAAAGATAAAGTTGAC 60
Langat      ATGGCAATGACTGACACAACCTGCATTCGGGCAGCAGAGGGTCTTTAAGGACAAAGTCGAC 60
Royal       ATGGCCATGACTGATACAACCGCATTTCGGGCAGCAGAGGGTCTTCAAAGGACAAAGTTGAT 60
Kadam       ATGGCTATGACAGACACCACGGCTTTTGGACAACAGAGAGTGTTCAAAGGAGAAAAGTCGAC 60
Yellow      ATGGCAATGACTGACACAACCCCTTTGGACAGCAGAGGGTGTTCAAAGAAAAAGTTGAC 60
Wesselsbron CTGGCCATGACTGACACAACCCCGTTTTGGGCAGCAAAGGGTGTTCAAAGAGAAAAGTTGAC 60
Zika        ATAGCCATGACTGACACCACACCATACGGCCAACAAAGAGTGTTCAAAGAAAAAGTTGAC 60
Dengue2     ATGGCAATGACAGACACGACTCCATTTGGACAACAGCGCGTTTTCAAAGAGAAAAGTTGAC 60
West        ATGGCCATGACTGACACCCTCTTTTTGGCCAGCAGCGAGTGTTTAAAGAGAAGTTGAC 60
JEVSAV      ATGGCCATGACTGACACCACCCCTTTTTGGACAGCAAAGAGTTTTTCAAAGGAGAAAAGTTGAC 60
            * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

LIV          ACAAAGCACAGGAGCCCCAACCGGGGCAAGAGTGATCACGAGAGCAGTGAATGATTGG 120
TBEV        ACAAAGGCACAGGAGCCTCAGCCCGGTACAAGAGTCATCATGAGAGCTGTAAATGATTGG 120
Langat      ACCAAGGCACAGGAGCCGCAACCCGGGACAAAATCATCATGAGAGCGGTGAATGACTGG 120
Royal       ACAAAGCTCAGGAGCCACAACCGGGAACCCGTGTCATCATGCGCTGCGTCAATGATTGG 120
Kadam       ACAAAGGTCATGAACCCAGGCCAGGCACACAGGTTATAATGCGAGCAACCAACGATTGG 120
Yellow      ACCAAGCACCAGGCTCCACCCGCGGAAACGAAAAATCATGAAAGTTGTCAACAGATGG 120
Wesselsbron ACCAAAGCCCGCCTCCACCTCCAGGAACACGTCGCGATCATGCGTGTGTCAATGCTGG 120
Zika        ACCAGGTTGCCAGATCCCCAAGAAGGCACTCGCCAGGTAATGAACATAGTCTCTTCCTGG 120
Dengue2     ACGAGAACCCAAGAACCAGAAAGGACGAAAAAACTAATGAAAATCACGGCAGAATGG 120
West        ACGAAAGTCCAGAGCCTCCAGAGGGAGTCAAGTACGTCCTCAATGAGACGACAACTGG 120
JEVSAV      ACGAAGGTCCTGAGCCACAGCTGGAGCCAAGGAAGTGTCAACGAGACCACCAACTGG 120
            ** * * * * * * * * * * * * * * * * * * * * * * * * * * *

LIV          ATTCTAGAGCGTCTGGCCAGAAAAGCAAACCACGCATGTGCAGCAGAGAGGAATTCATA 180
TBEV        ATTTTGGAAACGACTGGCGCAGAAAAGCAAACCAGCATGTGCAGCAGGGAAGAATTCATA 180
Langat      CTGCTTGAGCGACTGGTCAAGAAGAGTCGGCCACGCATGTGCTCCAGGGAGGAGTTTCATA 180
Royal       ATGATGGATAGACTGGCAAAAAGGAGCAAGCCGCGCTGTGCACAAAAGAAGAGTTTTGC 180
Kadam       CTACTTGAGAGACTAGTAAAGAAAAGGAAGCCAGGATGTGCTCCCGGGAGGAATTCAGA 180
Yellow      CTCTTCCGTCACTTGGCCAGGGAGAAGAATCCTAGACTGTGCACAAAAGAAGAATTCATT 180
Wesselsbron TTGTTC AACACCTCGCTCGTAAGAAGAAGCCCCGCATTTGCACGCGTGAAGAGTTTTGTG 180
Zika        TTGTGGAAGGAGCTGGGGAACGCAAGCGGCCACGCGTCTGCACCAAAGAAGAGTTTTATC 180
Dengue2     CTCTGGAAGAATTAGGAAAAGAAAAGACACCTAGGATGTGCACCAGAGAAGAATTCACA 180
West        TTGTGGGCTTACTTAGCTCGAGAGAAAAAACCGAGGATGTGCTCCCGAGAAGAGTTTTATC 180
JEVSAV      CTGTGGGCCACTTGTACGGGAAAAAAGACCCCGCTTGTGCACCAAAGAAGAATTCATA 180
            * * * * * * * * * * * * * * * * * * * * * * * * * * *

