UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

IMPROVED TOOLS FOR THE DIAGNOSIS OF RIFT VALLEY FEVER AND SEROLOGICAL EVIDENCE OF THE DISEASE IN SHEEP AND GOATS IN THE INTER-EPIDEMIC PERIOD IN THE ZAMBÉZIA PROVINCE, MOZAMBIQUE

BY

JOSÉ MANUEL FAFETINE

Submitted in partial fulfilment of the requirements for the degree of

Doctor of Philosophy in the Department of Veterinary Tropical Diseases,

Faculty of Veterinary Science, University of Pretoria

March, 2012

© University of Pretoria

Acknowledgements

I would like to express my sincere appreciation and gratitude to my supervisors Professor Koos Coetzer, Professor Victor P.M.G. Rutten, and Professor Janusz T. Paweska for their input, commitment, patience and guidance throughout the work and in writing of this thesis. My thanks are extended to Professor Luis Neves for the support and helpful advice that were crucial in different stages of the implementation of this study.

I am very grateful to all the staff of the Cytokine Centre, Utrecht University; Centre for Emerging and Zoonotic Diseases of the National Institute for Communicable Diseases; Institute of Hygiene and Tropical Medicine, Universidade Nova de Lisboa; Veterinary Faculty and Biotechnology Center, Eduardo Mondlane University; and Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria for the technical assistance. I would like to make a special mention to Prof. Peter Thompson, Prof. Ana Domingos and to Dr. Fernando Chanisso for their input and assistance in the serological survey and in the production of monoclonal antibodies.

I would also like to acknowledge the colleagues in the Zambézia Veterinary Services particularly to Dr. João Maria Severiano and to Mr. Vasco Nofre for their support in the implementation of the field work. I would particularly like to mention Dr Rogério Herinques for his invaluable help in providing the animals for the longitudinal study.

This work would not have been possible without the financial support of the International Foundation for Science -IFS (Sweden); SIDA/SAREC (Sweden); the Utrecht University; the Department of Veterinary Tropical Diseases – University of Pretoria and the Italian Project at the Biotechnology Center –UEM (AID 9397).

To my parents, brothers and sisters thanks for the friendship and support. Finally, I am endlessly grateful to Malaika, Eric and Natércia for their love.

© University of Pretoria

List of Contents

Acknowledgements 2
List of Contents 3
List of Figures 6
List of Tables 7
List of Abbreviations 8
Summary 9
1 . General introduction 12
1.1 Introduction 12
1.2 Aetiology 13
1.3 Vectors and maintenance of RVFV 14
1.4 Major recent epidemics 16
1.5 Rift Valley fever in Mozambique 17
1.6 Rift Valley fever in domestic animals 17
1.7 Rift Valley fever in humans 18
1.8 Diagnostic assays 18
1.8.1 Virus isolation and detection 19
1.8.2 Detection of RVFV specific antibodies 20
1.9 Outline of the thesis 22
1.10 References 22
2 . Cloning and expression of Rift Valley fever virus nucleocapsid (N) protein and evaluation of a N-protein based indirect ELISA for the detection of specific IgG and IgM antibodies in domestic ruminants. 33
2.1 Introduction 33
2.2 Materials and Methods 34
2.2.1 Virus 34
2.2.2 Construction of plasmids 34
2.2.3 Cloning and expression of the N-protein 35
2.2.4 Expression and purification of the his6-tagged NusA-N-protein 36
2.2.5 Sera 37
2.2.6 Virus neutralisation and haemagglutination-inhibition tests 38
2.2.7 ELISA internal controls 38

© University of Pretoria

2.2.8 Anti-ruminant immunoglobulin and recombinant protein G horseradish peroxidase (HRPO) conjugates ... 38

2.2.9 Procedures for the indirect ELISA (I-ELISA) ... 39

2.2.10 Selection of cut-offs and estimation of diagnostic accuracy ... 39

2.3 Results ... 40

2.3.1 Cloning, expression and purification of the N-protein ... 40

2.3.2 Analytical evaluation of the N-protein based indirect ELISA for detection of IgM and IgG anti-RVFV ... 41

2.3.3 Determination of cut-off values ... 45

2.3.4 Diagnostic accuracy ... 45

2.4 Discussion ... 46

2.5 References ... 48

3 . The detection of antibodies to Rift Valley Fever virus in small ruminants: comparison of an indirect recombinant nucleocapsid ELISA for IgG with an IgG sandwich ELISA utilizing inactivated virus ... 54

3.1 Introduction ... 54

3.2 Material and methods ... 55

3.2.1 Test sera ... 55

3.2.2 IgG indirect ELISA ... 55

3.2.3 IgG sandwich ELISA ... 56

3.2.4 Estimation of diagnostic accuracy ... 57

3.3 Results ... 57

3.4 Discussion ... 58

3.5 References ... 60

4 . Generation and characterization of monoclonal antibodies against Rift Valley fever virus nucleoprotein ... 63

4.1 Introduction ... 63

4.2 Materials and methods ... 65

4.2.1 Preparation of recombinant RVFV nucleocapsid protein ... 65

4.2.2 Production and purification of the monoclonal antibodies ... 65

4.2.3 Indirect ELISA ... 66

4.2.4 Antibody purification ... 66

4.2.5 Determination of the Ig class of hybridoma antibodies ... 67

4.2.6 Western blot assay ... 67

© University of Pretoria

4.2.7 Assessment of monoclonal antibodies cross-reactivity 68
4.3 Results 68
4.4 Discussion 70
4.5 References 72
5 . Serological evidence of Rift Valley fever virus circulation in Zambézia Province, Mozambique 77
5.1 Introduction 77
5.2 Materials and methods 78
5.2.1 Site description 78
5.2.2 Animals and sampling 80
5.2.3 Cross-sectional surveys 80
5.2.4 Longitudinal assessment of inter-epidemic transmission of RVF 80
5.2.5 Laboratory tests 81
5.2.6 Statistical analysis 82
5.3 Results 83
5.3.1 Cross-sectional surveys 83
5.3.2 Assessment of inter-epidemic transmission of RVFV 88
5.4 Discussion 88
5.5 References 90
6 . Concluding remarks and recommendations 95

© University of Pretoria

List of Figures

Fig.2.1...41
Fig.2.2 41-42
Fig.2.3 43
Fig.2.4 44
Fig.2.5 44
Fig.2.6. 45
Fig.4.1...69
Fig.4.2 70
Fig.5.1...79

© University of Pretoria

List of Tables

Table1.1.....15
Table2.1.....46
Table3.1.....58
Table5.1.....84
Table5.2.....85
Table5.3.....85
Table5.4.....86
Table5.5.....87
Table5.6.....88

© University of Pretoria

List of Abbreviations

ABTS - 2,2'-azinodiethylbenzothiazoline sulfonic acid

BHK - baby hamster kidney

CBD - chitine binding domain

CER - chicken embryo related

DNA – desoxyribonucleic acid

EDTA – ethylenediamine tetra acetic acid

ELISA - enzyme-linked immunosorbent assay

HI - haemagglutination-inhibition test

HRPO - horseradish peroxidase

IgM – immunoglobulin M

IgG – immunoglobulin G

IPTG - isopropyl-β-D-thiogalactopyranoside

mg – miligram

ml - mililitre

OD - optical density

OR – odds ratio

PBS - phosphate buffered saline

PCR – polymerase chain reaction

PP - percentage of the high-positive control serum

RNA – ribonucleic acid

RT-LAMP - reverse-transcription loop-mediated isothermal amplification

RT-PCR – reverse transcriptase polymerase chain reaction

RVF – Rift Valley fever

RVFV – Rift Valley fever virus

SDS - sodium dodecyl sulphate

SPU-NICD - Special Pathogens Unit, National Institute for Communicable Diseases

TG-ROC - two-graph receiver operating characteristics analysis

VN - virus neutralization test

μ – micro

© University of Pretoria

Summary

Title: *Improved tools for the diagnosis of Rift Valley fever and serological evidence of the disease in sheep and goats in the inter-epidemic period in the Zambézia Province, Mozambique*

By: José Manuel Fafetine

Promoter: Victor P.M.G. Rutten
Co-Promoters: Janusz T. Paweska and J.A.W. Coetzer
Degree: Doctor of Philosophy in Veterinary Science

Rift Valley fever (RVF) is primarily a mosquito-borne disease of domestic ruminants and humans caused by a virus of the family *Bunyaviridae*, genus *Phlebovirus.* The causative agente, RVF virus (RVFV), has been responsible for large epidemics in livestock in Africa and in the Arabic Peninsula with significant economic impact due to high mortality rates particularly in new-born lambs and kids and abortion in sheep, goats and cattle. The disease in humans is characterized by a mild to moderate febrile illness but severe complications such as ocular sequelae, encephalitis or a fatal haemorrhagic state, may occur in a low proportion of individuals. Apart from reports of the disease in 1969 and 1999 when RVF caused abortions and deaths in cattle and water buffalos respectively and the survey that was conducted in humans in 1981-83 that found a seroprevalence of 2% in pregnant woman, there are no other records of the disease in Mozambique.
At the beginning of this study the available enzyme-linked immunosorbent assay (ELISA) was based on whole inactivated virus which binds poorly to the immunoplates and requires containment facilities to reduce the risk of exposure of laboratory staff to infection with the virulent virus.
The objectives of this study were to:

- develop and evaluate new RVF reagents for safe, rapid and accurate diagnosis and surveillance of RVF; and

© University of Pretoria

- apply the newly developed assays to investigate the circulation of RVFV in sheep and goats in the Zambézia Province, Mozambique.

Chapter 1. This is the general introduction and deals particularly with aspects of the characteristic of RVFV; vectors and maintenance of RVFV; major recent epidemics; RVF in Mozambique; RVF in domestic animals and humans; and diagnostic assays.

Chapter 2 describes the cloning and expression of RVFV nucleocapsid (N) protein and evaluation of a N-protein based indirect ELISA for the detection of specific IgG and IgM antibodies in domestic ruminants. The recombinant nucleocapsid protein of RVFV was cloned, expressed in the bacterial expression vector pET43b and evaluated as antigen in an indirect ELISA using sera from experimentally infected sheep (n = 128), vaccinated sheep (n = 240), and field-collected sera from sheep (n = 251), goats (n =362) and cattle (n =100). Using virus neutralization (VN) as gold standard very high diagnostic sensitivity and specificity (both 100%) was found in goats when using the anti-species IgG conjugate. Protein G was also tested in the same ELISA format as a detection system, and the sensitivity and specificity in goats were 99,4% and 99,5%, in sheep field sera both 100%, and in cattle 100% and 98,3%, respectively. The indirect ELISA based on recombinant nucleocapsid protein has the potential to complement the conventional assays for serodiagnosis of RVF. Advantages of the N-protein are its safety, stability and cost-effectiveness in use and production.

Chapter 3 compares the performance of the indirect ELISA based on the recombinant nucleocapsid protein with the inactivated whole virus based IgG sandwich ELISA for the detection of RVFV antibodies in field-collected sera sample from sheep (n=605) and goat (n=657). A good agreement between both tests was found when testing for the presence of specific anti-RVFV IgG in the sheep (kappa=0,92) and goat sera (kappa = 0,99). Although the sandwich ELISA was slightly more sensitive than the indirect ELISA for the detection of IgG to RVFV the simpler and shorter procedure as well as the safety and high quality of the recombinant nucleocapsid protein antigen makes the indirect ELISA more suitable for surveillance studies.

Chapter 4 describes the generation and characterization of monoclonal antibodies against RVFV nucleoprotein to improve existing diagnostic assays. Monoclonal antibodies specific for the nucleocapsid protein of RVFV were produced, isotyped and

© University of Pretoria

characterized. Four antibodies selected that belonged to the class IgG and the subclass IgG2a, showed a strong reaction with denaturated nucleocapsid recombinant protein in Western blot and recognized the antigen in an inactivated preparation of whole RVFV in Western blot and in an ELISA. Cross-reactivity with extracts of genetically related and non-related viruses including Bunyamwera virus and Calovo virus (*Bunyaviridae* family), West Nile virus and Dengue virus II (*Flaviviridae* family) and Sindbis and Chikungunya viruses (*Togaviridae* family), could not be shown. These monoclonal antibodies represent a useful tool for the development of rapid diagnostic assays for early diagnosis of RVF.

Chapter 5 deals with the evidence of RVFV circulation in the inter-epidemic period in the Zambézia Province, Mozambique based on serological studies. During the last two decades several outbreaks of RVF have been reported in countries in Southern Africa. No clinical disease has been reported in Mozambique during this period. In a serological study conducted in 2007 in five districts of Zambézia Province, Mozambique of a total of 654 small ruminants sampled (277 sheep and 377 goats), 35,8% of sheep sera and 21,2% of goat sera were positive to RVFV. In 2010, a cross-sectional survey was conducted in 313 sheep and 449 goats in two districts (Mopeia and Nicoadala) of the same province. This study revealed an overall seropositivity rate of 9,2% in sheep and 11,6% in goat and an increased likelihood of being seropositive in older animals (OR = 7,3; $p < 0{,}001$) with a IgG ELISA. Twenty nine out of 240 animals assessed by IgM ELISA were positive, suggesting recent exposure to RVFV. However, a study carried out between September 2010 and April 2011 in a cohort of 125 animals (74 sheep and 51 goats) failed to demonstrate sero-conversion. The results of the study indicate that RVFV circulates subclinically in domestic small ruminants in Zambézia.

Chapter 6. deals with concluding remarks and recommendations.

© University of Pretoria

1 . General introduction

1.1 Introduction

Rift Valley fever (RVF) is a peracute or acute viral disease that is endemic in most parts of Africa and has also been reported in the Arabian Peninsula affecting mainly domestic ruminants but also humans (Swanepoel and Coetzer, 2004). The causative agent, RVF virus (RVFV) was first isolated in 1930 in an epidemic among sheep on a farm in the Rift Valley in East Africa (Gonzalez-Scarano et al., 1991) although a similar disease syndrome that may have been RVF, described as an acute and highly fatal disease in exotic wool sheep in the Rift Valley was reported as far back as 1913 (Davis, 2010). Rift Valley fever virus was only classified in 1973 when it was shown to have similar morphology of the bunyaviruses and antigenically related to phlebotomus fever virus (Gonzalez-Scarano et al., 1991).

Rift Valley fever virus is transmitted by different mosquito species of the genus *Aedes*, *Culex*, *Anopheles*, *Eritmapodites* and *Mansonia.* The infected animals develop necrotic hepatitis, widespread haemorrhages, abortion and high mortality is observed in new-born animals. Humans may be infected by contact with tissues of sick animals or by mosquito bites. Infection in humans is associated with symptoms ranging from mild to severe influenza-like illness. A haemorrhagic state, encephalitis, or retinal lesions (Swanepoel and Coetzer, 2004) may occur in 5% to 20% of RVF infected humans (Davies, 2010).

Apart of the significant impact on human and animal health associated with RVF outbreaks, the disease causes devastating socio-economic losses in sheep, goats and cattle. The negative impact of RVF is reflected not only among the producers in terms of food insecurity and reduction in income but also affects livestock trade, butchers, slaughterhouses, casual laborers and non-agricultural sectors. For example, the 2000/2001 outbreak in Saudi Arabia resulted in losses of around US$ 132 million in Ethiopia because of trade bans of life animals. During the Kenyan outbreak of 2007/2008 losses for the national economy were estimated as being over US$ 32 million (Rich and Wanyoike, 2010).

© University of Pretoria

Due to the ability of RVFV to cause unpredicted but recurrent epidemics, inefficient prophylactic and therapeutic measures and the existence of a wide range of competent mosquito vectors that potentially threaten areas beyond the range of countries where outbreaks occurred up to now there is a resurgence in the interest in the epidemiology of the disease and there have been increased efforts to develop more sensitive and specific diagnostic tools and improved vaccines during recent years.

1.2 Aetiology

Rift Valley fever virus belongs to the *Phlebovirus* genus, family *Bunyaviridae*. Like other bunyaviruses, it is spherical, 80-120 nm in diameter, has an envelope with glycoprotein peplomers and three circular helical nucleocapsid segments. The virus has a three-segmented, single stranded, RNA genome designated large (L), medium (M), and small (S) (Murphy et al., 1999). The L segment is a negative sense RNA and codes for the RNA-dependent RNA polymerase (transcriptase). The M segment is also negative sense and encodes the polyprotein that after cleavage generates two glycoproteins, Gn encoded by aminoterminal sequences of the precursor, Gc encoded by carboxy-terminal sequence as well as two nonstructural proteins NSm1 (78kDa) and NSm2 (14kDa). The S segment utilizes an ambisense strategy to code for the nucleocapsid (N) protein and a nonstructural (NSs) protein (Bouloy and Weber, 2010; Pépin et al., 2010).

The RVFV envelope glycoproteins are important for viral infection as they mediate particle entry into susceptible cells (Bouloy and Weber, 2010; Pépin et al., 2010) and serve as targets for neutralizing antibodies and haemagglutinin specific ones (Besselaar and Blackburn, 1991). The nucleopcapsid protein of the *Bunyaviridae* has been shown to be the immunodominant viral protein inducing rapid activation of strong humoral responses in infected hosts (Vapalahti et al., 1995; Schwarz et al., 1996). The nonstructural proteins are dispensable for viral multiplication in cell culture (Vialat et al., 2000; Won et al., 2006; Gerrard et al., 2007): however they play an important role in pathogenesis. The NSs protein is considered to be the major virulence factor of RVFV inducing the suppression of host innate immunological responses, including production

© University of Pretoria

of the alpha and beta interferons (Le May et al., 2004; 2008) and the specific degradation of the dsRNA dependent protein kinase (PRK) (Habjan et al., 2009; Ikegami et al., 2009). The NSm proteins have been reported to suppress virus-induced apoptosis (Won et al., 2007).

1.3 Vectors and maintenance of RVFV

Rift Valley fever virus has the potential to infect a variety of vectors including mosquitoes, ticks and various flies (Fontenille et al., 1998), however, its natural occurrence has been associated almost exclusively with mosquitoes (Turrel et al., 2008). In Southern Africa, the virus has been isolated from 12 mosquito species, five *Aedes*, three *Culex*, three *Anopheles* and one *Eretmapodites* (Swanepoel and Coetzer, 1994). The mosquito genera *Aedes*, *Culex*, *Anopheles* and *Mansonia* have been associated with some of the most devastating outbreaks. In the outbreak that occurred in 1977 in Egypt *Cx. pipiens* was considered the main vector (Meegan et al., 1979). In the 1998-99 and 2003 outbreaks in Senegal and Mauritania, *Cx. poicilipes* was incriminated in disease transmission (Diallo et al., 2005; Faye et al., 2007). In the outbreak that occurred in Saudi Arabia in 2000/2001, *Ae. vexans arabiensis* and *Cx. (cx) triteniorynchus* were incriminated as biological vectors of RVFV (Al-Alfaleq and Hussein, 2011). During the Kenyan outbreak of 2006/2007 four species of *Culex*, three species of *Aedes*, two species of *Mansonia* and one species of *Anopheles* tested positive for RVFV (Sang et al., 2010). The mosquito species associated most frequently with RVFV infection during the outbreak in Sudan in 2007 were *An. gambiae arabiensis* and *Cx. pipiens* (Seufi and Galal, 2010; Hassan et al., 2011). Table 1.1 summarises the mosquito species incriminated as vectors in some of the RVF outbreaks.

© University of Pretoria

Table 1.1. Mosquito species implicated in some of the RVF outbreaks

Species	**Year and country of outbreak**	**Reference**
Aedes (*Och.*) *caballus* s.l, *Culex* (*Cux.*) *theileri*	1953, South Africa	(Swanepoel and Coetzer, 1994)
Cx. (*Cux.*) *theileri*	1956, South Africa	(Swanepoel and Coetzer, 1994)
Ae. (*Adm.*) *dentatus*, *Ae.* (*Neo.*) *mcintoshi, Cx.* (*Cux.*) *theileri, Anopheles* (*Ano.*) *coustani*	1969, Zimbabwe	(Swanepoel and Coetzer, 1994)
Cx. (*Cux.*) *theileri*	1970, South Africa	(Swanepoel and Coetzer, 1994)
Eretmapodites quinquevittatus	1971, South Africa	(Swanepoel and Coetzer, 1994)
Cx. (*Cux.*) *theileri, An.* (*Cel.*) *cinereus*	1974, South Africa	(Swanepoel and Coetzer, 1994)
Ae. (*Och.*) *juppi, Ae.* (*Neo.*) *mcintoshi, Cx.* (*Cux.*) *theileri,, An.* (*Cel.*) *squamosus,*	1975, South Africa	(Swanepoel and Coetzer, 1994)
Cx. pipiens	1977, Egypt	(Meegan et al., 1979)
Ae. (*Neo.*) *circumluteolus*, *Cx.* (*Cux.*) *neavi*; *Cx.* (*Cux*) *zombaensis*,	1981, South Africa	(Swanepoel and Coetzer, 1994)
Cx. poicilipes	1998-99, 2003 Senegal and Mauritania	(Diallo et al., 2005; Faye et al., 2007);
Ae. vexans arabiensis, *Cx. (cx) tritenioryṅchus*	2000/2001, Saudi Arabia	(Al-Alfaleq and Hussein, 2011)
Ae. mcintoshi/circumluteolus, Ae. ochraceus, Ae. pembaensis, Cx. poicilipes, Cx. bitaeniorhynchus, Cx. univittatus, Cx. quinquefasciatus, Mansonia uniformis, M. africana, An. squamosus	2006/2007 Kenya	(Sang et al., 2010)
An. gambiae arabiensis, *Cx. pipiens, Ae. aegypti, An. coustani, Cx. poicilipes*	2007, Sudan	(Seufi and Galal; 2010; Hassan et al., 2011)

Mosquito vectors are not only associated with disease transmission but also play a role in maintenance of the virus during the inter-epidemic periods. For decades it has been accepted that the virus was endemic in indigenous forest circulating in mosquitoes and unknown vertebrates and spreading into bordering livestock rearing areas after heavy rains that favoured the breeding of mosquito vectors (Swanepoel and Coetzer, 2004). The currently accepted theory postulates that RVFV is maintained during inter-epidemic periods in eggs of aedine mosquitoes and epidemics occur when abnormally heavy rains lead to an increase in the epidemic vector population (Linthicum et al., 1985). According to this theory RVFV is maintained in dry mud for prolonged periods in eggs

© University of Pretoria

of *Aedes* mosquitoes that breed in water-lodged depressions called *dambos* or broad vleis. When the *dambos* are flooded after widespread rainfall the eggs of *Aedes* mosquitoes hatch resulting in infected mosquitoes that trigger an epidemic. Once initiated, epidemics may be sustained by many epidemic vectors including other mosquito species and biting flies (Swanepoel and Coetzer, 2004). Thus the vectors of RVFV can be classified as reservoir/maintenance vectors that include certain *Aedes* species and epidemic/amplifying vectors that incude *Culex* spp. and *Anopheles* spp.

