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EVALUATION OF A RECOMBINANT RIFT VALLEY FEVER VIRUS NUCLEOCAPSID PROTEIN AS A VACCINE AND AN IMMUNODIAGNOSTIC REAGENT

Petrus Jansen van Vuren

WitsETD

A thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Doctor of Philosophy

Johannesburg, October 2011

Promoter: Prof. Janusz T. Paweska

Co-promoter: Prof. Caroline T. Tiemessen

DECLARATION

I, Petrus Jansen van Vuren declare that this thesis is my own work. It is being submitted for the degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other university.

_____

Petrus Jansen van Vuren

_____ day of _____, _____

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For my loving wife

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PUBLICATIONS AND PRESENTATIONS ARISING FROM THE THESIS

Publications

Jansen van Vuren, P., Tiemessen, C.T. & Paweska, J.T. (2011). Anti-nucleocapsid protein immune responses counteract pathogenic effects of Rift Valley fever virus infection in mice. *PLoS One* **6(9)**: e25027

Jansen van Vuren, P., Tiemessen, C.T. & Paweska, J.T. (2010). Evaluation of a recombinant Rift Valley fever virus subunit nucleocapsid protein as an immunogen in mice and sheep. *Open Vaccine Journal* **3**, 114-126

Van Vuren, P. J. & Paweska, J. T. (2010). Comparison of enzyme-linked immunosorbent assay-based techniques for the detection of antibody to Rift Valley fever virus in thermochemically inactivated sheep sera. *Vector-Borne and Zoonotic Diseases* 10, 697-9.

Paweska, J.T., van Vuren, P.J., Kemp, A., Swanepoel, R., Buss, P., Bengis, R.G., Gakuya, F., Breimann, R.F. and Njenga, K. (2010). A recombinant nucleocapsid-based indirect ELISA for serodiagnosis of Rift Valley fever in African wildlife. In Symposium Proceedings: Odongo, N.E., Garcia, M. And Viljoen, G.J. (eds). *Sustainable Improvement of Animal Production and Health*. Vienna, Austria, 8 – 11 June 2009. Food and Agriculture Organization of the United Nations: FAO/IAEA, 309-12. ISBN 978-92-5-106697-3.

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Paweska, J. T., Jansen van Vuren, P., and 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. *Journal of Virological Methods* **146**(1-2)**,** 119-24.

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Jansen van Vuren, P., Kemp, A., Le Roux, C., Grobbelaar, A., Leman, P., Weyer, J., Paweska, J.T. (2010). The 2010 Rift Valley fever outbreak in humans in South Africa: A laboratory perspective. *Annual meeting of the ARBO-ZOONET,* Rabat, Morocco, 22-24 November 2010. (Oral presentation).

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Jansen van Vuren, P. & Paweska, J.T. (2008). Laboratory safe detection of nucleocapsid protein of Rift Valley fever virus in human and animal specimens by a sandwich ELISA. *National Institute for Communicable Diseases Academic Day*, 11 November 2008. (Poster presentation).

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ABSTRACT

The serodiagnosis of Rift Valley fever (RVF) relies on the use of inactivated whole virus based reagents which present biosafety, financial and operational constraints. There are no vaccines for humans, the availability of animal vaccines is limited and they have several drawbacks. The aim of this study was to evaluate a bacterially expressed recombinant RVF virus (RVFV) nucleocapsid protein (recNP) as a safe immunodiagnostic reagent, and an immunogen in a mouse and host animal model. Several enzyme-linked immunosorbent assays (ELISAs) were developed in this study, enabling sensitive and specific detection of antibodies and RVFV antigen in human and animal specimens. The recNP was combined with different adjuvants and used to immunize mice and sheep subsequently challenged with a virulent wild type RVFV strain. Depending on the recNP/adjuvant combination, protection against disease in mice ranged between 17 and 100%, with sterilizing immunity elicited in some experimental groups, compared to 100% morbidity/mortality and excessive viral replication in adjuvant and PBS control mice. Immunization with recNP combined with Alhydrogel, an adjuvant that biases immunity towards Th2 humoral immunity, that yielded 100% protection, induced an earlier and stronger type I interferon response in mice after challenge, compared to repression of the same gene in adjuvant and PBS control mice. There was massive activation of pro-inflammatory responses and genes with pro-apoptotic effects in the livers of control mice at the acute phase of infection, accompanied by high viral replication, possibly contributing to the pathology of the liver. There was also evidence of activation and repression of several genes involved in activation of B- and T-cell immunity in control mice, some indicating possible immune evasion by the challenge virus. Immunization of sheep with the same recNP/adjuvant combinations were, however, not able to decrease replication of challenge virus. The recNP based ELISAs are an important addition to and improvement of the currently available serodiagnostic tests for RVF. The mechanism by which recNP immunization protects mice from developing severe disease during the acute phase of infection is now better understood, but the mechanism for earlier clearance of the virus needs further investigation.

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WitsETD

TABLE OF CONTENTS

		Page
DECLARATION		ii
PUBLICATIONS AND PRESENTATIONS		iv
ABSTRACT		vii
ACKNOWLEDGEMENTS		viii
TABLE OF CONTENTS		ix
LIST OF FIGURES		xii
LIST OF TABLES		xiv
CHAPTER ONE		
1 INTRODUCTION AND LITERATURE REVIEW		**15**
1.1	Virus classification and characteristics	15
1.2	Epidemiology	17
1.3	Rift Valley fever in animals	18
1.4	Rift Valley fever in humans	19
1.5	Pathogenesis	19
1.6	Diagnostic techniques	22
1.6.1	Virus / antigen detection	22
1.6.2	Molecular biology	23
1.6.3	Serology	23
1.7	Vaccines and antivirals	24
1.7.1	Inactivated virus vaccines	25
1.7.2	Attenuated virus vaccines	25
1.7.3	Recombinant viruses by reverse genetics	26
1.7.4	DNA vaccines	27
1.7.5	Virus vectored / replicon vaccines	28
1.7.6	Virus like particles as vaccines	29
1.7.7	Recombinant subunit vaccines	30
1.8	Study objectives	31
CHAPTER TWO		
2 RECOMBINANT NUCLEOCAPSID PROTEIN AS IMMUNODIAGNOSTIC REAGENT – SEROLOGY		**33**
2.1	Detection of IgG antibody to Rift Valley fever virus in wild ruminants	33
2.1.1	Introduction	33
2.1.2	Materials and methods	34
2.1.3	Results	37
2.1.4	Discussion	42
2.2	Conjugation of the recombinant nucleocapsid protein with horseradish peroxidase for detection of IgM antibody to Rift Valley fever virus in humans	44
2.2.1	Introduction	44
2.2.2	Materials and methods	44
2.2.3	Results	45
2.2.4	Discussion	49

UKZN ETD

Page

2.3 Comparative evaluation of ELISA-based techniques for detection of antibodies against RVFV 51
2.3.1 Introduction 51
2.3.2 Materials and methods 52
2.3.3 Results 55
2.3.4 Discussion 56
2.4 Detection of IgG antibody to Rift Valley fever virus in humans 57
2.4.1 Introduction 57
2.4.2 Materials and methods 57
2.4.3 Results 58
2.4.4 Discussion 63

CHAPTER THREE

3 EVALUATION OF ANTI-RECOMBINANT NUCLEOCAPSID PROTEIN RABBIT AND SHEEP POLYCLONAL SERA AS IMMUNODIAGNOSTIC REAGENTS IN AN ANTIGEN DETECTION SANDWICH ELISA 65

3.1 Introduction 65
3.2 Materials and methods 66
3.2.1 Generation of rabbit and sheep hyperimmune sera against the RVFV recNP 66
3.2.2 Sandwich ELISA procedure 66
3.2.3 Inactivation of specimens and safety testing 67
3.2.4 Antigen detection in animal specimens 67
3.2.5 Antigen detection in human specimens 68
3.2.6 Monitoring viral growth *in vitro* 68
3.2.7 Antigen detection in decomposing tissues 69
3.2.8 ELISA performance, cut-off selection and IQC 69
3.3 Results 71
3.4 Discussion 80

CHAPTER FOUR

4 EVALUATION OF A RECOMBINANT NUCLEOCAPSID PROTEIN AS AN IMMUNOGEN IN A MOUSE MODEL 84

4.1 Introduction 84
4.2 Immunogenicity of the recombinant nucleocapsid protein alone and in combination with four adjuvants 86
4.2.1 Materials and methods 86
4.2.2 Results 87
4.2.3 Discussion 92
4.3 *In vivo* neutralizing ability of anti-nucleocapsid immune sera in mice 93
4.3.1 Materials and methods 93
4.3.2 Results 94
4.3.3 Discussion 95
4.4 Rift Valley fever virus challenge of mice immunized with the recombinant nucleocapsid protein 96
4.4.1 Materials and methods 96
4.4.2 Results 97
4.4.3 Discussion 102
4.5 Conclusion 104

UNISETD

CHAPTER FIVE **Page**
5 RECOMBINANT NUCLEOCAPSID PROTEIN AS AN IMMUNOGEN IN SHEEP **105**
5.1 Introduction 105
5.2 Immunogenicity of the recombinant nucleocapsid protein alone and in combination with four adjuvants in sheep 106
5.2.1 Materials and methods 106
5.2.2 Results 107
5.2.3 Discussion 108
5.3 Rift Valley fever virus challenge of immunized sheep 109
5.3.1 Materials and methods 109
5.3.2 Results 110
5.3.3 Discussion 113
5.4 Conclusion 115

CHAPTER SIX
6 HOST GENE EXPRESSION IN MICE IMMUNIZED WITH RECOMBINANT NUCLEOCAPSID PROTEIN AND CONTROL MICE AFTER RIFT VALLEY FEVER VIRUS INFECTION **116**
6.1 Introduction 116
6.2 Materials and methods 120
6.2.1 Immunization and Rift Valley fever virus challenge of mice 120
6.2.2 Measuring up- and downregulation of genes using qRT-PCR 122
6.3 Results 126
6.4 Discussion 145

CHAPTER SEVEN
7 CONCLUSIONS **151**

APPENDIX 1 **156**
Ethics approvals

APPENDIX 2 **162**
Reprints of publications originating from this study

REFERENCES **223**

WitsETD

LIST OF FIGURES

		Page
Chapter one		
Figure 1.1.	Rift Valley fever virus genome	15
Figure 1.2.	Rift Valley fever virus structure	16
Chapter two		
Figure 2.1.1.	Upper and lower internal quality limits for PP values of high positive, low-positive, negative serum and conjugate controls in recNP-based IgG I-ELISA	37
Figure 2.1.2.	Dose response curves of individual African buffalo sera in the recNP I-ELISA	38
Figure 2.1.3.	Dose response curves of wildlife sera in the recNP I-ELISA	39
Figure 2.1.4.	Optimisation of the cut-off for the recNP I-ELISA in African buffalo using the misclassification cost term option of the two-graph receiver operating characteristic analysis	40
Figure 2.1.5.	Distribution of recNP IgG I-ELISA PP values in African buffalo tested positive and negative in the virus neutralization test	42
Figure 2.2.1.	Titration curves of three different recNP-HRP preparations with strong positive human RVF IgM controls	46
Figure 2.2.2.	Titration curves of recNP-HRP with three different human IgM controls	47
Figure 2.2.3.	Optimisation of the cut-off for the recNP-HRPO IgM ELISA in humans using the misclassification cost term (MCT) option of the two-graph receiver operating characteristic analysis (TG-ROC)	49
Figure 2.3.1.	Comparison of mean immune responses in three experimentally infected sheep as measured by testing naïve versus thermo-chemically inactivated sera	55
Figure 2.4.1.	Upper and lower internal quality limits for PP values of high positive, low-positive, negative serum and conjugate controls in recNP-based IgG I-ELISA	60
Figure 2.4.2.	Cross-reactivity of recombinant nucleocapsid protein of RVFV in I-ELISA with mouse IgG antibody against selected viruses of the family *Bunyaviridae*	61
Figure 2.4.3.	The effect of different ELISA cut-off values on the discrimination between positive and negative human sera	63
Chapter three		
Figure 3.1.	Upper and lower internal quality control limits for PP values of high-, low-positive and negative antigen controls	72
Figure 3.2.	Dose response curves of recNP, RVFV Ar20368 RSA 81 and control antigen	73
Figure 3.3.	Dose response curves of human and animal sera, animal tissue and mosquito homogenates spiked with RVFV and their corresponding non-spiked controls	75
Figure 3.4.	RVFV replication kinetics in Vero cells inoculated with different concentrations of the virus measured by antigen detection ELISA	76

WitsETD

		Page
Figure 3.5.	Detection of infectious virus and viral nucleocapsid antigen in tissue homogenates spiked with RVFV followed by incubation at 37°C	77
Figure 3.6.	Detection of nucleocapsid protein by ELISA in RVFV-infected buffalo foetus and mouse organ tissues.	78
Figure 3.7.	Monitoring of viremia, antigenemia, anti-nucleocapsid IgM and IgG responses in a sheep experimentally infected with RVFV	79
Figure 3.8.	Blocking effect of increasing levels of anti-RVFV specific antibody on antigen detection in viremic sheep serum	79
Chapter four		
Figure 4.1.	Detection of total IgG, IgG1 and IgG2A specific antibodies against the RVFV recNP in individual mice after recNP immunization	88-92
Figure 4.2.	*In vivo* neutralization of RVFV with anti-recNP immune sera in mice	95
Figure 4.3.	Protection of mice immunized with the 35µg and 70µg recNP doses against disease or death after RVFV challenge compared to placebo control mice	99
Figure 4.4.	Mean RVFV $TCID_{50}$/gram of tissues from dead or sick mice compared to healthy mice	100
Chapter five		
Figure 5.1.	Average anti-NP responses in sheep after recNP first and booster immunizations	108
Figure 5.2.	Mean IgM responses in sheep after RVFV challenge on day 37 or 168	111
Figure 5.3.	Mean virus neutralizing antibody responses in sheep after RVFV challenge on day 37 or 168	112
Chapter six		
Figure 6.1.	Detection of total IgG, IgG1 and IgG2A specific antibodies against the RVFV recNP in mice after recNP immunization with Alhydrogel	127
Figure 6.2.	Mean viral loads in livers and spleens of RVFV infected mice.	131
Figure 6.3.	Fold changes in expression of IL10, IFNγ and IFNβ genes in tissues of mice after RVFV infection.	134
Figure 6.4.	Volcano plot displaying fold changes in expression of 84 genes in livers of recNP immunized mice	139
Figure 6.5.	Volcano plot displaying fold changes in expression of 84 genes in livers of Alhydrogel control mice	140
Figure 6.6.	Volcano plot displaying fold changes in expression of 84 genes in livers of PBS control mice	140
Figure 6.7.	The fold changes in expression of genes, with specific immune outcomes, in the liver of experimental groups at 72 hours after RVFV infection	141
Figure 6.8.	Heat maps showing fold changes in liver and spleen at 72 hours after RVFV infection	142

WITSETD

LIST OF TABLES

		Page
Chapter Two		
Table 2.1.1.	Number of field-collected wildlife sera tested in the virus neutralization test	35
Table 2.1.2.	Diagnostic accuracy of Rift Valley fever recNP-based I-ELISA in African wildlife	41
Table 2.2.1.	Diagnostic accuracy of Rift Valley fever recNP-HRPO IgM ELISA in humans	48
Table 2.3.1.	Antibody detection ELISAs for RVF diagnosis in humans	53
Table 2.3.2.	Antibody detection ELISAs for RVF diagnosis in livestock	54
Table 2.4.1.	Internal quality control data and repeatability estimates for Rift Valley fever IgG I-ELISA based on recombinant nucleocapsid antigen	59
Table 2.4.2.	Diagnostic accuracy of Rift Valley fever IgG I-ELISA based on recombinant nucleocapsid antigen	62
Chapter Three		
Table 3.1.	Identification, year of isolation, origin and concentration of RVFV strains used to evaluate the sAg-ELISA	70
Table 3.2.	Internal quality control limits and repeatability estimates of RVF s-Ag ELISA	71
Table 3.3.	Diagnostic accuracy of the sAg-ELISA for the detection of nucleocapsid protein of RVFV in sheep and human sera	77
Chapter Four		
Table 4.1.	Mouse survival rates after RVFV challenge	100
Table 4.2.	Viral load data in mice after RVFV challenge	101
Chapter Five		
Table 5.1.	Group assignments and immunization schedules of sheep	106
Table 5.2.	Viremia in sheep after RVFV challenge	114
Chapter Six		
Table 6.1.	Genes analyzed by Quantitect RT-PCR	123
Table 6.2.	Fold changes in gene expression, and viral loads in liver tissues from all treated groups at different time points	128
Table 6.3.	Fold changes in gene expression, and viral loads in spleen tissues from all treated groups at different time points	129
Table 6.4.	Fold changes in gene expression, and viral loads in brain tissues from all treated groups at different time points	130
Table 6.5.	Genes analyzed by SABiosciences PCR array, including fold changes and *P*-values	136-138

WITSETD

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 Virus classification and characteristics

Rift Valley fever virus (RVFV) is a mosquito borne member of the *Phlebovirus* genus in the *Bunyaviridae* family of viruses (Bishop et al., 1980). The *Bunyaviridae* family consists of spherical shaped enveloped viruses classified in five genera: *Hantavirus, Nairovirus, Orthobunyavirus, Phlebovirus* and *Tospovirus*. RVFV has a diameter of up to 120 nanometres (nm) with short glycoprotein spikes projecting through the lipid envelope (Gerdes, 2004). Even though strains of RVFV differ in their pathogenicity, they are structurally and serologically indistinguishable (Rice et al., 1980). The RVFV genome consists of three single-stranded ribonucleic acid (RNA) segments; large (L), medium (M) and small (S). The L segment, consisting of 6404 bases, has negative polarity and encodes the viral RNA-dependent RNA polymerase. The M segment, consisting of 3885 bases, has negative polarity and encodes the precursor of the viral envelope glycoproteins Gn and Gc, a 78-kilodalton (kDa) non-structural glycoprotein and a non-glycosylated 14-kDa protein. The S segment consists of 1690 bases and encodes the viral nucleocapsid protein (NP) and a non-structural protein NSs using an ambisense coding strategy (Ihara et al., 1984, Giorgi et al., 1991). The NP (length: 245 amino acid residues, weight: 27,431-kDa) is encoded by 738 bases of subgenomic viral-complementary messenger (m) RNA. The NSs protein (length: 265 amino acid residues, weight: 29,903-kDa) is encoded by 798 bases of subgenomic viral-sense mRNA (Suzich et al., 1990, Billecocq et al., 2004). Mature viral particles, however, have been shown to not only contain negative sense viral RNA but also a fraction of RNA complementary to viral RNA (cRNA) (Ikegami et al., 2005), allowing the virulence factor NSs to be expressed immediately after the virus enters the cell (Bouloy and Weber, 2010).

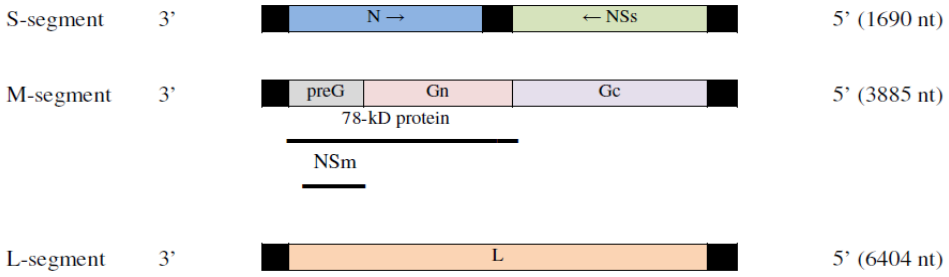


Figure 1.1. Rift Valley fever virus genome.

The gene encoding the NP of *Phleboviruses* is highly conserved (Giorgi et al., 1991, Vialat et al., 1997). The NP and viral polymerase proteins of negative sense RNA viruses associate with the

virus genome and form ribonucleoproteins (RNPs) which are necessary for transcription and replication (Gauliard et al., 2006). RNP molecules appear circular because of the complementary sequences of 5' and 3' non-coding regions which cause the formation of panhandle structures (Le May et al., 2005). It was recently shown that RNPs interact with the cytoplasmic tail of the glycoproteins, supposedly enabling successful packaging of the genome into virus particles (Bouloy and Weber, 2010). The NP is the most abundant protein in infected cells, and thus the immunodominant antigen during infections with viruses from the *Bunyaviridae* family (Swanepoel et al., 1986a, Magurano and Nicoletti, 1999, Gauliard et al., 2006). The RVFV NP is the first viral protein to be synthesized (Ikegami et al., 2005) and it has been shown that it can be released from infected cells independently of the glycoproteins (Liu et al., 2008). The NSs is the most variable protein among *Phleboviruses* (Sall et al., 1997). The RVFV NSs protein is different from those of other *Bunyaviruses* in that it is phosphorylated and found in the nucleus of infected cells, which is unique because all stages of the viral life cycle occurs exclusively in the cytoplasm. The NSs protein forms filamentous structures in the host nucleus and interacts with cellular nuclear proteins (Swanepoel and Blackburn, 1977, Yadani et al., 1999, Le May et al., 2004, Ikegami et al., 2006, Le May et al., 2008). The structural glycoproteins, Gn and Gc, are responsible for attachment and entry of the virus to cells and carries neutralizing epitopes (Besselaar and Blackburn, 1991, Besselaar and Blackburn, 1992, Bouloy and Weber, 2010).