LIV          GCAAAGTGAGATCAAATGCAGCCCTGGGAGCTTGGTGGATGAGCAGAACAGATGGGCA 240
TBEV        GCAAAGTGAAATCAAATGCAGCCTTGGGAGCTTGGTTCAGATGAGCAAACAGATGGGCA 240
Langat      GCGAAAGTCCGTTTCAATGCGGCCCTTGGCGCTTGGTTCAGATGAGCAAACAAATGGAAG 240
Royal       GCAAAGTGAGATCAAATGCAGCCTTGGGAGCATGGACAGATGAGCAGAATATATGGAAG 240
Kadam       GAGAAGTGCGGTCAAATGCCGCTCTGGGTGCCTGGCTTGATGAGCAGAACCAGTGGAAA 240
Yellow      GCAAAGTCCGCAGCCACGCAGCCATTGGAGCCTACCTGGAAGAACAAGAACAGTGGAAAG 240
Wesselsbron GCCAAAGTTTCGTAGCCATGCCGCCCTTGGAGCGTATCTCGAAGAGCAGGACAAATGGAAG 240
Zika        AACAAAGTGCGCAGCAATGCAGCACTGGGAGCAATATTGAAAGAGGAAAAAAGAATGGAAG 240
Dengue2     AGAAAGTGAGAAGCAATGCAGCCTTAGGTGCCATATTCAGTATGAGAACAAGTGGAAAG 240
West        AGTAAAGTCAACAGCAACGCGCTCTGGGAGCAATGTTTGAAGAACAGAACCAATGGAAA 240
JEVSAV      AAGAAAGTCAACAGCAACGCGCTCTTGGAGCAGTGTTCGCTGAACAGAATCAATGGAGC 240
            ** * * * * * * * * * * * * * * * * * * * * * * * * * * *

LIV          AGTGCGAGAGAGGCTGTGGTGGTCCCTGCATTCTGGGCCCTCGTGGATGAGGTGAGAGAG 300
TBEV        AGTGCAAGAGAGGCTGTAGAGGATCCTGCATTCTGGCGCCTCGTGGATGAAGAGAGAGAA 300
Langat      AGTGCGAGAGAAGCAGTGGAAAGATCCTGAGTTCTGGAGTCTTGTGAGGCTGAGAGAGAG 300
Royal       AACGCCAGGGAAGCTGTGGAAGACCCAGGATTTTGGAACTTGTGATGCTGAGCGTGAG 300
Kadam       ACCGCCCTGAGGCTGTGAGGACCCAGCTTCTGGAATCTTGTGAGAAAAGAACGTGAG 300
Yellow      ACTGCCAACGAGGCTGTCCAGGACCCGAAGTTTTGGGAATTGGTGGATGAAGAAAGGAGG 300
Wesselsbron AGTGCAAGTGAAGCTGTCCAGGATCCACAGTCTTGGAACTGGTTGATGATGAGAGGAAG 300
Zika        ACGGCTGTGGAAGCTGTGAATGATCAAGTTTTGGGCCCTAGTGGATAGGAGAGAGAA 300
Dengue2     TCGGCACGTGAGGCTGTTGAAGATAGTAGGATTTTGGGAAGTGGTTGACAAGGAAAGGAAT 300

```