1.4 Major recent epidemics

Rift Valley fever has been responsible for major outbreaks in livestock and humans during the last few decades. Apart from heavy losses due to high mortality and abortions in animals, approximately 200000 people were infected of which about 600 died in the 1977/78 epidemic in Egypt. In the 1987 outbreaks in Mauritania 200 people succumbed. During the 1997/98 epidemic in Kenya, Tanzania and Somalia more than 89000 of people were infected, about 500 people died and untold numbers of livestock died or aborted (Sall et al., 1998). In the first reported outbreak outside Africa in 2000/2001 in Saudi Arabia and Yemen, thousands of sheep died or aborted and more than 200 people died (CDC, 2000). During the outbreak of RVF in 2006/2007 in Kenya, Somalia and Tanzania more than 1000 infections and 323 human deaths were reported (WHO, 2007). In the same year, a large outbreak affected Sudan causing a total of 747 confirmed human cases and 230 deaths (Hassan et al., 2011). Madagascar, an island off shore of southern Mozambique, reported in 2008 an epidemic of RVF that was responsible for at least 476 suspected human cases, 19 deaths, more than 59 laboratory confirmed human cases and untold high deaths rates among cattle (Andriamandimby et al., 2010). In the same year, an outbreak of the disease affecting veterinarians and farmers was reported in South Africa. Among 53 persons potentially exposed to sick domestic ruminants that were included in a study, 15% revealed evidence of recent infection and 4% of past exposure to RVFV (Archer et al., 2011). Later in 2010 in the same country an epidemic of the disease resulted in 192 laboratory-confirmed human cases including 18 deaths (WHO, 2010). These recent outbreaks of

© University of Pretoria

RVF re-emphasized the importance and risks of the disease, not only as a cause of mortality and abortion in livestock, but also as a serious disease in humans.

1.5 Rift Valley fever in Mozambique

Rift Valley fever is thought to be endemic in Mozambique. The occurrence of the disease in the country dates back to 1960 when a seroprevalence of 2,8% was reported in cattle. There is also a report of an epidemic in 1969 in Gaza Province where 134 bovines died of which 106 deaths occurred in the capital Xai-Xai (Valadão, 1969). In 1999, in Zambezia Province, RVF was responsible for abortion in a herd of water buffaloes and 51% of the 140 animals were serologically positive to RVFV*.* A surveillance study of cattle in the Zambézia Province in 1996 and 2001 revealed a sero-prevalence of 37% (n=412) and 53% (n=95), respectively (DINAP, 2002). In humans, serological evidence of RVF was found in 1981-83 in a survey carried out in pregnant women from 8 of the 10 Mozambican provinces: 28 out of 1163 (2%) were positive for RVFV (Niklasson et al., 1987).

In 2002 a vaccination program in cattle was initiated in districts of the Zambézia Province. After severe flooding in the south of Mozambique in 2000 and in the central part of the country in 2001, RVF was considered a high risk disease and the vaccination program was extended in late 2002 to include also the Gaza Province and the Manica Province. After 2004, vaccination of livestock was limited to a few districts of the Zambézia Province (DINAP, 2002).

1.6 Rift Valley fever in domestic animals

Rift Valley fever virus is responsible for a severe disease in sheep, goats and cattle: new-born lamb, calf and kid being more susceptible than the adults (Peters and Meegan, 1981). In lambs, calves and kids less than one week old, the incubation period is usually 24 to 36 hours, but it may be as short as 12 hours. The animals develop fever that may exceed 41°C, are listless, anorectic, show abdominal pain, respiration is rapid and abdominal in the terminal phase of the illness. The disease is usually peracute

© University of Pretoria

resulting in death 24 to 36 hours after the onset of the illness. The mortality rates in new- born lambs and kids may be more than 90% (Swanepoel and Coetzer, 2004). In calves the mortality rate is usually between 10 to 20%. Lambs and kids older than two weeks are less susceptible and the disease may be inapparent, peracute or acute. In these animals the disease is most commonly acute characterized by an incubation period of 24 to 72 hours, fever (up to 42°C), inappetence, listlessness and sometimes nasal discharges that may be blood-tinged, and a foetid or bloody diarrhoea. Abortion occurs at any stage of pregnancy and may be as high as 80 - 100% in sheep and goats. In adult cattle, the infection is frequently inapparent but acute disease characterized by fever, anorexia, salivation, nasal discharge, reduction in milk production, and a foetid and blood-tinged diarrhoea may occur in some animals. The abortion rate may reach 80 to 100% (Peters and Meegan, 1981; Swanepoel and Coetzer, 2004).

1.7 Rift Valley fever in humans

Humans are usually infected by contact with tissues of sick animals or by mosquito bites. Infection in humans is associated with symptoms ranging from mild to severe influenza-like illness accompanied by fever, malaise, headaches, pain of the large joints (knees, elbows and shoulders) anorexia, nausea, vomition and mid-epigastric pain. The disease may progress in severity marked by renal impairment and a fatal haemorrhagic state, hepatomegaly, jaundice and delirium (Swanepoel and Coetzer, 2004; El Imam et al., 2009; Davies, 2010; Kahlon et al., 2010). Rift Valley fever may also result in meningoencephalitis (King et al., 2010) and eye lesions (LaBeaud et al., 2008).

1.8 Diagnostic assays

Different techniques are used for the diagnosis of RVF including virus isolation, virus detection and antibody detection.

© University of Pretoria

1.8.1 Virus isolation and detection

Rift Valley fever virus can be isolated from different tissues including blood, plasma, liver, spleen and brain. The samples should be collected during the febrile period or at necropsy and refrigerated or frozen until testing (Peters and Meegan, 1981; Swanepoel and Coetzer, 2004). The virus is readily isolated in various cell cultures such as Vero (African green monkey kidney), CER (chicken embryo related), BHK-21 (baby hamster kidney), mosquito cell lines, primary calf, lamb and goat kidney or testis cells, as well as by intracerebral or intraperitoneal inoculation of suckling and weaned mice or hamsters (Peters and Meegan, 1981; Anderson et al., 1989). Virus isolation to confirm a diagnosis of RVF is a time-consuming procedure with health risks for laboratory personnel (Soliman et al., 1988).

Viral antigen detection can be rapidly performed by complement fixation and immunodiffusion test on tissue suspensions, and by histochemical staining of tissue sections (Swanepoel and Coetzer, 2004). High titres of RVFV can be found in ovine brain, liver and in spleen (Yedloustschnig et al., 1981). Viral antigens can also be detected in serum using a reverse passive hemagglutination inhibition test (Swanepoel et al., 1983) and by enzyme-linked immunosorbent assay (ELISA) (Niklasson et al., 1983; Meegan et al., 1989; Jansen van Vuren and Paweska, 2009). ELISA was also reported to be useful in RVF viral antigens detection in infected mosquitoes (Niklasson et al., 1983; Niklasson and Gargan, 1985) and in culture supernatants (Fukushi et al., 2012). The sensitivity of the ELISA for detection of viral antigens in human serum was 29,3% when compared with virus isolation (Meegan et al., 1989), and in mosquitoes it was 93% compared with plaque assays. ELISA has the advantage of obtaining the results quicker and could be adapted for field use since it could be prepared, standardized and performed with inactivated antigens (Niklasson and Gargan, 1985). It is also a valuable tool for differential diagnosis of viral haemorrhagic fevers (Jasen van Vuren and Paweska, 2009). Although viraemic titres in infected animals and humans are usually high it is of short duration, limiting the usefulness of virus detection. Thus, the use of ELISA for the detection of recent RVFV should be run in parallel with other tests targeting both viral antigens and IgM antibodies (Pépin et al., 2010).

© University of Pretoria

Recently, considerable advances were made in the rapid detection and identification of RVFV with the use of nucleic acid techniques. Highly sensitive reverse transcriptase-polymerase chain reaction assays (RT-PCR) were developed to detect RVFV RNA in infected mosquitoes (Ibrahim et al., 1997) and in animal and human sera and other tissues (Espach et al., 2002; Sall et al., 2001; 2002). A method for quantifying parts of the RNA segment by a real-time detection reverse transcription using TaqMan technology was developed for rapid diagnosis of RVF and screening of efficacy of antiviral compounds (Garcia et al., 2001). A real-time reverse-transcription loop–mediated isothermal amplification (RT-LAMP) was developed and validated and has been shown to be highly sensitive and faster than traditional RT-PCR (Le Roux et al., 2009; Peyrefitte et al., 2008). However, a definitive diagnosis of RVF should not rely on a single PCR result and it is recommended that molecular techniques should be combined with other tests, such as detection of specific antibodies to RVFV, since the viraemia in individuals infected with RVFV is of very short duration but the IgM and IgG antibodies are readily demonstrable shortly after exposure of the virus and last for several months (Pépin et al., 2010).

1.8.2 Detection of RVFV specific antibodies

Several techniques such as complement fixation, indirect immunofluorescence, haemagglutination-inhibition, ELISA and neutralization tests have been developed for the detection of antibodies to RVFV (Swanepoel et al., 1986). Complement fixation is specific for detecting RVF viral antibodies but lacks sensitivity. Indirect immunofluorescence is slightly less specific in detecting antibodies against RVFV than haemagglutination-inhibition and neutralization tests and may cross-react with other phleboviruses. Therefore neutralization, haemagglutination-inhibition and ELISA are most suitable for detecting RVF viral antibodies (Scott et al., 1986). Virus neutralization is regarded as the gold standard test but it is laborious, expensive, holds heath risks and requires 5-7 days for completion. The test can only be performed when tissue culture and live virus facilities are available. The haemagglutination-inhibition is sensitive but it is laborious and like the indirect immunofluorescence cross-reacts with

© University of Pretoria

other phleboviruses (Scott et al., 1986; Swanepoel et al., 1986). Additionally the disadvantages of both methods are the risk to laboratory personnel (Smithburn et al., 1949), the restrictions to use outside RVF endemic areas (Scherer et al., 1980; Barnard and Gerdes, 2000) and the inability to distinguish different classes of immunoglobulins (Wright et al., 1993). Several ELISA were developed for the detection of RVF antibodies. An indirect ELISA based on a sucrose-acetone-extracted RVFV antigen was reported to be very sensitive (97,3%) and specific (97,4%) when compared with virus neutralization test to detect serum antibodies to RVFV in vaccinated sheep (Paweska et al., 1995). The same ELISA was later assessed using domestic and wild ruminant sera and its sensitivity and specificity ranged in different species from 84,31% to 99,18% and from 99,16% to 99,34% respectively (Paweska et al., 2003a). As this type of antigen binds poorly to the ELISA plates an IgG-sandwich and IgM-capture ELISA based on β-propiolactone inactivated and/or gamma-irradiated sucrose-acetone-extracted RVFV antigen was also validated using serum from domestic ruminants and reported to be highly sensitive (99,05% to 100%) and specific (97,4 to 99,9%) (Paweska et al., 2003b). However, the production of the antigen for the assays required containment facilities to reduce the risk of exposure of laboratory staff to infection. Recently, recombinant nucleocapsid protein has been used as an antigen in ELISA for the detection of antibody to RVFV in domestic ruminants, humans and African buffalo. Due to the high quality of the recombinant nucleocapsid protein it could be directly adsorbed into the immunoplate in an indirect ELISA format. Using virus neutralization as gold standard the ELISA's had a sensitivity and specificity ranging in domestic ruminants from 99,4% to 100% and from 98,3% to 100%, respectively (Fafetine et al., 2007). The sensitivity and specificity in humans was 99,7% and 99,6% and in African buffalo 98,7% and 99,4%, respectively (Paweska et al., 2007; 2008). The recombinant nucleocapsid protein used as antigen in ELISA proved to be a valuable diagnostic tool and renders a very sensitive and specific method for RVF diagnosis, avoiding the risk of laboratory infection of staff (Fafetine et al., 2007; Jansen van Vuren et al., 2007; Paweska et al., 2007; 2008). Further validation studies of the ELISA based on the nucleocapsid antigen in domestic ruminants is required. Production of monoclonal antibodies to the RVFV nucleocapsid protein may further contribute to the development of rapid, sensitive and specific

© University of Pretoria

diagnostic assays such as antigen detection by an antigen capture ELISA, chromatography based strip test, and the detection of specific RVFV antibodies in different animal species by capture ELISA. These diagnostic assays will play an important role in the implementation of surveillance programs in poor resourced countries as is the case of most endemic African countries.

1.9 Outline of the thesis

The studies had the following objectives:

- To develop and evaluate a new generation of RVF immunoreagents for development of safe laboratory methods for rapid and accurate diagnosis and surveillance of RVF.
- To apply the newly developed assays to investigate the endemicity of RVF in sheep and goats in the Zambézia Province, Mozambique.

Chapter 2 deals with the cloning and expression of RVFV nucleocapsid protein and its evaluation for the detection of specific IgG and IgM in domestic ruminants;

Chapter 3 compares the indirect ELISA based on a recombinant nucleocapsid protein with a sandwich ELISA based on inactivated whole virus for the detection of IgG to RVFV in field-collected sera of sheep and goats in Mozambique;

Chapter 4 describes the production and characterization of monoclonal antibodies against the RVFV recombinant nucleocapsid protein;

Chapter 5 investigates the hypothesis that RVFV is endemic in the Zambezia Province of Mozambique without noticeable clinical disease and may circulate in sheep and goats.

Chapter 6 deals with concluding remarks and recommendations for future studies.

1.10 References

Al-Alfaleq, A.I., Hussein, F. (2011). The status of Rift Valley fever in animals in Saudi Arabia: a mini review. Vector-Borne and Zoonotic Dis., 11(12), 1513-1520.

© University of Pretoria

Anderson Jr. G.W., Saluzzo, J.F., Ksiazek, T.G., Smith, J.F., Ennis, W., Thureen, D. (1989). Comparison of in vitro and in vivo systems for propagation of Rift Valley fever virus from clinical specimens. Res. Virol., 140: 129-138.

Andriamandimby, S.F., Randrianarivo-Solofoniaina, A.E., Jeanmaire, E.M., Ravalolomanana, L., Razafimanantsoa, L.T., Rakotojoelinandrasana, T., Razainirina, J., Hoffmann, J., Ravalohery, J.P., Rafisandratantsoa, J.T., Rollin, P.E., Reynes, J.M. (2010). Rift Valley fever during rainy seasons, Madagascar, 2008 and 2009. Emerg. Infect. Dis., 16 (6), 963-970.

Archer, B.N., Weyer, J., Paweska, J., Nkosi, D., Leman, P., Tint, K.S. Blumberg, L. (2011). Outbreak of Rift Valley fever affecting veterinarians and farmers in South Africa, 2008. S. Afr. Med. J., 101(4), 263-6.

Barnard, B.J.H., Gerdes, G.H. (2000). Rift Valley fever. In: OIE Manual of standards for diagnostic tests and vaccines. 4th ed. Ofiice Internacional des Epizooties, Paris France, pp 144-152.

Besselaar, T.G., Blackburn, N.K. (1991). Topological mapping of antigenic sites on the Rift Valley fever virus envelope glycoproteins using monoclonal antibodies. Arch. Virol., 121: 111-124.

Bouloy, M., Weber, F. (2010). Molecular biology of Rift Valley fever virus. The Open Virol. J., 4, 8-14.

CDC (2000). Update: outbrake of Rift Valley fever- Saudi Arabia, August-November, 2000. MMWR Morb. Mortal. Wkly Rep., 49 (43), 982-985.

Davies, F.G. (2010). The historical and recent impact of Rift Valley fever in Africa. Am J. Trop. Med. Hyg., 83 (Suppl. 2), 73-74.

© University of Pretoria

Diallo, M., Nabeth, P., Ba, K., Sall, A.A., Ba, Y., Mondo, M., Girault, L., Abdalahi, M.O., Mathiot, C. (2005). Mosquito vectors of the 1998-1999 outbreak of Rift valley fever and other arboviruses (Bagaza, Sanar, Wesselsbron and West Nile) in Mauritania and Senegal. Med. Vet. Entomol., 19, 119-126.

DINAP (2002). Ministério da Agricultura e de Desenvolvimento Rural (MADER) Relatório anual. Maputo, Moçambique
El Imam, M., El Sabiq, M., Omran, M. (2009). Acute renal failure associated with the Rift Valley fever: a single center study. Saudi J. Kidney Dis. Transpl., 20, 1047-1052.

Espach, A., Romito, M., Nel, L.H., Viljoen, G.J. (2002). Development of a diagnostic one-tube RT-PCR for the detection of Rift Valley fever virus. Onderstepoort J. Vet Res., 69, 247-252.

Fafetine, J.M., Tijhaar, E., Paweska, J.T., Neves, L.C.B.G., Hendriks, J., Swanepoel, R., Coetzer, J.A.W., Egberink, H.F., Rutten, V.P.M.G. (2007). Cloning and expression of Rift Valley fever virus nucleocapsid (N) protein and evaluation of a N-protein based indirect ELISA for the detection of specific IgG and IgM antibodies in domestic ruminants. Vet. Microb., 121 (1-2), 29-38.

Faye, O., Diallo, M., Diop, D., Bezeid, O., Bâ, H., Niang, M., Dia, I., Mhamed, S., Ndiaye K., Diallo, D., Ly, P.O., Diallo, B., Nabeth, Simon, P., Lo, B., Diop, O.M. (2007). Rift Valley Fever outbreak with East-Central African virus lineage in Mauritania, 2003. Emerg. Infect. Dis., 13 (7), 1016-1023.

Fontenille, D., Traore-Lamizana, M., Diallo, M., Thonnon, J., Digoutte, J.P., Zeller, H.G. (1998). New vectors of Rift Valley feve in West Africa. Emerg. Infect. Dis., 4, 289-293.

Fukushi, S., Nakauchi, M., Mizutani, T., Saijo, M., Kurane, I., Morikawa, S. (2012). Antigen-capture ELISA for the detection of Rift Valley fever virus nucleoprotein using new monoclonal antibodies. J. Virol. Methods, 180, 68-74. Garcia, S., Crance, J.M.,

© University of Pretoria

Billecocq, A., Peinnequin, A., Jouan, A., Bouloy, M., Garin, D. (2001). Quantitative real-time PCR detection of Rift Valley fever virus and its application to evaluation of antiviral compounds. J. Clin. Microbiol., 39 (12), 4456-4461.

Gerrard S.R., Bird, B.H., Albarino, C.G., Nichol, S.T. (2007). The NSm proteins of Rift Valley fever are dispensable for maturation, replication and infection. Virol., 359, 459-465.

Gonzalez-Scarano, F., Endres, M.J., Nathanson, N. (1991). Pathogenisis. Curr. Top. Microbiol. Immunol., 169, 217-249.

Habjan, M., Pilchlmair, A., Elliot, R.M., Overby, A.K., Glatter, T., Gstaiger, M., Superti-Furga, G., Unger, H., Weber, F. (2009). NSs protein of Rift valley fever virus induces the specific degradation of the double-stranded RNA-dependant protein kinase. J. Virol., 83 (9), 4365-4375.

Hassan, O.A., Ahlm, C., Sang, R., Evander, M. (2011). The 2007 Rift Valley fever outbreak in Sudan. PLOS Negl. Trop. Dis., 5(9), e1229

Ibrahim, M.S., Turell, M.J., Knauert, F.K., Lofts, R.S. (1997). Detection of Rift Valley fever virus in mosquitoes by RT-PCR. Mol.Cell. Probes, 11, 49-53.

Ikegami, T., Narayanan, K., Won, S., Kamitani, W., Peters, C.J., Makino, S. (2009). Rift Valley fever virus NSs protein promotes post-transcriptionla downregulation of protein kinase PKR and inhibis eIF2α phosphorylation. PLOS Pathog., 5(2).

Jasen Van Vuren, P., Potgieter, A.C., Paweska, J.T., Van Dijk, A.A. (2007). Preparation and evaluation of a recombinant Rift Valley fever virus N protein for the detection of IgG and IgM antibodies in humans and animals by indirect ELISA. J. Virol. Methods, 140, 106–114.

© University of Pretoria

Jansen, van Vuren, P., Paweska, J.T. (2009). Laboratory safe detection of nucleocapsid protein of Rift Valley fever virus in human and animal specimens by sandwich ELISA. J. Virol. Methods, 157, 15-24.

Kahlon, S.S., Peters, C.J., LeDuc, J., Muchiri, E.M., Muiruri, S., Njenga, M.K., Breiman, R.F., White Jr., A.C., King, C.H. (2010). Case report: severe Rift Valley fever may present with a characteristic clinical syndrome. Am. J. Trop. Med. Hyg., 82(3), 371-375.

King, C.H., Kahlon, S.S., Muiruri, S., LaBeaud, A.D. (2010). Facets of the Rift Valley fever outbreak in Northeastern Provine, Kenya, 2006-2007. Am. J. Trop. Med. Hyg., 82(3), 363.

LaBeaud, A.D., Muchiri, E.M., Ndzovu, M., Mwanje, M.T., Muiruri,S., Peters, C.J., King, C.H. (2008). Inter-epidemic Rift Valley fever seropositivity, Northeastern Kenya. Emerg. Infect. Dis., 14(8), 1240-1246.

Le May, N., Dubaele, S., de Santis, L.P., Billecocq, A., Bouloy, M., Egly, JM. (2004). TFIIH transcription factor, a target for the Rift Valley heamorrhagic fever virus. Cell, 116, 541-550.

Le May, N., Mansuroglu, Z., Léger, P., Josse, T., Blot, G., Billecocq, A., Flick, R., Jacob, Y., Bonnefoy, E. Bouloy, M. (2008). A SAP30 complex inhibits IFN-β expression in Rift Valley fever virus infected cells. PLOS Pathog., 4, 134-144.

Le Roux, C.A., Kubo, T., Grobbelaar, A.A., van Vuren, P.J., Weyer, J., Nel, L.H., Swanepoel, R., Morita, K.,Paweska, J.T. (2009). Development and evaluation of a Real-Time reverse transcription-loop-mediated isothermal amplification assay for rapid detection of Rift Valley fever virus in clinical specimens. J. Clin. Microb., 47 (3), 645-651.

© University of Pretoria

Linthicum, K.J., Davis, F.G., Kairo, A., Bailey, C.L. (1985). Rift Valley fever virus (family Bunyaviridae, genus Phlebovirus). Isolation from Diptera collected during an inter-epizootic period in Kenya. J. Hyg., 95, 197-209.

Meegan, J., Hoogstraal, H., Moussa, M. (1979). An epizootic of Rift Valley fever in Egypt in 1977. Vet. Rec., 53: 123-128.

Meegan, J., Leguenno, B., Ksiazek, T., Jouan, A., Knauert, F., Digoutte, J.P. Peters, C.J. (1989). Rapid diagnosis of Rift Valley fever: A comparision of methods for the direct detection of viral antigen in human sera. Res. Virol., 140: 59-65.

Murphy, F.A., Gibbs, E.P.J. Horzinek, M.C., Studdert, M.J. (1999). Veterinary Virology. 3rd ed. Elsevier, USA. Pp. 469-475.

Niklasson, B., Grandien, M., Peters, C.J., Gargan, T.P. (1983). Detection of Rift Valley fever virus antigen by enzyme-linked immunososrbent assay. J. Clin. Microbiol., 17(6), 1026-1031.

Niklasson, B., Gargan, T. (1985). Enzyme-linked immunosorbent assay for detection of Rift valley fever virus antigen in mosquitoes. Am. J. Trop. Med. Hyg., 34(2), 400-405.

Niklasson, B., Liljestrand, J., Bergstrom, S., Peters, C.J. (1987). Rift Valley fever: seroepidemiological survey among pregnant women in Mozambique. Epidemiol. Infect., 99, 517-522.

Paweska, J.T., Barnard, B.J.H., Williams, R. (1995). The use of sucrose-acetone-extracted ift Valley fever virus antigen derived from cell culture in an indirect enzyme-linked immunosorbent assay and haemagglutination-inhibition test. Onderstepoort J. Vet. Res., 62, 227-233.