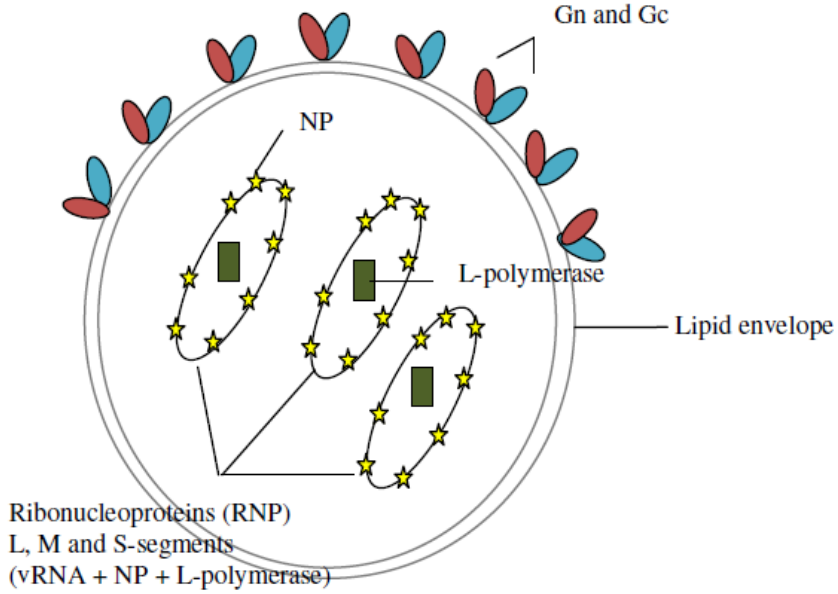


Figure 1.2. Rift Valley fever virus structure.

1.2 Epidemiology

Rift Valley fever virus was first isolated in 1931 in the Rift Valley of Kenya near Lake Naivasha from the blood of a newborn lamb during an outbreak in domestic livestock, after which the first human infections were also noted (Daubney et al., 1931). The first report of extensive human disease caused by RVFV was in 1951 in South Africa where an estimated 20 000 persons were infected (Mundel and Gear, 1951, House et al., 1996). Large outbreaks then occurred in Egypt 1977/1978 (between 18 000 and 200 000 human infections, 598 deaths), Mauritania 1987 (approximately 200 human deaths), East Africa and Madagascar 1991 (89 000 infections and more than 500 human deaths) and East Africa 1998 (98 000 humans infected and 250 deaths) (Swanepoel and Coetzer, 2004). The disease spread outside its endemic range, across the Arabian Peninsula, to Saudi Arabia and Yemen in 2000 affecting 882 humans and causing 124 deaths (Balkhy and Memish, 2003). More recently outbreaks were recorded in East Africa (2006-07), Sudan (2007/2008), Madagascar (2008) and South Africa (2008, 2009-2011)(Anonymous, 2007, Mohamed et al., 2010, Nguku et al., 2010). Although no RVF outbreaks have been confirmed in a number of African countries such as Mali, Gabon, Congo, Chad, Botswana, Angola, Nigeria, Uganda, antibodies to the virus were found in humans and livestock from these countries (Gerdes, 2004, Pourrut et al., 2010). Antibodies have also been found in domestic livestock from Senegal, Cameroon, Togo, Benin, Ivory Coast and Burkina-Faso (Zeller et al., 1995).

Outbreaks of RVF are usually associated with above average rainfall, occurring at irregular intervals of about 10 years (Swanepoel and Coetzer, 2004). Several possible explanations have been put forward to explain the survival and circulation of the virus between outbreaks. Initially it was thought that the virus was endemic in forests where it circulated between mosquitoes and vertebrate hosts, and spilled over into domestic livestock to cause outbreaks (Smithburn et al., 1948, Smithburn et al., 1949a). However, the RVF outbreak in South Africa in the 1950s occurred in grassland country where shallow and poorly drained depressions (pans) are abundant. These pans are usually dry but during heavy rains they fill up and allow mosquito eggs, possibly laid months or years before, to hatch. After RVFV was isolated from mosquitoes that were reared from eggs collected in a pan in South Africa, as well as unfed male and female mosquitoes in Kenya during an inter-epidemic period, it hinted that the virus might be transmitted transovarially by aedine mosquitoes (Linthicum et al., 1985).

RVFV is transmitted mostly by *Aedes* and *Culex* mosquitoes, but other mosquitoes (*Anopheles*, *Eretmapoites*, and *Mansonia*) have also been shown to be potential vectors of the virus (Smithburn et al., 1948, Smithburn et al., 1949a, Turell and Bailey, 1987, Turell et al., 1990, Turell et al., 2007, Turell et al., 2008a, Turell et al., 2008b, Sang et al., 2010). Interepidemic transmission of RVF in domestic livestock (Scott et al., 1956, Linthicum et al., 1985) and wildlife (Evans et al., 2008) have been shown, leading to the current theory that RVFV is maintained in interepidemic periods by

transovarial transmission and cycling between mosquitoes and ungulates. Outbreaks then occur after abnormally high rainfall due to an explosion in competent mosquito vector numbers.

1.3 Rift Valley fever in animals

Sheep are primarily affected by RVF but other ruminants including cattle, goats, camels and wildlife animals are also susceptible to infection (Swanepoel and Coetzer, 2004). Antibodies against RVFV have been found in African buffalo, black rhino, lesser kudu, impala, African elephant, Thompson's gazelle, gerenuk and waterbuck (Evans et al., 2008). It has been proposed that wild vertebrates might play a role in maintenance of the virus between epizootics, since wild animals repeatedly test positive for antibodies to RVFV in locations unrelated to any documented livestock or human outbreaks (Bengis et al., 2004). Fatality rates reach up to 100% in young lambs whereas 20-30% of infected adult sheep die. Up to 90% of pregnant ewes abort after being infected. This can be attributed to very high levels of viremia, which also increases the chances of transmission to humans that handle infected tissues (Woods et al., 2002).

In non-pregnant adult animals clinical signs include listlessness, abdominal pain, vomiting, diarrhoea, jaundice hepatitis, icterus, nasal discharge and death in some cases. Onset of abortions and high neonatal mortality are characteristic signs of a RVF outbreaks (Swanepoel and Coetzer, 2004). Animals like buffalo and camels do not exhibit disease but pregnant animals can abort when infected (Gerdes, 2004). It was recently shown that a European breed of sheep is also susceptible to RVFV infection, with experimentally infected sheep showing no mortality but mostly pyrexia and corneal opacity despite relatively high viral loads in their blood (Busquets et al., 2010).

Infected hepatocytes are probably the major source of high plasma viremia observed in infected animals (Ritter et al., 2000). RVFV has been found to cause extensive necrosis in the livers of aborted lambs, whereas livers from adult sheep are not affected as much. The spleen is not enlarged and is not affected by extensive haemorrhaging as seen in the liver, which is an important feature which distinguishes it from other sheep diseases with similar symptoms to RVF. Other organs also show signs of congestion or haemorrhaging including kidneys and the lymphatic system (Daubney et al., 1931).

Non-human primates are susceptible to RVF. Monkeys have been successfully infected with the RVFV ZH-501 pathogenic strain in the laboratory, resulting in poor appetite, anorexia and petechiation, as well as reduced activity 3 to 5 days after infection. The virus grew to titres reaching as high as $6.7\log_{10}$ PFU/ml between 3 and 5 days post infection, and could be isolated from brain, spinal cord, liver, spleen and mesenteric lymph node tissues of infected monkeys (Morrill and Peters, 2003).

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1.4 Rift Valley fever in humans

Humans are highly susceptible to infection with RVFV. Transmission of RVFV to humans can occur through bites from infected mosquitoes, contact with contaminated meat or through aerosols created during slaughtering. When the virus was isolated for the first time in 1931 in Kenya, all four European scientists/veterinarians involved in the outbreak were infected while working with aborted fetuses and other infected tissues. None of the infections were fatal but it showed clearly how easily humans can become infected. The group described their own symptoms as malaise at onset of the disease followed by rigors, headache, fever for a period of 12 to 36 hours and joint pains, with symptoms generally disappearing within four days. One of the scientists developed a second latent reaction which included headache and defective vision for a few weeks afterwards (Daubney et al., 1931).

Human infection may take on four forms: i) Uncomplicated, febrile, influenza-like illness; ii) hemorrhagic fever with liver involvement, thrombocytopenia, icterus and bleeding; iii) encephalitis following a febrile episode with confusion and coma or even death; iv) ocular involvement with reported blurred vision and loss of visual acuity due to retinal haemorrhage and macular oedema (Al-Hazmi et al., 2003, Gerdes, 2004, Mohamed et al., 2010). The more severe complications occur in up to 5% of the cases. Risk factors recognized for Rift Valley fever infection include consuming or handling products from sick animals and caretaking of animals, whereas touching aborted foetuses is associated with severe RVF complications and consuming or handling products from sick animals associated with death (Anyangu et al., 2010). Examination of blood from infected individuals show leucopaenia, elevated blood enzymes because of liver damage and thrombocytopenia. The main sites of viral replication are the liver, spleen and the brain (Ritter et al., 2000).

The incubation period of RVF ranges from 12 hours to six days in young and adult humans, with illness lasting up to eight days. The mortality rate is ±1%, but a 15% rate has been observed in hospitalized patients (Al-Hazmi et al., 2003, Gerdes, 2004). RVFV can be transmitted to a newborn child from its infected mother, as was shown in the 2000 Saudi Arabia outbreak when a 5-day-old newborn died from RVF, after onset of disease on day 2 after birth and presence of IgM antibodies (Arishi et al., 2006). The infant had sepsis, enlarged liver, coagulopathy, anemia and abnormal liver function.

1.5 Pathogenesis

The pathogenesis of RVFV infection after mosquito bite infection has been proposed to follow the following sequence: the virus spreads from the skin to draining lymph nodes where it initially replicates in the macrophages and then spreads to the circulatory system (Smith et al., 2010). The liver

is the first and major site of major RVFV replication after infection (Anderson et al., 1987, Shieh et al., 2010) where large amounts of virus is produced. This results in necrosis, apoptosis of the hepatic cells as early as day 2 after infection which, together with coagulation, is probably responsible for the hemorrhagic manifestation of the disease. Liver enzymes become elevated in infected mice on day 3 post infection (p.i.), and remain elevated until day 8 p.i. Infection also causes significant increases (neutrophils, red blood cells, eosinopils, basophils) or decreases (lymphocytes) in important circulating blood cells (Smith et al., 2010). The virus is also able to cross the blood-brain barrier as early as day 5 p.i. and cause meningoencephalitis and retinitis (Gonzalez-Scarano et al., 1991, Smith et al., 2010). Apart from these major organs, the virus has also been proved to show tropism to a variety of other organs including spleen, lymph nodes, heart, kidney, lung, pancreas and adrenal glands in a mouse model (Smith et al., 2010). Specific host genetic factors seem to play a very important role in the type and severity of disease caused by RVFV as shown by different responses to infection in different strains of inbred rats (Peters and Slone, 1982, Anderson et al., 1987).

Interferons (IFN) are a family of secreted proteins with many functions including antiviral defence, cell growth, and regulation and activation of the immune response. Interferons are regarded as a powerful defence mechanism and its effectiveness has led many viruses to develop mechanisms to counteract the production and actions of these proteins. In some degree all viruses that successfully infect an animal or human host must have some measure of evading IFN. Therefore the interaction between virus and host IFN is an important determinant of pathogenicity (Goodburn et al., 2000). Type I interferons (IFN-α/β) are produced in direct reaction to virus infection and are the products of two gene families. Leukocytes are mostly responsible for the production of the IFN-α multigene family, whereas IFN-β is synthesized in most cell types but predominantly in fibroblasts. On the other hand, type II IFN is the product of the IFN-γ gene and is produced by T lymphocytes and natural killer (NK) cells in response to the recognition of infected cells. Both type I and II IFN function by activating an antiviral state in target cells which interferes with viral and cellular processes. They also slow growth of the cells which induces apoptosis and thus limits the spread of a virus, and stimulate the acquired immune response (Goodburn et al., 2000).

There are two main pathways leading to the expression of IFN genes (Haller et al., 2006). The classical pathway is utilized by most cells in the body including fibroblasts, hepatocytes and conventional dendritic cells. Viral components are detected in the cytoplasm by intracellular sensors which lead to activation of interferon regulatory transcription factors (IRF-3) and nuclear factor kappa beta (NF-kB) which activates IFN-β expression. IFN-β is secreted as an initial response and IFN-α only later as a secondary response. Toll-like receptors (TLRs) are expressed on the surface of plasmacytoid dendritic cells or in endosomes where they sense extracellular or engulfed virus material.

The regulatory factor, IRF-7, serves as a regulator for IFN-α/β expression, and plasmacytoid dendritic cells express mainly IFN-α. Type I IFN activate a whole range of genes involved in antiviral activity which are grouped into three main systems, namely Protein Kinase R (PKR), 2-5 OAS/RNaseL and Mx protein systems. Viruses have found ways to interfere with the cellular IFN response in order to survive, which include interference with IFN induction, basic transcription, IFN signalling and IFN effectors (Haller et al., 2006).

Rift Valley fever virus is sensitive to interferon (Peters et al., 1986). The importance of IFN in the pathogenesis of *Phleboviruses* has been shown previously in experimental RVFV infection of rats and hamsters (Anderson and Peters, 1988, Perrone et al., 2007). It was found that some RVFV strains that were more capable of killing rats than other strains were not as sensitive to the antiviral effects of IFN in cell culture. The suppression of type I interferon by Punta Toro virus NSs protein results in uncontrolled viral replication and hamster death (Perrone et al., 2007). In rhesus monkeys it was found that IFN-α, administered as a prophylactic, was able to suppress viremia and subsequently disease (Morrill et al., 1990). Thus viruses or their specific strains that are able to counteract the induction of, or evade the action of, type 1 interferon (IFN-α/β) will be the more virulent ones. In a study to evaluate the effects of varying virulence of RVFV in mice, it was found that strains with lower virulence were better IFN -α/β inducers, with IFN being detected at a very early stage of infection, than more virulent strains where IFN was only detectable shortly before death (Higashihara et al., 1972). A study recently showed that a specific laboratory mouse breed MBT/Pas was more susceptible to RVFV infection and its embryonic fibroblasts (MEF) were able to propagate virus to higher titres when compared to a traditional laboratory mouse breed BALB/cByJ (do Valle et al., 2010). Through gene expression profiling of RVFV infected MEF cells from MBT/Pas mice it was shown that a delayed and incomplete type I interferon response was responsible for the increased virulence in these mice compared to BALB/cByJ MEF cells. This study showed the important role the innate immune response plays in susceptibility to RVFV infection (do Valle et al., 2010).

Understanding of the mechanism by which the virus inhibits the antiviral attacks of the host on the molecular level is important for the development of vaccines and anti-viral treatments. A large deletion in the NSs gene of the RVFV Clone 13 strain resulted in attenuation. This observation resulted in recent research exploring the role of the NSs in virulence and pathogenicity of RVFV. The determinants for RVFV virulence lies in the S segment (Vialat et al., 2000, Bouloy et al., 2001), with the NSs protein acting as an interferon antagonist. The NSs protein, which is neither stimulatory nor inhibitory to viral replication, inhibits the type I IFN response of the host by blocking virus-induced IFN-α/β production (Bouloy et al., 2001, Ikegami et al., 2006). Transcription factor II H (TFIIH), a basal transcription factor involved in deoxyribonucleic acid (DNA) repair and cell cycle regulation, is a

target of the NSs protein which interacts with the p44 subunit of TFIIH, blocking its assembly. This inhibits its helicase activities responsible for separating nucleic acid strands prior to transcription and the phosphorylation of RNA polymerase II, resulting in transcription shut down. (Dasgupta, 2004). The NSs protein also interacts with the host protein, Sin3A Associated Protein 30 (SAP30), which is an important regulator of IFN-β gene expression (Le May et al., 2008), thus blocking IFN-α/β production at the transcriptional level (Billecocq et al., 2004). The NSs also promotes the post-transcriptional downregulation of PKR, thus inhibiting the phosphorylation of important proteins in the interferon response (Habjan et al., 2009b, Ikegami et al., 2009, Bouloy and Weber, 2010).

The cytopathic effect of many viruses can be attributed to apoptosis, which can be regulated by viral gene products, host immune response or double stranded RNA mediated cell responses. The aim of apoptosis is to eliminate infected cells to limit further spread of the virus. The RVFV non-structural proteins from the M segment are not necessary for virus replication (Won et al., 2006, Bird et al., 2007a) but rats infected with a RVFV mutant lacking expression of both NSm proteins (14 kDa and 78 kDa) showed attenuated virulence which implies their possible involvement in RVF pathogenesis. The RVFV NSm proteins have been implicated as anti-apoptotic agents since it was found that NSm suppressed staurosporine (STP)-induced apoptosis in the absence of the other RVFV proteins, and cells infected with mutant virus lacking NSm genes underwent apoptosis earlier than cells infected with wild type virus (Won et al., 2007).

1.6 Diagnostic techniques

Rift Valley fever can be diagnosed by detecting antibodies, viral antigens or genetic material. Rapid diagnosis of RVF is essential in endemic areas but even more important in RVF-naïve countries at risk of introduction of the disease. Various classical laboratory methods for RVF diagnosis have been developed but are costly, time consuming and require biocontainment facilities since live virus is used, which hamper quick diagnosis. These tests also pose safety risks to laboratory personnel. There is an increasing demand for safe, accurate and simple diagnostic tools for RVF diagnosis because of the possibility of the virus spreading to previously non-endemic areas such as Europe and north-America where competent mosquito vectors are present.

1.6.1 Virus / antigen detection

The golden standard method for RVF virus isolation is intracranial (i.c.) inoculation of suckling mice which yields positive results within seven days after inoculation. The virus can also easily be propagated in a variety of mammalian cell lines including Vero (African green monkey

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kidney) and BHK (baby hamster kidney) cells (Swanepoel and Coetzer, 2004). These methods, however, are time consuming and involve the propagation of live virus, thus necessitating the use of biocontainment facilities. Viral antigen can also be detected by immunofluorescence, complement fixation, immunodiffusion or enzyme-linked immunosorbent assay (ELISA) (Niklasson et al., 1983, Peters et al., 1989, Swanepoel and Coetzer, 2004, Zaki et al., 2006). These methods are all based on whole virus antigen which still requires biocontainment facilities for production of antigens and performaing assays. The short duration of viremia in experimentally infected sheep (usually days 2 – 4 post infection) and monkeys (days 3 – 5 post infection) should, however, be taken into account when interpreting virus isolation, antigen detection or vRNA detection results (Olaleye et al., 1996, Morrill and Peters, 2003, Bird et al., 2011).

1.6.2 Molecular biology

Various molecular techniques have been developed and evaluated for the detection of RVFV RNA and have been found to be highly specific, sensitive and rapid. These assays include conventional and nested polymerase chain reaction (PCR) (Ibrahim et al., 1997, Jupp et al., 2000, Sall et al., 2001, Sall et al., 2002), real-time PCR (Garcia et al., 2001, Drosten et al., 2002, Njenga et al., 2009) and most recently loop-amplification-mediated-PCR (LAMP) (Peyrefitte et al., 2008, Le Roux et al., 2009). The molecular methods mentioned here have been optimized for the detection of RVF genetic material in livestock, human and insect specimens. Detection of virus genetic material, however, is of limited value once the virus has been cleared from the infected individual's system, especially for viruses with a short viremia when only blood or serum is available for testing. Molecular techniques are also highly specialized and might not be ideal for use in developing countries or in the field where conditions are less than ideal.

1.6.3 Serology

Methods that detect antigen or viral genetic material are highly sensitive shortly after infection but because of the short viremia in RVFV infected individuals, these methods have no use once the virus is cleared. Serological assays in general play a very important role in the field of infectious diseases. They are used for the diagnosis of suspected cases, sero-epidemiological studies, import-export certification of animals, disease eradication programmes and monitoring of vaccine efficacy. It is therefore critical to be able to detect antibodies against RVFV to show recent or past infections. In experimentally infected sheep IgM and IgG antibodies against RVFV can be detected as early as 4 days post infection. IgM responses are, however, transient and usually wane below detectable levels by day 60 post infection, whereas IgG does not have the same transient nature. A recent infection can

WitsETD

therefore be confirmed by detection of virus specific immunoglobulin-M (IgM) in serum as a result of the early production and transient nature of IgM, or by the indication of a four-fold increase in virus specific immunoglobulin-G (IgG). A past infection can be confirmed by the detection of virus specific IgG since infection of an individual with RVFV induces life-long immunity. The gold standard serological technique for RVF is the virus neutralization test (VNT) (Swanepoel et al., 1986a). The VN test detects neutralizing antibodies which are solely directed against the virus' glycoproteins, but cannot distinguish between IgM and IgG. It also involves the propagation of live virus.

Validation of a diagnostic assay essentially refers to the process of determining the fitness of the test for its intended use including assay accuracy, repeatability, reproducibility and stability. Once a diagnostic assay is validated, it can be used to identify the presence or absence of the specific analyte with high confidence. When taken seriously, validation is not a once-off experiment, but it is rather an ongoing process. However, certain steps have to be followed during initial validation which include i) a feasibility study; ii) development and standardization of reagents and protocols; iii) determination of assay performance by testing large numbers of reference or well characterized samples and calculation of cut-off values and accuracy estimates and iv) ongoing evaluation of performance (Crowther et al., 2006). Proper validation of diagnostic assays is therefore important since it results in the determination of reliable estimates of diagnostic specificity and sensitivity which in turn is important factors to take into account for disease diagnosis, risk-assessment and risk-factor studies (Paweska et al., 2003a). Various ELISAs have been developed and validated for the diagnosis of RVF in humans (Niklasson et al., 1984, Swanepoel et al., 1986a, Paweska et al., 2005a, Paweska et al., 2005b, Jansen van Vuren et al., 2007) and animals (Paweska et al., 1995, Paweska et al., 2003a, Paweska et al., 2003b, Paweska et al., 2005b, Fafetine et al., 2007, Jansen van Vuren et al., 2007, Cetre-Sossah et al., 2009, McElroy et al., 2009). Most of these ELISAs are based on whole inactivated virus antigens which still poses safety risks, but recently a few ELISAs were developed using recombinant antigens which are completely safe (Fafetine et al., 2007, Jansen van Vuren et al., 2007, McElroy et al., 2009). The ELISAs based on the recombinant nucleocapsid protein (recNP) were found to be highly sensitive and specific but has not yet been extensively validated. It would be important to develop and validate ELISAs based on recombinant antigens for detection of IgG and IgM in humans and also for detection of antibodies in animals because of the important role they play in disease maintenance and transmission.