```

West          AATGCCAGAGAGGCTGTAGAAGACCCGAAGTTTTGGGAGATGGTGGACGAGGAGCGCGAG 300
JEVSAV       ACGGCGCGTGAGGCTGTGGATGACCCGCGGTTTTGGGAGATGGTTGATGAAGAGAGGGAA 300
              **      ** ** ** *          ** ***      * ** ** *      *
LIV          AGGCATCTCGTGGGGTGGTGCACAC TGCGTGTACATCATGATGGGCA TGAGAGAAAAG 360
TBEV        AGGCACCTCATGGGGAGATGTGCGCACTGCGTGTACAACATGATGGGCAAGAGAGAAAAG 360
Langat      CGCCACTTGCAGGGGAGATGCGCCCATTTGTGTGTACAACATGATGGGAAAAGAGAGAAA 360
Royal      CTACACAGGCAGGGAAGATGTGCTCAATGCGTATAACAACATGATGGGAAAGCGTGAGAAG 360
Kadam      TTGCACCTCACGGGACGTTGCCAGCAGTGCCTGTATAACATGATGGGAAAAGAGAGAAA 360
Yellow     CTGCACCAACAAGGCAGGTGCCGGACTTGCCTGTACAACATGATGGGAAAAGAGAGAAG 360
Wesselsbron  CTGCATCTGCAAGGCCAATGCCGGACATGTGTG TACAACATGATGGGGAAACG TGAAAAG 360
Zika       CACCACCTGAGAGGAGAGTGTACAGCTGTGTGTACAACATGATGGGAAAAGAGAGAAAAG 360
Dengue2    CTTCATCTGAAGGAAAGTGTGAGACATGTGTGTACAACATGATGGGAAAAGAGAGAGAAG 360
West       GCTCACCTTCGCGGCGAATGTAACACCTGCATTTACAACATGATGGGCAAGCGTGAGAAG 360
JEVSAV     AACCATCTGCGAGGAGAGTGTACACATGTATC TACAACATGATGGG AAAAAGAGAGAAG 360
              **      **      **          ** * ** * ***** *      * ** **
LIV          AAACCTGGGAGAGTTCCGAGTGGCGAAGGGCAGCAGG GCCATCTGGTACATGTGG 414
TBEV        AAACCTGGGAGAGTTCCGAGTGGCGAAAGGAAGTCCGGCCATTTGGTACATGTGG 414
Langat      AAGCTTGGTGAATTTGGAGTAGCCAAGGGCAGCAGGGCCATCTGGTACATGTGG 414
Royal      AAGCTGGGAGAATTTGGCTCGGCCAAAGGAAGCAGAGCCATATGGTACATGTGG 414
Kadam      AAACGTGGGGAGTTTGGCGTCGCGAAGGGAAGCCGAGCCATCTGGTACATGTGG 414
Yellow     AAGTTGTCAGAGTTTGGGAAAGCAAAGGAAGCCGTGCCATCTGGTACATGTGG 414
Wesselsbron  AAACCTCTGAATTTGGCAAAGCAAAGGAAGCAGGGCGATCTGGTACATGTGG 414
Zika       AAGCAAGGAGAGTTCCGGGAAAGCAAAGGTAGCCGCGCCATCTGGTACATGTGG 414
Dengue2    AAGCTAGGGGAGTTCCGGCAAAGCAAAGGCAGCAGAGCCATATGGTACATGTGG 414
West       AAACCAGGAGAGTTTGGCAAAGCCAAGGTTAGTAGAGCCATTTGGTTTATGTGG 414
JEVSAV     AAGCCTGGGAGAGTTTGGAAAAGCTAAAGGAAGCAGGGCCATTTGGTTCATGTGG 414
              **      ** ** ** *          ** ** ** ** * ** ** *****

```

Forward primer is highlighted in yellow **ATGGCCATGACTGACAC**

Reverse primers are highlighted in blue **GCCATCTGGTACATGTGG**; **TGCGTGTACATCATGATGGGCA**;  
**TACAACATGATGGGGAAACG**

LNA probe is shown in red **TACAACATGATGGG**

## Appendix C. Alignment of hantavirus sequence data for design of primers and probes

Forward and reverse primers are highlighted in yellow