© University of Pretoria

Paweska, J.T., Smith, S.J., Wright, I.M., Williams, R., Cohen, A.S., van Dijk, A.A., Grobbelaar, A.A., Croft, J.E., Swanepoel, R., Gerdes, G.H. (2003a). Indirect enzyme-linked immunosorbent assay for the detection of antibody against Rift Valley fever virus in domestic and wild ruminant sera. Onderstepoort J. Vet. Res., 70, 49-64.

Paweska, J.T., Burt, F.J., Anthony, F., Smith, S.J., Grobbelaar, A.A., Croft, J.E., Ksiazek, T.G., Swanepoel, R. (2003b). IgG-sandwish and IgM-capture enzyme-linked immunosorbent assay for the detection of antibody to Rift Valley fever virus in domestic ruminants. J. Virol. Methods, 113, 103-112.

Paweska, J.T., Jansen van Vuren, P., Swanepoel, R. (2007). Validation of an indirect ELISA based on a recombinant nucleocapsid protein of Rift Valley fever virus for the detection of IgG antibody in humans., J. Virol. Methods, 146, 119-124.

Paweska, J.T., Jansen van Vuren, P., Kemp, A., Buss, P., Bengis, R.G., Gakuya, F., Breiman, R.F., Kariuki, M.N., Swanepoel, R. (2008). Recombinant nucleocapsid-based ELISA for detection of IgG antibody to Rift Valley fever virus in African buffalo. Vet Microbiol., 127, 21-28.

Pépin, M., Bouloy, M., Bird, B.H., Kemp, A., Paweska, J. (2010). Rift Valley fever virus (Bunyaviridae: Phlebovirus): an update on pathogenesis, molecular epidemiology, vectors, diagnostics and prevention. Vet. Res., 41:61.

Peters, C.J., Meegan, J.M. (1981). Rift Valley fever.In: CRC Handbook series in zoonoses. James H. Steele (ed. Chief). CRC Press, Inc. USA. Pp 403-420.

Peyrefitte, C.N., Boubis, L., Coudrier, D., Bouloy, M., Grandadam, M., Tolou, H.J., Plumet, S. (2008). Real- time reverse-transcription loop-mediated isothermal amplification for rapid detection of Rift Valley fever virus. J. Clin. Microb., 46 (11), 3653-3659.

© University of Pretoria

Rich, K.M., Wanyoike, F. (2010). An assessment of the regional and national socio-economic impacts of the 2007 Rift Valley fever outbreak in Kenya. Am. J. Trop. Med. Hyg., 83 (Suppl. 2), 52-57.

Sall, A.A., Zanotto, P.M.A., Vialat, P., Sene, O.K., Bouloy, M. (1998). Molecular epidemiology and emergence of Rift Valley fever. Memorias do Instituto Oswaldo Cruz, 93 (5), 609-614.

Sall, A.A., Thonnon, J., Sene, O.K., Fall, A., Ndiaye, M., Baudez, B., Mathiot, C., Bouloy, M. (2001). Single tube and nested reverse transcriptase-polymerase chain reaction for detection of Rift Valley fever virus in human and animal sera. J. Virol. Methods, 91, 85-92.

Sall, A.A., Macondo, E.A., Sene, O.K., Diagne, M., Sylla, R., Mondo, M., Girault, L., Marrama, L., Spiegel, A., Diallo, M., Bouloy, M., Mathiot, C. (2002), Use of reverse transcriptase PCR in early diagnosis of Rift Valley fever. Clinical and Diagnostic Laboratory Immunology, 9 (3), 713-715.

Sang, R., Kioko, E., Lutomiah, J., Warigia, M., Ochieng, C., O'Guinn, M., Lee, J.S., Koka, H., Godsey, M., Hoel, D., hanafi, H., Miller, B., Schnabel, D., Breiman, R.F., Richardson, J. (2010). Rift Valley fever virus epidemics in Kenya, 2006/2007: the entomologic investigations. Am. J. Trop. Med. Hyg., 83 (Suppl. 2) 28-37.

Scherer, W.F., Eddy, G.A., Monath, T.P., Walton, T.E., Richardson, J.H. (1980). Laboratory safety for arboviruses and certain other viruses of vertebrates. Am. J. trop. Med. Hyg., 29, 1359-1381.

Schwarz, T.F., Gilch, S., Pauli, C., Jager, G. (1996). Immunoblot detection of antibodies to Toscana virus. J. Med. Virol., 49, 83-86.

© University of Pretoria

Scott, R.M., Feinsod, F.M., Allam, I.H., Ksiazek, T.G., Peters, C.J., Botros, B.A.M., Darwish, M.A. (1986). Serological tests for detecting Rift valley fever viral antibodies in sheep from the Nile Delta. J. Clin. Microbiol., 24 (4), 612-614.

Seufi, A.M., Galal, F.H. (2010). Role of *Culex* and *Anopheles* mosquito species as potential vectors of Rift valley fever virus in Sudan outbreak, 2007. BMC Infect. Dis., 10:65.

Smithburn, K.C., Mahaffy, A.F., Haddow, A.J., Kitchen, S.F., Smith, J.F. (1949). Rift Valley fever accidental infections among laboratory workers. J. Immunol., 62, 213-227.

Soliman, A.K., Botros, B.A.M., Morril, J.C. (1988). Solid-phase immunosorbent technique for rapid detection of Rift valley fever virus immunoglobulin M by hemagglutination inhibition. J. Clin. Microbiol., 26 (9), 1913-1915.

Swanepoel, R., Struthers, J.K., McGillivray, G.M. (1983). Reversed passive hemagglutination and inhibition with Rift Valley fever and Crimean-Congo hemorrhagic fever virus. A. J. Trop. Med. Hyg., 32 (3), 610-617.

Swanepoel, R. Struthers, J.K., Erasmus, M.J., Shepherd, S.P., McGillivray, G.M., Erasmus, B.J., Barnard, B.J.H. (1986) Comparison of techniques for demonstrating antibodies to Rift Valley fever virus. J. Hyg. Camb., 97, 317-329.

Swanepoel, R., Coetzer, J.A.W. (1994). Rift Valley fever. In: Infectious diseases of livestock with special reference to Southern Africa. 1st ed. Ed. Coetzer, J.A.W., Thompson, G.R. Tustin, R.C Cape Town, Oxford University Press Southern Africa.

Swanepoel, R., Coetzer, J.A.W. (2004). Rift valley fever. In: Infectious diseases of livestock, 2nd ed. Ed. Coetzer, J.A.W. and Tustin, R.C. Vol II. Oxford University Press, Southern Africa. Pp 1037-1070.

© University of Pretoria

Turrel, M.J., Linthicum, K.J., Patrican, L.A., Davies, F.G., Kairo, A., Bailey, C.L. (2008). Vector competence of selected African mosquito (Diptera: Culicidae) species for Rift Valley fever virus. J. Med. Entomol., 45 (1), 102-108.

Valadão, F.G. (1969). Nota prévia sobre a ocorrência de uma nova doença em Moçambique – a doença do vale do Rift. Veterin. Moçamb., 2, 13-20.

Vapalahti, O., Kallio-Kokko, H., Narvanen, A., Julkunen, I., Lundkivist, A., Plyusnin, A., Lehvaslaiho, H., Brummer-Korvenkontio, M., Vaheri, A., Lankinen, H. (1995). Human B-cel epitopes of Puumala virus nucleocapsid protein, the major antigen in early serological response. J. Med. Virol., 46, 293-303.

Vialat, P., Billecocq, A., Kohl, A., Bouloy, M. (2000). The S segment of Rift Valley fever Phlebovirus (*Bunyaviridae*) carries determinants for attenuation and virulence in mice. J. Virol., 74 (3), 1538-1543.

Yedloutschnig, R.J., Dardiri, A.H., Walker, J.P. (1981). Persistence of Rift Valley fever virus in the spleen, liver, and brain of sheep after experimental infection. In: Swartz, T.A., Klinberg, M.A., Goldblum, N., Papier, C.M. (Eds.). Contributions to epidemiology and biostatistics: Rift Valley fever. S. Karger AG, Basel. Pp. 72-76.

Won S., Ikegami, T., Peters, C.J., Makino, S. (2006). NSm and 78-kilodalton proteins of Rift Valley fever virus are non essential for viral replication in cell culture. J. Virol., 80, 8274-8278.

Won, S., Ikegami, T., Peters, C.J., Makino, S. (2007). NSm protein of Rift Valley fever virus suppresses virus-induced apoptosis. J. Virol., 81 (24), 13335-13345.

WHO (2007). Outbrakes of rift Valley fever in Kenya, Somalia and United Republic of Tanzania, December 2006 - April 2007. Weekly epidemiological news, 20 (82), 169-178.

© University of Pretoria

WHO (2010). Rift Valley fever, South Africa – update1. Weekly epidemiological news, 21 (85), 185-186.

Wright, P.F., Nilsso, E., Van Rooij, E.M.A., Lelenta, M., Jeggo, M.H. (1993) standardization and validation of enzyme-linked immunosorbent assay techniques for the detection of antibody in infectious disease diagnosis. Rev. Sci. Tech.Off. Int. Epiz., 12, 435-450.

© University of Pretoria

2 . Cloning and expression of Rift Valley fever virus nucleocapsid (N) protein and evaluation of a N-protein based indirect ELISA for the detection of specific IgG and IgM antibodies in domestic ruminants.

2.1 Introduction

Rift Valley fever (RVF) is primarily a mosquito-borne viral disease that results in severe economic losses due to high mortality particularly in new-borne lambs and kids and abortion in adult sheep, goats and cattle. It causes mild to moderate febrile illness in humans but severe complications such as ocular sequelae, encephalitis or a fatal haemorrhagic state, may occur in a low proportion of infected individuals (Swanepoel and Coetzer, 1994). The first confirmed outbreaks of RVF among humans and livestock outside Africa, occurred in the Arabian Peninsula (Shoemaker et al., 2002), implying that spread of infection into non-endemic areas is most likely to occur, may be transmitted by a wide range of mosquito vectors (Turrel et al., 1998).

Considerable efforts have been made recently to develop nucleic acid techniques for rapid detection and identification of RVFV (Ibrahim et al., 1997; Turrel et al., 1997; Garcia et al., 2001). However, conventional and molecular procedures for diagnosis of RVF may be beyond the resources and capabilities of many laboratories, particularly in developing African countries. Accurate diagnosis of RVF can be achieved when serological tests are used in combination with clinical observation and epidemiological data and/or when seroconversion to the virus is demonstrated.

The three most useful methods for the detection of antibodies to RVFV are haemagglutination-inhibition (HI), virus neutralization (VN) tests and the enzyme-linked immunosorbent assay (ELISA). Although regarded as a gold standard, the VN test is laborious, expensive, and requires 5-7 days for completion. It can be performed only when a standardized stock of live virus and tissue culture facilities are available. Consequently, it is only performed in highly specialized laboratories. The HI test is sensitive but laborious and cross-reactive with other phleboviruses (Scott et al., 1986; Swanepoel et al., 1986a). In addition, disadvantages of the conventional diagnostic

© University of Pretoria

methods include health risk to laboratory personnel (Kitchen, 1934; Smithburn et al., 1949), restrictions to their use outside RVF endemic areas (Scherer et al., 1980; Barnard and Gerdes, 2000), their inability to distinguish between different classes of immunoglobulins, lack of standardization and unsuitability for screening large numbers of sera (Wright et al., 1993).

A highly sensitive and specific ELISA based on β-propiolactone inactivated and/or gamma-irradiated, sucrose-acetone-extracted RVFV antigen derived from tissue cultures or mouse brain has recently been developed and validated (Paweska et al., 1995; 2003a; b). However, this type of RVFV antigen has to be produced in a containment facility and it poorly binds to the ELISA plates. To overcome this problem a sandwich ELISA that utilizes immobilized antibodies to trap crude or semi-purified preparations of antigen can be used (Meegan et al., 1987; Paweska et al., 2003a).

Recombinant nucleocapsid proteins have successfully been used for the detection of antibodies to other viruses in the family *Bunyaviridae* (Kallio-Kokko et al., 1993; Marriott et al., 1994; Elgh et al., 1997; Ciufolini et al., 1999). This paper describes the cloning, expression and purification of nucleocapsid protein of RVFV and its evaluation as an antigen in indirect ELISA (I-ELISA) for the detection of RVFV specific IgM and IgG antibodies in ruminant sera.

2.2 Materials and Methods

2.2.1 Virus

Inactivated RVFV strain FVR 1976 was kindly supplied by Dr J. Groen, Institute of Virology, The University Hospital Rotterdam, The Netherlands.

2.2.2 Construction of plasmids

Vector pET43b (Novagen) enables purification of fusion proteins by immobilised metal affinity chromatography. The vector was adapted to GateWay cloning (Invitrogen) and a second purification tag (a chitine binding domain -CBD) was introduced as follows. Using pTYB11 (New England Biolabs) as a template, the CBD gene was amplified by

© University of Pretoria

polymerase chain reaction and suitable restriction sites were introduced using the forward primer 5'-CGC GTC TAG *ACT AGT* GGT GCT AGC ACA AAT CC-3' and reverse primer 5'-CGA CAT ATG ACC ACC ATG GCC ACC TTG-3'. The PCR product was digested with *XbaI* and *NdeI* (underlined in primer sequences) and ligated into the Gateway cassette of pDEST17 (Invitrogen), digested with the same enzymes. The resulting plasmid was purified from carbenicillin and choramphenicol resistant colonies obtained after transformation of *E coli* DB3.1 (Invitrogen) that is resistant to the toxic protein encoded by the *ccdB* gene present on the GateWay cassette of pDEST17. The purified plasmid was digested with *SpeI* (italics in the forward primer sequence) and *HindIII.* The fragment containing the CBD and GateWay cloning cassette was ligated into *SpeI* - *HindIII* digested pET43b. After transformation of DB3.1 cells, plasmid DNA was purified from selected colonies and sequenced to confirm that the correct plasmid, designated pET43GW, had been obtained. The plasmid was digested with *SpeI*, blunted with T4 DNA polymerase and, subsequently, digested with *HindIII*. The smaller fragment, containing the CBD sequence and GateWay cassette, was cloned into *SmaI*-*HindIII* digested pET43b to yield pETH2GW. Genes cloned into this vector are expressed with an additional hexa-histidine tag.

2.2.3 Cloning and expression of the N-protein

The RNA of RVFV was extracted using the RNeasy kit (Qiagen) and subjected to RT-PCR. Based on published sequences of the S segment (Gene Bank # 9632365) two primers, the forward primer RVF-F2 (5'-GCC GCG CGG GAG CAT GGA CAA CTA TCA AGA CGT TGC GAT CC-3) and the reverse primer RVF-R (5'-GCG GCG GCG GGC CCC CTG GGC AGC CAC-3') were designed to amplify the RVFV gene encoding the nucleocapsid (N) protein. The underlined sequences were not encoded by the virus, but were included as annealing sites for a second PCR performed with the forward primer GW2-F (5'-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT GGT GCC GCG CGG GAG C-3') and the reverse primer GW-R (5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTG CGG CGG CGG G-3'). This second PCR introduced the *att B1* and *att B2* sites, which enabled subsequent GateWay cloning. The PCR product was harvested

© University of Pretoria

from the low melting point agarose gel and inserted into the vector pDONR201 (Invitrogen) by the BP Gateway reaction performed according to the instructions of the manufacturer. After transformation of *E. coli* strain DH5α, plasmid DNA was purified from selected colonies and sequenced to check the cloned fragment. Subsequently, the fragment was subcloned into the expression vector pETH2GW by the LR Gateway reaction. Carbenicillin resistant colonies obtained after transformation of *E.coli* DH5α were checked by PCR, using primers GW2-F and GW-R. Plasmid DNA of the resulting vector, designated pETH2-NP was purified from a culture grown from a PCR positive colony.

2.2.4 Expression and purification of the his6-tagged NusA-N-protein

The expression strain *E.coli* BL 21DE3 (RIL) Codon Plus (Stratagene) was transformed with pETH2-NP using carbenicillin and chloramphenicol resistance for selection. Twenty-five milliliter cultures of carbenicillin (25 µg/ml) and chloramphenicol (34 µg/ml) containing LB were incubated shaking overnight at 37°C. Then the culture was diluted 1:20 in 500 ml LB containing the same antibiotics and incubated at 37°C, with shaking, till the optical density at 600 nm (OD600) reached 0,6-0,9. At that time, expression of the NusA-N-fusion protein was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1mM/L. The culture was incubated for an additional 4 hours at 37°C with vigorous shaking. Cells were harvested by centrifugation for 15 minutes at 5000g. The pellet was resuspended in 25 ml Buffer B (20 mM Tris/HCl (pH 8,0), 500 mM NaCl) containing 0.1 mg/ml lysozym, and the suspension incubated for 30 minutes at room temperature. Subsequently 2,5 ml of a solution containing 50 mM EDTA, 10 mM 2-Mercaptoethanol and 10% Triton-X100, was added. After three freeze-thaw cycles, 1,5 ml 0.5 M $MgCl_2$ and 3 µl benzonase (Novagen) were added to the lysate to break down the released DNA. Once the lysate was no longer viscous (approximately 15 minutes at room temperature) it was centrifuged at 5000g for 15 minutes. The supernatant was collected and the pellet (inclusion bodies) was washed once with Buffer B containing 1% Triton-X100 and subsequently dissolved in Buffer B containing 8 M urea and 20 mM imidazole. After

© University of Pretoria

incubation at room temperature for 25 minutes the solution was centrifuged at 5000g for 15 minutes at room temperature to remove insoluble material. The clear supernatant was purified on a column packed with Ni2+- charged chelating Sepharose (Amersham). After washing the column with the starting buffer (20mM Tris-HCl pH 8,0, 500mM NaCl, 20 mM imidazole and 8 M urea), the sample was eluted with Buffer B containing 8 M urea and 50 mM EDTA and dialysed overnight at 4°C against PBS to allow refolding of the protein. Any material precipitated during the buffer exchange was removed by spinning at 5000g at 4°C for 20 min. The resulting supernatant was used as antigen in ELISA. The protein concentration of the supernatant was 0,34 mg/ml as determined by ND-1000 Spectrophotometer (NanoDrop Technologies, Inc.). Samples taken during the purification process were subjected to SDS-PAGE analysis.

2.2.5 Sera

All test sera were obtained from the Special Pathogens Unit, National Institute for Communicable Diseases (SPU-NICD), South Africa.

Sera (n=128) from eight sheep inoculated subcutaneously with 1 ml of cell culture supernatant fluid containing $10^{6.5}$ $TCID_{50}$/ml of the AR 20368 strain of RVFV isolated in 1981 from *Culex zombaensis* in South Africa (Paweska et al., 2003a), taken on days 0, 1-14, 21, 70 and 77 after inoculation and sera (n = 240) from 10 sheep vaccinated subcutaneously with 1 ml of tissue-culture preparation of modified live vaccine of the Smithburn strain of RVFV, taken on days 0, 1-18, and 20 after vaccination, were used to study the kinetics of antibody production and to compare the analytical sensitivity and specificity of the ELISA with those of VN and HI tests under control conditions.

A total of 713 field-collected sera from goats (n = 362), sheep (n = 251), and cattle (n = 100) was used. The sera were collected in East Africa (Kenya and Somalia) to monitor the 1997-1998 outbreak of RVF in this region (Woods et al., 2002). The VN was used as a relative standard of comparison to categorize animals as infected and uninfected with RVFV, consequently individual sera from test animals were grouped as positive and negative and subsequently analyzed in the indirect ELISA. ELISA results obtained

© University of Pretoria

in VN-positive and VN-negative sera were used for the selection of the ELISA cut-off value.

2.2.6 Virus neutralisation and haemagglutination-inhibition tests

The virus microneutralization and haemagglutination-inhibition tests were conducted as described previously (Paweska et al., 2003b).

2.2.7 ELISA internal controls

Freeze-dried, gamma-irradiated serum controls were obtained from the SPU-NICD, SA. Selection, inactivation, and characterization of high, low and negative controls followed recommended guidelines for the preparation of reference standards for an ELISA (Wright et al., 1993; 1997) and was described elsewhere (Paweska et al., 2003a).

2.2.8 Anti-ruminant immunoglobulin and recombinant protein G horseradish peroxidase (HRPO) conjugates

Using a checkerboard titration and standard direct ELISA procedures (Crowther, 1995), commercially available anti-species-Ig-HRPO conjugates were tested for their capacity to detect antibody bound to the recombinant N-protein of RVFV. Rabbit antiserum to the μ chain of sheep IgM (ICN Pharmaceuticals. Inc., Aurora, Ohio, USA) was selected for detection of IgM. For the detection of IgG two different anti-IgG-HRPO conjugates were used: rabbit anti-sheep IgG(H + L) (Zymed Laboratories, Inc., San Francisco, CA, USA) to detect sheep and goat IgG, and goat anti-bovine IgG(H + L) (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, USA) to detect cattle IgG. In addition, peroxidase-conjugated protein G (HRPO-(rec) Protein G, Zymed, USA) was used. This conjugate can be applied to detect IgG antibody of different species (Bjorck and Blomberg, 1987; Abdu-Zeid, 2002; Paweska et al., 2003b; Saegerman et al., 2004).

© University of Pretoria

2.2.9 Procedures for the indirect ELISA (I-ELISA)

Immunoplates (Maxisorb, Nunc, Denmark) were coated directly with 100 µl stock recombinant N-protein diluted 1:200 for the IgM and 1:100 for the IgG I-ELISA in PBS pH 7,4 and incubated overnight at 4°C. After washing three times with PBS containing 0,1% Tween 20 (washing buffer) the plates were blocked with 200 µl of 10% fat-free milk powder (“Elite”, Clover SA, Pty Ltd) in PBS, then incubated in a moist chamber for 1h at 37°C and washed as described before. Control and test sera were diluted 1:400 in PBS containing 2% milk powder (diluting buffer) and 100 µl were added to the plates. Each test serum was tested in duplicate and the control sera in quadruplicate. After incubation in a moist chamber for 1h at 37°C, plates were washed 6 times with the washing buffer and 100 µl of the peroxidase conjugated anti-IgM (diluted 1:500) or anti-IgG (diluted 1:3000) or Protein G (diluted 1: 4000) were added. Plates were incubated at 37°C for 1h and after washing 6 times, 100 µl 2,2’-Azino di-ethyl-benzothiazoline-sulfonic acid (ABTS) substrate was added to each well. Subsequently plates were incubated in the dark at room temperature for 30 minutes. The reactions were stopped by the addition of 100 µl 1% sodium dodecyl sulphate (SDS), and the optical densities (OD) determined at 405 nm. The results were expressed as percentages of the high-positive control serum (PP) using the formula: (mean OD of the test serum /mean OD of the high positive control) x 100.

Sera from experimentally infected and vaccinated sheep were tested for the presence of antibodies to RVFV using rabbit anti-sheep IgM and rabbit anti-sheep IgG, and sera from vaccinated sheep were additionally tested using protein G. The field serum panel was tested using protein G, in addition goat sera were tested using rabbit anti-sheep IgG.