1.7 Vaccines and antivirals

In RVFV enzootic areas vaccination is the only practical method of preventing the disease. Various types of vaccines have been developed for RVFV, but none are commercially available for human use and those available for animal use have several drawbacks. Recently a broad-spectrum

WitsETD

antiviral (so called "LJ001", derived from an organic compound called rhodanine), was shown to inhibit virus-cell fusion of lipid enveloped viruses without inhibiting host cell-cell fusion (Wolf et al., 2010). It was specifically shown to prevent death in 100% of RVFV challenged mice that had been pre-treated with the compound.

1.7.1 Inactivated virus vaccines

Inactivated vaccines are relatively expensive to produce, require multiple inoculations because of weak immunogenicity and pose a safety risk due to possible incomplete inactivation of the virus. A laboratory adapted neurotropic RVF virus strain and the pantropic Entebbe RVF virus strain were used to generate a range of formalin inactivated experimental vaccines in chick embryo, mouse brain and monkey kidney cell cultures (Randall et al., 1962, Randall et al., 1964). The experimental vaccine produced in monkey kidney cells from the pantropic Entebbe strain was safe and relatively effective for the immunization of mice, monkeys and humans. This vaccine (TSI-GSD 200) produced under strict quality control conditions, had been used to immunize many veterinarians and laboratory workers at risk of infection (Niklasson et al., 1985, Pittman et al., 1999). Only one batch of TSI-GSD 200 was, however, produced by the US Army (USAMRID) and it is therefore in short supply and very expensive. It also requires three initial inoculations and a booster after 6 months, making it impractical (Bouloy and Flick, 2009).

The same formalin-inactivated vaccine used for humans (TSI-GSD 200) was evaluated in sheep (Harrington et al., 1980). It induced neutralizing immunity that resulted in protection from disease and decreased viral replication after challenge. A formalin inactivated RVF vaccine is available from Onderstepoort Biological Products (OBP, South Africa) for use in livestock (Barnard and Botha, 1977, Barnard, 1979, Bouloy and Flick, 2009). It induces protective responses but is not very immunogenic, neccesitating multiple inoculations which could be problematic during outbreaks when rapid induction of protective immunity is required.

1.7.2 Attenuated virus vaccines

Attenuated vaccines are less expensive to produce, and more immunogenic compared to inactivated vaccines, but still carry a safety risk because of possible reversion to virulence and possible spread by mosquito vectors (Swanepoel and Coetzer, 2004). The attenuated Smithburn neuroadapted strain is also commercially available from OBP (South Africa) for use in livestock but has adverse side effects like teratology, liver pathology and abortions in pregnant animals (Coetzer and Barnard, 1977, Botros et al., 2006, Kamal, 2009).

WitsETD

Another attenuated RVF strain, MP12, which was generated by mutagenesis with 5-fluorouracil of the ZH548 strain, has been extensively evaluated as a possible vaccine candidate (Caplen et al., 1985, Morrill et al., 1991a, Hunter et al., 2002, Morrill and Peters, 2003). The MP12 strain contains nine, 12 and four mutations in the L, M and S segments of the virus' genome respectively, with at least one mutation in each segment playing a role in attenuation (Takehara et al., 1989, Vialat et al., 1997). The MP12 attenuated strain induced strong neutralizing antibody responses but is also teratogenic and abortogenic in sheep when administered in the first trimester of pregnancy (Morrill et al., 1991a, Hunter et al., 2002). Despite these negative effects in sheep, MP12 was evaluated in rhesus monkeys and shown to be markedly attenuated, causing only minor neurovirulence comparable to that seen with the widely used 17D yellow fever vaccine (Morrill and Peters, 2003). Humans immunized with MP12 remained asymptomatic and 95% developed neutralizing antibodies against RVFV (Bettinger et al., 2009, Bouloy and Flick, 2009).

Clone 13, a small plaque naturally attenuated RVFV strain that was isolated in the Central African Republic from a human patient, lacks approximately 70% of the open reading frame coding for the NSs protein preventing it from evading the host IFN pathway (Muller et al., 1995, Bouloy et al., 2001, Billecocq et al., 2004). It was shown to be highly immunogenic in animals, elicited protective immune responses against subsequent challenge and did not cause teratogenesis in sheep during early pregnancy (Muller et al., 1991, Swanepoel and Coetzer, 2004, Bouloy and Flick, 2009, Dungu et al., 2010). Clone 13 was very recently commercialized and is currently under mass production at Onderstepoort Biological Products. Mice immunized with a reassortant virus (R566), containing the S segment of clone 13 and the L and M segments of MP-12, were protected from subsequent viral challenge and sheep did not show any side effects or abortions as a result of vaccination (Bouloy and Flick, 2009).

1.7.3 Recombinant viruses by reverse genetics

The development of various reverse genetics systems for the rescue of recombinant RVF viruses has allowed for the determination of certain factors/proteins involved in pathogenesis and virulence of the virus (Billecocq et al., 2004, Won et al., 2006, Bird et al., 2007a, Gerrard et al., 2007, Won et al., 2007, Billecocq et al., 2008, Habjan et al., 2008a, Habjan et al., 2008b). The 14-kDa (NSm) and 78-kDa non-structural proteins encoded by the M segment were shown to be dispensable for replication and recombinant virus without these proteins remained highly virulent *in vivo* even though it suppressed virus-induced apoptosis (Bird et al., 2007a, Gerrard et al., 2007, Won et al., 2007).

The NSs is the major virulence factor of RVFV. Various recombinant viruses have been generated with the NSs completely omitted, or where it was replaced by a reporter gene like the green

fluorescent protein(GFP) (Billecocq et al., 2004, Ikegami et al., 2006, Bird et al., 2008, Habjan et al., 2008b, Ikegami et al., 2009). Only one recombinant virus generated by reverse genetics has been evaluated as a possible vaccine candidate (Bird et al., 2008). A ZH501 strain derived mutant virus, lacking the NSm gene and carrying a GFP gene in the place of the NSs gene (rRVF-ΔNSs:GFP-ΔNSm), was shown to be highly attenuated but still immunogenic in rats, and protected rats from lethal RVFV challenge. Additionally this vaccine candidate allows for differentiation of naturally infected and vaccinated animals (DIVA) because of the missing viral genes and the insertion of a non-viral gene (Bird et al., 2008). This vaccine candidate shows much promise but has not been evaluated in a host animal model.

Attenuated viruses, either naturally or by reverse genetics, still pose a possible safety risk due to recombination in nature where the missing gene(s) conferring attenuation could be replaced again by a gene from wild type virus, thus reverting it back to virulence. Therefore vaccines of this nature would not be ideal during outbreaks and should be used before seasonal activity of mosquito vectors.

1.7.4 DNA vaccines

DNA-based vaccines are completely safe and are usually more immunogenic when compared to inactivated or subunit vaccines since their target proteins are expressed *in vivo* by the host cells resulting in correct protein folding, they result in longer term expression of target proteins compared to once-off inoculation with subunit proteins and they are able to induce cellular and humoral immunity (Lorenzo et al., 2008). Various DNA vaccine candidates have been developed against RVF. A DNA vaccine, administered by gene gun and expressing the M segment without the NSm gene, was highly immunogenic in mice after three inoculations and elicited 100% protection against lethal challenge (Spik et al., 2006). Immunization of sheep with a DNA construct expressing the M segment and the NP was not able to elicit detectable humoral responses, but low level antigen-specific cellular responses were induced (Lorenzo et al., 2008). A construct expressing only the NP, however, was able to induce strong anti-NP IgG1 isotype responses as well as cellular responses in sheep (Lorenzo et al., 2008), although the protective ability of this response is not known. DNA constructs based on a pCMV (cytomegalovirus) vector backbone expressing both the RVFV glycoproteins (pCMV-M4), or the nucleocapsid protein (pCMV-N), were evaluated as vaccines in a transgenic mouse model with an impaired type I interferon response using the attenuated RVFV MP12 strain as a challenge virus (Lorenzo et al., 2010). The mice vaccinated with pCMV-M4 were completely protected from challenge, whereas mice immunized with pCMV-N, or pCMV-N combined with pCMV-M4, were not.

Gene-gun immunization of mice with a cDNA construct encoding the RVFV NP induced high anti-NP antibody titres and strong proliferative cellular responses, but no neutralizing antibodies

WitsETD

(Lagerqvist et al., 2009). Fifty percent of NP cDNA immunized mice were protected from viral challenge, most likely due to cell-mediated immunity. Immunization with a cDNA construct expressing both glycoproteins (Gn/Gc) simultaneously resulted in neutralizing antibody responses and 62.5% protection from RVFV challenge (Lagerqvist et al., 2009). A DNA vaccine expressing the RVFV Gn protein, coupled to the molecular adjuvant C3d, induced increased neutralizing antibody titres compared to one expressing only Gn, and also increased survival from lethal challenge (Bhardwaj et al., 2010). DNA vaccines have an added advantage of not needing stringent shipping conditions because of high stability, making it ideal for use in countries with sub-optimal infrastructure. The disadvantage, however, is that DNA vaccines require multiple immunizations to induce protective responses.

1.7.5 Virus vectored / replicon vaccines

Virus vectored and replicon vaccines are capable of expressing high levels of inserted genes, can be easily produced in large quantities and are highly immunogenic. The earliest report of an unrelated virus being used to express RVFV proteins shows the insertion of RVF glycoprotein genes into vaccinia virus (Collett et al., 1987). Mice inoculated with the live recombinant RVF/vaccinia virus developed strong neutralizing responses and protection of 90-100% of challenged mice, depending on immunization route. Despite these promising results and the fact that mice immunized with the recombinant RVF/vaccinia virus did not develop any side effects from immunization, this vaccine candidate has not been further evaluated or commercialized possibly due to the fact that vaccinia virus poses a threat to immunocompromised individuals.

A recombinant lumpy skin disease virus (LSDV) containing both RVF glycoprotein genes yielded strong neutralizing responses and 100% protection from subsequent lethal challenge in mice, and 100% protection from clinical disease in sheep (Wallace and Viljoen, 2005, Wallace et al., 2006). It also allows for differentiation of RVF naturally infected and vaccinated animals since only RVF glycoproteins are expressed by the construct. Despite these promising results, this vaccine candidate has not been commercialized.

Alphaviruses, such as Sindbis virus (SINV) and Venezualan equine encephalitis (VEEV), have been evaluated as virus vectors, or replicons, to express RVFV glycoprotein genes (Gorchakov et al., 2007). VEEV was able to express glycoproteins to such a level that protective immunity against lethal challenge was induced in mice (Gorchakov et al., 2007). A SINV replicon based RVF vaccine produced neutralizing responses in mice and sheep, and protected mice from lethal challenge (Heise et al., 2009). Alphaviruses are, however, widespread and it is not known to what effect the background immunity against these viruses could have an influence on efficacy of vaccines.

WitsETD

Recently the RVF glycoprotein genes were incorporated into a non-replicating complex adenovirus (CAdVax) vector and evaluated as a possible vaccine in mice (Holman et al., 2009). The CAdVax-RVF construct induced long-lasting humoral immunity that protected 100% of mice against lethal challenge. Background immunity against adenovirus does, however, have an effect on protection with only 25-75% of mice with pre-existing adenoviral immunity surviving challenge (Holman et al., 2009).

A capripoxvirus recombinant expressing the two structural RVFV glycoproteins was evaluated as a possible vaccine in a mouse and sheep model (Soi et al., 2010), and was shown to induce up to 100% protection against lethal challenge in mice, depending on the dose, route and number of immunizations, and induced sterilizing immunity in sheep.

A recombinant Newcastle disease virus (NDV) expressing the RVFV glycoproteins has been evaluated as a vaccine in mice, lambs and calves, showing promising protection against disease/death and viral replication as a result of the development of neutralizing antibodies (Kortekaas et al., 2010a, Kortekaas et al., 2010b).

1.7.6 Virus like particles as vaccines

Virus-like particles (VLP) are formed when the virus' structural proteins self-assemble into replication deficient particles that resemble wild type virus in structure. VLPs are usually more immunogenic when compared to subunit recombinant proteins since the conformational epitopes of structural proteins are presented in a more natural way, similar to wild type virus. Immune responses against VLPs, therefore, are also thought to more accurately represent the immune responses elicited against natural infections (Noad and Roy, 2003, Grgacic and Anderson, 2006). VLP production can be easily upscaled and VLPs have been successfully evaluated as vaccines for various other viruses including *Bunyaviruses* and *Filoviruses* (Grgacic and Anderson, 2006). Indeed, various groups have recently successfully generated Rift Valley fever VLPs in mammalian and insect cell systems (Liu et al., 2008, Habjan et al., 2009a, Mandell et al., 2009, Naslund et al., 2009, de Boer et al., 2010, Mandell et al., 2010b).

Immunization of mice with RVFV VLPs containing glycoproteins and the NP, produced in mammalian cells (293T) resulted in high neutralizing titres and 50-92% protection from lethal challenge, depending on VLP dose (Habjan et al., 2009a, Naslund et al., 2009). Moreover, none of these mice developed any detectable anti-NP antibodies after immunization, despite the NP being present in the VLP. Chimeric RVFV VLPs containing the RVF NP, glycoproteins and the Moloney murine leukaemia gag protein were shown to be highly immunogenic in two murine animal models, yielding high neutralizing titres and strong cytokine responses which protected mice (68%) and rats

WitsETD

(100%) from lethal RVFV challenge (Mandell et al., 2009). Interestingly VLPs that were modified to actively express the NP were also able to induce 100% protection against lethal challenge (Pichlmair et al., 2010). These results clearly indicate that the NP does play a role in protection despite it having no neutralizing epitopes. Recently, however, VLPs based on both glycoproteins but without NP, produced in *Drosophila* insect cells, were shown to be immunogenic in mice and conferred 100% protection from lethal RVFV challenge (de Boer et al., 2010).

Because of the advantages offered by VLPs, they seem like ideal vaccine candidates for RVF. However, efforts to prepare RVFV VLPs were only very recently undertaken and therefore are in the very early stages of evaluation as vaccine candidates. Based on promising results obtained in murine models, it would be interesting to see how efficient they would be as immunogens in a RVF host animal or humans.

1.7.7 Recombinant subunit vaccines

Recombinant subunit immunogens are the least explored method for producing vaccines against RVF. This is most likely due to the fact that recombinant subunit proteins are generally weak immunogens, requiring multiple immunizations and the use of adjuvants, and are usually expressed as inclusion bodies thus not presenting conformational epitopes in a natural way (O'Hagan et al., 2001).

Partially purified recombinant Gn protein, bacterially expressed as inclusion bodies and subsequently solubilized with urea, induced low neutralizing titres in mice which protected 56-70% of animals from lethal RVF challenge, whereas the Gc protein did not elicit a neutralizing response or protection against challenge at all (Collett et al., 1987).

Recombinant Gn protein, expressed using *Autographa californica* nuclear polyhedrosis viral recombinants in SF9 insect cells, or a combination of Gc/Gn is immunogenic and resulted in protection from lethal challenge after two immunizations. Passive immunization of mice with mouse anti-serum generated against these recombinant antigens protected a proportion of the animals, further indicating that humoral antibodies against the glycoproteins play a major role in protection (Schmaljohn et al., 1989).

Recombinant Gn protein, expressed in *Drosophila* insect cells and adjuvanated with Stimune, was shown to be immunogenic in mice and conferred 100% protection against lethal challenge (de Boer et al., 2010). Immunity, however, was not sterilizing since anti-NP antibodies were detected in the mice sera after challenge, an evidence of viral replication.

In a preliminary study using a limited number of experimental animals, Wallace et. al. evaluated a bacterially expressed RVF nucleocapsid protein as an immunogen in mice (Wallace et al., 2006). The protein was expressed as inclusion bodies and solubilized with urea. Mice immunized with

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the protein and QuilA adjuvant did not develop any detectable antibodies, yet 60% were protected from lethal RVFV challenge. Immunization of animals with recombinant subunit N proteins from related B*unyaviruses* resulted in complete protection against viral challenge (Schmaljohn et al., 1990, Maes et al., 2008).

Recombinant subunit immunogens are easy to produce, relatively cheap, completely safe and production can be easily upscaled. Their disadvantages of weak immunogenicity and expression as incorrectly folded proteins can be overcome by using adjuvants (O'Hagan et al., 2001, Lautze et al., 2007) and optimizing expression conditions respectively. A bacterially expressed recombinant RVFV nucleocapsid protein (recNP) was recently produced in a completely soluble form, and thus assumed to be correctly folded (Jansen van Vuren et al., 2007). The fact that it recognizes RVFV specific antibodies from naturally or experimentally infected individuals with very high efficiency is further proof that conformational epitopes are presented correctly (Jansen van Vuren et al., 2007, Paweska et al., 2007, Evans et al., 2008).

1.8 Study objectives

This study had two major objective. The first objective was to evaluate the recNP as an immunodiagnostic reagent. From the literature it became apparent that most serological techniques for RVF diagnosis are based on reagents that are expensive and time consuming to produce, and pose safety risks to laboratory personnel. In order to address this it was decided to develop and validate safe ELISAs, based on the recNP, for the detection of RVFV specific antibodies and antigens in animals and humans. To achieve this, laboratory animals were immunized to generate immune sera and clinical specimens from RVF cases were tested to evaluate diagnostic accuracy of the assays. This study undertook the first development of an ELISA based on a horseradish-peroxidase conjugated RVFV recombinant nucleocapsid protein, and extensive validation of recombinant nucleocapsid protein based ELISAs for use in RVF serodiagnosis in humans and wildlife.

The second objective was to evaluate a bacterially expressed RVFV nucleocapsid protein (recNP) as an immunogen in two animal models and attempt to understand the mechanism of protection against viral challenge. From the literature it became apparent that the role of the anti-NP response in immunity against viral infection is not well understood. A preliminary study in mice by another group showed that the RVFV recNP was able to induce protection against lethal RVFV challenge despite the absence of neutralizing antibodies. The same was shown for related viruses from the *Bunyaviridae* family. It was decided to expand the investigation on immunogenicity in mice using the soluble recNP and the protection it confers against lethal challenge when used in combination with different adjuvants that enhance immune responses by varying mechanisms. In an effort to understand

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the mechanism of protection elicited by recNP immunization the expression levels of selected genes in immunized versus naïve mice after viral challenge were compared. The recNP was also evaluated as an immunogen in a host animal species, sheep. This study is the first evaluation of a subunit RVFV nucleocapsid protein as an immunogen in a RVF host species.

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CHAPTER TWO

RECOMBINANT NUCLEOCAPSID PROTEIN AS IMMUNODIAGNOSTIC REAGENT – SEROLOGY

2.1 Detection of IgG antibody to Rift Valley fever virus in wild ruminants*

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Paweska, J.T., van Vuren, P.J., Kemp, A., Swanepoel, R., Buss, P., Bengis, R.G., Gakuya, F., Breimann, R.F. and Njenga, K. (2010). A recombinant nucleocapsid-based indirect ELISA for serodiagnosis of Rift Valley fever in African wildlife. In Symposium Proceedings: Odongo, N.E., Garcia, M. And Viljoen, G.J. (eds). *Sustainable Improvement of Animal Production and Health*. Vienna, Austria, 8 – 11 June 2009. Food and Agriculture Organization of the United Nations: FAO/IAEA, 309-12. ISBN 978-92-5-106697-3.

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2.1.1 Introduction

Antibodies against the virus have been found in many wildlife species including African buffalo, black rhino, lesser kudu, impala, African elephant, Thompson's gazelle, gerenuk and waterbuck (Anderson and Rowe, 1998, Fischer-Tenhagen et al., 2000, Evans et al., 2008). Experimental infection of African buffalo with RVFV results in fever, malaise and abortion as a result of transient viremia (Davies and Karstad, 1981). The role of wildlife in the epidemiology of RVF is not well understood but they are thought to maintain the virus together with mosquitoes in a sylvatic cycle during inter-epizootic periods (Swanepoel and Coetzer, 2004, Evans et al., 2008). Because there is low level transmission of the virus during inter-epizootic periods, and most infected animals are probably

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asymptomatic, this transmission goes undetected without the aid of proper diagnostic techniques. Properly validated assays that are safe, cheap and easy to use could be very useful in gaining more information about the prevalence of RVF in wildlife during inter-epizootic periods.

Various ELISAs have been developed and validated recently for the detection of antibodies in domestic livestock and humans (Paweska et al., 2003a, Paweska et al., 2005a, Paweska et al., 2007), and one for humans, domestic livestock and African buffalo (Paweska et al., 2005b). At the time of this study no ELISA had been validated for the detection of anti-RVFV antibodies in wildlife, with the exception of African buffalo. The ELISA available for detection in African buffalo is, however, based on inactivated whole virus antigen which requires it to be produced in biocontainment, making its production expensive and unsafe (Paweska et al., 2005b). To address the needs highlighted above, an ELISA was developed and validated for the detection of IgG antibodies in wildlife.