```

HTNV      TAGTAGTAGACTCCCTAAAGAGCTACTGTAACAACGATG-----GCAACTATGGAG 51
SANGV     TAGTAGTAGACTCCCTAAAGAGCCTGCAAACAAAGAAGGAAAAAATGGCAACTAGAG 60
SEOV      TAGTAGTAGACTCCCTAAAGAGCTACTCCACTAACAAGAG---AAATGGCAACTATGGAG 57
ANDV      TAGTAGTAGACTCCTTGAGAAGCTACTGCTGCGAAAGCTG---GAATGAGCACCTCCAA 57
SNV       TAGTAGTAGACTCCTTGAGAAGCTACTACGACTAAAGCTG---GAATGAGCACCTCAA 57
PUUV      TAGTAGTAGACTCCTTGAAAAGCTACTACGAGAACAACCTG---GAATGAGTGACTTGACA 57
          ***** *..**:. *.. . . * ..

HTNV      GAACTGCAGAAGGAAATCAATGCCCATGAGGGCCAATTGGTAATAGCCAGGCAGAAGGTG 111
SANGV     GAGATCCAAAAGGAAATCAACATCCATGAGGGTCAACTAATTATAGCAAAGCAAAGGTG 120
SEOV      GAAATCCAGAGAGAAATCAGTGTCTACGAGGGGAGCTTGTGTATAGCACGCCAGAAGGTC 117
ANDV      GAATTGCAGGAAAACATCACAGCACACGAACAACAGCTCGTGACTGCTCGGCAAAAGCTT 117
SNV       GAAGTGCAGACAACATCACTCTCCACGAACAACAACCTCGTGACTGCCAGGCAGAAGCTC 117
PUUV      GACATCCAAGAGGAGATAACCCGCGATGAGCAACAACCTTGTGTGCCAGACAAAACCTC 117
          ** * .. . * ** * ** * .. * .. : ** .. * .. *

HTNV      AGGGATGCAGAAAAGCAGTATGAGAAGGACCCAGATGAGCTGAATAAGAGAGCATTACA 171
SANGV     AAGGATGCTGAAAACAGTATGAGAAGGATCCTGATGACCTTAATAAGAGGGCACTCAGT 180
SEOV      AAGGATGCAGAAAAGCAGTATGAGAAGGATCCTGATGACTTAAACAAGAGGGCACTGCAT 177
ANDV      AAGGATGCCGAGAAGGCAGTGGAGGTGGACCCGGATGACGTTAACAAAGAGCACACTACA 177
SNV       AAGATGCAGAAAAGAGCGGTGGAATTGGACCCCGATGATGTTAACAAAAGCACATTACAG 177
PUUV      AAGGATGCAGAGAGAGCAGTGGAAAGTGGACCCGGATGACGTTAACAAAACACACTACA 177
          ..***** **.*.. .. : ** : ** * * * * * * * * * * * * * *

```

Probes are highlighted in following sequences in yellow

```

HTNV      TAGTAGTAGACTCCCTAAAGAGCTACTG---TAACAACG---ATGGCAACTATGGAGGAA 54
SEOV      TAGTAGTAGACTCCCTAAAGAGCTACTCCACTAACAAGAGAAATGGCAACTATGGAGGAA 60
          ***** . *****

HTNV      CTGCAGAAGGAAATCAATGCCCATGAGGGCCAATTGGTAATAGCCAGGCAGAAGGTGAGG 114
SEOV      ATCCAGAGAGAAATCAGTGTCTACGAGGGGAGCTTGTGTATAGCACGCCAGAAGGTCAAG 120
          .* *****.*****.*** ** ***** *. * **.******.* ***** **

HTNV      GATGCAGAAAAGCAGTATGAGAAGGACCCAGATGAGCTGAATAAGAGAGCATTAAACAGAC 174
SEOV      GATGCAGAAAAGCAGTATGAGAAGGATCCTGATGACTTAAACAAGAGGGCACTGCATGAT 180
          ********** ** : ***** *..* ***** .*** *..: **

ANDV      TAGTAGTAGACTCCTTGAGAAGCTACTGCTGCGAAAGCTGGAATGAGCACCTCCAAGAA 60
SNV       TAGTAGTAGACTCCTTGAGAAGCTACTACGACTAAAGCTGGAATGAGCACCTCAAAGAA 60
PUUV      TAGTAGTAGACTCCTTGAAAAGCTACTACGAGAACAACCTGGAATGAGTGACTTGACAGAC 60
          **********.* . *..***** . * * ..**