2.2.10 Selection of cut-offs and estimation of diagnostic accuracy

ELISA cut-off values at 95 % accuracy level were selected using the two-graph receiver operating characteristics analysis (TG-ROC) (Greiner, 1995; Greiner et al., 1995). The following statistical approaches (Greiner and Gardner, 2000) were used to estimate: relative diagnostic sensitivity (D-Se) = [Tp/(Tp+Fn)] x 100 and relative diagnostic

© University of Pretoria

specificity (D-Sp) = [Tn/(Tn+Fp)] x 100; where: Tp represents the true-positive sera Fn the false-negative sera, Tn the true-negative sera, Fp the false positive sera.

2.3 Results

2.3.1 Cloning, expression and purification of the N-protein

Amplification of the RVFV gene encoding the N-protein by RT-PCR yielded a fragment of the expected size of approximately 800 bp. After addition of the GateWay cloning sites *attB1* and *attB2* by a second PCR round, the fragment was inserted into pDONR201 and subsequently transferred to the expression plasmid pETH2GW, yielding plasmid pETH2-NP. Sequence analysis demonstrated 100% homology with the published gene for the N-protein of RVFV (results not shown). Analysis by SDS-PAGE demonstrated that *E.coli* BL 21DE3 (RIL) Codon Plus cells transformed with pETH2-NP, expressed, considerable amounts of a 97 kDa protein (Fig. 2.1: lanes 2 and 3) after IPTG induction. The majority of the fusion protein was in the insoluble fraction (inclusion bodies) after cell lysis (Fig. 2.1: lanes 4 and 5) It was dissolved in 8 M urea and purified on Ni2+ chelating sepharose, employing its two his6-tags. Most of the fusion protein bound to the column (Fig. 2.1: lanes 6 and 7) and was successfully eluted by the addition of EDTA (Fig. 2.1: lane 8). Once purified, the fusion protein remained soluble after urea removal by extensive dialysis against PBS (Fig. 2.1 lane 9).

© University of Pretoria

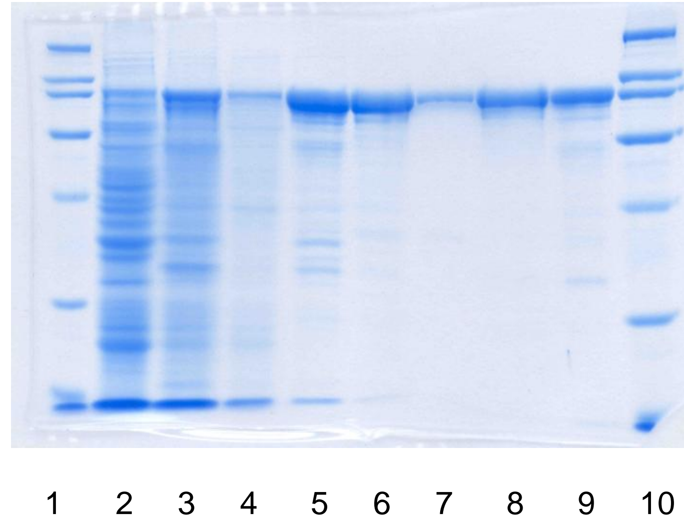
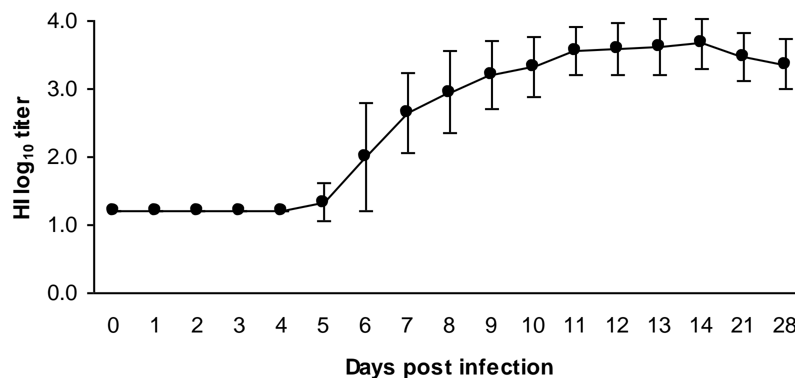


Fig. 2.1. SDS-PAGE analysis of the N-protein of RVFV - Lanes: **1.** Mw-marker **2.** not induced bacteria. **3**. induced bacteria (total lysate) **4**. soluble fraction **5.** insoluble fraction (inclusion bodies) **6.** inclusion bodies solved in 8 M urea **7.** flow-through Ni2+ column **8.** protein eluted from Ni2+ column **9.** soluble protein after dialysis against PBS **10.** Mw-marker

2.3.2 Analytical evaluation of the N-protein based indirect ELISA for detection of IgM and IgG anti-RVFV

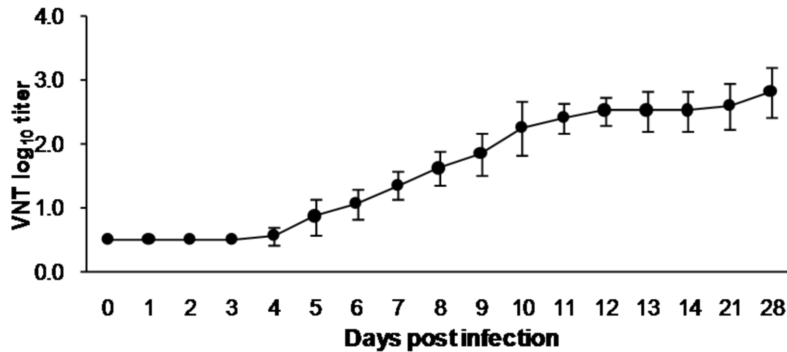
Comparison of the HI, VN and I-ELISA (using anti-species IgM and IgG conjugates) in RVF-experimentally infected sheep is shown in Fig. 2.2A-C. The ELISA and the VN test detected antibodies earlier than the HI.

(A)



© University of Pretoria

(B)



(C)

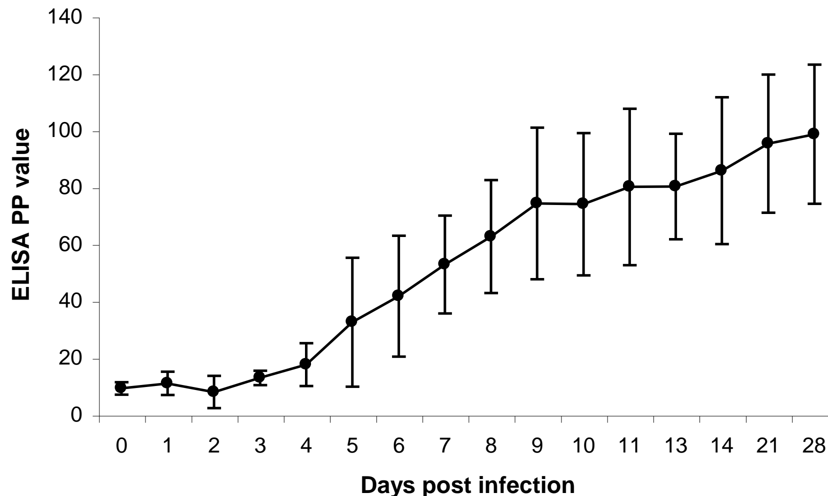


Fig. 2.2. (A) Mean + 1SD. Immune response of eight sheep experimentally infected with wild type of RVF as measured by HI. (B) Mean + 1SD. Immune response of eight sheep experimentally infected with wild type of RVFV as measured by VN. (C) Immune response of eight sheep experimentally infected with wild type of RVFV as measured by N-based I-ELISA.

The dynamics of IgM and IgG responses in experimentally infected and vaccinated sheep using the recombinant N-protein I-ELISA are shown in Fig. 2.3 and Fig. 2.4, respectively. In experimentally infected sheep the IgM PP values started to rise from day 3 onwards and reached the maximum value at day 9. At day 77 the IgM PP values had returned to values recorded before inoculation. The IgG PP values started to rise at day 5 and were still high at day 77 (Fig. 2.3). In vaccinated sheep the IgM levels started to rise from day 4, reached the highest PP values at day 10 followed by their gradual

© University of Pretoria

decrease. The IgG PP values rose from day 6 onwards and were still high at day 34 after vaccination (Fig. 2.4).

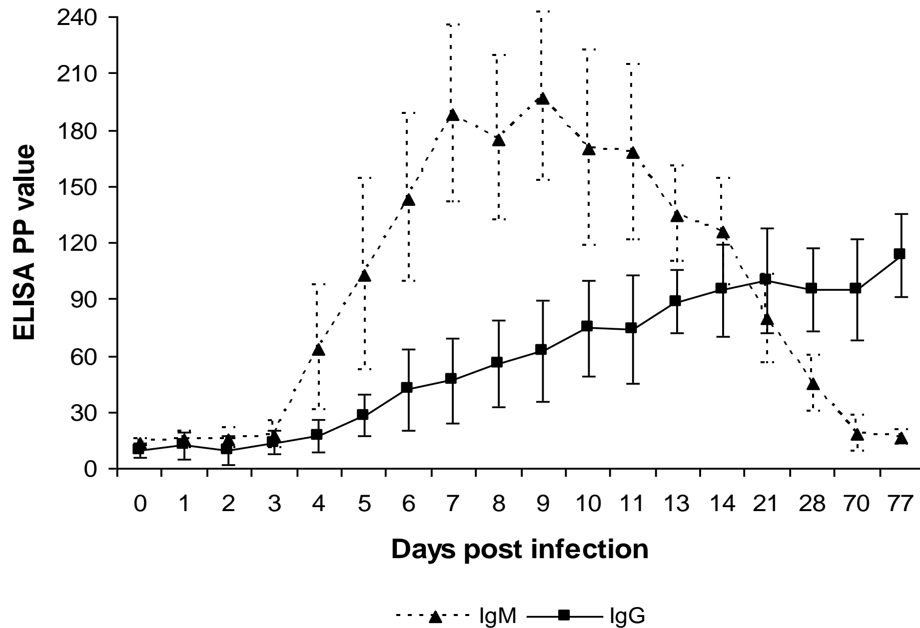


Fig. 2.3. Mean ± 1S.D. IgM and IgG responses in sheep (*n* = 8) infected with wild type AR 20368 strain of RVFV using the recombinant N-protein I-ELISA .

© University of Pretoria

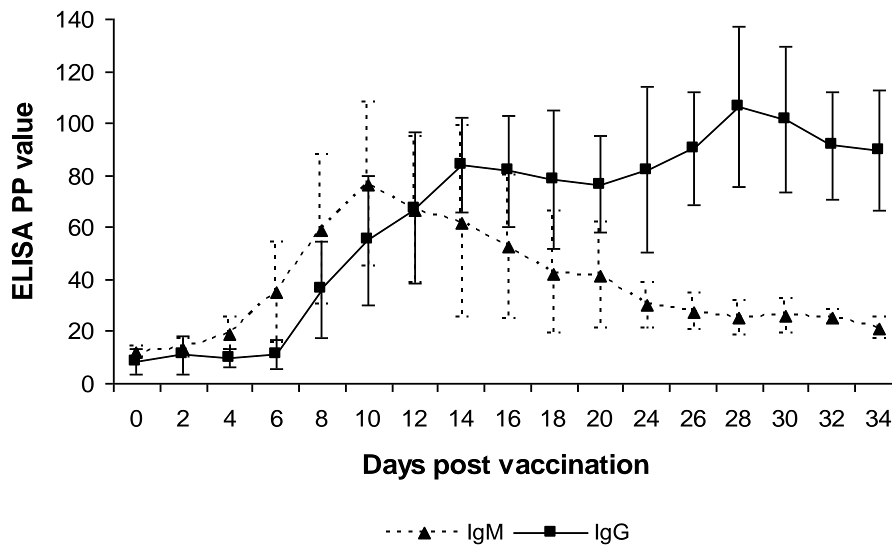


Fig. 2.4. Mean ± 1S.D. IgM and IgG responses in sheep (*n* = 10) vaccinated with live-attenuated Smithburn strain of RVFV using the recombinant N-protein I-ELISA.

In the vaccinated sheep IgG responses were also measured by the N-protein based I-ELISA using protein G as a detection system, which produced higher PP values than the assay run with anti-species IgG (Fig. 2.5). However, the correlation coefficient between the two data sets was high (0.823).

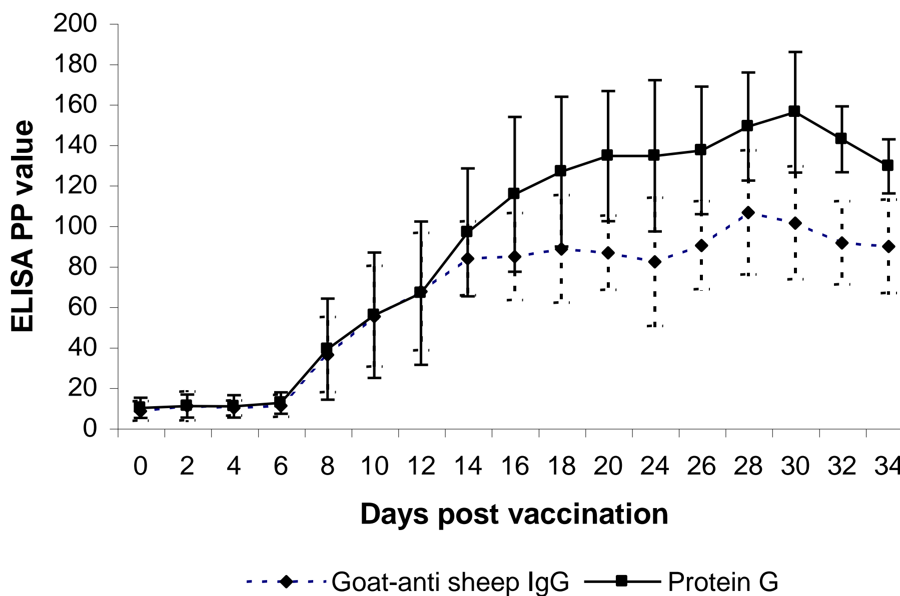


Fig. 2.5. Mean ± 1S.D. IgG responses in sheep (*n* = 10) vaccinated with live-attenuated Smithburn strain of RVFV as measured by the N-protein based RVFV IgG I-ELISA using anti-sheep IgG and protein G HRPO conjugates

© University of Pretoria

2.3.3 Determination of cut-off values

Cut-off values derived from the TG-ROC analysis using VN as reference test are given in Table 1. Optimisation of cut-off values using the TG-ROC analysis was based on the non-parametric programme option (Greiner, 1995) due to departure from a normal distribution of data sets analysed. The optimal cut-off PP values were different for the distinct subpopulations of species tested. An example of the graphic presentation produced by the TG-ROC analysis is shown in Fig. 2.6.

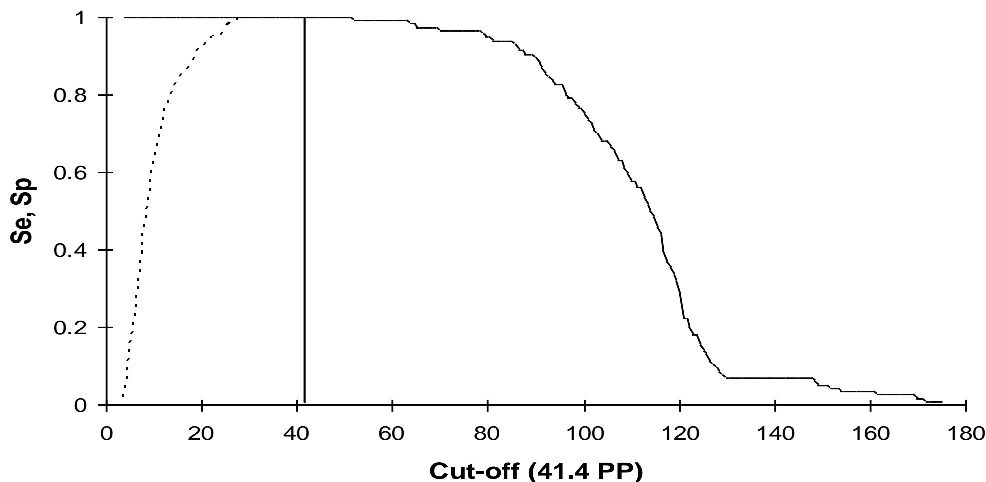


Fig. 2.6. Selection of cut-off for N-protein based RVFV IgG I-ELISA using anti-sheep conjugate in goats by TG-ROC analysis. The insertion point of the sensitivity (Se, smooth line) and specificity (Sp, dashed line) graphs represents a cut-off PP value (41.4) at which the highest and equivalent test parameters are achieved at 95% accuracy level.

2.3.4 Diagnostic accuracy

Very high estimates of the relative D-Sn and D-Sp were derived from the study data sets both when using anti-species IgG and protein G conjugates for each of the species (Table 2.1).

© University of Pretoria

Table 2.1. Diagnostic accuracy of the Rift Valley fever recombinant N-based I-ELISA

Measure	Cattle	Goat	Sheep
Cut-off[a]	35.3 PP	73.5 PP; 41.4PP[b]	48.3 PP
Sensitivity (%)	100 (VNT+[c] =42)	99.4; 100 (VNT+ =162; VNT+ =197)	100 (VNT+ =51)
95% C.I.	[91.6;100%]	[96.6;99.9%]; [98.1;100%]	[93;100%]
Specificity (%)	98.3 (VNT-[d] =58)	99.5; 100 (VNT- =200; VNT- =116)	100 (VNT- =200)
95% C.I.	[90.8;99.9%]	[99.3;99.9%]; [96.9;100%]	[98.2;100%]

[a] Cut-off values expressed as a percentage positivity (PP) of an internal high positive serum control was optimized at 95 % accuracy level by the TG-ROC analysis.

[b]Non-underline data: ELISA run with protein G, underline data: ELISA run with anti-sheep IgG

[c] Number of sera tested positive in the virus neutralisation test. [d] Number of sera tested negative in the virus neutralisation test.

2.4 Discussion

Many countries where RVF is endemic, and where disease outbreaks occur in both ruminants and humans, do not have adequate containment facilities required to work with RVFV. For this reason and the fact that the virus has recently been shown to spread beyond its traditional endemic areas (Shoemaker et al., 2002) there is an urgent need for the development of diagnostic methods allowing for safe and cost effective production and application of RVF immunoreagents. The N-protein of RVFV was chosen as an antigen because it was shown to be the immuno-dominant protein in other members of the *Bunyaviridae* family (Swanepoel et al., 1986b; Vapalahti et al., 1995; Schwarz et al., 1996).

The use of unpurified whole virus preparations in the I-ELISA (Forghani and Schmidt, 1979) gave rise to high background absorbance and purification of the virus is laborious and expensive. Recombinant antigens lack infectivity and have shown to be very stable (Maree and Paweska, 2005) which makes them suitable for wide distribution in ELISA kit format. In the present study the I-ELISA using HRPO-conjugated anti-species IgM,

© University of Pretoria

IgG and protein G were used to assess the recombinant N-protein as potential diagnostic immunoreagent for serodiagnosis of RVF.

In the pETH2-NP vector the gene for the N-protein, confirmed by sequence analysis, was cloned in-frame with gene segments encoding the NusA protein, the Chitin binding domain (CBD) and two hexa-histidine-tags.

Good solubility as previously shown for proteins expressed in *E. coli* as a fusion with the NusA protein, (De Marco et al., 2004) could not be shown in the present approach. However, the CBD and his6-tags allowed purification of the fusion protein dissolved in 8M urea on columns of immobilised Ni2+ ions that resulted in a soluble fraction that could be used in ELISA. The molecular mass of the 97 kDa recombinant protein produced by E*. coli* BL 21 DE3 (RIL) Codon Plus cells transformed with pETH2-NP (Fig.2 lanes 2 and 3) corresponded well to the calculated mass of NusA-NP fusion protein.

Results obtained with sera from experimentally infected and vaccinated sheep show high analytical sensitivity of the N-protein I-ELISA in detection of RVFV specific antibodies. The I-ELISA detected RVFV specific IgG earlier than the HI test, and its sensitivity was comparable to the VN test. Results in this study were similar to those obtained by Paweska et al. (2003 b) using an indirect or a sandwich ELISA (Paweska et al., 2003a) based on inactivated sucrose-acetone extracted antigen.

The diagnostic accuracy of the N-protein based I-ELISA to correctly classify animals as infected or uninfected was assessed by testing field-collected goat, sheep and cattle sera known to be positive or negative in the VN test. Very high estimates of the relative D-Sn and D-Sp of the assay were obtained when using both anti-species IgG and protein G conjugates. In goats, the relative D-Sn and D-Sp was 100% when using the anti-species IgG conjugate. Using protein G as a detection system, the relative D-Sn and D-Sp in goats were 99,4% and 99,5%, in sheep field sera both 100%, in cattle 100% and 98,3% respectively. There was a good degree of correlation (0,823) between results obtained with the N-protein based I-ELISA using anti-species IgG and protein G in vaccinated sheep the latter showing lower diagnostic accuracy (Fig. 2.5). However, the use of protein G in I-ELISA has the advantage of detecting anti-RVFV antibodies in different ruminant species with one set of reagents. This would provide a very practical

© University of Pretoria

diagnostic tool, especially during outbreaks of RVF involving different domestic and wild ruminant species and humans.

Central to any serological assay is determination of the diagnostic threshold or cut-off, for which appropriateness of data used is important as this impacts on D-Sn and D-Sp (Jacobson, 1998). The TG-ROC analysis provides a simple graphical means of evaluating sensitivity and specificity and allows selection of cut-off values to obtain a minimum desired level of accuracy (Greiner, 1995). In the present study, the sera used for this evaluation were from animals with unknown vaccination or infection status. Therefore, the virus neutralisation test (current gold standard) was used to categorize individuals according to their RVF infection status. It is important to note that infection with RVFV induces life long virus neutralising immunity in animals (Barnard, 1979), and that there is no evidence of serological subgroups or major antigenic variation between virus isolates of disparate temporal or geographic origins (Swanepoel and Coetzer, 1994). Antigenic cross-reactivity studies in sheep (Swanepoel et al., 1986b) and field studies in cattle (Swanepoel, 1976) failed to provide evidence that other African phleboviruses could hamper the serodiagnosis of RVF.

The estimates of diagnostic accuracies of the N-protein based I-ELISA are similar to those reported previously (Paweska et al., 2003b) using the sucrose-acetone extracted antigen for ELISA. Hence, the results presented here confirm the potential value of the recombinant N-protein as an antigen in I-ELISA for detection of specific anti-RVFV antibodies. Additional advantages of the N-protein are its safety, stability and cost-effectiveness in use and production.

2.5 References

Abdu-Zeid, Y.A. (2002). Protein G ELISA for detection of antibodies against Toxoplasma SAG1 in dromedaries. J. Egypt. Soc. Parasitol., 32, 247-257.

Barnard, B.J.H. (1979). Rift Valley fever vaccine – antibody and immune response in cattle to a live and an inactivated vaccine. J. S. Afr. Vet. Assoc., 50, 155-157.

© University of Pretoria

Barnard, B.J.H., Gerdes, G.H. (2000). Rift Valley Fever. In: OIE Manual of Standards for Diagnostic Tests and Vaccines, fourth ed. Office International des Epizooties, Paris, France, Pp.144-152.

Bjorck, L., Blomberg, J. (1987). Streptococcal protein G: a sensitive tool for detection of antibodies to human immunodeficiency virus protein in Western blot analysis. Eur. J. Clin. Microbiol., 6 (4), 428-429.