2.1.2 Materials and methods

2.1.2.1 Serum controls and internal quality control (IQC)

Freeze-dried, gamma-irradiated serum controls from experimentally infected sheep produced previously were used (Paweska et al., 2003a).

Internal quality control upper and lower control limits for the controls were established as described previously (Paweska et al., 2003a) by testing each control 24 times on five plates on five separate occasions (24 x 5 x 5 = 600 determinants). The upper control limit (UCL) for the controls (high positive C++, low positive C+, negative C- and conjugate control) was determined by calculating the percentage positivity (PP) from the mean optical density (OD) value from the 600 replicates, plus two standard deviations (+ 2 S.D.). PP values were calculated as follows: PP = (net OD serum / net mean OD C++) x 100. The lower control limit (LCL) was determined similarly by calculating the mean values minus 2 standard deviations (- 2 S.D.). During routine runs of the assay, four replicates of each control (high positive C++, low positive C+, negative C- and conjugate control CC) were included on each plate. The means and standard deviations of OD values and PP values were calculated from the replicates on 40 routine runs of the assay over a period of three months (4 replicates x 40 runs = 160 determinants) to assess intra- and interplate variation. The coefficient of variation (CV = standard deviation of replicates / mean of replicates x 100) was determined for positive control sera. Assay repeatability was determined from these results.

2.1.2.2 Serum specimens

A total of 1900 individual wildlife sera collected in 1978-2008 in Kenya, South Africa and Zimbabwe were used. Sera which tested negative in the virus neutralization test were regarded as a

reference panel from non-infected animals, and sera which tested positive as a reference panel from animals infected with RVFV (Table 2.1.1).

Table 2.1.1. Number of field-collected wildlife sera tested in the virus neutralization test.

Species	Total Tested	VNT-[a]	VNT+[b]
African buffalo	1023	946	77
Black rhinoceros	43	29	14
Common zebra	24	24	0
Elephant	73	69	4
Giraffe	81	81	0
Grevy zebra	78	77	1
Warthog	49	47	2
Eland	66	63	3
Gerenuk	6	1	5
Hartebeest	10	10	0
Impala	324	315	9
Kudu	73	66	7
Waterbuck	42	40	2
Thomson gazelle	8	1	7
Grand Total	**1900**	**1769**	**131**

[a] Number of sera tested negative in virus neutralization test

[b] Number of sera tested positive in virus neutralization test

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2.1.2.3 Virus neutralization test

Duplicates of serial two-fold dilutions of sera inactivated at 56°C for 30 min were tested as previously described (Paweska et al., 2003a). Titres were expressed as the reciprocal of the serum dilution that inhibited ≥ 75 % of viral cytopathic effect. A serum sample was considered positive when it had a titre of ≥ $\log_{10}$ 1.0, equivalent to a serum dilution ≥ 1:10.

2.1.2.4 ELISA antigen production and recNP I-ELISA procedure

Production of the recNP and the assay procedure was carried out as described previously with minor modifications (Jansen van Vuren et al., 2007). Maxisorb immunoplates (Nunc, Denmark) were coated with stock antigen, diluted 1:2000 in carbonate–bicarbonate buffer pH 9.6 and incubated overnight at 4°C. After washing three times with a washing buffer consisting of phosphate-buffered saline (PBS) pH 7.2 and 0.1% Tween 20, the plates were blocked with 200 µl of 10% fat-free milk

powder in PBS and incubated in a moist chamber for 1 h at 37°C and then washed as described before. Control and test sera were diluted 1:400 in PBS containing 2% milk powder (diluting buffer) and 100 µl of diluted sera was added to the plates. Each test serum was assayed in duplicate and each internal control was tested in quadruplicate. After incubation in a moist chamber for 1 h at 37°C, plates were washed three times with the washing buffer and 100 µl of a 1:5000 dilution of the horseradish peroxidase (HRPO) conjugated Protein G (Zymed Laboratories, Inc.) was added. Plates were incubated for 1 h at 37°C, washed three times, and 100 µl of 2,2'-azino di-ethyl-benzothiazoline-sulfonic acid substrate was added to each well. Plates were then incubated in the dark at room temperature for 30 minutes. The reactions were stopped by the addition of 100 µl of 1% sodium dodecyl sulphate (SDS) and OD values were determined at 405 nm. The results were expressed as PP values. PP values were calculated as follows: PP = (net OD serum / net mean OD C++) x 100.

2.1.2.5 Selection of cut-off values and determination of ELISA diagnostic accuracy

Cut-off values at 95 % accuracy level were optimised using the misclassification cost term option of the two-graph receiver operating characteristics (TG-ROC) analysis (Greiner, 1996). Optimization of cut-off values was based on the following equation: misclassification cost term = (1 - p) (1 - Sp) + rp (1 - Se), where p (prevalence) = 0.5 and r (costs of false-positive and false-negative results) = 1.0. In addition, cut-off values were determined by mean plus 2S.D.s. and by mean plus 3S.D.s derived from PP values in uninfected animals.

Estimates of diagnostic sensitivity and specificity and other measures of combined diagnostic accuracy were calculated as previously described (Paweska et al., 2003a). Sensitivity $(D - Se) = [Tp/(Tp + Fn)] \times 100$; specificity $(D - Sp) = [Tn/(Tn+Fp)]\times100$; Youden's index $(J) = [Sn+(Sp-1)]$; efficiency $(Ef) = [PSe + Sp(1 - P)]$; positive predictive value $(PPV) = Pse/[Pse + (1 - P)(1 - Sp)] \times 100$; negative predictive value $(NPV) = [(1 - P)Sp]/[(1 - P)Sp + P(1 - Se)] \times 100$; apparent prevalence $(AP) = [(Tp + Fp)/N] \times 100$; and true prevalence $(TP) = [AP + (Sp-1)]/[Sn+(Sp-1)] \times100$, where Tp is the true-positive sera, Fn the false-negative sera, Tn the true-negative sera, Fp the false-positive sera, P the prevalence, and N the number of sera tested.

2.1.2.6 Antibody dose response curves

Antibody dose response curves were generated by testing increasing dilutions of wildlife sera known to be positive or negative for anti-RVFV antibodies, based on VNT results, using the recNP based IgG indirect ELISA (I-ELISA).

2.1.3 Results

2.1.3.1 Assay repeatability and internal quality control (IQC)

The recNP IgG I-ELISA was able to differentiate clearly between the internal controls used and generated minimal background. Variation between and within runs were minimal (Figure 2.1.1).

2.1.3.2 Antibody dose response curves

Dose response curves using different dilutions of sera known to be positive or negative in the virus neutralisation test had the expected analytical slope and the recNP IgG I-ELISA clearly differentiated between different levels of specific IgG antibody against RVFV in African buffalo (Figure 2.1.2) and other wildlife (Figure 2.1.3).

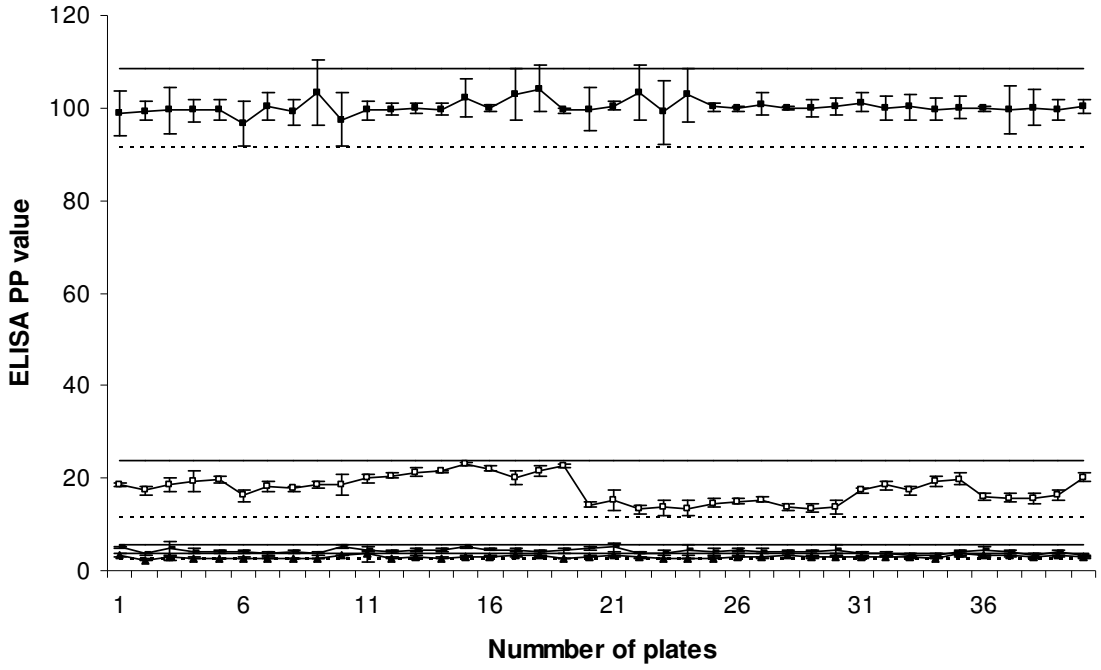


Figure 2.1.1 Upper (—) and lower (- - -) internal quality limits for PP values of high positive (C++), low-positive (C+), negative serum (C-) and conjugate (Cc) controls in recNP-based IgG I-ELISA and the results for these controls (mean ± SD) on 40 plates during routine runs of the assay over a period of 3 months. Each plate includes four replicates of each of the internal controls.

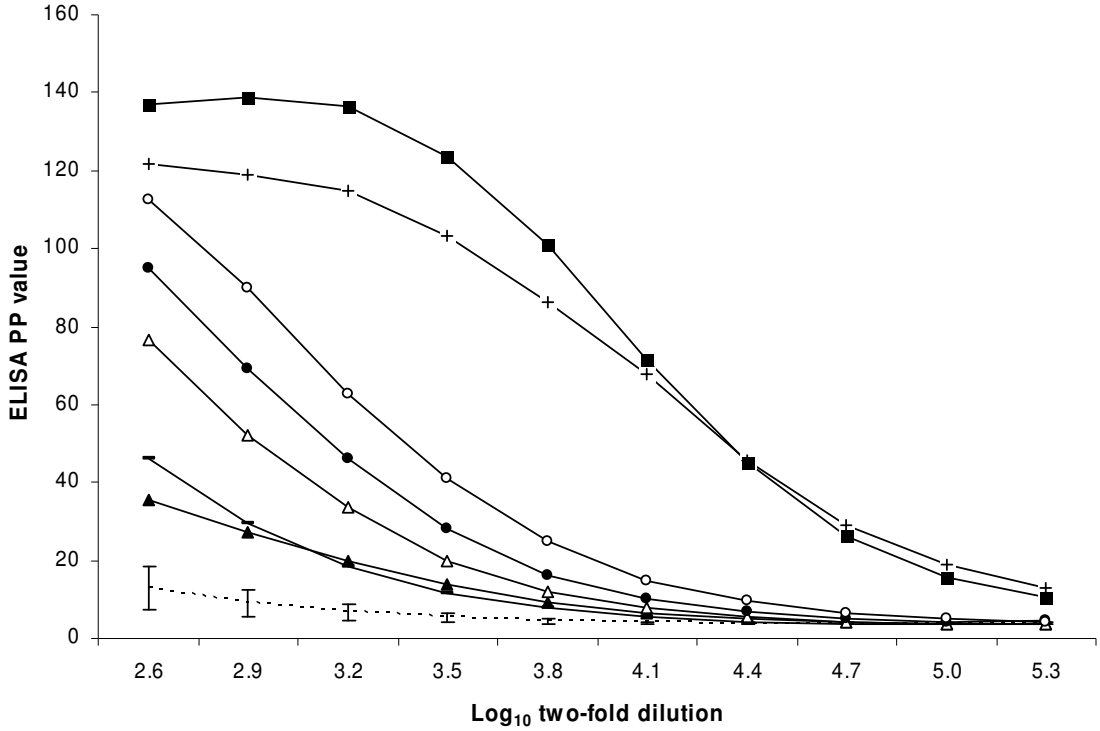


Figure 2.1.2 Dose response curves of individual African buffalo sera in the recNP I-ELISA. Sera were collected from 16 animals in RVF endemic areas in Kenya and South Africa of which 8 tested positive and 8 negative in virus neutralization test. Positive sera (—) represent different levels of virus neutralizing antibody ranging in titres from $log_{10}10^{1.6}$ (▲) to $log_{10}10^{3.1}$ (■). Mean ± SD of 8 negative sera (- - -) with VNT titres < $log_{10}10^{1}$.

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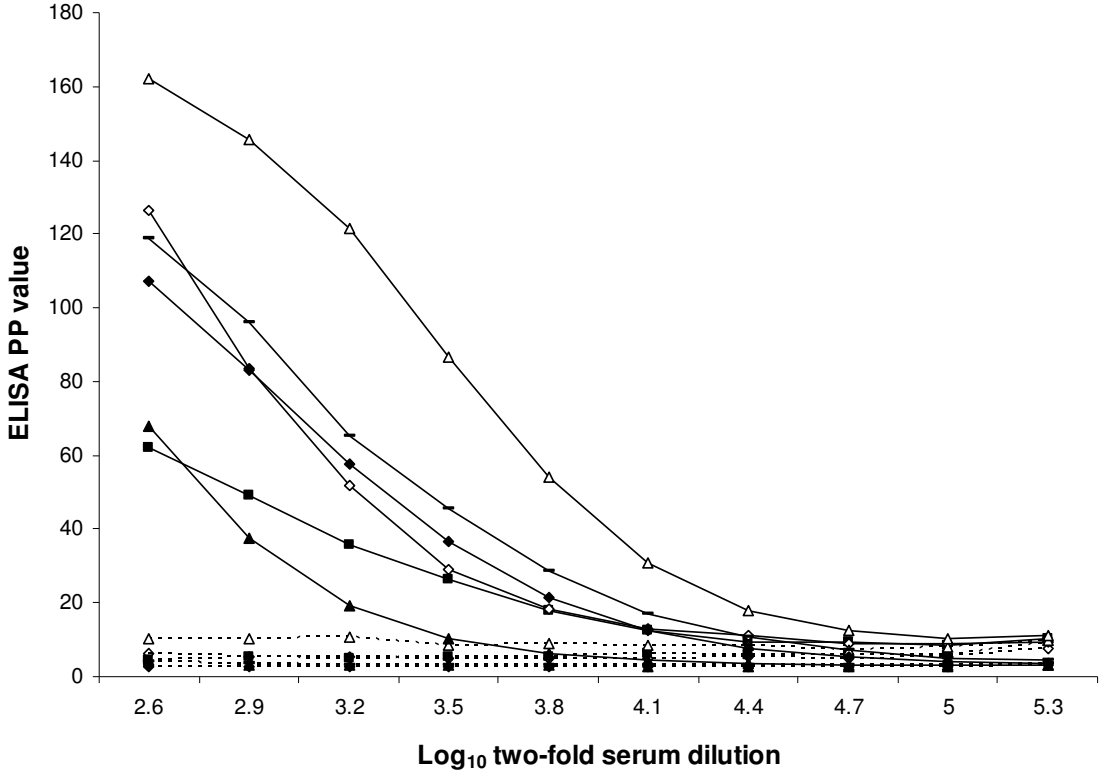


Figure 2.1.3 Dose response curves of wildlife sera in the recNP I-ELISA. Sera were collected from individuals that tested positive (—) or negative (- - -) in the virus neutralization test (VNT): Black rhinoceros (♦), eland (▲), gerenuk (■), kudu (◊), impala (Δ), Thomson gazelle (▬). VNT titres in positive sera ranging from $log_{10}10^{1.9}$ (■) to $log_{10}10^{3.1}$ (Δ).

2.1.3.3 Cut-off values and diagnostic accuracy

Threshold values for the recNP IgG I-ELISA were derived from data sets dichotomised according to the results of the VN test (Table 2.1.1). The effect of differently determined cut-off values on distinguishing between sera which tested negative or positive in this assay, and consequently on estimates of sensitivity, specificity, and other estimates of diagnostic accuracy is given in Table 2.1.2 and figure 2.1.5. Optimisation of cut-off values using the misclassification cost term option of the TG-ROC analysis was based on the non-parametric programme option (Greiner, 1996) due to departure from a normal distribution of data sets analysed. Graphical presentation of the TG-ROC analysis for African buffalo is shown in figure 2.1.4.

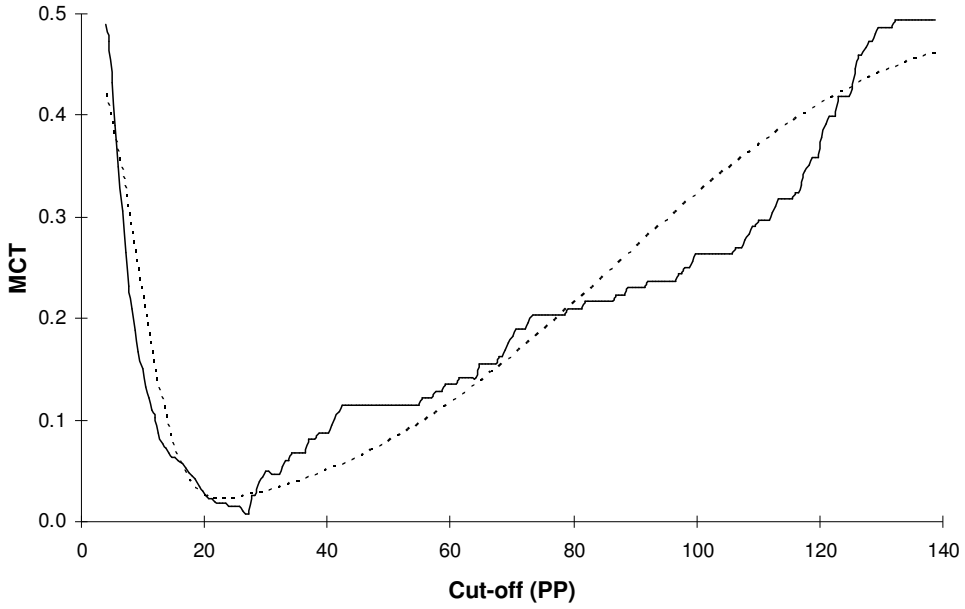


Figure 2.1.4. Optimisation of the cut-off for the recNP I-ELISA in African buffalo using the misclassification cost term (MCT) option of the two-graph receiver operating characteristic analysis (TG-ROC). The two curves represent MCT values based on non-parametric (—) or parametric (- - -) estimates of sensitivity and specificity derived from data sets in field-collected sera. Optimisation of the cut-off value was based on the non-parametric (—) program option due to departure from a normal distribution of data sets analysed.

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Table 2.1.2. Diagnostic accuracy of Rift Valley fever recNP-based I-ELISA in African wildlife.

Species	Cut-off[a]	D-Sn[b]	D-Sp[c]	Y[d]	Ef[e]	PPV[f]	NPV[g]
African buffalo	26.94[h]	98.7	99.4	0.98	99.3	92.5	99.9
	17.73[i]	100	94.2	0.94	94.6	55.0	100
	22.23[j]	100	97.8	0.98	97.9	77.9	100
Black rhinoceros	33.6	100	100	1	100	100	100
	27.5	100	91.3	0.91	93.7	81.5	100
	35.5	100	100	1	100	100	100
Common zebra	-[k]	-	-	-	-	-	-
	13.9	-	100	-	-	-	-
	17.9	-	100	-	-	-	-
Elephant	28	100	100	1	100	100	100
	10.6	100	95.8	0.96	99.7	99.7	100
	13.6	100	97.2	0.97	99.8	99.8	100
Giraffe	-	-	-	-	-	-	-
	11.7	-	100	-	-	-	-
	14.3	-	100	-	-	-	-
Gravy zebra	-	-	-	-	-	-	-
	17.3	-	100	-	-	-	-
	22.5	-	100	-	-	-	-
Warthog	27.7	100	100	1	100	100	100
	13.5	100	95.9	0.96	96	49.7	100
	17.5	100	97.9	0.98	98	66.4	100
Antelopes[l]	20.4	100	99.8	0.99	99.7	95.6	100
	8.4	100	88.1	0.88	88.7	32.4	100
	14.4	100	97.0	0.97	97.1	67.9	100

Animals were categorized according to the results of virus neutralization test (VNT)

[a] Cut-off value expressed as percentage positivity (PP) of an internal high-positive serum control.

[b] Diagnostic sensitivity (%).

[c] Diagnostic specificity (%).

[d] Youden's index.

[e] Efficiency (%).

[f] Positive predictive value (%).

[g] Negative predictive value (%).

[h] Cut-off value optimised by TG-ROC analysis.

[i] Cut-off value based on mean + 2 S.D. of ELISA PP values in VNT-negative population.

[j] Cut-off value based on mean + 3 S.D. of ELISA PP values in VNT-negative population.