ANDV      TTGCAGGAAAACATCACAGCACACGAACAACAGCTCGTGACTGCTCGGCAAAAGCTTAAG 120
SNV       GTGCAAGACAACATCACTCTCCACGAACAACAACCTCGTGACTGCCAGGCAGAAGCTCAA 120
PUUV      ATCCAAGAGGAGATAACCCGCGATGAGCAACAACCTTGTGTGCCAGACAAAACCTCAAG 120
          * **.* * * * * * * * * * * * * * * * * * * * * * * * * * *

ANDV      GATGCCGAGAAGGCAGTGGAGGTGGACCCGGATGACGTTAACAAAGAGCACACTACAAGT 180
SNV       GATGCAGAAAAGAGCGGTGGAATGGACCCCGATGATGTTAACAAAAGCACATTACAGAGC 180
PUUV      GATGCAGAGAGAGCAGTGGAAAGTGGACCCGGATGACGTTAACAAAACACACTACAAGCA 180
          *****.*.*..*..*****.***** ***** **********.*.***** *****

SANGV     TAGTAGTAGACTCCCTAAAGAGCACTGCAAACAAAGAAGGAAAAATGGCAACTAGAG 60

SANGV     GAGATCCAAAAGGAAATCAACATCCATGAGGGTCAACTAATTATAGCAAAGCAAAGGTG 120

SANGV     AAGGATGCTGAAAACAGTATGAG----- 144

```

## Appendix D Sequence determined for CCHF from patient 24

Sequence determined for CCHF from patient 24

5' to 3' from positions 299 to 533 of S gene relative to South Africa isolate SPU407/88 with 91% identity