Ciufolini, M.G., Fiorentini, C., Di Bonito, P., Mochi, S., Giorgi, C. (1999). Detection of Toscana virus-specific immmunoglobulins G and M by enzyme-linked immunosorbent assay based on recombinant viral nucleoprotein. J. Clin. Microbiol., 37 (6), 2010-2012.

Crowther, J.R. (1995). ELISA theory and practice. In: Crowther, J.R. (Ed.), Methods in Molecular Biology. Humana Press, Totowa, NJ.

De Marco, V., Stier, G., Blandin, S., de Marco, A. (2004). The solubility and stability of recombinant proteins are increased by their fusion to NusA. Biochem. Biophys. Res. Commun., 322 (3), 766-771.

Elgh, F., Lundkvist, A., Alexeyev, O.A., Stenlund, H., Avsic-Zupanc, T., Hjelle, B., Lee, H.W., Smith, K.J., Vainionpaa, R., Wiger, D., Wadell, G., Juto, P. (1997). Serological diagnosis of Hantavirus infections by an enzyme-linked immunosorbent assay based on detection of immunoglobulin G and M responses to recombinant nucleocapsid proteins of five viral serotypes. J. Clin. Microbiol., 35 (5), 1122-1130.

Forghani, B., Schmidt, R.E. (1979). Detection of antibodies to alphavirus by enzyme-linked immunosorbent assay. J. Clin. Microbiol., 10, 583-585.

Garcia, S., Crance, J.M., Billecocq, A., Peinnequin, A., Jouan, A., Bouloy, M., Garin, D. (2001). Quantitative real-time PCR detection of Rift Valley fever virus and its application to evaluation of antiviral compounds. J. Clin. Microbiol., 39, 4456-4461.

© University of Pretoria

Greiner, M. (1995). Two-graph receiver operating characteristics (TG-ROC) - a Microsoft-Excel template for the selection of cut-off values in diagnostic tests. J. Immunol. Methods., 185, 145-146.

Greiner, M., Sohr, D., Gobel, P. (1995). A modified ROC analysis for the selection of cut-off values and the definition of intermediate results of serodiagnostic tests. J. Immunol. Methods., 185, 123-132.

Greiner, M., Gardner, I.A. (2000). Application of diagnostic tests in veterinary epidemiological studies. Prev. Vet. Med., 45, 43-59.

Ibrahim, M.S., Turell, M.J., Knauert, F.K., Lofts, R.S. (1997). Detection of Rift Valley fever virus in mosquitos by RT-PCR. Mol. Cell. Probes., 11, 49-53.

Jacobson, R.H. (1998). Validation of serological assays for diagnosis of infectious diseases. Rev. Sci. Tech. Off. Int. Epiz., 17, 469-486.

Kallio-Kokko, H., Vapalahti, O., Hedman, K., Brummer-Korvenkontio, M., Vaheri, A. (1993). Puumala virus antibody and immunoglobulin G avidity assays based on a recombinant nucleocapsid antigen. J. Clin. Microbiol., 31 (3), 677-680.

Kitchen, S.F. (1934). Laboratory infections with the virus of Rift Valley fever. Am. J. Trop. Med. Hyg., 14, 547-564.

Maree, S., Paweska, J.T. (2005). Preparation of recombinant African horse sickness virus VP7 antigen via a simple method and validation of a VP7-based indirect ELISA for the detection of group-specific IgG antibodies in horse sera. J. Virol. Methods., 125, 55-65.

© University of Pretoria

Marriott, A.C., Polyzoni, T., Antoniadis, A., Nuttall, P.A. (1994). Detection of human antibodies to Crimean-Congo haemorrhagic fever virus using expressed viral nucleocapsid protein. J. Gen. Virol., 75, 2157-2161.

Meegan, J.M., Yedlontschnig, R.J., Peleg, B.A., Shy, J., Peters, C.J., Walker, J.S., Shope, R.E. (1987). Enzyme-linked immunosorbent assay for detection of antibodies to Rift Valley fever virus in ovine and bovine sera. Am. J. Vet. Res., 48 (7), 1138-1141.

Paweska, J.T., Barnard, B.J.H., Williams, R. (1995). The use of sucrose-acetone-extracted Rift Valley fever virus antigen derived from cell culture in an indirect enzyme-linked immunosorbent assay and haemagglutination-inhibition test. Onderstepoort J. Vet. Res., 62, 227-233.

Paweska, J.T., Burt, F.J., Anthony, F., Smith, S.J., Grobbellaar, A.A., Croft, J.E., Ksiazek, T.G., Swanepoel, R. (2003a). IgG-sandwich and IgM capture enzyme-linked immunosorbent assay for the detection of antibodies to Rift Valley fever virus in domestic ruminants. J. Virol. Methods., 113, 103-112.

Paweska JT, Smith SJ, Wright IM, Williams R, Cohen AS, Van Dijk AA, Ksiazek, T.G., Swanepoel, R. (2003b). Indirect enzyme-linked immunosorbent assay for the detection of antibody against Rift Valley fever virus in domestic and wild ruminant sera. Onderstepoort J. Vet. Res., 70, 49-64.

Saegerman, C., De Waele, L., Gilson, D., Godfroid, J., Thiange, P., Michel, P., Limbourg, B., Vo, T.K., Limet, J., Letesson, J.J., Berkvens, D. (2004). Evaluation of three serum i-ELISA using monoclonal antibodies and protein G as peroxidase conjugate for the diagnosis of bovine brucellosis. Vet. Microbiol., 100 (1-2), 91-105.

Scherer, W.F., Eddy, G.A., Monath, T.P., Walton, T.E., Richardson, J.H. (1980). Laboratory safety for arboviruses and certain other viruses of vertebrates. Am. J. Trop. Med. Hyg., 29, 1359-1381.

© University of Pretoria

Schwarz, T.F., Gilch, S., Pauli, C., Jager, G. (1996). Immunoblot detection of antibodies to Toscana virus. J. Med. Virol., 49, 83-86.

Scott, R.M., Feinsod, F.M., Allam, I.H., Ksiazek, T.G., Peters, C.J., Botros, B.A.M., Darwish, M.A. (1986). Serological tests for detecting Rift Valley fever viral antibodies in sheep from the Nile Delta. J. Clin. Microbiol., 24 (4), 612-614.

Shoemaker, T., Boulianne, C., Vincent, M.J., Pezzanite, L., Al-Qahtani, M.M., Al-Mazrou, Y., Khan, A.S., Rollin, P.E., Swanepoel, R., Ksiazek, T.G., Nichol, S.T. (2002). Genetic analysis of viruses associated with emergence of Rift Valley fever in Saudi Arabia and Yemen, 2000-2001. Emerg. Infect. Dis., 12, 1415.

Smithburn, K.C., Mahaffy, A.F., Haddow, A.J., Kitchen, S.F., Smith, J.F. (1949). Rift Valley fever accidental infections among laboratory workers. J. Immunol., 62, 213-227.

Swanepoel, R. (1976). Studies on the epidemiology of Rift Valley fever. J. S. Afr. Vet. Ass., 3, 42-52.

Swanepoel, R., Struthers, J.K., Erasmus, M.J., Shepherd, S.P., McGillivray, G.M. (1986a). Comparison of techniques for demonstrating antibodies to Rift Valley fever virus. J. Hyg., 97, 317-329.

Swanepoel, R., Struthers, J.K., Erasmus, M.J., Shepherd, S.P., McGillivry, G.M., Shepherd, A.J., Hummitzsch, D.E., Erasmus, B.J., Barnard, B.J.H. (1986b). Comparative pathogenicity and antigenic cross-reactivity of Rift Valley fever and other African phleboviruses in sheep. J. Hyg., 97, 331-346.

Swanepoel, R., Coetzer, J.A.W. (1994). Rift Valley fever. In: Coetzer, J.A.W, Thomsom, R.C. (Eds.), Infectious diseases of livestock with special reference to Southern Africa, 1st ed., Vol. 1, Cape Town, Oxford University Press, Southern Africa, Pp. 688-717.

© University of Pretoria

Turrel, M.J., Bailey, C.L., Beaman, J.R. (1998). Vector competence of a Houston, Texas strain of *Aedes albopictus* for Rift Valley fever virus. J. Am. Mosq. Control Assoc., 4, 94-98.

Vapalahti, O., Kallio-Kokko, H., Narvanen, A., Julkunen, I., Lundkvist, A., Plyusnin, A., Lehvaslaiho, H., Brummer-Korvenkontio, M., Vaheri, A., Lankinen, H. (1995). Human B-cell epitopes of Puumala virus nucleocapsid protein, the major antigen in early serological response. J. Med. Virol., 46, 293-303.

Woods, C.W., Karpati, A.M., Grein, T., McCarthy, N., Gaturuku, D., Muchiri, E. Dunster, L., Henderson, A., Khan, A.S., Swanepoel, R., Bonmarin, I., Martin, L., Mann, P., Smock, B.L., Ryan, M., Ksiazek, T.G., Arthur, R.R., Ndikuyeze, A., Agata, N.N., Peters, C.J. (2002). An outbreak of Rift Valley fever in Northeastern Kenya, 1997-1998. Emerg. Infect. Dis., 8 (2), 138-144.

Wright, P.F., Nilsson, E., Van Rooij, E.M.A., Lelenta, M., Jeggo, M.H. (1993). Standardisation and validation of enzyme-linked immunosorbent assay techniques for the detection of antibody in infectious disease diagnosis. Rev. Sci. Tech. Off. Int. Epiz., 12, 435-450.

Wright, P.F., Tounkara, K., Lelenta, M., Jeggo, M.H. (1997). International reference standards: antibody standards for the indirect enzyme-linked immunosorbent assay. Rev. Sci. Tech. Off. Int. Epiz. 16, 824-832.

© University of Pretoria

3 . The detection of antibodies to Rift Valley Fever virus in small ruminants: comparison of an indirect recombinant nucleocapsid ELISA for IgG with an IgG sandwich ELISA utilizing inactivated virus

3.1 Introduction

There is an increasing international demand for safe and accurate laboratory procedures for early diagnosis of RVF and for use in surveillance programs. In this context, a number of serological (Paweska et al., 2003; 2005; 2007; Fafetine et al., 2007; Sorbaso et al., 2007; Jansen van Vuren et al., 2007; 2010; Kim et al., 2011; Williams et al., 2011) and nucleic acid detection techniques (Garcia et al., 2001; Sall et al., 2001; 2002; Espach et al., 2002; Peyrefitte et al., 2008; Le Roux et al., 2009) for human and animal use have been the subject of development during the last decade.

Amongst serological tests for the detection of antibodies against RVFV, the virus neutralization (VN) test is reported to have the highest sensitivity and specificity and is regarded as the gold standard (Swanepoel et al., 1986; Paweska at al., 2003). However, there are a number of practical constraints related to the use of this assay: it is expensive and laborious; it can only be performed when a standardized stock of live virus and tissue culture facilities are available, and it requires biocontainment facilities. To overcome these problems enzyme-linked immunosorbent assays (ELISA) have been developed in various formats for the detection of RVFV specific IgM and IgG antibodies but their diagnostic accuracy has not been directly compared in targeted populations of domestic ruminants. Sandwich IgG and IgM-capture ELISAs using β-propiolactone inactivated and/or gamma-irradiated, sucrose-acetone-extracted RVFV derived from tissue culture or mouse brains have been developed, validated and reported to be highly sensitive and specific for the detection of RVFV antibodies in humans and domestic livestock species (Paweska et al., 2003; 2005). However, since the production of the antigen poses a health risk to laboratory workers, this procedure is restricted to a few laboratories in Africa with biocontainment facilities. The shortcomings of these

© University of Pretoria

conventional techniques were overcome by the application of recombinant technology in the process of antigen production. Recombinant nucleocapsid protein of RVFV was successfully applied in an indirect ELISA format for the detection of antibodies to RVFV in domestic ruminants, humans and wildlife (Fafetine et al., 2007; Jansen van Vuren et al., 2007; Paweska et al., 2007; 2008; 2010). More recently, recombinant nucleocapsid protein was applied in an IgM antibody capture ELISA (Williams et al., 2011) and in a competitive ELISA format (Kim et al., 2011) providing a safe, reliable and highly accurate diagnostic tool for the detection of RVFV specific antibodies.

The aim of the present study was to compare efficacy of the detection of IgG antibodies to RVF virus, in the indirect ELISA based on recombinant nucleocapsid protein antigen with that in the sandwich ELISA based on an inactivated whole virus antigen, in sheep and goat sera collected in Mozambique.

3.2 Material and methods

3.2.1 Test sera

A total of 1262 serum samples (657 from goats and 605 from sheep) collected during a field survey of RVF in 2010 and 2011 in different localities of the Mopeia district, Zambézia Province, Mozambique were tested.

3.2.2 IgG indirect ELISA

The IgG indirect ELISA based on recombinant nucleocapsid protein was performed essentially as described elsewhere (Fafetine et al., 2007; Jansen van Vuren et al., 2007). One hundred micro litres of the recombinant nucleocapsid RVFV diluted 1:2000 in carbonate-bicarbonate buffer were coated overnight at 4°C on ELISA plates (Maxisorb, Nunc, Denmark). After incubation, the plates were washed 3 times with 0,1% Tweeen-20 in phosphate buffered saline (PBS) (washing buffer). The same washing procedure was used after each subsequent stage of the assay. Plates were incubated in a moist chamber for 1h at 37°C with 200 µl PBS with 10% skim milk. After incubation, plates were washed and 100 µl test, positive and negative control sera diluted 1:400 in

© University of Pretoria

2% skim milk in PBS (diluting buffer) were added and plates were incubated in a moist chamber for 1h at 37°C. After washing recombinant Protein G HRPO conjugate (Zymed Lab., Inc), was added at a dilution of 1:15000, in 100 µl and incubated for 1h at 37°C. After incubation, plates were washed and 100 µl 2,2'-azinodiethylbenzothiazoline sulfonic acid (ABTS, KPL Laboratories, Inc.) added. Plates were then incubated in the dark for 30 minutes at room temperature, after which 100 µl of the stop reagent, 1% sodium dodecyl sulphate (SDS), was added to each well and optical densities (OD) were measured at 405 nm. The results were subsequently expressed as percentage of a high positive control serum (PP) using the formula [(mean OD of test serum/mean OD of high positive control)] x 100.

3.2.3 IgG sandwich ELISA

The IgG sandwich ELISA based on whole inactivated antigen was performed according to the manufacturer's instructions (BDSL, UK) and procedure published by Paweska et al. (2003). ELISA plates (Maxisorb, Nunc, Denmark) were coated with 100 µl polyclonal mouse anti-RVFV antibody (NICD-SPU) diluted 1:5000 in PBS overnight at 4°C. Plates were washed 3 times with 0.1% Tweeen-20 in PBS (washing buffer) and blocked with 200 µl PBS with 10% skim milk in a moist chamber for 1h at 37°C. Plates were washed again as described before and 100 µl RVFV infected mouse brain (NICD-SPU) and control mouse brain antigen (NICD-SPU) diluted 1:400 in PBS containing 2% skim milk were added to rows of the top half of the plate (rows A-D: 1-12) and to the rows of the bottom half of the plate (rows E-G: 1-12) respectively. After incubation for 1h at 37°C, the plates were washed 3 times with the washing buffer. Subsequently duplicate volumes of 100 µl of each test, positive and negative control sera diluted 1:400 were added to the wells of the top half and the bottom half of the plate. Plates were incubated in a moist chamber at 37°C for 1h, washed 6 times with the washing buffer and 100 µl anti-sheep IgG HRPO conjugate (Zymed Lab., Inc) diluted 1:8000 was added. After incubation for 1h at 37°C, the plates were washed 6 times with the washing buffer and 100 µl ABTS (KPL Laboratories, Inc.) was added to each well. Plates were then incubated in the dark at room temperature for 30 minutes and the reaction was stopped

© University of Pretoria

by the addition of 100 µl of 1% SDS. Optical densities (OD) were determined at 405 nm. The net OD values were first recorded for each serum as the value determined with the RVFV antigen minus the value determined with the control antigen and subsequently converted into a percentage of the OD value of a high positive control serum (PP) using the same formula as in the indirect ELISA.

3.2.4 Estimation of diagnostic accuracy

Since the IgG sandwich ELISA was validated against the VN (Paweska et al., 2003), it was used in this study for evaluation of the indirect ELISA using the recombinant nucleocapsid protein. The cut-off values previously optimized for the assays (positive sample if: PP values greater than 25 in the indirect ELISA and PP values greater than 11.1 in the sandwich ELISA) were used to estimate the sensitivity, specificity and the agreement between the tests (Henken et al., 1997). The analyses were done using MedCal 12.2.1.

3.3 Results

A total of 657 field sera from goat and 605 field sera from sheep were used in this study to compare the ability of the IgG sandwich ELISA based on inactivated virus and the IgG indirect ELISA based on recombinant nucleocapsid protein to detect antibodies to RVFV. In both species, the IgG sandwich ELISA was able to detect more positive animals than the IgG indirect ELISA (Table 3.1), but the overall agreement between the tests in sera of small ruminants was high, *kappa* values of 0,99 and 0,92 in goats and sheep respectively. Using the IgG sandwich ELISA as gold standard, the sensitivity of the IgG indirect ELISA was 88,0% [95% confidence intervals (C.I): 79.6; 93,9] and 98,5% [95% C.I: 91,7; 99,9] and the specificity 99,8% [95% C.I.: 98,9; 100,0] and 100% [95% C.I.: 99,4; 100,0] in sheep and goats respectively.

© University of Pretoria

Table 3.1. Comparative results of the IgG sandwich ELISA and the IgG indirect ELISA in goats (A) and sheep (B)

A. Goats				
		IgG Indirect ELISA		
		+	-	Total
IgG Sandwich	+	64	1	65
ELISA	-	0	592	592
	Total	64	593	657
B. Sheep				
		IgG Indirect ELISA		
		+	-	Total
IgG Sandwich	+	81	11	92
ELISA	-	1	512	513
	Total	82	523	605

3.4 Discussion

Conventional immunodiagnostic assays for RVFV required virus culture for production of antigen. The need for biocontainment facilities hampered the application of these assays hence other sources of antigen to limit the risk of exposure for laboratory personnel were explored. In this context, RVFV recombinant nucleocapsid protein has been tested in indirect ELISA's for detection of specific RVFV IgG antibodies.

A RVF recombinant nucleocapsid protein indirect ELISA described by Fafetine et al. (2007) had a diagnostic sensitivity and diagnostic specificity in goats of 99.4% and 99,5%, in sheep both 100%, in cattle 100% and 98,3%, respectively. Diagnostic sensitivity (98,7%) and specificity (99,4%) in a similar range was also reported for an indirect recombinant nucleocapsid protein ELISA using African buffalo sera (Paweska et al., 2007). Jansen van Vuren et al. (2007) described a RVF recombinant nucleocapsid indirect ELISA as more sensitive than the virus neutralization test and the haemaglutination-inhibition test in detecting early immune response in experimentally

© University of Pretoria

infected sheep. A similar ELISA format applied for the detection of RVFV specific IgG in human sera had a diagnostic sensitivity of 99,7% and a diagnostic specificity of 99,6% (Paweska et al., 2007).

The IgG sandwich ELISA employing whole inactivated virus as antigen source has been widely used, validated and reported to have a sensitivity of 99,0 to 100% and specificity of 99,1 to 99,9% in domestic ruminants (Paweska et al., 2003). Although both assays used in this study were previously validated their performance has not been compared directly in field sera. Given that the IgG sandwich ELISA has been validated against the virus neutralization test, its commercial kit was used as standard reference for evaluating the indirect ELISA based on recombinant nucleocapsid protein.

The specificity of the indirect ELISA for goats (100%) and sheep (99,8%) and the sensitivity in goats (98,5%) found in this study are similar to the ones previously reported (Fafetine et al., 2007; Jansen van Vuren et al., 2007). In sheep the sensitivity of the indirect ELISA was lower than reported in previous studies. The adjustment of cut-off values using mean plus three-fold standard deviation and TG-ROC (16.0 or 15.95 for sheep and 15.5 or 17.80 for goats, respectively) did not significantly change the results obtained. The higher sensitivity of the IgG sandwich ELISA might be explained by the use of a species specific HRPO conjugate in this assay which possibly has higher affinity to IgGs generated early after infection than Protein G. The very good agreement between the RVF recombinant nucleocapsid IgG indirect ELISA and the IgG sandwich ELISA for the detection of IgG to RVFV in sheep (0,92) and in goats (0,99) demonstrates that these ELISAs are suitable diagnostic tools for the detection of specific anti-RVF IgG.

Although the IgG sandwich ELISA was more sensitive than the indirect ELISA, advantages of the latter assay include much easier and shorter procedures that render it more suitable for disease surveillance programs concerned with testing large amounts of sera. Other major advantage is cost-effective production of recombinant nucleocapsid protein as antigen in ELISA, its safety, stability and the fact that high quality antigen could be produced in large amounts outside high bio-containment facilities, thus enabling active surveillance programs in poor resource African countries.

© University of Pretoria

3.5 References

Espach, A., Romito, M., Nel, L.H., Viljoen, G.J. (2002). Development of a diagnostic one-tube RT-PCR for the detection of Rift Valley fever virus. Onderstepoort J. Vet Res., 69, 247-252.

Fafetine, J.M., Tijhaar, E., Paweska, J.T., Neves, L.C.B.G., Hendriks, J., Swanepoel, R., Coetzer, J.A.W., Egberink, H.F., Rutten, V.P.M.G. (2007). Cloning and expression of Rift Valley fever virus nucleocapsid (N) protein and evaluation of a N-protein based indirect ELISA for the detection of specific IgG and IgM antibodies in domestic ruminants. Vet. Microbiol., 121 (1-2), 29-38.

Garcia, S., Crance, J.M., Billecocq, A., Peinnequin, A., Jouan, A., Bouloy, M., Garin, D. (2001). Quantitative real-time PCR detection of Rift Valley fever virus and its application to evaluation of antiviral compounds. J. Clin. Microbiol., 39 (12), 4456-4461.

Henken, A.M., Graat, E.A.M.,Casal, J. Measurement of disease frequency. In: Application of quantitative methods in veterinary epidemiology (1997). Noordhuizen, J.P.T.M., Frankena, K., van der Hoofd, C.M., Graat, E.A.M. eds. Wageningen Pers, The Netherlands, Pp, 63-97.

Jansen Van Vuren, P., Potgieter, A.C., Paweska, J.T., Van Dijk, A.A. (2007). Preparation and evaluation of a recombinant Rift Valley fever virus N protein for the detection of IgG and IgM antibodies in humans and animals by indirect ELISA. J. Virol. Methods; 140: 106–114.

Jansen van Vuren, P., Paweska, J.T. (2010). Comparison of ELISA-based techniques for the detection of antibody to Rift Valley fever virus in thermo-chemically inactivated sheep sera, Vector-Borne and Zoonotic Dis., 10 (7), 697-699.

© University of Pretoria

Kim, H-J., Nah, J-J., Moon, J-S, Ko, Y-J, Yoo, H-S., Kweon, C-H. (2011). Competitive ELISA for the detection of antibodies to Rift Valley fever virus in goat and cattle. J. Vet. Med. Sci.[Epub ahead of print].