[k] Not determined due to unavailability or very limited number of VNT-positive sera

[l] Eland, gerenuk, hartebeest, impala, kudu, Thomson gazelle, waterbuck

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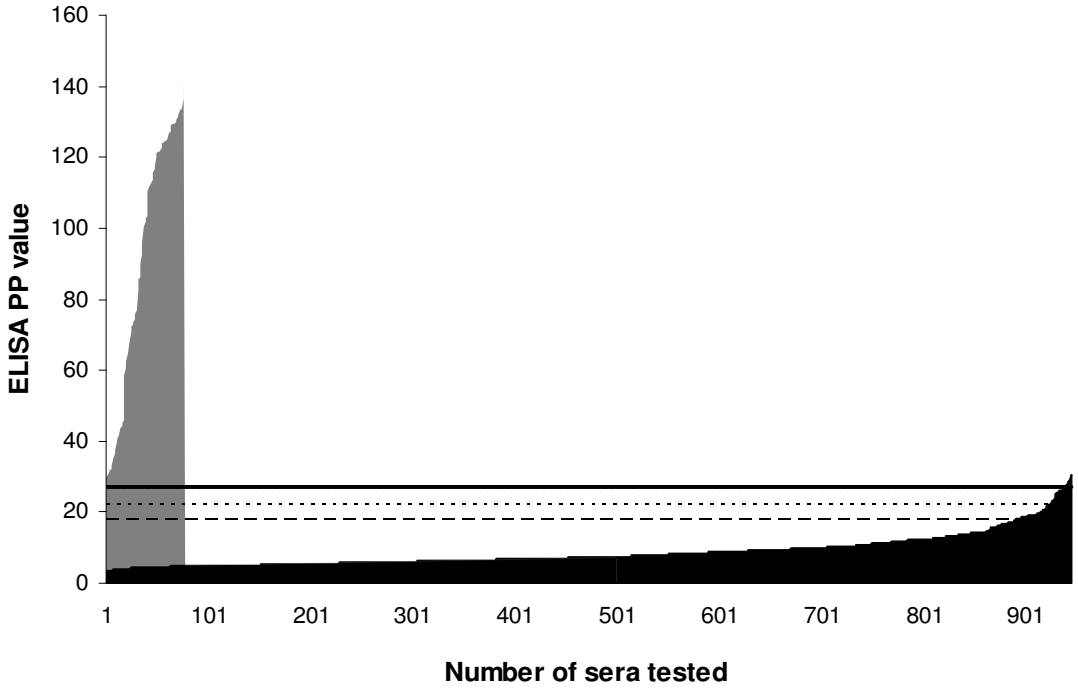


Figure 2.1.5. Distribution of recNP IgG I-ELISA PP values in African buffalo tested positive (n = 77, gray area) and negative (n = 946, black area) in the virus neutralisation test. Sera ordered according to ELISA PP values. Horizontal lines indicate the ELISA cut-off values determined by the TG-ROC analysis (▬), and as a mean plus three (- - -) and two (— — —) standard deviations of the ELISA PP values observed in the VNT-negative population.

2.1.4 Discussion

The VN test is the golden standard test for serological diagnosis of RVF because it can be used for any species and is highly sensitive and specific (Swanepoel et al., 1986a). It is, however, laborious, expensive and requires the amplification of live virus which poses a significant safety risk to laboratory personnel and thus restricts its use to high biocontainment facilities. For these reasons it is not widely used which creates the need for alternative, safer assays. The ELISA offers a quick, safe and less expensive alternative to the VNT. In particular the I-ELISA is one of the simplest immunoassays for antibody detection but requires highly pure antigen preparations for coating plates. A recNP based I-ELISA was recently developed and so-far validated for the detection of RVF specific IgG antibodies humans (Jansen van Vuren et al., 2007, Paweska et al., 2007). Because of the important role wildlife seem to play in the inter-epizootic maintenance of RVFV, there was a need for a safe, reliable and properly validated assay for detection of IgG in wildlife animals.

In this study the recNP IgG I-ELISA had high estimates of sensitivity and specificity for the detection of antibodies in wildlife animals. The diagnostic performance of the assay was consistently lower when using traditional methods for cut-off determination (mean values of known negatives plus 2 or 3 standard deviations) when compared to the TG-ROC method. It was necessary to determine separate cut-off values for each species, or group of similar animals (e.g. antelopes), to optimize diagnostic accuracy for a targeted wildlife species. Establishing a single cut-off for all species would make the assay less complex to interpret and use but would have a negative effect on diagnostic performance which is more important. Apart from assay sensitivity and specificity, other statistical parameters were also used to evaluate assay performance. The Youden's index is another statistic that aims to capture assay performance by including sensitivity and specificity. Positive and negative predictive values indicate the ability of the test to accurately identify true positives and negatives as positives and negatives respectively. Although diagnostic sensitivity and specificity is the most used parameters in diagnostic assays, these other statistical values can give further confidence in the performance of the assay.

Based on the results from this study, the recNP IgG I-ELISA has the potential to be a safe, quick and cheap tool for the detection of IgG antibodies in African wildlife species to aid in the monitoring of inter-epizootic transmission of RVFV.

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2.2 Conjugation of the recombinant nucleocapsid protein with horseradish peroxidase for detection of IgM antibody to Rift Valley fever virus in humans

2.2.1 Introduction

The symptoms of RVF in humans are not very specific and can therefore not be easily diagnosed clinically which emphasizes a need for quick and accurate techniques for RVF diagnosis in humans. Molecular techniques are sensitive and rapid but viremia in RVFV infected individuals is transient and therefore they are only useful for a limited time and very shortly after infection when testing blood samples. It is therefore recommended that serological tests for detection of specific IgM be done concurrently with antigen/RNA detection techniques. RVFV-infected patients usually develop anti-RVFV IgM antibodies within 6 days of exposure, making IgM detection a useful tool for diagnosis of recent infections (Paweska et al., 2005a).

To expand on the successful development and validation of an indirect IgG ELISA based on the RVFV recNP (refer to chapter 2.4), this chapter describes the development and evaluation of an IgM detecting ELISA based on the recNP antigen conjugated to the HRPO enzyme.

2.2.2 Materials and methods

2.2.2.1 Horseradish peroxidase conjugation of the recNP

The recNP was produced as described before (section 2.1.2.4) but additionally the protein was concentrated and salts removed by using a Vivaspin 5000 kDa molecular weight cut-off (MWCO) ultrafiltration spin column (Sartorius-Stedim Biotech, Germany) as recommended by the manufacturer. The recNP, at 0.8 mg/ml in PBS buffer, pH 7.2, was conjugated to the horseradish peroxidase enzyme by using the LYNX Rapid HRP conjugation kit (ABD Serotec, United Kingdom) as prescribed by the manufacturer. Briefly, LYNX Modifier reagent was added to the recNP at a ratio of 1:10 (v/v) and gently mixed. The resulting mixture was added to lyophilized HRPO at different ratios to determine the optimal conjugation ratio [4:1, 2:1 and 1:1 (weight recNP/weight HRPO)]. The mixture was incubated at room temperature for 4 hours, and after incubation LYNX Quencher reagent was added at a ratio of 1:10 (v/v) of the original recNP volume, mixed and stored at 4°C until use. Once the optimal recNP/HRPO ratio was determined, conjugation was repeated to produce bulk recNP-HRPO for further testing.

2.2.2.2 Enzyme-linked immunosorbent assay (recNP-HRP IgM ELISA)

Maxisorb immunoplates (Nunc, Denmark) were coated with 100µl goat anti-human IgM µ-chain (Zymed Laboratories, Inc.) diluted 1:500 in phosphate-buffered saline (PBS) pH 7.2 and

incubated overnight at 4°C. After washing three times with a washing buffer consisting of phosphate-buffered saline (PBS) pH 7.2 and 0.1% Tween 20, the plates were blocked with 200 µl of 10% fat-free milk powder in PBS and incubated in a moist chamber for 1 h at 37°C and then washed as described before. Control and test sera were diluted 1:400 in PBS containing 2% milk powder (diluting buffer) and 100 µl of diluted sera was added to the plates. Each test serum was assayed in duplicate and each internal control was tested in quadruplicate. After incubation in a moist chamber for 1 h at 37°C, plates were washed six times with the washing buffer and 100 µl of a 1:100 dilution of the recNP-HRPO added to the plates. Plates were incubated for 1 h at 37°C, washed six times, and 100 µl of 2,2'-azino di-ethyl-benzothiazoline-sulfonic acid substrate was added to each well. Plates were then incubated in the dark at room temperature for 30 minutes. The reactions were stopped by the addition of 100 µl of 1% SDS and OD values were determined at 405 nm. OD values of test sera were converted into percentages of the high-positive control serum (PP value). PP values were calculated as follows: PP = (OD serum / mean OD C++) x 100.

2.2.2.3 Serum controls

Internal serum controls were prepared as described previously (Paweska et al., 2005a).

2.2.2.4 Human serum specimens

A total of 257 individual human sera collected in Kenya in 2007, and South Africa in 2008/09 were used. Sera which tested negative in the RVF IgM Capture ELISA (Paweska et al., 2005a) were regarded as a reference panel from non-infected individuals (n = 219 humans), and sera which tested positive as a reference panel from individuals recently infected with RVFV (n = 38 humans).

2.2.2.5 Selection of cut-off values and determination of diagnostic performance

Cut-off values of the recNP-HRP IgM ELISA was determined as described before (section 2.1.2.5). The following criteria were used to evaluate the diagnostic performance: sensitivity (D-Sn) = [Tp/(Tp + Fn)] × 100; specificity (D-Sp) = [Tn/(Tn+Fp)]×100; Youden's index (Y) = [Sn+(Sp−1)]; efficiency (Ef) = (D-Sn+D-Sp)/2; positive predictive value (PPV) = TP/(TP + FP); negative predictive value (NPV) = TN/(FN + TN) where TP is true positives, FP is false positives, FN is false negatives and TN is true negatives.

2.2.3 Results

2.2.3.1 Optimal conjugation ratio and recNP-HRP dilution for ELISA

The optimal ratio at which to conjugate recNP with the peroxidase enzyme was determined to be 1:1 (weight recNP / weight HRP) (Figure 2.2.1). Dilution of the recNP-HRP at 1:100 yielded the best discrimination between high positive, low positive and negative control sera (Figure 2.2.2).

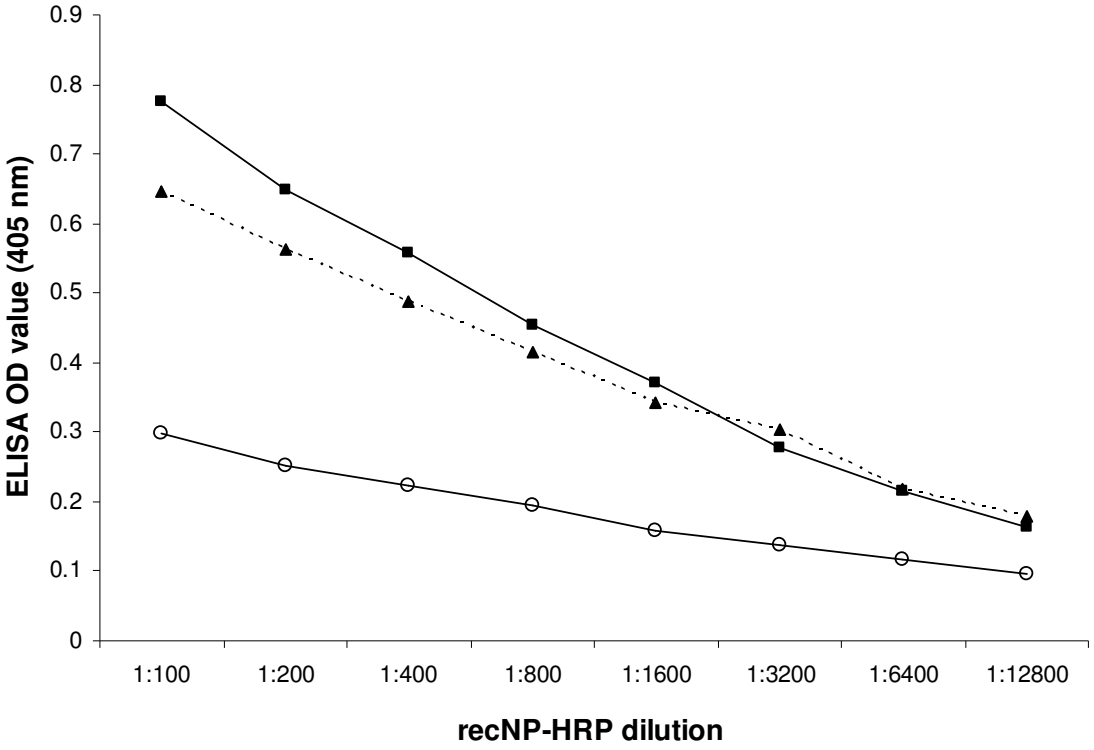


Figure 2.2.1. Titration curves of three different recNP-HRP preparations with RVF IgM strong positive human serum. The recNP/HRP ratios during conjugation (w/w) were as follows: ratio 4:1 (─○─); ratio 2:1 (--▲--); and ratio 1:1 (─■─).

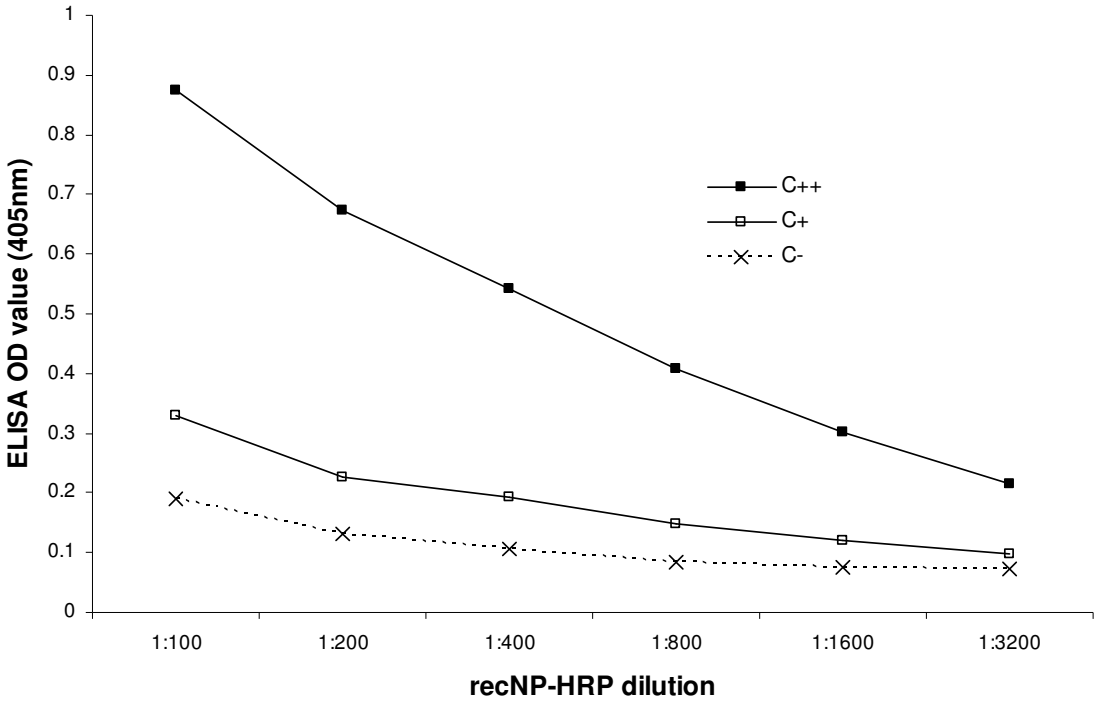


Figure 2.2.2. Titration curves of recNP-HRP with three different human IgM controls: C++ strong positive control (—■—); C+ low positive control (—□—); and negative control (--x--).

2.2.3.2 Cut-off values and diagnostic accuracy

Threshold values for the recNP IgG I-ELISA were derived from data sets dichotomised according to the results of the IgM capture ELISA (Paweska et al., 2005a). The effect of differently determined cut-off values on distinguishing between sera which tested negative or positive in this assay, and consequently on estimates of sensitivity, specificity, and other estimates of diagnostic accuracy is given in table 2.2.1. Optimisation of cut-off values using the misclassification cost term option of the TG-ROC analysis was based on the non-parametric programme option (Greiner, 1996) due to departure from a normal distribution of data sets analysed. Graphical presentation of the TG-ROC analysis is shown in figure 2.2.3.

Table 2.2.1. Diagnostic accuracy of Rift Valley fever recNP-HRPO IgM ELISA in positive (n = 38) and negative (n = 219) human specimens as catagorized according to the results of the IgM capture ELISA.

Cut-off[a]	D-Sn[b]	D-Sp[c]	Y[d]	Ef[e]	PPV[f]	NPV[g]
27.06[h]	81.58	95.90	0.77	88.73	80.85	96.90
29.40[i]	78.95	97.72	0.77	88.33	88.37	96.48
36.10[j]	71.05	98.17	0.69	84.61	90.48	95.22

[a] Cut-off value expressed as a percentage positivity (PP) of an internal high-positive serum control.

[b] Diagnostic sensitivity (%).

[c] Diagnostic specificity (%).

[d] Youden's index.

[e] Efficiency (%).

[f] Positive predictive value (%).

[g] Negative predictive value (%).

[h] Cut-off value optimised by TG-ROC analysis.

[i] Cut-off value based on mean + 2 S.D. of ELISA PP values in negative population.

[j] Cut-off value based on mean + 3 S.D. of ELISA PP values in negative population.

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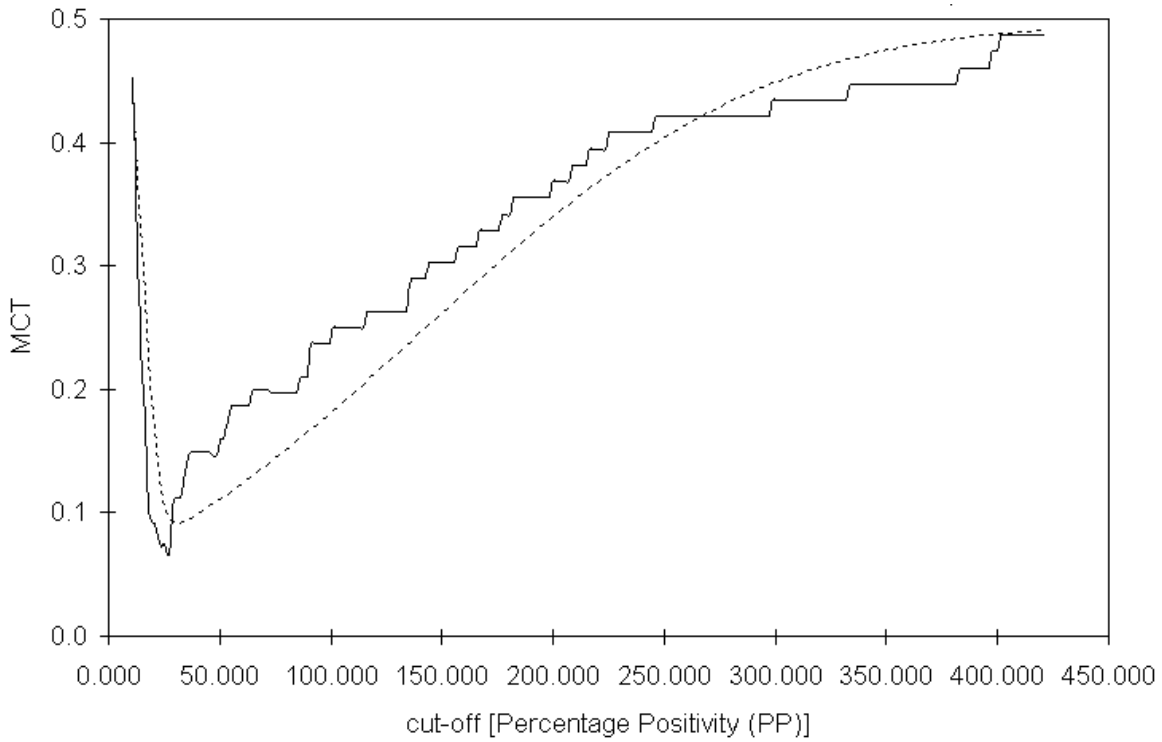


Figure 2.2.3. Optimisation of the cut-off for the recNP-HRPO IgM ELISA in humans using the misclassification cost term (MCT) option of the two-graph receiver operating characteristic analysis (TG-ROC). The two curves represent MCT values based on non-parametric (—) or parametric (- - -) estimates of sensitivity and specificity derived from data sets in field-collected sera. Optimisation of the cut-off value was based on the non-parametric (—) program option due to departure from a normal distribution of data sets analysed.

2.2.4 Discussion

A capture ELISA format was developed recently for the confirmation of a recent infection with RVFV in humans by detection of IgM. This capture ELISA was validated against the golden standard method, virus neutralization, and displayed high diagnostic sensitivity and specificity (Paweska et al., 2005a). It is, however, based on whole virus antigen which needs to be prepared in high biocontainment laboratory. An indirect ELISA based on a completely safe recombinant antigen of RVFV, the nucleocapsid protein, was validated recently for the detection of IgG in humans (Paweska et al., 2007). Although the indirect ELISA format, with recNP coated on the plate, was initiall evaluation for the detection of IgM in human sera, it yielded a high false-positivity rate, probably because of interfering rheumatoid factor (results not shown). This study describes attempts to conjugate the recNP with the HRPO enzyme and the subsequent development and preliminary validation of a RVFV recNP-HRPO IgM ELISA. In this format IgM is first captured from the sera to

minimize rheumatoid factor interference, after which HRPO conjugated recNP antigen is added as a detection system.

The best specificity of the assay was achieved when using the mean plus 3 standard deviations method, but this was at the cost of lower sensitivity. The method yielding the most sensitive cut-off was the misclassification cost term option (MCT) of the two-graph receiver operating characteristic analysis (TG-ROC) method but this was at the cost of specificity. Although this assay has some promise as a diagnostic assay based on its high specificity, it still requires further optimization to improve the sensitivity. The assay can be further improved by further improving the conjugation of the RVFV recNP to HRPO to obtain a more concentrated product, or using monoclonal antibodies to capture human IgM from specimens.