```
CCTGGGTTAGGTCCCCTGGCCTTGTGAAGAGGGGGCTTGAATGGTTTGAAAAAATGCGGGC  
CCCCTTAAGTTTTGGGGTGAAAGTTATACTGAGCTAAAAGTTGGCGTCCCGAAAATAGAACAAC  
TTGCCAATTCCAACAAGCTGCCTTGAAATGGAGAAAGGACATAGGTTTCCGGGTCAATGCAA  
CACCAGCGGTTTTAACCAACAAACTCCTCAAAAATTACAGGGTTCCTG
```

## Appendix E Raw data for hantavirus ELISA

HANTAVIRUS AMERICA												
Dual wave data (difference)												
<>	1	2	3	4	5	6	7	8	9	10	11	12
A	0.2730	0.0220	0.0790	0.1030	0.0360	0.1230	0.0100	0.0160	0.0970	0.0140	0.0110	1.4470
B	0.0240	0.0210	0.0130	0.0040	0.0080	0.1150	0.0020	0.0870	0.0270	0.0190	0.0570	0.3260
C	0.0860	0.0390	0.0580	0.0410	0.0360	0.1510	0.1610	0.0880	0.0140	0.0250	0.0130	0.0620
D	0.1160	0.1340	0.0520	0.0990	0.0690	0.0240	0.0860	0.1240	0.0430	0.1830	0.0280	1.0170
E	0.4190	0.0710	0.0550	0.1590	0.0160	0.0650	0.0960	0.0430	0.0310	0.0320	0.0490	1.0280
F	1.5510	0.1460	0.0550	0.0910	0.1040	0.0270	0.1630	0.1350	0.0130	0.0310	0.1720	0.0110
G	0.0270	0.0630	0.0780	0.0170	0.1190	0.0280	0.0940	0.0510	0.1000	0.0130	0.0570	0.0120
H	0.1500	0.0420	0.2420	0.0690	0.0840	0.1460	0.0550	0.0680	0.4200	0.0780	0.1100	0.0050
	<b>Ratio</b>											
	0.837	0.067	0.242	0.316	0.110	0.377	0.031	0.049	0.298	0.043	0.034	4.439
	0.074	0.064	0.040	0.012	0.025	0.353	0.006	0.267	0.083	0.058	0.175	1.000
	0.264	0.120	0.178	0.126	0.110	0.463	0.494	0.270	0.043	0.077	0.040	0.190
	0.356	0.411	0.160	0.304	0.212	0.074	0.264	0.380	0.132	0.561	0.086	3.120
	<b>1.285</b>	0.218	0.169	0.488	0.049	0.199	0.294	0.132	0.095	0.098	0.150	3.153
	<b>4.758</b>	0.448	0.169	0.279	0.319	0.083	0.500	0.414	0.040	0.095	0.528	0.034
	0.083	0.193	0.239	0.052	0.365	0.086	0.288	0.156	0.307	0.040	0.175	0.037
	0.460	0.129	0.742	0.212	0.258	0.448	0.169	0.209	<b>1.288</b>	0.239	0.337	0.015
			pos	3								
			eqv	1								
HANTAVIRUS AMERICA												
Dual wave data (difference)												
<>	1	2	3	4	5	6	7	8	9	10	11	12
A	0.1770	0.0420	0.1420	0.4370	0.0420	0.0420	0.0510	0.0390	0.0110	0.0360	0.0300	1.3170
B	0.1070	0.1780	0.0580	0.1060	0.0390	0.0150	0.2860	0.0650	0.1690	0.0320	0.1870	0.2620
C	0.0130	0.3090	0.0610	0.0260	0.0680	0.0180	0.0830	0.0330	0.2530	0.0710	0.0770	0.0560
D	0.2910	0.0940	0.0930	0.0630	0.1470	0.0090	0.1820	0.1020	0.1480	0.0250	0.0560	0.8540
E	0.0480	0.0250	0.0830	0.3090	0.0150	0.1740	0.1440	0.0180	0.1380	0.0280	0.0110	0.9300
F	0.4610	0.5300	0.1340	0.1060	0.0320	0.0890	0.0210	0.0490	0.0290	0.0390	0.0350	0.0120
G	0.0880	0.2530	0.0800	0.1130	0.1500	0.0140	0.0550	0.0080	0.0200	0.0480	0.0280	0.0120
H	0.0320	0.0960	0.0400	0.0270	0.0060	0.4670	0.0550	0.0210	0.0210	0.1380	0.0420	0.0050
	<b>Ratio</b>											
	0.676	0.160	0.542	<b>1.668</b>	0.160	0.160	0.195	0.149	0.042	0.137	0.115	5.027
	0.408	0.679	0.221	0.405	0.149	0.057	<b>1.092</b>	0.248	0.645	0.122	0.714	1.000
	0.050	<b>1.179</b>	0.233	0.099	0.260	0.069	0.317	0.126	0.966	0.271	0.294	0.214
	<b>1.111</b>	0.359	0.355	0.240	0.561	0.034	0.695	0.389	0.565	0.095	0.214	3.260
	0.183	0.095	0.317	<b>1.179</b>	0.057	0.664	0.550	0.069	0.527	0.107	0.042	3.550
	<b>1.760</b>	<b>2.023</b>	0.511	0.405	0.122	0.340	0.080	0.187	0.111	0.149	0.134	0.046
	0.336	0.966	0.305	0.431	0.573	0.053	0.210	0.031	0.076	0.183	0.107	0.046
	0.122	0.366	0.153	0.103	0.023	<b>1.782</b>	0.210	0.080	0.080	0.527	0.160	0.019
		pos	<b>8 total</b>		<b>11</b>	<b>6.3%</b>						
		eqv	1		<b>2</b>	<b>1.1%</b>						