Le Roux, C.A., Kubo, T., Grobbelaar, A.A., van Vuren, P.J., Weyer, J., Nel, L.H., Swanepoel, R., Morita, K.,Paweska, J.T. (2009). Development and evaluation of a Real-Time reverse transcription-loop-mediated isothermal amplification assay for rapid detection of Rift Valley fever virus in clinical specimens. J. Clin. Microb., 47 (3), 645-651.

Paweska, J.T., Burt, F.J., Anthony, F., Smith, S.J., Grobbelaar, A.A., Croft, J.E., Ksiazek, T.G., Swanepoel, R. (2003). IgG-sandwish and IgM-capture enzyme-linked immunosorbent assay for the detection of antibody to Rift Valley fever virus in domestic ruminants. J. Virol. Methods; 113, 103-112.

Paweska, J.T., Burt, F.J., Swanepoel, R. (2005). Validation of IgG-sandwich and IgM-capture ELISA for the detection of antibody to Rift Valley fever virus in humans. J. Virol. Methods, 124, 173-181.

Paweska, J.T., Jansen van Vuren, P., Swanepoel, R. (2007). Validation of an indirect ELISA based on a recombinant nucleocapsid protein of Rift Valley fever virus for the detection of IgG antibody in humans. J. Virol. Methods. 146, 119-124.

Paweska, J.T., Jansen van Vuren P., Kemp, A., Buss, P., Bengis, R.G., Gakuya, F., Breiman, R.F., Kariuki, M.N., Swanepoel, R. (2008). Recombinant nucleocapsid-based ELISA for detection of IgG antibody to Rift Valley fever virus in African buffalo. Vet Microbiol.; 127: 21-28

Paweska, J.T., Jansen van Vuren, P., Kemp, A., Swanepoel, R., Buss, P., Roy G., Bengis, R.G., Gakuya, F., Robert F. Breiman, E.R., M. Kariuki Njenga, M. (2010). A recombinant nucleocapsid-based indirect ELISA for serodiagnosis of Rift Valley fever in

© University of Pretoria

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

African wildlife. In: Proceedings of Sustainable Improvement of Animal Production and Health (eds. N.E. Odongo, M. Garcia & G.J. Viljoen), FAO, Rome 2010, ISBN 978-92-5-106697-3, Pp. 309-312.

Peyrefitte, C.N., Boubis, L., Coudrier, D., Bouloy, M., Grandadam, M., Tolou, H.J., Plumet, S. (2008). Real- time reverse-transcription loop-mediated isothermal amplification for rapid detection of Rift Valley fever virus. J. Clin. Microb., 46 (11), 3653-3659.

Sall, A.A., Thonnon, J., Sene, O.K., Fall, A., Ndiaye, M., Baudez, B., Mathiot, C., Bouloy, M. (2001). Single tube and nested reverse transcriptase-polymerase chain reaction for detection of Rift Valley fever virus in human and animal sera. J. Virol. Methods, 91, 85-92.

Sall, A.A., Macondo, E.A., Sene, O.K., Diagne, M., Sylla, R., Mondo, M., Girault, L., Marrama, L., Spiegel, A., Diallo, M., Bouloy, M., Mathiot, C. (2002), Use of reverse transcriptase PCR in early diagnosis of Rift Valley fever. Clinical and Diagnostic Laboratory Immunology, 9 (3), 713-715.

Sorbazo, A., Paweska, J.T., Herrman, S., Amir, T., Marks, R.S., Lobel, L. (2007). Optical fiber immunosensor for the detection of IgG antibody to Rift Valley fever virus in humans. J. Virol. Methods, 146, 327-334.

Swanepoel, R. Struthers, J.K., Erasmus, M.J., Shepherd, S.P., McGillivray, G.M., Erasmus, B.J., Barnard, B.J.H. (1986). Comparison of techniques for demonstrating antibodies to Rift Valley fever virus. J. Hyg. Camb.; 97: 317-329.

Williams, R., Ellis, C.E., Smith, S.J., Potgieter, C.A., Wallace, D., Mareledwane, V.E. Majiwa, P.A.O. (2011). Validation of an IgM antibody capture ELISA based on a recombinant nucleoprotein for identification of domestic ruminants infected with Rift Valley fever virus. J. Virol. Methods. 177, 140-146.

© University of Pretoria

4 . Generation and characterization of monoclonal antibodies against Rift Valley fever virus nucleoprotein

4.1 Introduction

Rift Valley fever virus (RVFV) is an important animal and human pathogen that belongs to the genus *Phlebovirus*, family *Bunyaviridae* (Murphy et al., 1999). It contains a three-segmented genome, comprising a large (L), medium (M) and small (S) genome segment. The L segment encodes the viral RNA-dependent RNA polymerase (L), and the M segment encodes two glycoproteins (Gn and Gc) and two non-structural proteins collectively referred to as NSm. The L and M genome segments are of negative-sense polarity, whereas the S segment is of ambisence polarity. This genome segment contains the non-structural NSs gene in the antigenomic orientation and the gene encoding the viral nucleocapsid (N) protein in genomic orientation (Bouloy and Friedemann, 2010).

A rapid and accurate diagnosis of RVF is essential in the control of the disease. Different serological techniques have been employed in the diagnosis of RVF. Several ELISA formats employing whole inactivated virus as antigen have been widely used and validated and reported as highly sensitive and specific (Paweska et al., 1995; 2003a; 2003b; 2005). However, the production of whole antigen requires containment facilities to reduce the risk of exposure of laboratory staff to infection with the virulent virus in culture. Moreover the whole antigen has shown to bind poorly to the ELISA plates. Recently, recombinant nucleocapsid protein has been used successfully as an antigen in an indirect ELISA for the detection of antibodies to RVFV and provided a very sensitive and specific method for RVF diagnosis, without risk of laboratory infection. The recombinant nucleocapsid protein has been applied as antigen in indirect ELISA format that requires either a species-specific conjugate, alternatively a protein G conjugate that recognizes immunoglobulin of various species (Fafetine et al., 2007; Jansen van Vuren et al., 2007; Paweska et al., 2007; 2008).

ELISA has also been used to detect RVFV antigens in serum (Niklasson et al., 1983; Meegan et al., 1989) but the production of reagents has been difficult and posed health

© University of Pretoria

risks to laboratory staff due to the need of virus culture. A sandwich ELISA for the detection of RVFV nucleocapsid protein based on hyperimmune serum has recently been reported as a safe and valuable tool for the detection of RVFV (Jansen van Vuren and Paweska, 2009). Disadvantages of using hyperimmune serum in immunochemical assays relate to the presence of a mixed population of antibodies that can create a variety of diagnostic problems (Harlow and Lane, 1988). Furthermore, polyclonal antibodies are subject to intra- and inter-laboratory variability that results from the batch-to-batch variations in sera while monoclonal antibodies enable a continuous supply of large quantities of well characterized antibodies which can be easily standardized between different laboratories as they are usually from stable hybridoma clones (Qui et al., 2009). Due to the high specificity for antigens, monoclonal antibodies are widely used as capture antibodies for the detection of different important pathogens in antigen capture ELISAs (Saijo et al., 2007; Cai et al., 2009; Qui et al., 2009; Liu et al., 2010) and in chromatographic strip-tests (Bruning et al., 1999; Bruning-Richardson et al., 2011). Monoclonal antibodies are also used for the detection of antibodies in competitive ELISAs (Martin-folgar et al., 2010) offering the advantage, over indirect ELISA, of allowing the screening of sera from different species with only one anti-mouse immunoglobulin conjugate.

Since the nucleocapsid protein was shown to be the most immunodominant protein in different members of the *Bunyaviridade* family (Swanepoel et al., 1986; Vapalathi et al., 1995; Schwarz et al., 1996) and it was used in ELISA as antigen with very promising results (Fafetine et al., 2007), it represents a very good target for use in the detection of RVFV infection. The monoclonal antibodies against the nucleocapsid protein may be applied for the development of different diagnostic tools to RVF, for example a competitive ELISA that can be validated for detection of RVFV specific antibodies in different animal species. This is particularly useful in surveillance studies. The monoclonal antibodies can also be used in the chromatographic strip-tests for a rapid detection of RVFV antigen and/or in an antigen capture ELISA format to capture RVFV nucleocapsid protein in different specimens. Hence, the objective of the present study was to generate and characterize monoclonal antibodies specific for the recombinant nucleocapsid protein of RVFV.

© University of Pretoria

4.2 Materials and methods

4.2.1 Preparation of recombinant RVFV nucleocapsid protein

The nucleocapsid protein of the RVFV isolate ZIM688/78 was expressed and purified as previously described (Fafetine et al., 2007; Jansen van Vuren et al., 2007). Briefly, the recombinant nucleocapsid protein was expressed in the bacterial expression vector pET32(a)+ as an inclusion body and refolded in an appropriate buffer. The refolded protein was purified using metal affinity chromatography, and stored at -20°C until use for immunization and in the different assays.

4.2.2 Production and purification of the monoclonal antibodies

Monoclonal antibodies were produced as previously described (Caldeira et al., 2009) by immunization of 2 female BALB/c mice, aged 4 to 6 weeks, with the recombinant RVFV nucleocapsid protein. Prior to the immunization the animals were bled for collection of pre-immune serum. The mice were then given 100µg of the recombinant RVFV nucleocapsid protein in complete Freund's adjuvant (Sigma) intraperitoneally followed by three boosters with the same amount of protein in incomplete adjuvant (Sigma) at three week intervals. Animals were bled two weeks after the third booster and the final immunization was carried out three days later with 100µg of the recombinant RVFV nucleocapsid protein. Three days after the last immunization animals were bled again. The pre-immune serum and the titre of the polyclonal antiserum after the final booster were assessed by indirect ELISA using recombinant RVFV nucleocapsid protein as antigen. The animal with the highest titre was sacrificed and its spleen cells used to fuse with Sp2/0 myeloma cells at a ratio of 1:1 in the presence of polyethylene glycol (PEG, Sigma). The hybridoma cells were selected in DMEM media (Gibco) with HAT (Sigma) and 5% (v/v) foetal calf serum (Gibco) and subsequently cloned by limiting dilution. Clones producing the highest titres of RVFV nucleocapsid protein specific antibodies, as assessed in the indirect ELISA, were selected for further use.

© University of Pretoria

4.2.3 Indirect ELISA

The detection of secreted antibodies was done by indirect ELISA. Briefly, immunoplates (Costar 3590, Corning Incorporated, USA) were coated overnight at 4°C with 0,25 µg/well of the recombinant RVFV nucleocapsid protein diluted in PBS. After washing three times with TBS containing 0,05% (v/v) Tween 20 (washing buffer) the plates were blocked with 100 µl washing buffer containing 5% (w/v) skim milk (Difco) then incubated 1h at room temperature and washed as mentioned before. Fifty microlitres of hybridoma supernatant were added to each well and incubated for 1h at 37°C. The pre-immune serum and the polyclonal RVFV recombinant nucleocapsid protein specific antiserum were added as negative and positive controls, respectively. The plates were washed as described above followed by the addition to each well of 50 µl of alkaline phosphatase conjugated anti-mouse polyvalent immunoglobulins (G, A, M) (Sigma). After incubation for 1h at 37°C the plates were washed 5 times with washing buffer and 50 µl 4-nitrophenylphosphate disodium salt hexahydrate (AppliChem) substrate was added. Subsequently the plates were incubated at room temperature in the dark for 30 min and the optical densities (OD) determined at 415 nm in a TIM 200 Inter Med plate reader.

4.2.4 Antibody purification

The supernatants of the selected clones were collected, clarified by centrifugation (20,000 x g, 10 min, 4°C), filtered through a 0,22 µm membrane filter (Millipore) and further purified on a HiTrap Protein A HP affinity column (GE HealthCare). The samples were first adjusted to the composition of the binding buffer (20 mM sodium phosphate, pH 7,0) using PD-10 columns (GE HealthCare). Before sample loading, the column was washed with 10 volumes of binding buffer. Ten column volumes of the binding buffer were further used to remove contaminants. Bound antibodies were eluted stepwise with five column volumes of elution buffer (0,1 M citric acid, pH 3,5). Eluates were collected and pH neutralized by adding 100 µl of 1 M Tris-HCl, pH 9,0 per ml of fraction to be collected and purity was analyzed by SDS-PAGE. The antibody containing fractions were pooled and stored for further use.

© University of Pretoria

4.2.5 Determination of the Ig class of hybridoma antibodies

Isotype identification was performed by using the MonoAb ID isotyping Kit (Zymed). A 96-well microtitre plate (Costar) was coated with recombinant RVFV nucleocapsid protein overnight at 4ºC. After 3 washes with PBS containing 0,05% (v/v) Tween 20 (washing buffer), all the wells were filled with 200 µl of washing buffer containing 5% (w/v) skim milk (Difco) and incubated 1h at room temperature. Culture supernatants containing the selected MoAb (100 µl) were added and further incubated for 1h at 37°C. After incubation, the plate was washed as previously described and 100 µl of rabbit antisera specific for mouse, µ, y_1,y_{2a}, y_{2b}, y_3 heavy chains and *k* and λ light chains supplied in the kit added to appropriate wells. The plate was incubated 1h at 37°C and washed 5 times with washing buffer. Alkaline phosphatase conjugated goat anti-rabbit IgG was subsequently added in all wells (100 µl) and incubated at 37°C for 1h. After washing 5 times with washing buffer the substrate solution p-nitrophenyl phosphate containing 5% (w/v) skim milk (Difco), supplied in the kit) was added and the optical density read in a microplate reader as described above.

4.2.6 Western blot assay

A Western blot assay was used to confirm the specificity of the clones using the recombinant RVFV nucleocapsid protein as antigen on 12,5% (w/v) sodium dodecyl sulfate (SDS)-polyacrylamide gel. The recombinant RVFV nucleocapsid protein preparation (100µg/ml) was run on the gel under denaturating circumstances and electrotransferred to a nitrocellulose membrane. The membranes were stained using the Ponceau staining solution, cut into strips that were placed in a multichannel reservoir and blocked at room temperature for 1h with PBS containing 0,05% Tween 20 (v/v) and 5% (w/v) skim milk (Blocker non-fat dry milk- BioRad). Membranes were incubated with hybridoma supernatant and positive and negative controls (the same as used in ELISA), for 1,5 h at room temperature and then washed three times with 0,05% (v/v) Tween 20 in PBS. After incubation for 1h at room temperature with alkaline phosphatase conjugated anti-mouse polyvalent immunoglobulins (G, A, M) (Sigma) the membranes were washed

© University of Pretoria

as previously described and further incubated, in the dark, with AP color development buffer (BioRad).

4.2.7 Assessment of monoclonal antibodies cross-reactivity

The Western blot assay described above was also used, to assess whether the monoclonal antibodies recognized antigens from other arboviruses of the families *Flaviviridae* (West Nile virus and Dengue virus II) and *Togaviridae* (Sindbis virus and Chikungunya virus) and other members of the *Bunyaviridae* family, namely from the genus *Orthobunyavirus* (Bunyamwera virus and Calovo virus). Briefly, 10^7 sub-confluent Vero cells were infected at a m.o.i. 0,1-1 PFU/cell of these viruses and collected when a generalized cytophatic effect became evident (24-48 hr p.i.). Infected cells were lysed in in 20 mM Tris-HCl, pH 7,0, 150 mM NaCl, 1 mM EDTA and 1% (v/v) Triton X-100. Extracts from cells infected with different virus were tested by Immunoblot as described above using the inactivated whole RVFV as a positive control (SPU 00201) and non-infected cell extract as a negative control.

4.3 Results

Twenty three hybridoma cell lines produced recombinant RVFV nucleocapsid proteins specific antibodies based on indirect ELISA data using the recombinant RVFV nucleocapsid protein as antigen. Hybridomas were cloned by limiting dilution and those clones showing a higher optical density when analyzed by ELISA were selected for further assays. The specificity of the monoclonal antibodies as confirmed by Western blot analysis has shown that ten out of the twenty three monoclonal antibodies bound to the antigen of which four (Fig. 4.1: lanes 3, 6, 7 and 9) showed strongest reaction with the recombinant RVFV nucleocapsid protein. The antiserum against the recombinant RVFV nucleocapsid protein used as positive control did also reveal a strong single band of approximately 62 kDa (Fig. 4.1, lane 1). Some of the monoclonal antibodies that showed positive results in the indirect ELISA did not recognize nucleocapsid protein in the Western blot assay (Fig. 4.1). The monoclonal antibody isotype determination,

© University of Pretoria

showed that the clones named (A12G, 4D3, B3H and D3G) all belonged to the class IgG and the subclass IgG2a. These results were further used for the definition of the purification strategy; a protein A affinity column allowed the purification of the selected clones without major contaminations of other immunoglobulins that might be present, namely IgM, IgA or IgE. The monoclonal antibodies were further tested for recognition of RVF inactivated virus using Western blot, as referred before. The same assay was used to assess the cross-reactivity of the generated monoclonal antibodies with extracts from cells infected with West Nile virus, Dengue virus II, Sindbis virus, Chikungunya virus, Bunyamwera virus and Calovo virus. As can be seen in Fig. 4.2, the monoclonal antibodies 4D3 recognized specifically the RVF inactivated virus producing an expected band of approximately 62 kDa, but no reaction was observed neither with other viral extracts nor on the control extract. Hence all four monoclonal antibodies proved specific for RVFV nucleocapsid protein.

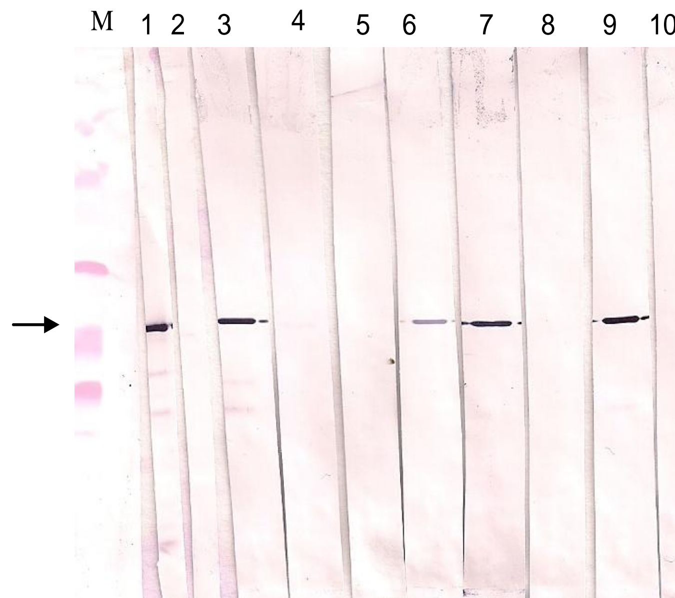


Fig. 4.1. Western bloting analisys of the clones against recombinant RVFV nucleocapsid protein.**Lane M**, protein marker; **lane 1**anti-recombinant RVFV nucleocapsid protein polyclonal antibodies; **lane 2** mouse negative serum; lane **3 to 10** different clones. The arrow indicates positive reaction (62kDa recombinant RVFV nucleocapsid protein band)

© University of Pretoria

Fig. 4.2. Western blotting analysis of the clone 4D3 against different viral extracts. **Lane M** protein markers, **lane 1** positive control; **lane 2** RVF inactivated virus; **lane 3** control extract; **lane 4 to 9** different viral extracts: West Nile, Dengue virus II, Sibindis, Chikungunya, Bunyamwera and Calovo viruses. The arrow indicates positive reaction (62kDa band)

4.4 Discussion

The nucleocapsid protein is the most immunodominant protein in different members of the *Bunyaviridae* family (Swanepoel et al., 1986; Vapalathi et al., 1995; Schwarz et al., 1996) and has been applied successfully in indirect ELISAs formats for the detection of RVF specific antibodies (Fafetine et al., 2007; Jansen van Vuren et al., 2007; Paweska et al., 2007; 2008). Production of specific monoclonal antibodies against this structural protein may further contribute to the development of improved assays for the detection of the disease.

The monoclonal antibodies selected in the present study revealed a specific ability to recognize the recombinant nucleocapsid protein both in an indirect ELISA and in the Western blot assay. However, only four out of ten chosen monoclonal antibodies based on ELISA results gave a strong reaction in Western blot. This could be explained by the different reactivity against hidden epitopes, weak affinity of the monoclonal antibodies

© University of Pretoria

(Daginakatte et al., 1999) or different conformation adopted by the recombinant nucleocapsid protein in the solid phase (Al-Yousif et al., 2000).

Serological techniques applied for the detection of antibodies to RVFV have been reported to be cross-reactive with other viruses (Scott et al., 1986; Swanepoel et al., 1986). To assess whether the produced monoclonal antibodies were specific for RVFV only, they were tested against extracts of cell infected with other members of the *Bunyaviridae* family (Bunyamwera virus and Calovo virus from the genus *Orthobunyavirus*) and other RNA viruses from the families *Flaviviridae* (West Nile virus and Dengue virus II) and *Togaviridae* (Sindbis virus and Chikungunya virus). No cross-reactivity was found using Western blot for analyzing the four selected monoclonal antibodies. Recently Raymond et al. (2010) reported that the phlebovirus nucleocapsid protein has similar structure but differs substantially from the other negative-sense RNA virus a fact that can explain the absence of cross-reaction with other viruses.

Monoclonal antibodies against RVFV were previously described by Zaki et al. (2006) and by Martin-Folgar et al. (2010). Zaki et al. (2006) produced monoclonal antibodies after immunization of BALB/c mice with the whole inactivated RVFV and tested them against two RVFV strains (the KEN97 and the ZH548) representative of two phylogenic lineages. The monoclonal antibodies could successfully detect RVFV from cell culture. Martin-Folgar et al. (2010) developed monoclonal antibodies against RVFV nucleocapsid protein, evaluated them against different strains of RVFV and applied them effectively in ELISA for detection of antibodies and RVFV antigen. Compared to these studies in which monoclonal antibodies against RVFV were developed the monoclonal antibodies described in our study were also tested against other viruses of the *Bunyaviridae, Flaviviridae* and *Togaviridae* family adding value to its assessment.

Based on the results described previously, the monoclonal antibodies generated and characterized in this study could be used in a competitive ELISA to detect IgG in the sera from different animal species with the use of one anti-mouse immunoglobulin conjugate in contrast to the currently available indirect ELISA that requires species-specific immunoglobulin conjugates. The assay would be particularly important in studying the role of different domestic and wildlife species in the epidemiology of the disease (Anderson and Rowe, 1998; Swanepoel and Coetzer, 2004; Evans et al., 2008; LaBeaud

© University of Pretoria

et al., 2011). The high specificity of the monoclonal antibodies for antigens could also be exploited to capture RVFV nucleocapsid protein in an antigen capture ELISA or/and chromatographic strip-test for rapid diagnosis of RVF.

4.5 References

Al-Yousif, Y., Al-Majhdi, F., Chard-Bergstrom, C., Anderson, J., Kapil, S. (2000). Development, characterization, and diagnostic applications of monoclonal antibodies against bovine Rotavirus. Clin. Diagn.Lab. Immunol., 7(2), 288-292.

Anderson, E.C., Rowe, L.W. (1998). The prevalence of antibodies to viruses of bovine virus diarrhea, bovine herpes virus 1, Rift Valley fever, ephemeral fever and bluetongue and to *Leptospira* sp in free-ranging wildlife in Zimbabwe. Epidemiol. Infect., 121, 441-449.

Bouloy, M., Friedemann, W. (2010). Molecular biology of Rift Valley fever virus. The Open Virol. J., 4, 8-14.