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2.3 Comparative evaluation of ELISA-based techniques for detection of antibodies against RVFV*

* Partially published as:

Van Vuren, P. J. & Paweska, J. T. (2010). Comparison of enzyme-linked immunosorbent assay-based techniques for the detection of antibody to Rift Valley fever virus in thermochemically inactivated sheep sera. *Vector-Borne and Zoonotic Diseases* **10**, 697-9.0

* Presented at international conferences as:

Jansen van Vuren, P., Paweska, J.T. (2009). A comparative evaluation of ELISA-based techniques for serodiagnosis of Rift Valley fever. *Annual meeting of the ARBO-ZOONET network,* St. Raphael, France, 30 September 2009. (Oral presentation).

Jansen van Vuren, P., Paweska, J.T. (2009). Comparison of ELISA-based techniques for serodiagnosis of Rift Valley fever. *5th European Meeting on Viral Zoonoses*, St. Raphael, France, 26 – 29 September 2009. (Poster presentation).

2.3.1 Introduction

In recent years numerous new RVF diagnostic techniques have been developed and validated, including molecular assays (Le Roux et al., 2009) for RNA detection and ELISAs for antibody detection (Paweska et al., 2003a, Paweska et al., 2005a, Paweska et al., 2005b, Jansen van Vuren et al., 2007, Paweska et al., 2007). The abovementioned ELISAs were all developed and validated separately and direct comparison was never undertaken. The diagnostic performance various ELISAs, as taken from the published literature, is summarized in table 2.3.1 (ELISAs for human diagnosis) and table 2.3.2 (ELISAs for livestock diagnosis). These ELISAs are based on gamma-irradiated reagents and/or recombinant antigens and are thus regarded safe. A simple thermo-chemical inactivation method for RVFV was developed (section 3.2.3) which would render these tests completely safe to conduct outside biocontainment facilities. The effect of the inactivation on detectable antibodies, however, is not known.

This sub-chapter describes the direct comparison of four livestock ELISAs for anti-RVFV antibody detection using a well characterized panel of sera collected from experimentally infected sheep, as well as an evaluation of the effect of a thermo-chemical RVFV inactivation step on detectable antibodies.

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2.3.2 Materials and methods

2.3.2.1 Enzyme-linked immunosorbent assays

The following ELISAs were directly compared using serial bleeds from experimentally infected sheep: IgG-sandwich ELISA (Paweska et al., 2003a), IgM-capture ELISA (Paweska et al., 2003a), inhibition ELISA (Paweska et al., 2005b) and an indirect ELISA based on the recombinant RVFV N protein (Jansen van Vuren et al., 2007) (also see section 2.2 of this thesis).

2.3.2.2 Experimental sheep sera

Serial sera were obtained from three sheep experimentally infected with wild type RVF virus as described previously (Le Roux et al., 2009).

2.3.2.3 Thermo-chemical inactivation of sera

Sheep sera were inactivated as described in chapter 3 (section 3.2.3). Briefly, an equal volume of 1% Tween20 in PBS was added to each serum and incubated at 56°C for 1 hour. Inactivated sera were tested for complete inactivation on 24 - 48h old Vero cell monolayers and in 2-3 day old suckling mice. Cells were monitored for cytopathic effect (CPE) until 14 days after inoculation and mice until 10 days p.i.

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Table 2.3.1 Antibody detection ELISAs for RVF diagnosis in humans

ELISA set-up	Indirect ELISA	Sandwich ELISA	Capture ELISA	Inhibition ELISA
Diagram[a]	Species specific, Anti-IgG HRPO conjugated antibody | IgG antibody in specimen | Pure RVFV antigen bound to plate surface	Species specific, Anti-IgG HRPO conjugated antibody | IgG antibody in specimen | Crude RVFV antigen | Hyperimmune anti-RVFV serum	Species specific, Anti-IgG HRPO conjugated antibody | Hyperimmune anti-RVFV serum | Crude RVFV antigen | IgM antibody in specimen | Anti-IgM capturing antibody	Species specific, Anti-IgG HRPO conjugated antibody (unbound) Hyperimmune anti-RVFV serum (out-competed) IgG/IgM antibody in specimen | Crude RVFV antigen | Hyperimmune anti-RVFV capturing serum
Antigen	Bacterially expressed recombinant nucleocapsid	Sucrose acetone extracted whole virus from mouse liver	Sucrose acetone extracted whole virus from mouse liver	RVF infected Vero cell supernatant
Antibody detected	IgG	IgG	IgM	Total Antibody
Number of specimens	n = 2969	n = 2400	n = 1396	n = 1367
Cross-reactivity evaluated[b]	Yes	No	No	No
TG-ROC cut-off[c]	**28.98 PP**[h]	**13.21 PP**	**7.1 PP**	**38.6 PI**[h]
Sensitivity[d]	99.72 %	100.0 %	96.47 %	99.47 %
Specificity[e]	99.62 %	99.95 %	99.44 %	99.66 %
Positive predictive value[f]	97.14 %	99.50 %	96.55 %	97.97 %
Negative predictive value[g]	99.66 %	100.0 %	99.54 %	99.91 %

[a] Indicates the complete immunocomplex formed on the ELISA plate in the case of a positive reaction

[b]Mouse anti-serum against: Saint-Floris (*Phlebovirus*), Gordil (*Phlebovirus*), Arumowot (*Phlebovirus*), Gabek Forest (*Phlebovirus*), Nairobi sheep disease (*Nairovirus*), Hazara (*Nairovirus*), Crimean-Congo hemorrhagic fever (*Nairovirus*), Akabane (*Orthobunyavirus*), Bunyamwera (*Orthobunyavirus*), Shuni (*Orthobunyavirus*) and Bhanja viruses (unassigned, Bunyaviridae family).

[c] Cut-off value at 95% accuracy level optimized using the misclassification cost term option (Greiner, 1996) of the two-graph receiver operating characteristics analysis (Greiner, 1995; Greiner, Sohr and Göbel, 1995).

[d] Se = [Tp/(Tp + Fn)] x 100 where Tp is true positives and Fn is false negatives.

[e] Sp = [Tn/(Tn + Fp)] x 100 where Tn is true negatives and Fp is false positives.

[f] PPV = [(P)(Se)]/[(P)(Se)] + [(1 – P)(1 – Sp)] x 100 where P is the prevalence

[g] NPV = [(1 – P)(Sp)]/[(1 – P)(Sp)] + [(P)(1 – Se)] x 100

[h] Percentage positivity

[i] Percentage inhibition

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Table 2.3.2 Antibody detection ELISAs for RVF diagnosis in livestock

Species	**Ovine**			**Caprine**			**Bovine**			**Camel**
ELISA set-up	**Sandwich ELISA**	**Capture ELISA**	**Inhibition ELISA**	**Sandwich ELISA**	**Capture ELISA**	**Inhibition ELISA**	**Sandwich ELISA**	**Capture ELISA**	**Inhibition ELISA**	**Inhibition ELISA**
Antigen	Sucrose acetone whole virus	Sucrose acetone whole virus	RVF Vero cell supernatant	Sucrose acetone whole virus	Sucrose acetone whole virus	RVF Vero cell supernatant	Sucrose acetone whole virus	Sucrose acetone whole virus	RVF Vero cell supernatant	RVF Vero cell supernatant
Antibody detected	IgG	IgM	Total Antibody	IgG	IgM	Total Antibody	IgG	IgM	Total Antibody	Total Antibody
Number of specimens	n =1321	n =1321	n = 493	n =1459	n =1459	n = 806	n = 997	n = 997	n = 694	n = 156
TG-ROC cut-off	**13.2 PP**	**7.9 PP**	**38.4 PI**	**18.8 PP**	**9.5 PP**	**41.4 PI**	**16.4 PP**	**14.3 PP**	**41.9 PI**	**36.1 PI**
Sensitivity	99.05 %	100.0 %	100.0 %	100.0 %	97.40 %	99.56 %	96.34 %	99.20 %	100.0 %	100.0 %
Specificity	99.10 %	99.40 %	99.29 %	99.90 %	-	99.65 %	99.67 %	-	99.52 %	100.0 %
Positive predictive value	91.19 %	-	95.78 %	99.69 %	-	99.15 %	96.61 %	-	95.35 %	100.0 %
Negative predictive value	99.90 %	-	100.0 %	100.0 %	-	99.81 %	99.66 %	-	100.0 %	100.0 %

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2.3.3 Results

The immune responses in sheep after experimental infection with wild-type RVFV were monitored using the IgG sandwich, indirect IgG, IgM capture and an inhibition ELISA. There was no significant difference in detection of antibodies between untreated versus inactivated serum using any of the ELISAs but these assays differed in their ability to detect the early humoral responses to infection with RVFV (Figure 2.3.1). The IgM-capture ELISA was able to detect seroconversion on day 4 post-infection (p.i.) compared to day 5 p.i. with the IgG-sandwich ELISA. The inhibition ELISA yielded false-positive results on day 2 and 3 p.i. as a result of the capturing of viral antigen in highly viremic sera on days 2 and 3 p.i. (circle in figure 2.3.1). The recombinant N protein-based IgG ELISA, using Protein G HRPO, was less sensitive in detecting seroconversion (day 9 p.i.) as compared to the IgG-sandwich ELISA (day 5 p.i.). This problem was alleviated when replacing Protein G with anti-sheep IgG HRPO (Figure 2.3.1).

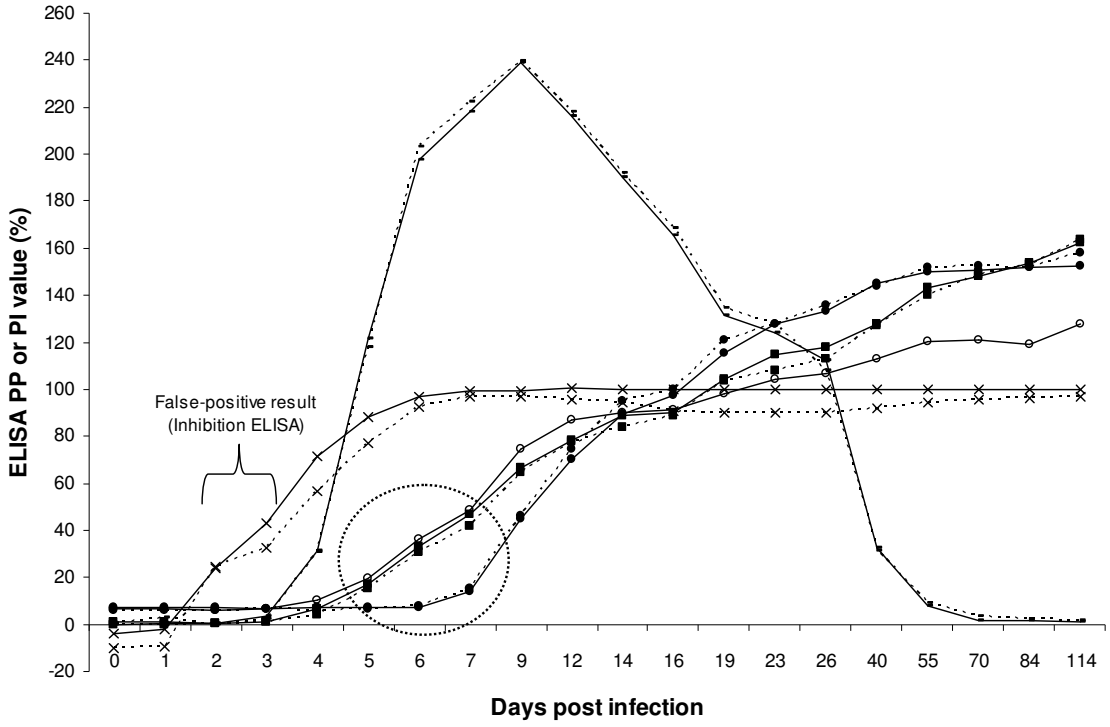


Figure 2.3.1. Comparison of mean immune responses in three experimentally infected sheep as measured by testing naïve (solid lines) versus thermo-chemically inactivated (dotted lines) sera by IgM- capture ELISA (—), inhibition ELISA (x), indirect ELISA Protein G HRPO (●), indirect ELISA anti-sheep IgG HRPO (○), and IgG- sandwich ELISA (■).

2.3.4 Discussion

The thermo-chemical inactivation step had no adverse effect on the detection of antibody in any of the ELISAs tested and renders RVFV completely inactive, as evidenced by safety testing in suckling mice and tissue culture. This makes it a practical method for use in the field or in laboratories with limited biocontainment facilities.

The most sensitive assay for detection of antibodies early after infection was the IgM capture ELISA. This was expected since the early humoral response to infection is IgM. The IgM capture ELISA would therefore be the most suitable test for diagnosis of recent RVFV infections.

There was a slight decrease in sensitivity of the recNP based indirect ELISA when the pseudoimmunogen Protein G was used as an HRPO conjugate in the assay instead of the species specific conjugate. It is possible that the early IgG antibody has lower afinity for Protein G. Using Protein G, which is not species specific, instead of a species specific HRPO conjugate, however, offers the opportunity to use the same assay format for detection of antibodies in various species. In addition the likelyhood of testing a large number of samples that were collected so early after infection is low. The recNP based indirect ELISA would therefore be suitable for serological surveys, testing of immune status after vaccination and import/export testing. An additional advantage of the recNP based I-ELISA is that it can be used for the differentiation of naturally infected and vaccinated animals (DIVA) when vaccines are based on the other structural proteins of RVFV (McElroy et al., 2009, de Boer et al., 2010).

The RVFV inhibition ELISA, which detects total antibodies IgG/IgM, was recently evaluated by a European group and found to be highly sensitive and specific for testing European ruminant sera (Cetre-Sossah et al., 2009). It was surprising, however, to note false-positive results with the inhibition ELISA when testing sera collected early after infection of sheep. Upon closer inspection of the assay format, it was concluded that the ELISA set up allows for capture any RVFV antigens present in the experimental sera during viremia and thus it will yield false-positive results before seroconvertion. This intrinsic characteristic of the test has an impact on how the results should be interpreted, especially if it is used during RVF outbreaks where viremic individuals are likely to be encountered. It also stresses the importance of understanding the basis of each assay for correct interpretation of results. However, taking into account the short viremia during RVFV infection the practical consequence of this seems to be neglible.

This is the first direct comparison of validated ELISA techniques for RVF serological diagnosis which highlights the difference in characteristics and design of each assay and their impact on the interpretation of results.

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2.4 Detection of IgG antibody to Rift Valley fever virus in humans*

* Published as:

Paweska, J. T., Jansen van Vuren, P., and 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. *Journal of Virological Methods* **146**(1-2)**,** 119-24.

2.4.1 Introduction

An IgG-sandwich ELISA was recently developed and validated using extended panels of well-characterized human sera (Paweska et al., 2005a). This ELISA is based on sucrose acetone extracted RVF whole virus and hyperimmune sera that were generated against live RVFV. Although these reagents are gamma-irradiated for safety before usage, they still need to be prepared in high biocontainment facilities before inactivation. The use of a recombinant antigen circumvents some of the issues that hamper the safe production of immunoreagents for serological diagnostic assays. The indirect ELISA is also a less time consuming and simple assay when compared to the sandwich format, which makes it more user-friendly and cost-effective.

2.4.2 Materials and methods

2.4.2.1 ELISA serum controls and internal quality control

Freeze-dried, gamma-irradiated serum controls were produced as described previously (Paweska et al., 2005a). To assess inter- and intra-plate variation the means and standard deviations (S.D.) of the ELISA optical density and percentage positivity values (PP) were determined from replicates of the internal controls included in each plate and run of the assay during validation. Coefficient of variation values (CV%) were also determined for the positive serum controls [CV% = (S.D. of replicates / means of replicates) x 100]. Estimates of the assay repeatability and the upper and lower control limits for each internal control were determined from the resultant data. During routine runs of the assay each plate had four replicates of high positive (C++), low positive (C+), negative (C-) and the conjugate control (Cc).

2.4.2.2 Human serum panels

A total of 2967 sera collected in Kenya (n = 982), South Africa (n = 1255), Tanzania (n = 360), Uganda (n = 210) and Zimbabwe (n = 160) were used. The South African and Zimbabwean sera represented post RVF outbreak specimens collected in the late 1970s and routine diagnostic submissions to the Special Pathogens Unit of the National Institute for Communicable Diseases (National Health Laboratory Services) for the period 1999 to 2005. East African sera were taken to

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monitor the 1997-98 outbreak of RVF in the region (Woods et al., 2002). Sera which tested negative in the virus neutralization test were regarded as a reference panel from non-infected individuals, whereas sera which tested positive as a reference panel from previously infected individuals. Cut-off value calculation and diagnostic accuracy determination were done using the IgG I-ELISA results obtained from these field collected sera.

2.4.2.3 Mouse ascetic fluids for cross-reactivity testing

Hyperimmune mouse ascetic fluids generated against viruses representing the genus *Phlebovirus*, *Nairovirus*, *Orthobunyavirus* and Bhanja virus of the family *Bunyaviridae* as described before (Burt et al., 1993) were obtained from the serum bank of the Arbovirus section of the SPU-NICD/NHLS.

2.4.2.4 Virus neutralization test

The virus neutralization test was done as described previously (section 2.1.2.3)

2.4.2.5 Antigen production and IgG I-ELISA procedure

Antigen was produced and the I-ELISA procedure done as described previously (section 2.1.2.4), except that HRPO conjugated to goat anti-human IgG (H+L chain) was used for human specimens and HRPO conjugated to recombinant Protein G (Zymed Laboratories, Inc.) was used for mouse ascitic fluid.

2.4.2.6 Selection of cut-off values and determination of ELISA diagnostic accuracy

Cut-off values were determined as described previously (section 2.1.2.5). Parameters of diagnostic accuracy were determined as described previously (section 2.1.2.5).

2.4.3 Results

2.4.3.1 Internal quality control and repeatability

The upper and lower internal quality control limits and estimates of repeatability of the assay are summarized in Table 2.4.1. There was no excessive variation within and between routine runs of the assay, and the internal controls were constantly within upper and lower control limits during routine runs of the assay (Figure 2.4.1).

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2.4.3.2 Cross-reaction with hyperimmune mouse ascitic fluid

The I-ELISA optical density (OD) value of the mouse IgG positive RVFV ascitic fluid was 1.52 while that of normal mouse ascitic fluid and the conjugate control was 0.072 and 0.068 respectively. The OD readings of hyperimmune ascitic fluids from mice experimentally infected with different viruses from the genus *Phlebovirus*, *Nairovirus*, *Orthobunyavirus* and Bhanja virus of the family *Bunyaviridae* were within the OD values for negative controls (Figure 2.4.2). These results demonstrate highly specific binding affinity of mouse IgG antibody against RVFV and the recNP of the virus and lack of cross-reaction between the recNP and IgG antibody against other Bunyaviruses assayed.

Table 2.4.1. Internal quality control data and repeatability estimates for Rift Valley fever IgG I-ELISA based on recombinant nucleocapsid antigen

	IQC parameters	
IQC[a] limits	UCL[b]	LCL[c]
OD C++	1.7	0.81
PP[d] C++	117	82
PP C+	39	24
PP C-	9.8	5.4
PP Cc	7.2	3
Repeatability[e]		
Intra-plate variation		
C++		5.98 ± 2.4 S.D. (2.72-9.83)[f]
C+		6.19 ± 3.25 S.D. (2.73-13.65)
Inter-plate variation		
C++		6.01 ± 1.54 S.D. (3.35-7.7)
C+		6.19 ± 2.33 S.D. (3.98-11.03)

[a] Internal quality control (IQC) data were calculated from the mean ± 2 S.D. of 420 replicates of each control over seven runs including five plates.

[b] Upper control limit

[c] Lower control limit

[d] Percentage positivity

[e] Repeatability estimates for high positive (C++) and low positive (C+) serum controls were calculated as the %CV.

[f] Range of %CV values

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2.4.3.3 Cut-off values and diagnostic accuracy

Cut-off values were optimized using the TG-ROC as described before (section 2.1.3.3). At a cut-off value of 28.98 PP the overall misclassification costs were minimal under assumption of 50% disease prevalence and equal costs of false-positive and false-negative test results. Graphical presentation of the effect of three differently determined threshold values on distinguishing between positive or negative sera is shown in Figure 2.4.3. At a cut-off optimized by TG-ROC at 95% accuracy level, the diagnostic sensitivity of the I-ELISA was 99.72% and diagnostic specificity 99.62% while estimates for the J and Ef were 0.993 and 99.62% respectively. When cut-off values were determined by traditional statistical approaches, the diagnostic sensitivity was 100% but estimates of J, Ef, PPV and NPV values were lower compared to those based on the TG-ROC cut-off (Table 2.4.2).