HANTAVIRUS EURASIA												
Dual wave data (difference)												
<>	1	2	3	4	5	6	7	8	9	10	11	12
A	0.1800	0.0430	0.0780	0.1820	0.0610	0.0560	0.1000	0.0610	0.0120	0.0450	0.0730	1.2430
B	0.1140	0.1250	0.0800	0.0880	0.0760	0.0160	0.5940	0.1350	0.3010	0.0430	0.1310	0.2190
C	0.0240	0.3590	0.0530	0.0640	0.1550	0.0420	0.1040	0.0420	0.1860	0.0570	0.0740	0.0350
D	0.4730	0.1350	0.1240	0.0980	0.0450	0.0130	0.1430	0.0490	0.1050	0.0390	0.0410	0.8830
E	0.0410	0.0340	0.0770	0.4850	0.0210	0.1980	0.2150	0.0460	0.0590	0.0470	0.0140	0.8920
F	0.5770	0.1400	0.1060	0.1080	0.0690	0.1140	0.0360	0.0590	0.0380	0.0810	0.0330	0.0130
G	0.1460	0.2450	0.1080	0.1760	0.1630	0.0280	0.1050	0.0160	0.0220	0.0620	0.0410	0.0120
H	0.0450	0.2430	0.0590	0.0430	0.0090	0.3100	0.0570	0.0320	0.0150	0.2580	0.0330	0.0070
	<b>Ratio</b>											
	0.822	0.196	0.356	0.831	0.279	0.256	0.457	0.279	0.055	0.205	0.333	5.676
	0.521	0.571	0.365	0.402	0.347	0.073	2.712	0.616	1.374	0.196	0.598	1.000
	0.110	1.639	0.242	0.292	0.708	0.192	0.475	0.192	0.849	0.260	0.338	0.160
	2.160	0.616	0.566	0.447	0.205	0.059	0.653	0.224	0.479	0.178	0.187	4.032
	0.187	0.155	0.352	2.215	0.096	0.904	0.982	0.210	0.269	0.215	0.064	4.073
	2.635	0.639	0.484	0.493	0.315	0.521	0.164	0.269	0.174	0.370	0.151	0.059
	0.667	1.119	0.493	0.804	0.744	0.128	0.479	0.073	0.100	0.283	0.187	0.055
	0.205	1.110	0.269	0.196	0.041	1.416	0.260	0.146	0.068	1.178	0.151	0.032
		pos		10								
		equ		6								
Dual wave data (difference)												
<>	1	2	3	4	5	6	7	8	9	10	11	12
A	0.2490	0.0360	0.1760	0.2170	0.0920	0.2530	0.0150	0.0290	0.2540	0.0240	0.0200	1.4440
B	0.0610	0.0100	0.0330	0.0090	0.0330	0.1140	0.0060	0.1170	0.0280	0.1670	0.2320	0.2910
C	0.1280	0.0980	0.1610	0.0920	0.0440	0.1890	0.1570	0.0830	0.0250	0.1410	0.0570	0.0430
D	0.1570	0.3070	0.1000	0.1800	0.0880	0.0260	0.1680	0.1660	0.4770	0.6470	0.0370	0.9630
E	0.4400	0.1540	0.1340	0.1560	0.0250	0.1360	0.2470	0.0990	0.0660	0.0640	0.1500	0.9930
F	1.0170	0.1860	0.1060	0.3500	0.1600	0.1400	0.3320	0.3070	0.0140	0.0390	0.1670	0.0150
G	0.0360	0.1420	0.0770	0.0450	0.1410	0.0330	0.0780	0.1080	0.1240	0.0510	0.0840	0.0140
H	0.2550	0.0310	0.3460	0.0990	0.1250	0.0810	0.0580	0.0720	0.0920	0.0940	0.0810	0.0100
	<b>Ratio</b>											
	0.856	0.124	0.605	0.746	0.316	0.869	0.052	0.100	0.873	0.082	0.069	4.962
	0.210	0.034	0.113	0.031	0.113	0.392	0.021	0.402	0.096	0.574	0.797	1.000
	0.440	0.337	0.553	0.316	0.151	0.649	0.540	0.285	0.086	0.485	0.196	0.148
	0.540	1.055	0.344	0.619	0.302	0.089	0.577	0.570	1.639	2.223	0.127	3.309
	1.512	0.529	0.460	0.536	0.086	0.467	0.849	0.340	0.227	0.220	0.515	3.412
	3.495	0.639	0.364	1.203	0.550	0.481	1.141	1.055	0.048	0.134	0.574	0.052
	0.124	0.488	0.265	0.155	0.485	0.113	0.268	0.371	0.426	0.175	0.289	0.048
	0.876	0.107	1.189	0.340	0.430	0.278	0.199	0.247	0.316	0.323	0.278	0.034
		pos		7	total	17	9.7%					
		equ		6		12	6.8%					

## Appendix F Ethics statement and approval

Ethics approval was obtained from the University of the Free State Ethics Committee: Ethics number 118/06

The animal work was approved by the University of the Free State Animal Ethics Committee. All animal experiments were carried out in accordance with the regulations and guidelines stipulated by the University of the Free State Animal Ethics Committee. Approval number: 24/2011