Bruning, A., Bellamy, K., Talbot, D., Anderson, J. (1999). A rapid chromatographic strip test for the pen-side diagnosis of rinderpest virus. J. Virol. Methods, 81, 143 -154.

Bruning-Richardson, A., Akerblom, L., Klingeborn, B., Anderson, J. (2011). Improvement and development of rapid chromatographic strep-tests for the diagnosis of rinderpest and pest des petit ruminants virus. J. Virol. Methods, 174, 42-46.

Caldeira, R.L., Gonçalves, L.M.D., Martins, T.M., do Rosário, V.E., Novo, C., Domingos, A. (2009). *Plasmodium chabaudi*: expression of active recombinant chabaupain-1 and localization studies in *Anopheles sp*. Exp. Parasitol., 122: 97–105.

Cai, J.-P., wanga, Y-D., Tse, H., Xianga, H., Yuen, K-Y., Che, X-Y. (2009). Detection of asymptomatic antigenemia I pigs infected by porcine reproductive and respiratory

© University of Pretoria

syndrome virus (PRRSV) by a novel capture immunoassay with monoclonal antibodies against the nucleocapsid protein of PRRSV. Clin. Vaccine. Immunol., 16(12), 1822-1828.

Daginakatte, G.C., Chard-Bergstrom, C., Andrews, G.A., Kapil, S. (1999). Production, characterization and uses of monoclonal antibodies against recombinant nucleoprotein of elk coronavirus. Clin. Diagn.Lab. Immunol., 6 (3), 341-344.

Evans, A., Gakuya, F., Paweska, J.T., Rostal, M., Akoolo, L., Jansen van Vuren P.J., Manyibe, T., Macharia, J.M., Ksiazek, T.K., Feikin, D.R., Breiman, R. F., Karriuki Njenga, M. (2008). Prevalence of antibodies against Rift Valley fever virus in Kenya wildlife. Epidemiol. Infect., 136, 1261-1269.

Fafetine, J.M., Tijhaar, E., Paweska, J.T., Neves, L.C.B.G., Hendriks, J., Swanepoel, R., Coetzer, J.A.W., Egberink, H.F., Rutten, V.P.M.G. (2007). Cloning and expression of Rift Valley fever virus nucleocapsid (N) protein and evaluation of a N-protein based indirect ELISA for the detection of specific IgG and IgM antibodies in domestic ruminants. Vet. Microb., 121 (1-2), 29-38.

Harlow, E., Lane, D. (1988). Antibodies, a laboratory manual. Cold Spring Harbor Laboratory, Pp. 141-243.

Jansen van Vuren, P., Potgieter, A.C., Paweska, J.T., Van Dijk, A.A. (2007). Preparation and evaluation of a recombinant Rift Valley fever virus N protein for the detection of IgG and IgM antibodies in humans and animals by indirect ELISA. J. Virol. Methods, 140, 106–114.

Jansen van Vuren, P., Paweska, J.T. (2009). Laboratory safe detection of nucleocapsid protein of Rift Valley fever virus in human and animal specimens by a sandwich ELISA. J. Virol. Methods, 157, 15-24.

© University of Pretoria

LaBeaud, A.D., Cross, P.C., Getz, W.M., Glinka, A., King, C.H. (2011). Rift Valley fever virus infection in African Buffalo (*Syncerus caffer*) herds in rural South Africa: evidence of inter-epidemic transmission. Am. J. Trop. Med. Hyg., 84 (4), 641-646.

Liu, M., Chen, X., Wang, Y., Zhang, Y., Li, Y., Wang, Y., Shen, N., Chen, H. (2010). Characterization of monoclonal antibodies agains Muscovy duck reovirus σB protein. Virol. J., 7:133.

Martin-Folgar, R., Lorenzo, G., Boshra, H., Iglesias, J., Mateos, F., Borrego, B., Brun, A. (2010). Development and characterization of monoclonal antibodies against Rift Valley fever virus nucleocapsid protein generated by DNA immunization. mAbs, 2 (3), 275-284.

Meegan, J., Le Guenno, B., Ksiazek, T., Jouan, A., Knauert, F., Digoutte, J.P., Peters, C.J. (1989). Rapid diagnosis of Rift Valley fever: a comparision of methods for the direct detection of viral antigens in human sera. Res. Virol., 140, 59-65.

Murphy, F.A., Gibbs, E.P.J. Horzinek, M.C., Studdert, M.J. (1999). Veterinary Virology. 3rd ed. Elsevier, USA. Pp. 469-475.

Niklasson, B., Grandien, M., Peters, C.J., Gargan, T.P. (1983). Detection of Rift Valley fever virus antigen by Enzyme-linked immunosorbent assay. J. Clin. Microbiol., 17, 1026-1031.

Paweska, J.T., Barnard, B.J.H., Williams, R. (1995). The use of sucrose-acetone-extracted ift Valley fever virus antigen derived from cell culture in an indirect enzyme-linked immunosorbent assay and haemagglutination-inhibition test. Onderstepoort J. Vet. Res., 62, 227-233.

Paweska, J.T., Burt, F.J., Anthony, F., Smith, S.J., Grobbelaar, A.A., Croft, J.E., Ksiazek, T.G., Swanepoel, R. (2003a). IgG-sandwish and IgM-capture enzyme-linked

© University of Pretoria

immunosorbent assay for the detection of antibody to Rift Valley fever virus in domestic ruminants. J. Virol. Methods, 113, 103-112.

Paweska, J.T., Smith, S.J., Wright, I.M., Williams, R., Cohen, A.S., van Dijk, A.A., Grobbelaar, A.A., Croft, J.E., Swanepoel, R., Gerdes, G.H. (2003b). Indirect enzyme-linked immunosorbent assay for the detection of antibody against Rift Valley fever virus in domestic and wild ruminant sera. Onderstepoort J. Vet. Res., 70, 49-64.

Paweska, J.T., Mortimer, E., Leman, P.A., Swanepoel, R. (2005). An inhibition enzyme-linked immunosorbent assay for the detection of antibody to Rift Valley fever virus in humans, domestic and wild ruminants. J. Virol. Methods, 127, 10-18.

Paweska, J.T., Jansen van Vuren, P., Swanepoel, R. (2007). Validation of an indirect ELISA based on a recombinant nucleocapsid protein of Rift Valley fever virus for the detection of IgG antibody in humans., J. Virol. Methods, 146, 119-124.

Paweska, J.T., Jansen van Vuren P., Kemp, A., Buss, P., Bengis, R.G., Gakuya, F., Breiman, R.F., Kariuki, M.N., Swanepoel, R. (2008). Recombinant nucleocapsid-based ELISA for detection of IgG antibody to Rift Valley fever virus in African buffalo. Vet Microbiol., 127, 21-28.

Qiu, L-w.; Di, Biao, Wen, K., Wang, X-s., liang, W-h., wang, Y-d., Pan, Y-x, wang, M., Ding, Y-q., Che, Xiao-y. (2009). Development of an antigen capture immunoassay based on monoclonal antibodies specific for dengue virus serotype 2 nonstructural protein 1 for early and rapid identification of Dengue virus serotype 2 infections. Clin. Vaccine. Immunol., 16 (1), 88-95.

Raymond, D.D., Piper, M.E., Gerrard, S.R., Smith, J.L. (2010). Structure of the Rift Valley fever virus nucleocapsid protein reveals another architecture for RNA encapsidation. In: www.pnas.org/cgi/doi/10.1073/pnas.10001760107

© University of Pretoria

Saijo, M., Georges-Courbot, M.C., marianneau, P., Romanowski, V., Fukushi, S., Mizutani, T., Georges, A.J., Kurane, I., Morikawa, S. (2007). Development of recombinant nucleoprotein-based diagnostic systems for Lassa fever. Clin. Vaccine Immunol., 14, 1182-1189.

Schwarz, T.F., Gilch, S., Pauli, C., Jager, G. (1996). Immunoblot detection of antibodies to Toscana virus. J. Med. Virol., 49, 83-86.

Scott, R.M., Feinsod, F.M., Allam, I.H., Ksiazek, T.G., Peters, C.J., Botros, B.A.M., Darwish, M.A. (1986). Serological tests for detecting Rift valley fever viral antibodies in sheep from the Nile Delta. J. Clin. Microbiol., 24 (4), 612-614.

Swanepoel, R., Coetzer, J.A.W. (2004). Rift Valley fever. In: Infectious diseases of livestock, 2nd ed. Ed. Coetzer, J.A.W. and Tustin, R.C. Vol II. Oxford University Press. Southern Africa. Pp 1037-1070.

Swanepoel, R. Struthers, J.K., Erasmus, M.J., Shepherd, S.P., McGillivray, G.M., Erasmus, B.J., Barnard, B.J.H. (1986). Comparison of techniques for demonstrating antibodies to Rift Valley fever virus. J. Hyg. Camb., 97, 317-329.

Vapalathi, O., Kallio-Kokko, H., Narvanen, A., Jalkunen, I., Lundkvist, A., Plyusnin, A., Lehvaslaiho, H., Brummer-Korvenkontio, M., Vaheri, A., Lankinen, H. (1995). Human B-cell epitopes of Puumala virus nucleocapsid protein, the major antigen in early serological response. J. Med. Virol., 46, 293-303.

Zaki, A., Coudrier, D., Yousef, A.I., Fakeeh, M., Bouloy, M., Billecocq, A. (2006). Production of monoclonal antibodies against Rift Valley fever virus . Application for rapid diagnosis test (virus detection and ELISA in human sera. J. Virol. Methods, 131, 34-40.

© University of Pretoria

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

5 . Serological evidence of Rift Valley fever virus circulation in Zambézia Province, Mozambique

5.1 Introduction

Rift Valley fever is a disease caused by a RNA virus of the family *Bunyaviridae*, genus *Phlebovirus* (Murphy et al., 1999). The disease is of considerable economic importance due to high abortion rates, high mortality in young animals, trade restriction and the negative impact on other non-agricultural sectors (Rich and Wanyoike, 2010). The disease is also a serious public health hazard resulting in mild to moderately severe influenza-like illness that may be complicated by ocular lesions, encephalitis or a fatal haemorrhagic state in a low percentage of patients (Swanepoel and Coetzer, 2004).

In the last decade Eastern and Southern African countries have experienced several RVF epidemics responsible for severe losses both in animals and humans. In 2006/2007 in Kenya, Somalia and Tanzania the disease caused more than 1 000 infections in humans and 323 deaths (WHO, 2007). In 2008, Madagascar reported an epidemic of RVF that was responsible for at least 476 suspected human cases and 19 deaths: more than 59 laboratory confirmed human cases and untold high death rates among cattle were also reported (Andriamandimby et al., 2010). In the same year, an outbreak of the disease affecting veterinarians and farmers was reported in South Africa. Of the 53 humans that contracted RVF following exposed to the tissues of sick domestic ruminants, 15% revealed evidence of recent infection and 4% of past exposure to RVFV. Transmission by direct contact as a result of performing necropsies on infected animals were found to be the main risk factor (Archer et al., 2011). During an epidemic of RVF in 2010 in South Africa 192 laboratory-confirmed human cases including 18 deaths were reported (WHO, 2010).

Despite the occurrence of several epidemics of RVF in the last 2 decades in neighboring countries such as South Africa no epidemics have been reported in Mozambique during this time. There are only a few reports of RVF in Mozambique. In 1960 RVFV antibodies were found in 2,8% of cattle. Nine years later 134 cattle died of

© University of Pretoria

RVF in Gaza Province (Valadão, 1969). In 1999 in the Zambézia Province, cases of abortion were reported in a herd of water buffaloes that were seropositive to RVFV. Surveillance of cattle in the same province in 1996 and 2001 revealed a sero-prevalence of 37% and 53%, respectively (DINAP, 2002). A seroprevalence of 2% (28 out of 1163) in humans was found in a study conducted from 1981 to 1983 in 8 of the 10 provinces of Mozambique (Niklasson et al., 1987).

The current theory is that RVFV is maintained in aedine mosquito eggs during the inter-epidemic period and epidemics occur subsequently to an increase in the mosquito population following abnormally heavy rains (Linthicum et al., 1985). It is postulated that the virus is maintained in eggs of *Aedes* mosquitoes that breed in water-logged depressions called *dambos.* Several mosquito species and biting-flies may act as vectors during an epidemic (Swanepoel and Coetzer, 2004; Pepin et al., 2010). Different wildlife species may become infected by RVFV (Anderson and Rowe, 1998; Evans et al., 2008). Evidence of inter-epidemic transmission of RVFV has been shown to occur in African buffalo (*Syncerus caffer*) (LaBeaud et al., 2011), humans (LaBeaud et al., 2008) and sheep (Chevalier et al., 2009).

Since surveys that could indicate the activity of RVFV are not regularly carried out it is believed that the disease is underreported in Mozambique. There are indications that RVF is endemic in certain parts of the country and that infection occurs in sheep, goats and cattle during the inter-epidemic period. Some districts of the Zambézia Province located in the central part of the country seem to have suitable agro-ecological conditions for the maintenance of the disease. The objective of the present study was to determine the circulation of RVFV in sheep and goats in the Zambézia Province in the inter-epidemic period by cross-sectional and longitudinal serological studies.

5.2 Materials and methods

5.2.1 Site description

Zambézia Province is located in the central coastal region of Mozambique (17°0'S; 37°0'E), south of Nampula and north of Sofala Province. It has a total area of 103 127

© University of Pretoria

km^2, much of it drained by the Zambezi River. The coast consists mainly of mangrove swamps and inland forest. The monthly average minimum temperature in the capital of the province between 1971 and 2000 ranged from 15,3^{o}C to 23,4^{o}C and the average maximum temperature from 26,3^{o}C to 32,4^{o}C. In the same period the average monthly rainfall was 189,4 mm. January, February and March are the months with higher precipitation and July, August, September and October are considered to be the drier months (INAM, 2010). The province has a total cattle population of 32629, 1308 water buffaloes, 194052 goats and 47603 sheep of which the majority are kept in rural areas by subsistence farmers with less than 20 animals each (SPPZ, 2009). Five out of 16 administrative districts of the Province, namely, Maganja da Costa, Mocuba, Mopeia, Morrumbala and Nicoadala were chosen for the baseline study in 2007. In September 2010 samples were collected only in Mopeia and Nicoadala districts (Fig. 5.1).

Fig. 5.1. Location of the study areas

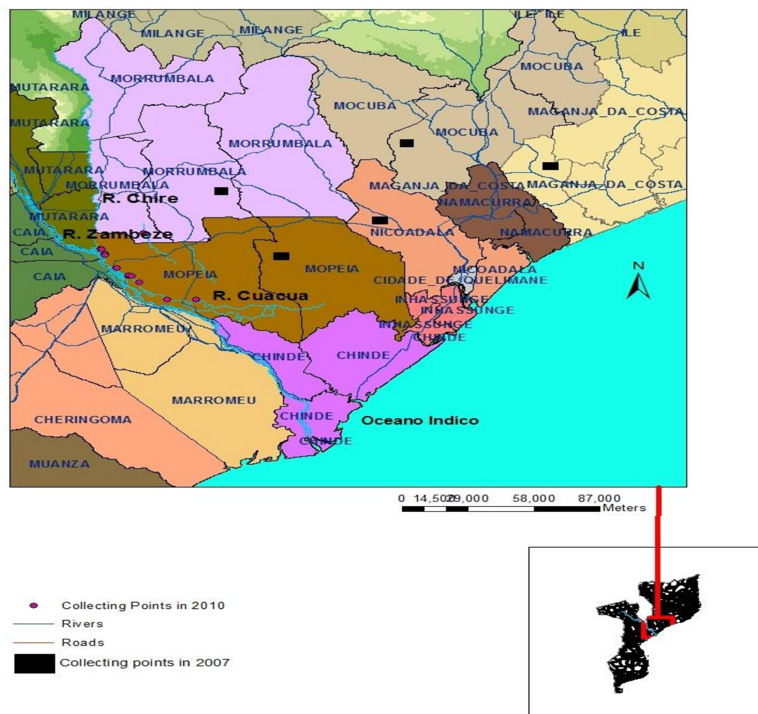


Fig. 5.1. Location of the study areas

© University of Pretoria

5.2.2 Animals and sampling

This study site was selected because no vaccination was performed in sheep and goats and was identified by the local Veterinary Services as high risk for RVFV activity based on ecological receptiveness for the vector, proximity to rivers, presence of *dambos*, historical ocurrence of seropositive animals and relatively high concentration of animals. The study consisted of cross-sectional and longitudinal surveys of the animals.

5.2.3 Cross-sectional surveys

In 2007 a cross-sectional survey was conducted as a baseline study in sheep and goats in the five districts previously mentioned.

For the estimation of RVF prevalence in Mopeia and Nicoadala districts in 2010, three age groups in sheep and goats were studied, namely 0-6 months (group I), 6-12 months (group II) and more than 12 month old (group III). The sample size was calculated based on an estimated seroprevalence of 50%, confidence level of 95% and a maximum allowable error of 10%, giving a required sample size of 97 for each group in both species. Animals were selected based on a simple random sampling strategy, mainly in small scale farms where the animal owners showed willingness to take part in the survey.

5.2.4 Longitudinal assessment of inter-epidemic transmission of RVF

In Mopeia district 125 animals (74 sheep and 51 goats) between 1 and 4 months of age were individually identified using ear-tags. These animals were monitored from September 2010 until April 2011, and bled at 45-day intervals.

Blood samples were collected from the jugular vein in plain tubes. Clotted blood samples were separated by centrifugation and the sera stored at -20°C until use.

© University of Pretoria

5.2.5 Laboratory tests

5.2.5.1 Virus neutralization test

The virus neutralization (VN) test was conducted in 96-well microplate using Vero cells as described previously (Paweska et al., 2003a). Briefly, duplicates of 25 µl serial two-fold diluted heat-inactivated serum was mixed with an equal volume of 100 $TCID_{50}$ of RVFV and incubated at 37°C for 30 minutes. A total of 50 µl of Vero cells in MEM medium containing 10% foetal bovine sera were then added to each well. The microplates were incubated at 5% CO_2 and checked daily for the presence of cytopathic effecst. The titre was expressed as the reciprocal of the serum dilution that inhibited ≥ 75% of viral cytopathic effects. A serum sample was considered seropositive when it had a titre equivalent to ≥ 1:10.

5.2.5.2 IgG indirect ELISA

The IgG indirect ELISA was conducted as described by Fafetine et al. (2007) and Jansen van Vuren et al. (2007). ELISA plates (Maxisorb, Nunc, Denmark) were coated with 100 µl of the recombinant nucleocapsid RVFV diluted 1:2000 in carbonate-bicarbonate buffer overnight at 4°C. Plates were washed 3 times with 0,1% Tweeen-20 in phosphate buffer saline (PBS) (washing buffer) and blocked with 200 µl PBS with 10% skim milk. After incubation in a moist chamber for 1h at 37°C, plates were washed 3 times with washing buffer and 100 µl test, positive and negative control sera diluted 1:400 in 2% skim milk in PBS were added. Plates were incubated in a moist chamber for 1h at 37°C, washed as previously described and 100 µl recombinant Protein G HRPO conjugate diluted 1:15000 added (Zymed Lab., Inc). After incubation of 1h at 37°C, plates were washed 3 times with washing buffer followed by the addition of 100 µl 2,2'-azinodiethylbenzothiazoline sulfonic acid (ABTS, KPL Laboratories, Inc.). Plates were then incubated in dark for 30 minutes at room temperature, 10
0 µl of the stop reagent, 1% sodium dodecyl sulphate (SDS) was added to each well and optical densities (OD) were measured at 405 nm. The results were subsequently expressed as percentage of the high positive control serum using the formula [(mean net OD of test serum/mean net OD of high positive control)] x 100.

© University of Pretoria

5.2.5.3 IgM ELISA

The IgM capture ELISA was performed following a method previously described by Paweska et al. (2003b). Briefly, plates were coated overnight at 4°C with 100 µl rabbit anti-sheep IgM (Zymed Laboratories, Inc.) diluted 1:500 in PBS. After incubation plates were washed three times with the washing buffer (0,1% Tweeen-20 in PBS) and incubated with 10% skim milk in PBS in a moist chamber for 1h at 37°C. Plates were washed 3 times with the washing buffer and duplicate volumes of 100 µl of test and control sera diluted 1:400 added in rows A-D; 1-12 respectively to the corresponding wells in the bottom half of the plate (rows E-G: 1-12). After incubation at 37°C for 1h and washing 6 times with washing buffer, 100 µl of the virus (NICD-SPU) and control antigen (NICD-SPU) diluted 1:200 in PBS containing 2% skim milk were added to both the rows of the top half of the plate (rows A-D: 1-12) and of the bottom half of the plate (rows E-G: 1-12) respectively. Plates were incubated for 1h at 37°C, washed 3 times with the washing buffer and mouse anti-RVF serum diluted 1:3000 added to each well of the plate. Plates were incubated again for 60 minutes at 37°C, washed 3 times with the washing buffer and goat anti-mouse IgG conjugated with peroxidase (Zymed laboratories, Inc.) diluted 1:4000 added to each well for 1h at 37°C. Plates were washed 6 times with the washing buffer and the reaction was developed by the addition of ABTS (KPL Laboratories, Inc.). After incubation in a dark at room temperature for 30 minutes the reaction was stopped by the addition of 100 µl 1% SDS. Optical densities (OD) were determined at 405 nm. The net OD values were first recorded for each serum as the value determined with the RVFV antigen minus the value determined with the control antigen and subsequently converted into percentage of the OD value of a high positive control serum as previously mentioned.

5.2.6 Statistical analysis

Seroprevalence, with 95% confidence intervals, was calculated for each district and overall, taking into account the different sampling weight in each district. Seroprevalence was compared between districts, and the overall seroprevalence was compared between species, using the chi-square test with second order correction to

© University of Pretoria

account for the survey design (Rao and Scott, 1984). A multiple logistic regression model was used to measure the association of age, sex and locality with the outcome (seropositive to RVFV) while controlling for possible confounding. For this purpose, age was categorized into less than 6 months, 7 - 12 months and more than 12 months, and locality was modelled as a fixed effect. Separate models were used for sheep and for goats. The fit of each logistic regression model was assessed using the Hosmer-Lemeshow goodness-of-fit test. A significance level of 5% was used. Analyses were done using Stata 11 (StataCorp, College Station, TX, USA).

5.3 Results

5.3.1 Cross-sectional surveys

Serum samples randomly collected in 2007 in a cross-sectional survey of 377 goats and 277 sheep in five different district of the Zambézia Province were tested with the VN test and indirect IgG ELISA. Antibodies to RVFV were detected in all the districts in both species (except in Mocuba for goats) with the higher seroprevalence values in Nicoadala and Mopeia districts in both species. The corrected total prevalence in sheep was higher (35,8%) than in goats (21,2%) (Table 5.1).

In 2010 a total of 449 serum samples from goats and 313 from sheep were collected in seven different localities of the Mopeia and Nicoadala districts. The results of the IgG ELISA are shown in Tables 5.2, 5.3 and 5.4. IgG specific for RVFV was detected in all localities where samples were collected with seroprevalence values varying from 4,3 - 50% and the total corrected seroprevalence in sheep lower (9,2%) than in goats (11,6%) (Table 5.2). Mopeia district recorded a higher seroprevalence than Nicoadala district in goats (13,1 versus 10,6%) and in sheep (22,8 versus 4,3%) (Table 5.3). However, this difference was not statistically significant in goats (p = 0,584), while in sheep it was marginally significant (p=0,051). In females higher seroprevalences occurred than in males in both species. The seroprevalences were also higher with increasing age of the animals (Table 5.4).