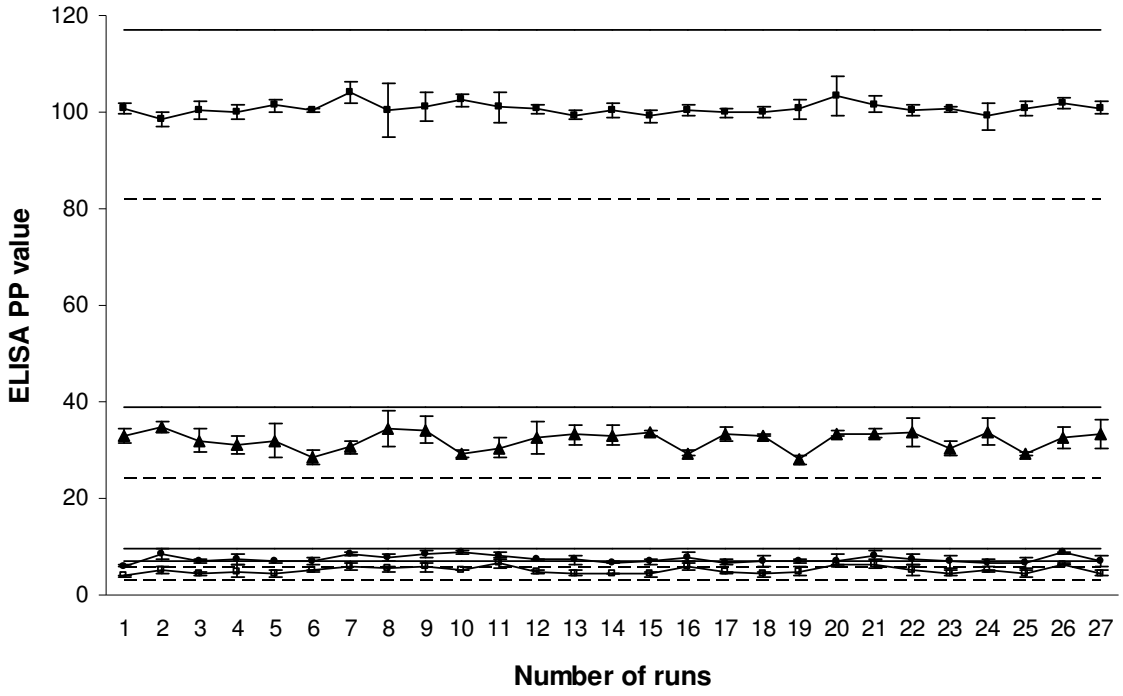


Figure 2.4.1. Upper (____) and lower (----) internal quality control limits for PP values of high-positive (■), low positive (▲), negative (●) serum controls and conjugate control (□) and means ± S.D. for these controls during 27 routine runs of the assays over a period of 12 weeks. Two or three plates were used during each run with four replicates of each control on each plate.

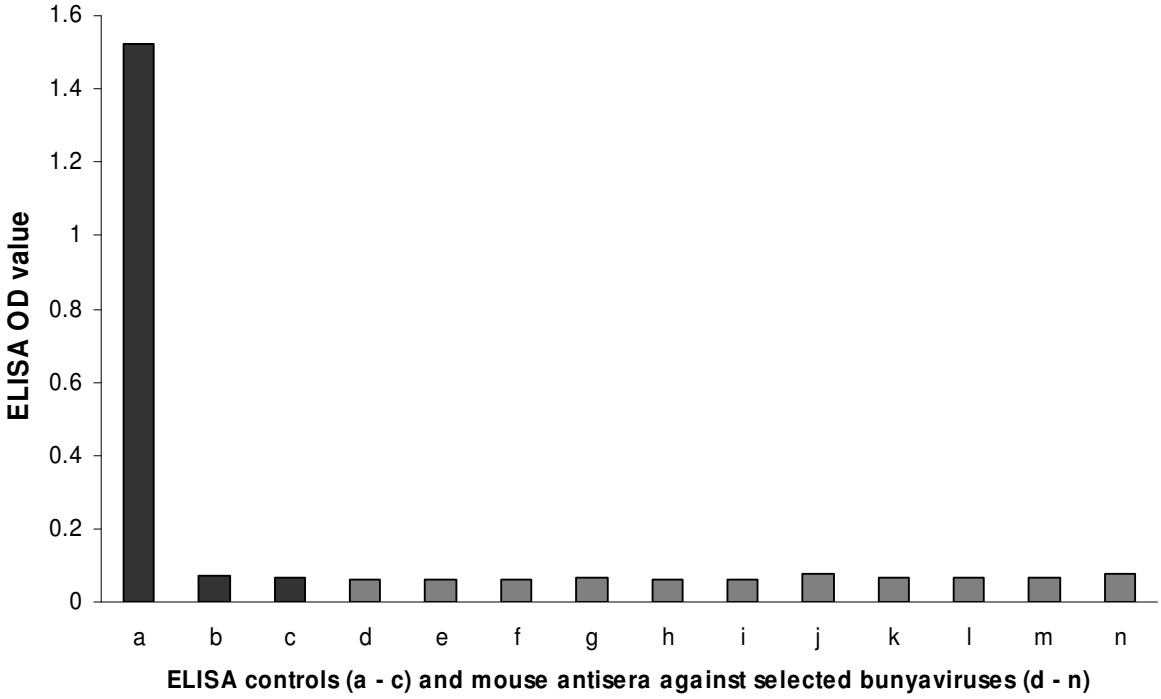


Figure 2.4.2. Cross-reactivity of recombinant nucleocapsid protein of RVFV in I-ELISA with mouse IgG antibody against selected viruses of the family Bunyaviridae. (a) Mouse IgG anti RVFV (Phlebovirus), (b) normal mouse ascitic fluid and (c) conjugate control. Mouse IgG anti: (d) Saint-Floris (Phlebovirus), (e) Gordil (Phlebovirus), (f) Arumowot (Phlebovirus), (g) Gabek Forest (Phlebovirus), (h) Nairobi sheep disease (Nairovirus), (i) Hazara (Nairovirus), (j) Crimean-Congo hemorrhagic fever (Nairovirus), (k) Akabane (Orthobunyavirus), (l) Bunyamwera (Orthobunyavirus), (m) Shuni (Orthobunyavirus) and Bhanja viruses (not assigned to a recognized genera of the family Bunyaviridae).

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Table 2.4.2. Diagnostic accuracy of Rift Valley fever IgG I-ELISA based on recombinant nucleocapsid antigen

Measure[a]	Cut-off 28.98 PP[b]	Cut-off 17.18 PP[c]	Cut-off 21.38 PP[d]
Sensitivity (%) VNT+=350[e]	99.72	100	100
Specificity (%) VNT-=2617[f]	99.62	95.3	97.54
Youden's index	0.993	0.953	0.975
Efficiency (%)	99.62	95.84	97.82
Positive predictive value (%)	97.14	73.23	84.12
Negative predictive value (%)	99.66	100	100

Individuals were categorized according to the results of the virus neutralization test (VNT).

[b] Cut-off value optimized by the misclassification cost term option of the two-graph receiver operating characteristics analysis at 95% accuracy level.

[c] Cut-off value determined by mean plus two standard deviations derived from PP values in uninfected reference population.

[d] Cut-off value determined by mean plus three standard deviations derived from PP values in uninfected reference population.

[e] Number of sera tested positive in the VN test.

[f] Number of sera tested negative in the VN test.

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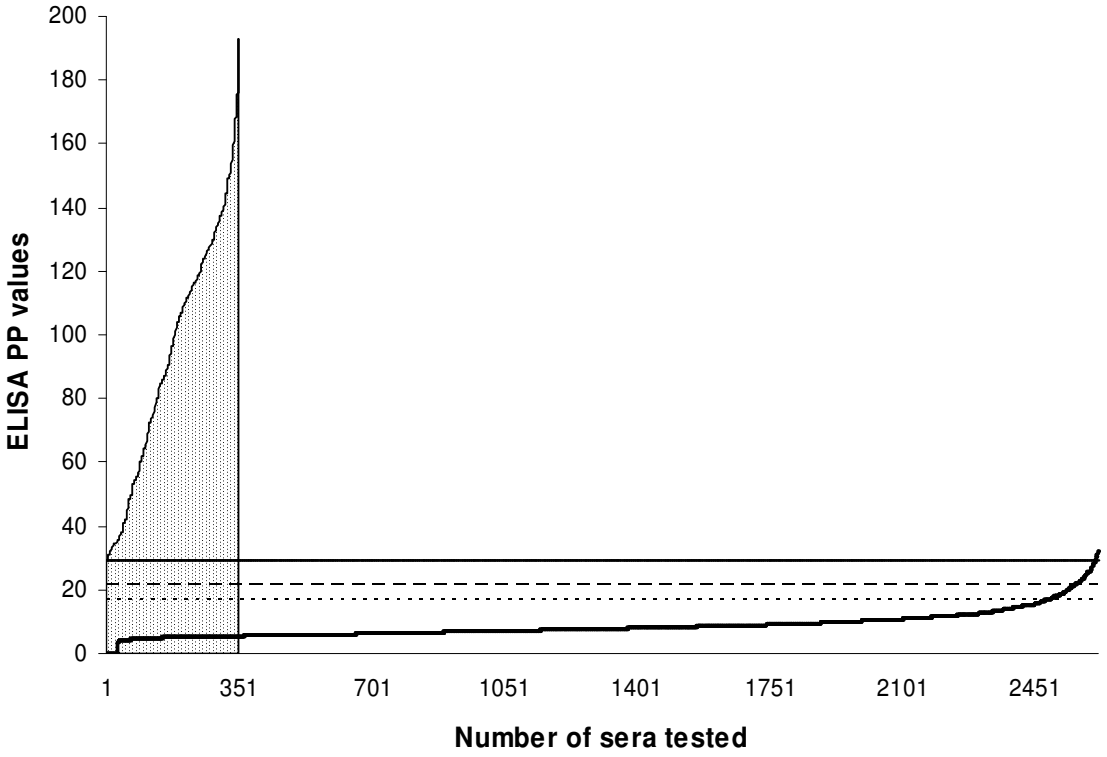


Figure 2.4.3. The effect of different ELISA cut-off values on the discrimination between human sera tested negative or positive in the VN test for antibodies against RVFV. Distribution of IgG I-ELISA PP values in human sera tested positive (n = 350, ▒) or negative (n = 2617, ▬) in the VN test. Sera ordered according to ELISA PP values. Horizontal lines: (–) cut-off value of 28.98 PP determined by TG-ROC analysis, (---) cut-off value of 21.37 PP determined by mean plus 3 S.D., and (···) cut-off value of 17.18 PP determined by mean plus 2 S.D. of ELISA PP values observed in negative sera.

2.4.4 Discussion

The indirect ELISA is one of the simplest immunoassay techniques for the detection of antibodies, but its routine application is impeded by non-specific signals arising from the use of crude or semi-purified antigens (Gravell et al., 1977, Frazier and Shope, 1979). RVFV does replicate to high titres in cell cultures but production of purified and concentrated virus stocks by classical virological methods is expensive, time consuming and requires high biocontainment. The results from this chapter confirm earlier findings (Jansen van Vuren et al., 2007) that the recNP of RVFV binds readily to ELISA plates, generates minimal background and effectively differentiates sera with varying concentrations of IgG antibodies to the virus in humans. The I-ELISA presented here achieved high repeatability estimates within statistically pre-determined IQC limits.

In this study the virus neutralization test was used to classify individuals according to their RVF infection status. Infection with RVFV induces life long virus neutralizing antibody in humans (Findlay and Howard, 1951), and only one serotype of RVFV is known (Swanepoel and Coetzer, 2004). The NP is one of the most immunodominant viral proteins and appears to be highly conserved among members of the *Bunyaviridae* family (Swanepoel et al., 1986b, Vapalahti et al., 1995, Schwarz et al., 1996, Magurano and Nicoletti, 1999, Gauliard et al., 2006). The serological cross-reactivity results from this study indicates that infection with related African Phleboviruses and other viruses from the *Bunyaviridae* family should not hamper serodiagnosis of RVF based on the recNP, which corresponds with previous reports on cross-reaction between known African *Phleboviruses* (Swanepoel et al., 1986b).

The antigenic specificities of antibodies measured by ELISA and the virus neutralization test differ (Swanepoel et al., 1986b), therefore it is expected that the ELISA based on inactivated whole virus will be more sensitive than the virus neutralization test which detects only antibodies against the RVFV glycoproteins. This may explain the slightly lower specificity of the I-ELISA in this study compared to virus neutralization test in the study population. The recNP I-ELISA has been shown to be more sensitive than the virus neutralization test in detecting early immune responses in experimentally infected sheep (Jansen van Vuren et al., 2007).

The recNP I-ELISA had a higher estimate of diagnostic sensitivity when cut-offs were determined by traditional statistical methods (mean + 2S.D. and 3S.D.), but lower specificity and combined measurements of assay performance characteristics when compared to the cut-off determined by TG-ROC analysis. In conclusion, the I-ELISA based on the recNP is highly accurate and robust for the detection of specific IgG antibody against RVFV in human sera and can be used in the diagnosis of infection and sero-epidemiological studies.

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CHAPTER THREE

EVALUATION OF ANTI-RECOMBINANT NUCLEOCAPSID PROTEIN RABBIT AND SHEEP POLYCLONAL SERA AS IMMUNODIAGNOSTIC REAGENTS IN AN ANTIGEN DETECTION SANDWICH ELISA *

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Jansen van Vuren, P. & Paweska, J.T. (2008). Laboratory safe detection of nucleocapsid protein of Rift Valley fever virus in human and animal specimens by a sandwich ELISA. *National Institute for Communicable Diseases Academic Day*, 11 November 2008. (Poster presentation).

3.1 Introduction

Various methods exist for the detection of antibodies against RVFV, but detection of antibodies only become possible after seroconversion which is usually between 2-4 days after the onset of viremia. There is therefore a period after RVFV infection where individuals would test negative with serological methods when in fact they are infected. Virus isolation is expensive, laborious and requires the propagation of live virus, necessitating the use of biocontainment facilities. Various molecular techniques have been developed to enable detection of virus genetic material (Jupp et al., 2000, Drosten et al., 2002, Peyrefitte et al., 2008, Le Roux et al., 2009). Molecular methods are highly sensitive and specific but also expensive and highly specialized.

ELISA is a robust technique that requires less specialized equipment and training, and is probably in use in most diagnostic laboratories around the world. Some ELISAs for the detection of RVFV antigens have been reported (Niklasson et al., 1983, Meegan et al., 1989, Zaki et al., 2006) but these assays are based on reagents that are difficult and expensive to produce, pose a biohazard risk to

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laboratory personnel, and have not been properly validated. This chapter describes the first development and validation of a sandwich ELISA for antigen detection based on polyclonal antibodies specifically generated against the major viral antigen, the nucleocapsid protein, and the use of thermo-chemical inactivation of specimens to ensure safety of laboratory personnel.

3.2 Materials and methods

3.2.1 Generation of rabbit and sheep hyperimmune sera against the RVFV recNP

The recNP was produced as described before (Chapter 2.1.2.4). The pET32 control antigen was produced in the same way but using wild-type pET32(a)+ vector without the RVFV NP-gene insert.

Three New Zealand white rabbits were immunized subcutaneously (s.c.) with 140µg recNP emulsified in an equal volume of TiterMax Gold® adjuvant (Sigma-Aldrich, USA) according to the manufacturer's instructions. Rabbits received an identical booster inoculation 14 days later followed by another booster of 375µg recNP without adjuvant on day 33 after the first immunization.

Two Dorper cross sheep were immunized s.c. with 350µg recNP in TiterMax Gold adjuvant, emulsified as described above. Sheep received an identical booster inoculum on day 21 after the first immunization. Blood was taken regularly from immunized animals to monitor their responses to immunization. When the animals' responses yielded optical density (OD at 405nm) readings higher than 2.0 on a recNP I-ELISA at 1:400 dilution they were regarded as hyperimmune and bulk serum collected. Bulk sera from individual animals were pooled together to obtain homogenous un-purified preparations of polyclonal rabbit- and sheep anti-recNP respectively.

3.2.2 Sandwich ELISA procedure

The top half of a high protein binding plate (Maxisorb, Nunc, Denmark) was coated with sheep anti-recNP hyperimmune serum (capture antibody) and the bottom half with normal sheep serum, both at dilution 1:400 in PBS, pH 7.2 and incubated overnight at 4°C. After washing three times with washing buffer (PBS, pH 7.2 and 0.1% Tween-20), plates were blocked with 200µl of 10% fat-free milk powder ("Elite", Clover SA, Pty, Ltd.) in PBS, incubated in a moist chamber at 37°C for 60 minutes and washed as described above. RVFV recNP stock antigen diluted 1:3000 in 2% milk powder (diluting buffer) was used as a high positive control; 100µl of the diluted antigen was added in quadruplicate to the top and bottom half of the plates. RVFV recNP stock antigen, diluted 1:30,000 was used as a low positive control antigen and pET32 antigen diluted 1:3000 as negative control antigen; 100µl of each was added in duplicate to the top and bottom halves of each plate. A volume of 100µl of each specimen, inactivated as determined in 3.2.3, was added undiluted and in duplicate to the

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top and bottom halves of each plate. The plates containing internal controls and specimens were incubated at 37°C for 60 minutes in a moist chamber, washed as before and 100µl of hyperimmune rabbit anti-recNP serum (detecting antibody), diluted 1:3000 in diluting buffer, added to each well. After incubation at 37°C for 60 minutes in a moist chamber, plates were washed as before and 100µl of goat anti-rabbit IgG (H+L) HRPO conjugate (Zymed Laboratories, USA) diluted 1:8000 added to each well. After incubating as before plates were washed as before and 100µl of 2'2-azinodiethylbenzthiazoline sulfonic acid (ABTS, KPL Laboratories, USA) peroxidase substrate added to each well. Plates were incubated at room temperature in the dark for 30 minutes after which the reaction was stopped by the addition of 100µl of 1% sodium dodecyl sulphate (SDS, Sigma-Aldrich, USA) to each well. Optical density (OD) was determined at 405nm wavelength and results expressed as percentage positivity of the mean high-positive control antigen (PP) using the formula: (mean net OD of duplicate test specimen/mean net OD of high positive control) x 100.

3.2.3 Inactivation of specimens and safety testing

Three regularly used laboratory detergents were evaluated together with heat for their ability to increase antigen detection efficiency in the sAg-ELISA. The detergents TritonX-100, NP40 and Tween-20 were mixed, each at 1%, with PBS pH 7.2 or carbonate/bicarbonate buffer pH 9.6 and evaluated as inactivation buffers. Normal sheep serum was then spiked with RVFV Ar20368 RSA 81 strain to a final concentration of $10^{5.8}$ $TCID_{50}$/ml and inactivated with each inactivation buffer (equal volumes of spiked serum and inactivation buffer) for 60 minutes at 56°C. Spiked serum was also inactivated at 56°C for 60 minutes without the presence of detergent. As a no-treatment control, RVFV spiked serum was added to an equal volume of PBS without detergent and not subjected to heat inactivation. As a negative control, negative serum without virus was treated the same as the no-treatment control. These preparations were tested on the sAg-ELISA.

The optimal inactivation protocol (1% Tween-20 in PBS + 56°C for 60 minutes) was safety tested in 2-3 days old suckling mice and Vero cell monolayers. Mice were monitored for clinical symptoms until day 10 p.i. and Vero cells were monitored for CPE for a period of 14 days p.i. To control for all conditions, the following controls were also set-up: RVFV spiked sheep serum inactivated just by heat and spiked serum treated just with Tween-20.

3.2.4 Antigen detection in animal and insect specimens

Heart, lung, liver, kidney and brain tissues were harvested from three female BALB/c mice on day 2 after subcutaneous inoculation with the SPU22/118 KEN 07 strain of RVFV. The same tissues were harvested from a mock inoculated BALB/c mouse. Diagnostic submissions of liver, heart, kidney,

lung, and brain tissues from three aborted buffalo fetuses during the 2008 RVF outbreak in South Africa (Paweska et al., 2008a) were used. Animal tissues were homogenized as 10% (w/v) suspensions in Eagles Minimal Essential Medium (EMEM) (BioWhitaker,MD, USA) containing L-glutamine, non-essential amino acids and antibiotics (100 IU penicillin, 100µg streptomycin, and 0.25µg amphotericin B). After centrifugation at 3000×*g*, supernatants were harvested and stored at −70°C.

Homogenates (10%, w/v) of uninfected ovine and bovine liver and spleen tissues, prepared as described above, and uninfected human, sheep and cattle sera were spiked with the Ar20368 RSA 81 strain of RVFV to a final virus concentration of $10^{6.5}$ TCID50/ml. Half $\log_{10}$ dilutions of these preparations were used to determine the sAg-ELISA analytical detection limit in tissues and sera. As controls, the unspiked homogenates and sera were used with EMEM in place of virus suspension.

Homogenates (10%, w/v) of mosquito pools, each containing 100 individuals of *Anopheles arabiensis*, *A. gambiae* and *A. funestus,* obtained from laboratory mosquito colonies at Vector Control Unit of the National Institute for Communicable Diseases, were prepared in EMEM and spiked with the Ar20368 RSA 81 strain of RVFV as described above.

A total of 105 sheep sera were used of which 20 were from sheep inoculated subcutaneously with the SPU22/118 KEN 07 strain of RVFV, and the remaining 85 were from naïve sheep.

3.2.5 Antigen detection in human specimens

A total of 130 human sera submitted to the Special Pathogens Unit of the National Institute for Communicable Diseases, Sandringham, South Africa (SPU-NICD) for routine testing were used; 70 specimens were from suspected RVF cases sampled during the 2006–2008 disease outbreaks in Southern Africa.

3.2.6 Monitoring viral growth *in vitro*

Tenfold dilutions of the Ar20368 RSA 81 strain of RVFV in EMEM (from $10^{5.8}$ to$10^{0.8}$ TCID50/ml) were used for inoculation of 25cm^2 tissue culture flasks containing 48 h confluent Vero cell monolayers. Inoculated flasks were incubated on a rotating platform for 1h at 37°C. Two mock inoculated flasks were included as controls. After 1 h of incubation, inoculated flasks were removed, cells were washed with PBS and supplemented with 10 ml of EMEM containing 1% fetal calf serum and antibiotics. Inoculated cells were maintained at 37°C in a CO_2 incubator. One ml aliquots of tissue culture medium were collected hourly for the first 8 h, and thereafter at 12, 24, 30, 48, 54, 72, 78, 96 and 102 h after inoculation for testing on the sAg-ELISA. The collected aliquots of tissue culture medium were replaced each time with the same volume of fresh medium. Appearance of cytopathic effect (CPE) was documented at each collection time.