© University of Pretoria

Table 5.5 shows the results of the multiple logistic regression model that was applied to determine the effect of multiple variables on the outcome and to control for possible confounding. No significant difference of RVF prevalence was encountered between males and females. However, the effect of age was found to be highly significant, with older animals (more than 12 months) much more likely to be seropositive than the younger animals (0-6 months) (OR = 7,3; p < 0,001). There was substantial variation in seroprevalence between the localities; compared to Chimuara, goats in Nhamirere had lower odds of being seropositive (OR=0,23; p=0,021) and sheep in Deda had higher odds of being seropositive (OR=5,85; p=0,035).

To test for recent infection, the IgM ELISA was performed only in the 240 samples that were low positive in the IgG ELISA. Twenty samples from sheep were IgM positive (17 from Chimuara, 2 from Nuere and 1 from Deda) and 9 goat samples were IgM positive (6 from Chimuara, 1 from Nuere, 1 from Nzanza and 1 from Deda).

Table 5.1. RVF seroprevalence in goats and sheep in Zambézia districts, Mozambique, in 2007, as determined by virus neutralization test and IgG ELISA

		District					**Total**
		Maganja da Costa	Mocuba	Mopeia	Morrumbala	Nicoadala	
Goats	*N*	92	59	53	131	42	377
	Seroprevalence (%)	39.1^{c}	0^{a}	50.9^{cd}	7.6^{b}	61.9^{d}	21.2^{A}
	95% C.I.	[29.7,49.5]	[0.0,4.9]	[37.6,64.1]	[4.1,13.7]	[46.3,75.4]	[17.9,24.9]
Sheep	*N*	11	181	60	-	25	277
	Seroprevalence (%)	54.6^{b}	13.8^{a}	93.3^{c}	-	80^{bc}	35.8^{B}
	95% C.I.	[25.6,80.7]	[9.5,19.7]	[83.4,97.5]		[59.4,91.6]	[30.7,41.1]

^{a,b,c,d}Values within a row with no superscripts in common differ significantly (p < 0.05)

^{A,B} Seroprevalence differs between goats and sheep (P = 0.0002)

© University of Pretoria

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Table 5.2. RVF seroprevalence in goats and sheep in different localities of Mopeia and Nicoadala districts, Zambézia, in 2010, as determined by IgG ELISA

		Place								Total
		Bras	Chimuara	Deda	Massan-cara	Nhamirere	Nuere	Nzanza	Nicoadala	
Goats	*N*	29	140	48	6	53	53	54	66	449
	Seroprevalence (%)	6.9[ab]	21.4[b]	12.5[ab]	16.6[ab]	5.7[a]	9.4[ab]	5.6[a]	10.6[ab]	11.6[A]
	95% C.I.	[1.7,23.9]	[15.4,29.0]	[5.7,25.2]	[2.3,63.3]	[1.8,16.2]	[3.9,20.8]	[1.8,15.9]	[5.1,20.8]	[7.7,17.2]
Sheep	*N*	-	254	8	-	-	21	7	23	313
	Seroprevalence (%)		21.7[ab]	50[b]			28.6[b]	14.3[ab]	4.3[a]	9.2[A]
	95% C.I.		[16.9,27.2]	[19.9,80.1]			[13.4,50.9]	[1.9,58.4]	[0.6,26.2]	[4.5,17.9]

[a,b,c,d] Values within a row with no superscripts in common differ significantly ($P < 0.05$)

[A] Seroprevalence does not differ between goats and sheep ($P = 0.578$)

Table 5.3. RVF seroprevalence in goats and sheep in Mopeia and Nicoadala districts in 2010, as determined by IgG ELISA

		District	
		Mopeia	Nicoadala
Goats	*N*	383	66
	Seroprevalence (%)	13.1	10.6
	95% C.I	[10.0,16.8]	[5.1,20.8]
Sheep	*N*	252	25
	Seroprevalence (%)	22.8	4.3
	95% C.I.	[18.3,27.9]	[0.58,26.2]

© University of Pretoria

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Table 5.4. RVF seroprevalence for each sex and age group in goats and sheep in Mopeia and Nicoadala districts, Zambézia, in 2010

			Total sampled	No. positive	Seroprevalence (%)
Goats	Sex				
		Female	345	54	15.7
		Male	104	3	2.9
	Age				
		0-6 months	128	4	3.1
		7-12 months	124	8	6.5
		> 12 months	197	45	22.8
Sheep	Sex				
		Female	248	59	23.8
		Male	65	8	12.3
	Age				
		0-6 months	90	4	4.4
		7-12 months	53	6	11.3
		>12 months	170	57	33.5

© University of Pretoria

Table 5.5. Effect of sex, age and locality on seropositivity to RVFV in sheep and goats in Mopeia and Nicoadala districts, Zambézia, in 2010: results of multiple logistic regression models

Species	Variable	Level	Odds ratio (*OR*)	95% confidence interval (*OR*)	*P*-value
Goats	Sex				
		Female	1*	–	–
		Male	0.34	0.10, 1.20	0.094
	Age				
		0-6 months	1*	–	–
		7-12 months	2.20	0.60, 8.07	0.234
		> 12 months	7.32	2.42, 22.12	<0.001
	Locality				
		Chimuara	1*	–	–
		Bras	0.30	0.07, 1.40	0.127
		Deda	1.02	0.35, 2.96	0.975
		Massancara	0.60	0.06, 5.65	0.657
		Nhamirere	0.23	0.06, 0.80	0.021
		Nuere	0.44	0.15, 1.25	0.123
		Nzanza	0.30	0.08, 1.09	0.068
		Nicoadala	0.80	0.28, 2.25	0.672
Sheep	Sex				
		Female	1*	–	–
		Male	0.91	0.37, 2.27	0.853
	Age				
		0-6 months	1*	–	–
		7-12 months	1.79	0.44, 7.34	0.420
		>12 months	9.50	3.15, 28.67	<0.001
	Locality				
		Chimuara	1*	–	–
		Deda	5.85	1.13, 30.30	0.035
		Nuere	1.44	0.50, 4.18	0.503
		Nzanza	1.47	0.15, 14.82	0.744
		Nicoadala	0.23	0.03, 1.79	0.158

* Reference level

© University of Pretoria

5.3.2 Assessment of inter-epidemic transmission of RVFV

The number of animals positive in IgM and IgG ELISA are summarized in Table 5.6. Nine animals out of 125 were positive at the beginning of September 2010 by IgG ELISA. Of these, five were also positive by IgM ELISA. In mid October all nine animals were still positive in IgG ELISA but only one positive in IgM ELISA. In the beginning of December one young animal was no longer positive and one was slaughtered for human consumption giving only 7 IgG positive animals and the only animal IgM positive was still positive. In mid-January one more animal was slaughtered for consumption (was IgM and IgG positive) and one more was no longer positive giving a total of 5 IgG positive animals. The same picture was found in April. None of the animals negative at the beginning of the study sero-converted during the 7 month study period.

Table 5.6. Number of RVF positive animals in the longitudinal study

	No. positive	
	IgM	IgG
September	5	9
October	1	9
December	1	7*
January	-	5**
April	-	5

* 1 animal slaughtered and 1 animal no longer positive

** 1 animal slaughtered and 1 animal no longer positive

5.4 Discussion

In recent years severe outbreaks of RVF have been reported in humans and animals in southern Africa (Samui et al., 1997; Perez at al., 2010; Jeanmaire et al., 2011; Nderitu et al., 2011). Apart from reports of the disease in 1969 (Valadão, 1969) and in 1999 (DINAP, 2002), as a cause of abortions and deaths in cattle and water buffalo, there are no other records of RVFin Mozambique.

© University of Pretoria

The five districts where the cross-sectional survey was conducted in 2007 were chosen based on reports of the Mozambican Veterinary Services on the possible occurrence of RVFV activity in these districts. The overall detected seroprevalence of RVF in the five districts was 35,75% in sheep and 21,75% in goats. These prevalences are slightly lower than the prevalence of 39% in sheep and 33,5% in goats reported in Comoros in 2009 (Roger et al., 2011). A lower prevalence of 24,7% was reported in small ruminants after the 2008 outbreak in Madagascar (Jeanmaire et al., 2011). Since the diagnostic tests used in the above studies were the same as used in our study, the difference in the prevalence can be attributed to differences in factors related to climate, agro-ecological conditions and/or sampling strategies.

The 2010 survey reported on in this study was restricted to Mopeia and Nicoadala because these districts had the highest prevalences in the 2007 study. The overall seroprevalences in both species in the 2010 survey were 9,2% in sheep and 11,6% in goats. The Zambézia Province is characterized by high temperature and humidity, with the rainy season starting in November and ending in April. The rainfall records from the closest meteorological station to the study site show that the average monthly rainfall in 2007 was 150 mm and in 2010 it was 80 mm. The average monthly rainfall from January to April 2007 was 210 mm, compared to 114 mm in 2010. These differences in precipitation may have influenced the different sero-prevalence rates obtained.

The overall seroprevalence (Table 5.3) initially suggested a possible influence of sex on the seroprevalence, with a much higher seroprevalence observed in females than in males. However, in the multiple logistic regression model the apparent effect of sex disappeared since it was due to confounding by age. Relatively more of the older animals were females, particularly amongst goats, and adult animals were far more likely to be seropositive than young animals ($p < 0.001$). The differences in seroprevalence observed between different localities of Mopeia district in 2010 as well as in different districts in 2007 are difficult to explain because the weather, the agro-ecological conditions, management and other risk factors were similar.

The IgM ELISA was used to detect recent infections. It has been demonstrated that after experimental infection with RVFV IgM starts to rise after Day 3 and at 77 days the levels decrease to that of non-infected animals while IgG was detected from Day 5 and

© University of Pretoria

remained high until 77 days after infection (Paweska et al., 2003b; Fafetine et al., 2007). In our study, 29 of the 240 animals were IgM ELISA positive indicating that infection of the animals probably occurred during the dry season. No outbreak of RVF or apparent clinical signs of the disease were reported during the dry season.

The Zambézia Province has suitable ecological conditions for the circulation of RVFV. It has extensive wetlands and dambos that provide suitable habitat for mosquito breeding. The Chimuara district is also drained by three rivers, namely the Zambeze, Cuacua and Chire.

To test the hypothesis of endemicity of RVF 125 animals were bled at approximately 45-day intervals in a longitudinal study from September 2010 until April 2011. None of the animals sero-converted. These results show that no active viral transmission occurred in the animals during the study period. Evidence of inter-epidemic transmission has been shown in African buffalo (*Syncerus caffer*) in South Africa where 9 out of 126 seronegative animals seroconverted between 2001 and 2003/4 (LaBeaud et al., 2011). The animals in our study were bled for only a 7 month period (middle of the dry season and the end of the rainy season) that was drier than usual, may have had an effect on our results.

In summary, the presence of antibodies to RVFV in sheep and goats in different districts of Zambézia Province is evidence of inter-epidemic circulation of RVFV. Additional studies including the isolation of the virus from mosquito vector and longitudinal studies in sheep, goats and cattle are necessary to further elucidate the inter-epidemic state of the disease.

5.5 References

Anderson, E.C., Rowe, L.W. (1998). The prevalence of antibody to the viruses of bovine virus diarrhoea, bovine herpes virus 1, Rift Valley fever, ephemeral fever and bluetongue and to Leptospira sp in free-ranging wildlife in Zimbabwe. Epidemiol. Infect., 121, 441-449.

© University of Pretoria

Andriamandimby, S.F., Randrianarivo-Solofoniaina, A.E., Jeanmaire, E.M., Ravalolomanana, L., Razafimanantsoa, L.T., Rakotojoelinandrasana, T., Razainirina, J., Hoffmann, J., Ravalohery, J.P., Rafisandratantsoa, J.T., Rollin, P.E., Reynes, J.M. (2010). Rift Valley fever during rainy seasons, Madagascar, 2008 and 2009. Emerg. Infect. Dis., 16 (6), 963-970.

Archer, B.N., Weyer, J., Paweska, J., Nkosi, D., Leman, P., Tint, K.S. Blumberg, L. (2011). Outbreak of Rift Valley fever affecting veterinarians and farmers in South Africa, 2008. S. Afr. Med. J., 101 (4), 263-6.

Chevalier, V., Thiongane, Y., Lancelot, R. (2009). Endemic transmission of Rift Valley fever in Senegal. Transbound Emer. Dis., 56, 372-374.

DINAP (2002). Ministério da Agricultura e Desenvolvimento Rural. Direcção Nacional de Pecuária. Relatório Anual.

Evans, A., Gakuya, F., Paweska, J.T., Rostal, M., Akoolo, L., Van Vuren, P.J., Manyibe, T., Macharia, J.M., Ksiazek, T.G., Feikin, D.R., Breiman, R.F., Njenga, M.K. (2008). Prevalence of antibodies against Rift Valley fever virus in Kenyan wildlife. Epidemiol. Infect., 136, 1261-1269.

Fafetine, J.M., Tijhaar, E., Paweska, J.T., Neves, L.C.B.G., Hendriks, J., Swanepoel, R., Coetzer, J.A.W., Egberink, H.F., Rutten, V.P.M.G. (2007). Cloning and expression of Rift Valley fever virus nucleocapsid (N) protein and evaluation of a N-protein based indirect ELISA for the detection of specific IgG and IgM antibodies in domestic ruminants. Vet. Microb., 121 (1-2), 29-38.

Instituto Nacional de Metereologia - INAM (2010). http://www.inam.gov.mz

Jansen Van Vuren, P., Potgieter, A.C., Paweska, J.T., Van Dijk, A.A. (2007). Preparation and evaluation of a recombinant Rift Valley fever virus N protein for the

© University of Pretoria

detection of IgG and IgM antibodies in humans and animals by indirect ELISA. J. Virol. Methods, 140, 106–114.

Jeanmaire, E.M., Rabenarivahiny, R., Biarmann, M., Rabibisoa, L., Ravaomanana, F., Randriamparany, T., Andriamandimby, S.F., diaw, C.S., Fenozara, P., de La Rocque, S., reynes, J.M. (2011). Prevalence of Rift Valley fever infection in ruminants in Madagascar after the 2008 outbreak.Vector Born Dis., 11 (4), 395-402.

LaBeaud, A.D., Muchiri, E.M., Ndzovu, M., Mwanje, M.T., Muiruri, S., Peters, C.J., King, C.H. (2008). Interepidemic Rift Valley fever virus seropositivity, Northeastern Kenya. Emerg. Infect. Dis., 14 (8), 1240-1246.

LaBeaud, A.D., Cross, P.C., Getz, W.M., Glinka, A., King, C.H. (2011). Rift Valley fever virus infection in African Buffalo (*Syncerus caffer*) herds in rural South Africa: evidence of inter-epidemic transmission. Am. J. Trop. Med. Hyg., 84 (4), 641-646.

Linthicum, K.J., Davis, F.G., Kairo, A., Bailey, C.L. (1985). Rift Valley fever virus (family Bunyaviridae, genus Phlebovirus). Isolation from Diptera collected during an inter-epizootic period in Kenya. J. Hyg., 95, 197-209.

Murphy, F.A., Gibbs, E.P.J. Horzinek, M.C., Studdert, M.J. (1999). Veterinary Virology. 3rd ed. Elsevier, USA. Pp. 469-475.

Nderitu, L., Lee, J.S., Omolo, J., Omulo, S., O'Guinn, M.L., Hightower, A., Mosha, F., Mohamed, M., Munyua, P., Nganga, Z., Hiett, K., Seal, B., Feikin, D.R., Breiman, R.F. , Njenga, M.K. (2011) Sequential Rift Valley fever outbreaks in eastern Africa caused by multiple lineages of the virus. J. Infect. Dis., 203: 655-665.

Niklasson, B., Liljestrand, J., Bergstrom, S., Peters, C.J. (1987). Rift Valley fever: seroepidemiological survey among pregnant women in Mozambique. Epidemiol. Infect., 99, 517-522.

© University of Pretoria

Paweska, J.T., Smith, S.J., Wright, I.M., Williams, R., Cohen, A.S., Van Dijk, A.A., Grobbelaar, A.A., Croft, J.E., Swanepoel, R., Gerdes, G.H. (2003a). Indirect enzyme-linked immunosorbent assay for the detection of antibody against Rift Valley fever virus in domestic and wild ruminant sera. Onderstepoort J. Vet. Res., 70, 49-64.

Paweska, J.T., Burt, F.J., Anthony, F., Smith, S.J., Grobbelaar, A.A., Croft, J.E., Ksiazek, T.G., Swanepoel, R. (2003b). IgG-sandwish and IgM-capture enzyme-linked immunosorbent assay for the detection of antibody to Rift Valley fever virus in domestic ruminants. J. Virol. Methods, 113, 103-112.

Pepin, M., Bouloy, M., Bird, B., Kemp, A., Paweska, J. (2010). Rift Valley fever virus (*Bunyaviridae: Phlebovirus*): an update on pathogenesis, molecular epidemiology, vectors, diagnostics and prevention, Vet. Res., 41:61.

Perez, A.M., Medanic, R.C., Thurmond, M.C. (2010). Rift Valley fever outbreaks in South Africa. Vet. Rec., 166: 798.

Rao, J.N.K., Scott, A.J. (1989). On chi-squared tests for multiway contingency tables with cell proportions estimated from survey data. Annals of Statistics, 12, 46-60.

Rich, K.M., Wanyoike, Francis (2010). An assessment of the regional and national socio-economic impacts of the 2007 Rift Valley fever outbreak in Kenya. Am. J. Trop. Med. Hyg., 83 (Suppl 2), 52-57.

Roger, M., Girard, S., Faharoudine, A., Halifa, M., Bouloy, M., Cetre-Sossah, Cardinale, E. (2011). Rift valley fever in ruminants, republic of Comoros, 2009. Emerg. Infect. Dis., 17 (7), 1319-1320.

Samui, K.L., Inoue, S., Mweene, A.S., Nambota, A.M., Mlangwa, J.E., Chilonda, P., Onuma, M., Morita, C. (1997) Distribution of Rift Valley fever among cattle in Zambia. Jpn. J. Med. Sci. Biol., 50 (2), 73-77.

© University of Pretoria

Serviços Provinciais de Pecuária da Zambézia - SPPZ (2009). Arrolamento de espécies pecuárias.

Swanepoel, R., Coetzer, J.A.W. (2004). Rift Valley fever. In: Infectious diseases of livestock, 2nd ed. Ed. Coetzer, J.A.W. and Tustin, R.C. Vol II. Oxford University Press. Southern Africa. Pp 1037-1070.

Valadão, F.G. (1969). Nota prévia sobre a ocorrência de uma nova doença em Moçambique – a doença do vale do Rift. Veterin. Moçamb., 2, 13-20.

WHO (2007). Outbrakes of rift Valley fever in Kenya, Somalia and United Republic of Tanzania, December 2006-April 2007. Weekly epidemiological news, 20 (82), 169-178.

WHO (2010). Rift Valley fever, South Africa – update1. Weekly epidemiological news, 21 (85), 185-186.

© University of Pretoria

6 . Concluding remarks and recommendations

Rift Valley fever is an economically important high impact disease endemic in most countries of Africa resulting in high mortality and abortion rates particularly in sheep, goats and cattle. It is also an important zoonotic disease. Outside Africa it has been reported in the Arabian Peninsula and has the potential for spread to other parts of the world. There is still some controversy regarding the maintenance of RVFV during the inter-epidemic periods. One of the hypotheses is that the virus is transmitted transovarially to *Aedes* floodwater mosquito eggs where it can survive for long periods in dry mud until the next flooding of dambos or wetlands occurs. However, field and experimental evidence to support this hypothesis is limited; transovarial transmission has been demonstrated only once and could not be reproduced experimentally so far. Another possible explanation for maintenance of RVFV during the inter-epidemic period is that there is continuous low level circulation of the virus between mosquitoes and livestock (sheep, goats and cattle) and wildlife species. Although the role of wildlife in maintenance and perpetuation of RVFV has not been well studied the presence of anti-RVFV antibodies in various wildlife species such as African buffalo supports this theory. Further work is necessary to prove both the “egg” hypothesis and maintenance of RVF in livestock and wildlife.

At the advent of this study, available laboratory techniques for the detection of antibodies against RVFV relied on the use of immunoreagents that were prepared from infectious virus, thus requiring a high biocontainment facility which restricts their production to a limited number of laboratories in Africa.

Accordingly, the major objectives of the present study were to develop and evaluate new diagnostic reagents for the detection of anti-RVFV antibodies and viral antigen. An indirect ELISA based on recombinant antigen was developed in this study and applied to investigate RVFV activity during the inter-epidemic period in sheep and goats in the Zambézia Province, Mozambique. This recombinant nucleocapsid protein-based indirect ELISA performed as well as the virus neutralization and heamagglutination-inhibition tests readily detecting serum IgM levels early after infection and IgG levels for

© University of Pretoria

several months thereafter. The results obtained with sera from vaccinated and experimentally infected sheep showed high sensitivity in the detection of RVFV specific antibodies. Therefore, RVFV recombinant nucleocapsid protein represents a good alternative for the conventional inactivated whole virus antigen because it is safe, stable and cost-effective in use and production. To further assess the use of the recombinant nucleocapsid protein-based indirect ELISA a comparison was made with the conventional IgG sandwich ELISA employing whole inactivated virus as antigen. The results reveal a very good agreement between the RVF recombinant nucleocapsid IgG indirect ELISA and the IgG sandwich ELISA. The slightly higher sensitivity of the IgG sandwich ELISA could have been due to the use of a species-specific horse-radish peroxidase (HRPO) conjugate in this assay that might have affinity to early IgGs than Protein G. Since the indirect ELISA encompasses much easier and shorter procedures it could be considered to be more suitable in surveillance programs of RVF that usually involves testing sera of large numbers of animals. Another major advantage is the fact that high quality recombinant antigens can be produced in large amounts in laboratories without high bio-containment facilities. The IgG ELISA developed in this study expands the repertoire of safe diagnostic assays for RVF and can be a useful tool in future large-scale surveillance studies such as those that are anticipated to be carried out in the Zambezia Province.

The monoclonal antibodies selected in the study specifically recognize the recombinant nucleocapsid protein of RVF, as shown in both an indirect ELISA and in a Western blot assay and did not cross-react with extracts of cells infected with other members of the *Bunyaviridae* family such as the Bunyamwera virus and Calovo virus and other RNA viruses from the families *Flaviviridae* (West Nile virus and Dengue virus II) and *Togaviridae* (Sindbis and Chikungunya viruses). High specificity and the possibility of reproducible production in large quantities from stable hybridoma clones, render these monoclonal antibodies valuable diagnostic reagents.

The seroprevalence findings in this study showed that RVFV circulates in sheep and goats in the inter-epidemic period in the Zambézia Province. This strongly suggests an endemic status of the disease and active transmission of the virus to sheep and goats but most probably also in cattle and wildlife species during inter-epidemic periods in

© University of Pretoria

study area. Further studies in the Zambezia and other provinces in Mozambique including the characterization of the mosquito population, detection and isolation of the virus in mosquito vectors, longitudinal serological studies in sentinel herds and searching for other possible reservoir hosts of the virus during the inter-epidemic periods will further elucidate the epidemiology of RVF in Mozambique.

© University of Pretoria