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3.2.7 Antigen detection in decomposing tissues

To mimic clearance of NP protein in decomposing tissues from RVFV-infected animals, homogenates (5%, w/v) of fresh normal ovine and bovine liver tissues were prepared as described above and mixed with an equal volume of tissue culture supernatant containing $10^{7.9}$ TCID50/ml of RVFV Zim688/78 strain, and then incubated at 37°C for a period of 8 days during which aliquots were taken for testing immediately after mixing, and 5, 24, 48, 72, 168 and 192 h thereafter. Supernatants were collected after centrifugation at 3000×*g* at 4°C and tested by sAg-ELISA and virus titration.

Virus titrations of clinical and laboratory generated specimens were performed as described previously (Swanepoel et al., 1986a). Briefly, four 100µl replicates of 10-fold dilutions (10^{-1} to 10^{-7}) of specimens in EMEM were transferred into flat bottomed 96- well cell culture microplates (Nunc, Denmark) and equal volumes of Vero cell suspension in EMEM, containing 2×10^5 cells/ml, 8% fetal bovine serum/ml (Gibco) and antibiotics, were added. The inoculated microplates were incubated at 37°C in a CO_2 incubator and observed under a microscope for CPE for 10 days post-infection (p.i.). Virus concentrations, calculated by the method of Kärber (Kärber, 1931), were expressed as median tissue culture infectious dose ($TCID_{50}$) per ml of specimen.

3.2.8 ELISA performance, cut-off selection and IQC

Seventeen RVFV isolates recovered over a period of 53 years (1955–2008) in African countries, Madagascar and Saudi Arabia (Table 3.1), four African Phleboviruses (Arumowot, Gabek Forest, Gordil and Saint-Floris) and two other members of the family *Bunyaviridae* (Akabane and Bunyamwera viruses) were used to evaluate the analytical sensitivity and specificity of the sAg-ELISA.

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Table 3.1. Identification, year of isolation, origin and concentration of RVFV strains used to evaluate the sAg-ELISA.

Strain	Year of isolation	Source	Country of origin	Concentration $Log_{10}TCID_{50}$/ml	ELISA PP value[a]
Lunyo UGA 55	1955	Mosquito	Uganda	$10^{6.3}$	112.0
ZH 501	1977	Human	Egypt	$10^{5.8}$	121.9
Zim 688/78	1978	Bovine	Zimbabwe	$10^{8.5}$	137.8
VRL-825-ZIM79	1979	Bovine foetus	Zimbabwe	$10^{7.0}$	123.3
Ar20368 RSA 81	1981	Mosquito	South Africa	$10^{6.8}$	110.3
Ank-6087	1984	Bat	Guinea	$10^{7.0}$	108.1
ArD38661 SEN 83	1983	Mosquito	Senegal	$10^{7.3}$	134.2
ArD38388 BF 83	1983	Mosquito	Burkina Faso	$10^{7.5}$	126.1
CAR R1662	1985	Human	Central African Republic	$10^{6.5}$	131.5
SPU-204-ANGL85	1985	Human	Angola	$10^{6.0}$	115.0
900085MAU88	1988	Human	Mauritania	$10^{7.3}$	125.3
An991-MAD91	1991	Bovine	Madagascar	$10^{7.5}$	121.7
SPU12002-SOM98	1998	Caprine	Somalia	$10^{8.0}$	124.6
AR21229-SA00	2000	Mosquito	Saudi Arabia	$10^{7.3}$	117.2
SPU 77/04	2004	Human	Namibia	$10^{7.0}$	132.3
SPU22.118KEN 07	2007	Human	Kenya	$10^{6.8}$	131.2
AR 52/08	2008	Human	South Africa	$10^{6.8}$	127.5

[a] Percentage positivity of ELISA high positive antigen control.

The internal quality control data were generated as described before (2.1.2.1). Means and standard deviations (S.D.) of the ELISA optical density readings and the percentage positivity (PP) of high-positive antigen control were calculated from replicates of all internal controls in each plate and each run of the assay to assess intra- and inter-plate variation. Additionally, coefficients of variation (CV = standard deviation of replicates/mean of replicates×100) were calculated for positive antigen controls. Data obtained from this analysis were used to estimate the assay repeatability and to establish the upper and lower control limits for each of the internal controls. Upper and lower control limits together with CV values (≤10 for high-positive serum and ≤15 for low-positive serum) were applied as IQC rules for further analysis. During routine runs of the ELISA each plate had four replicates of high-positive antigen control (Ag++) and two replicates each of low-positive antigen (Ag+) and negative control antigen (Ag−).

Cut-off values at the 95% accuracy level were optimized using the misclassification cost term option (Greiner, 1996) of the two-graph receiver operating characteristics (TG-ROC) analysis (Greiner, 1995). Additionally, cut-off values were determined by mean plus 2 standard deviations (S.D.s) and

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mean plus 3 S.D.s derived from PP values in known RVFV-negative human and sheep sera. Estimates of diagnostic sensitivity, specificity, and efficiency were calculated as described before (2.1.2.5).

3.3 Results

3.3.1 Internal quality control and assay repeatability

The s-Ag ELISA was able to differentiate clearly between the internal controls used and generated minimal background. Variation between and within runs were minimal (Figure 3.1). CV values for intra- and inter-plate runs were below 5% (Table 3.2) demonstrating high repeatability of the assay.

Table 3.2
Internal quality control limits and repeatability estimates of RVF s-Ag ELISA

Internal controls	IQC[a] limits		Repeatability[e]	
	UCL[b]	LCL[c]	Intra-plate variation	Inter-plate variation
OD Ag++	2.0	1.1		
PP[d] Ag++	129	71	1.04 ± 0.9 S.D.	1.28 ± 0.63 S.D.
PP Ag+	51	28	1.85 ± 1.83 S.D.	2.55 ± 1.72 S.D.
PP Ag-	4.9	1.4		

[a] Internal quality control - data were calculated from the mean ± 2 S.Ds. of 180 replicates of high positive antigen control (Ag++), 90 replicates of low positive (Ag+) and negative (Ag-) antigen controls over 5 runs each including 3 plates.
[b] Upper control limit.
[c] Lower control limit.
[d] Percentage positivity of high positive antigen control.
[e] Repeatability estimates for high (Ag++) and low (Ag+) positive antigen controls were calculated as the percentage coefficient of variation [%CV = (PP S.D. of replicates / PP mean of replicates) x 100]

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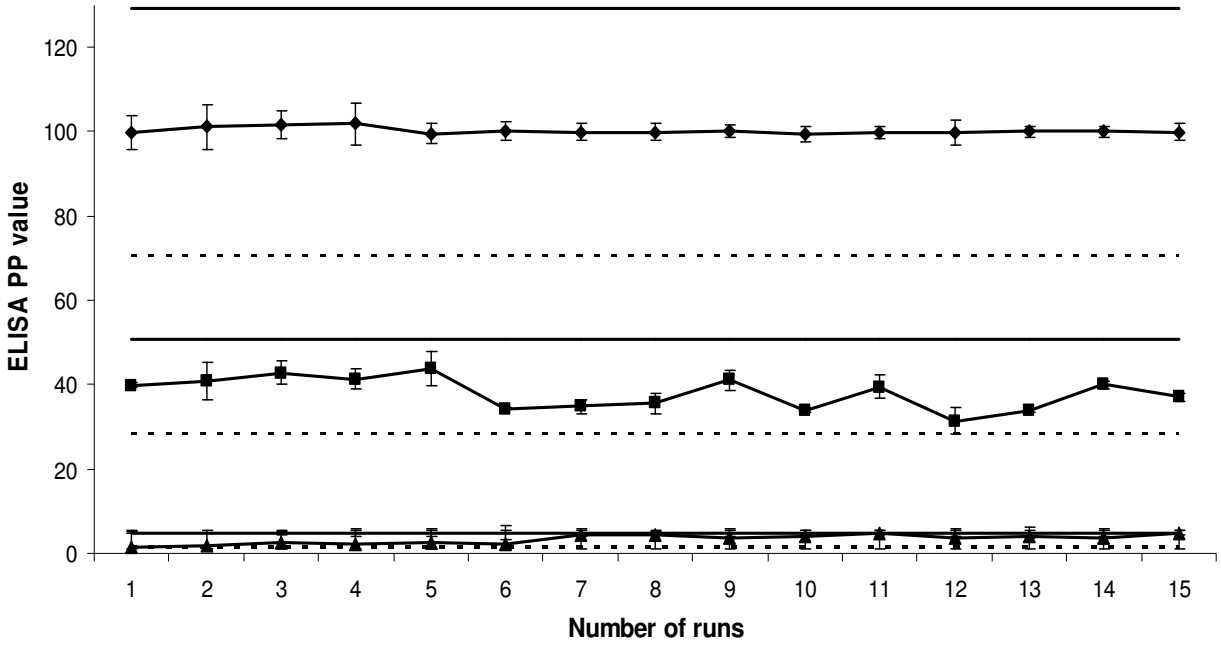


Figure 3.1. Upper (□) and lower (---) internal quality control limits for PP values of high-positive (♦), low-positive (■), negative (▲) antigen controls and means ±2 S.D. for these controls during 15 routine runs of the assays over a period of 16 weeks. Two to six plates were used during each run with four replicates of high positive, two replicates of low positive and negative controls on each plate.

3.3.2 Efficiency of different inactivation buffers and safety testing

The 1% Tween-20 in PBS inactivation buffer together with 56°C for 60 minutes yielded the highest signal of antigen detection (results not shown). Mice and tissue cultures inoculated with spiked samples that were inactivated according to this method did not develop any signs of infection in the specified monitoring period, indicating that it renders samples completely safe. In comparison, spiked samples only inactivated by heat or Tween-20 separately were not rendered safe since Vero cell monolayers developed CPE and suckling mice developed typical RVFV clinical signs after inoculation.

3.3.3 Analytical detection limit, specificity and sensitivity

The sAg-ELISA was able to detect as little as 110 pg of recNP, corresponding to $10^{2.2}$ $TCID_{50}$ of RVFV per 100µl of Vero-derived infectious tissue culture supernatant (Figure 3.2). Analysis of the ELISA readings for sera and tissue homogenates spiked with different concentrations of RVFV shows

that the detection limit in most assayed samples was approximately $\log_{10}10^{2.2}$ $TCID_{50}$ per ELISA reaction volume (100µl) except for bovine liver where the detection limit was 10 times lower (Figure 3.3) at $\log_{10}10^{3.2}$ $TCID_{50}$ per ELISA reaction volume (100µl).

When testing infectious tissue culture supernatant containing related African Phleboviruses (Arumowot, Gabek Forest, Gordil and Saint-Floris) and two other members of the family *Bunyaviridae* (Akabane and Bunyamwera viruses), ELISA readings ranged from 0 to 0.63 PP (mean 0.41±0.26) (results not shown) whereas non-specific background noise of normal tissue culture fluid was 0.44 PP. These results demonstrate the highly specific binding affinity of anti-recNP RVFV polyclonal hyperimmune sheep and rabbit sera and the absence of detectable cross-reactions between these anti-sera and nucleocapsid proteins of the other Bunyaviruses assayed. All of the 17 RVFV strains were easily detected by ELISA (Table 3.1).

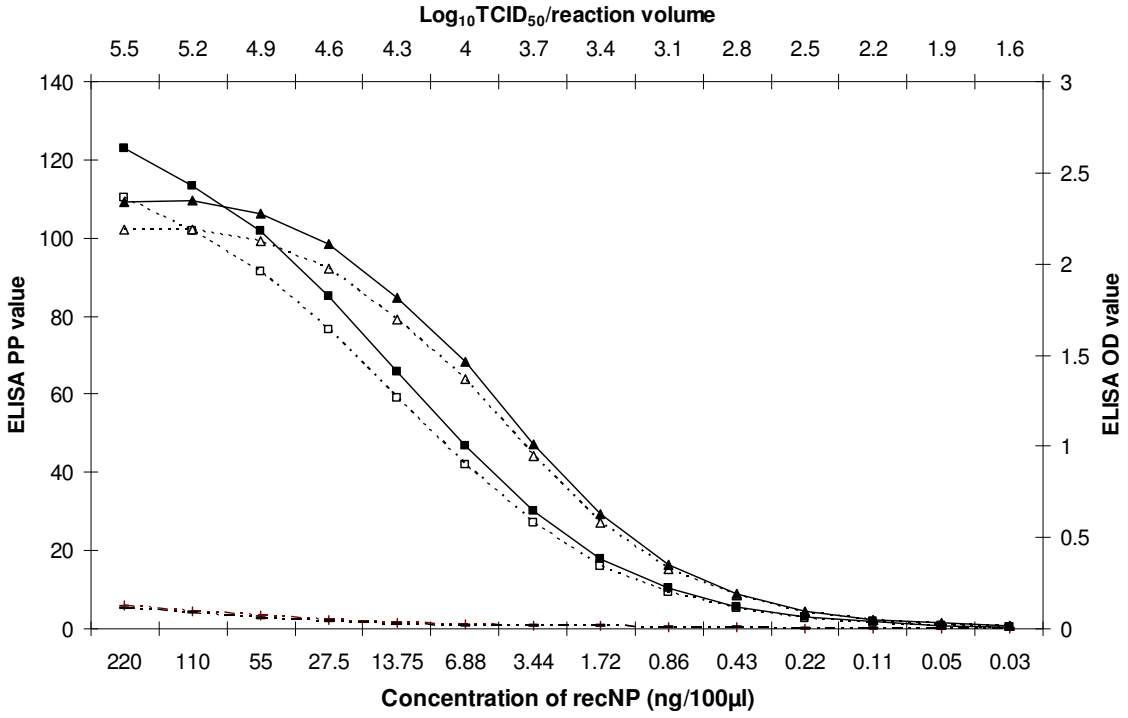


Figure 3.2. Dose response curves of recNP (PP ■, OD □), RVFV Ar20368 RSA 81 (PP ▲, OD Δ), and control antigen (PP +, OD -).

3.3.4 Monitoring viral growth *in-vitro*

The detection of antigen in infected tissue culture supernatants after specific incubation times are shown in figure 3.4. Irrespective of the inoculum used, the sAg-ELISA yielded positive results earlier than CPE was observed. For example, in the flasks inoculated with $10^{5.8}$ and $10^{0.8}$ $TCID_{50}$/ml RVFV the ELISA detected antigen 8 and 48 h after inoculation respectively, whereas CPE could only be observed 16 and 24 h later.

3.3.5 Antigen detection in decomposing tissues

The sAg-ELISA was able to detect nucleocapsid antigen equally until the last collection time (192 hours) in spiked sheep liver incubated at 37°C, whereas antigen detection ability decreased in spiked bovine liver from 168 hours onwards (Figure 3.5). In contrast the simulated decomposition of tissues resulted in rapid inactivation of infectious virus particles as shown by negative results in the same organs after 48 hours incubation at 37°C by virus titration.

3.3.6 Diagnostic cut-off values and accuracy

The effect of three differently determined cut-off values on the estimates of diagnostic sensitivity, specificity, and efficiency of the sAg-ELISA in human and sheep sera are given in Table 3.3. The highest diagnostic accuracy for human and sheep serum data sets was achieved when threshold values (5.6 PP and 1.23 PP) determined as mean plus 3 S.D. were used. However, estimates of the assay diagnostic performance based on cut-off determined as mean plus 2 S.D or derived from the TG-ROC analysis were similar (Table 3.3). In ELISA positive human sera at the optimal cut-off, mean $TCID_{50}$/ml of the virus was 5.6±0.83, and in ELISA negative sera, it was 3.7±1.61. At cut-off determined as mean plus 2 S.D. derived from PP values of normal mice and ruminant tissues (2.4 PP), the sAg-ELISA had 100% sensitivity and specificity in detecting the nucleocapsid protein of RVFV in various tissues of experimentally infected mice and naturally infected buffalo foetuses (Figure 3.6).

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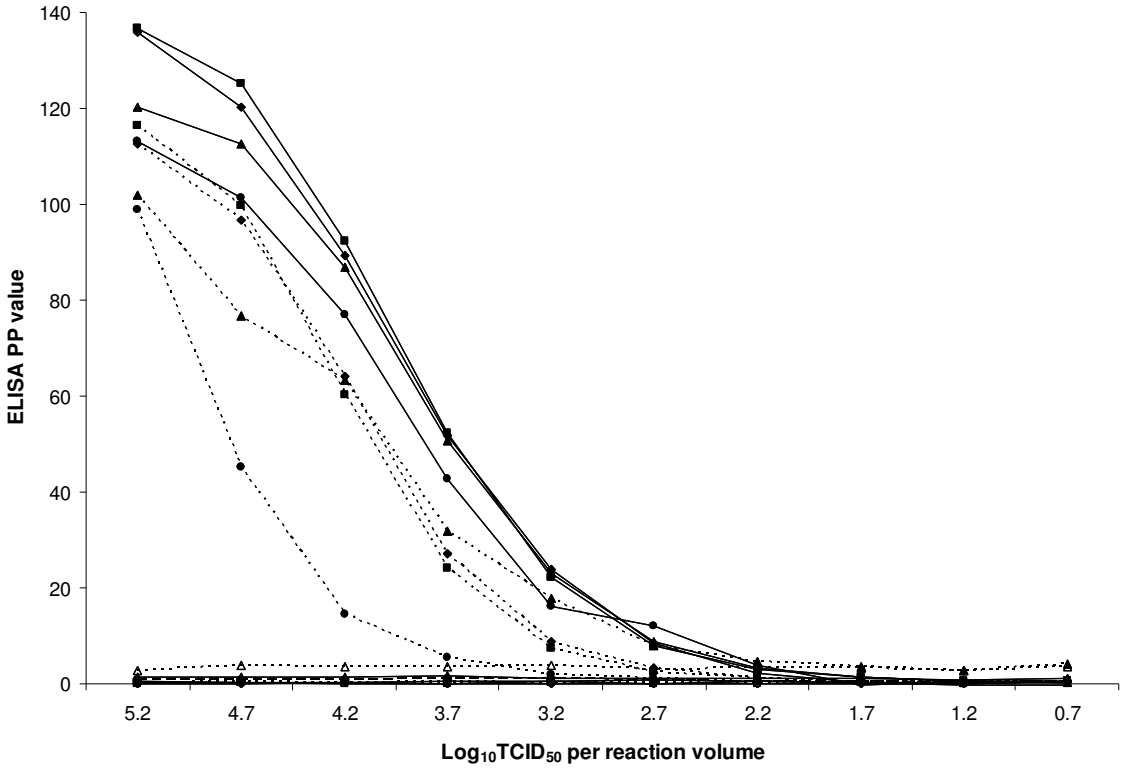


Figure 3.3. Dose response curves of human and animal sera, animal tissue and mosquito homogenates spiked with RVFV and their corresponding non-spiked controls. Virus-spiked samples: human (—●—), sheep (- -♦- -), bovine (- -■- -) serum; sheep (—♦—), bovine (—■—) spleen; sheep (--▲--), bovine (--●--) liver; Anopheles mosquito (—▲—). Uninfected samples: human (—○—), sheep (- -◊- -), bovine (- ǂ -) serum; sheep (—◊—), bovine (————) spleen; sheep (--Δ--), bovine (--○--) liver; Anopheles mosquito (—Δ—).

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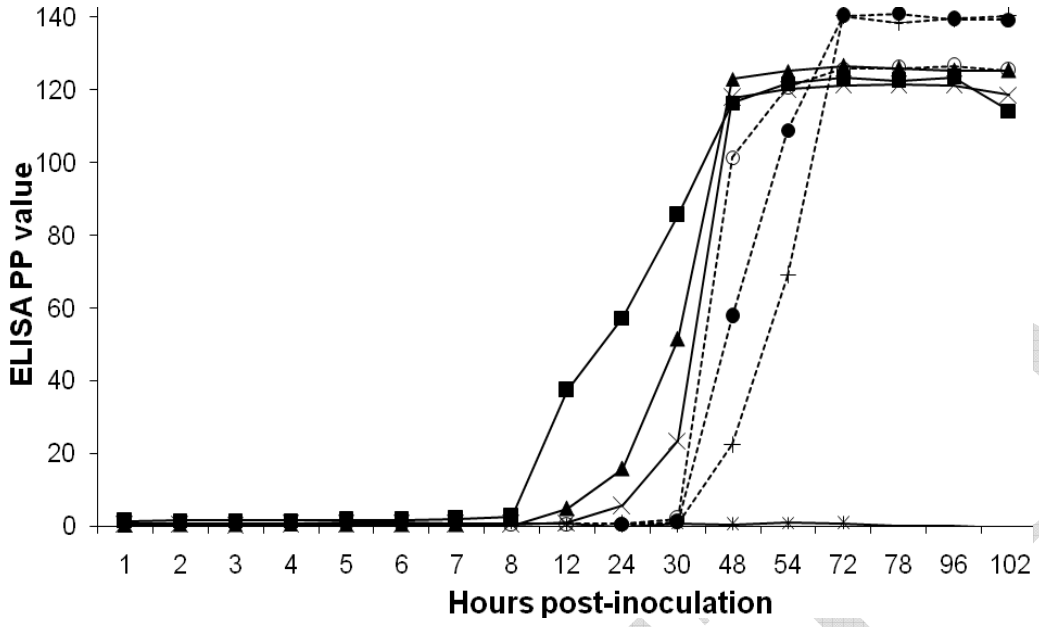


Figure 3.4. RVFV replication kinetics in Vero cells inoculated with different concentrations of the virus measured by antigen detection ELISA. $Log_{10}TCID_{50}$ virus concentrations in 1 ml of inoculum were $10^{5.8}$ (■), $10^{4.8}$ (▲), $10^{3.8}$ (x), $10^{2.8}$ (○), $10^{1.8}$ (●) and $10^{0.8}$ (+), mock control (*).

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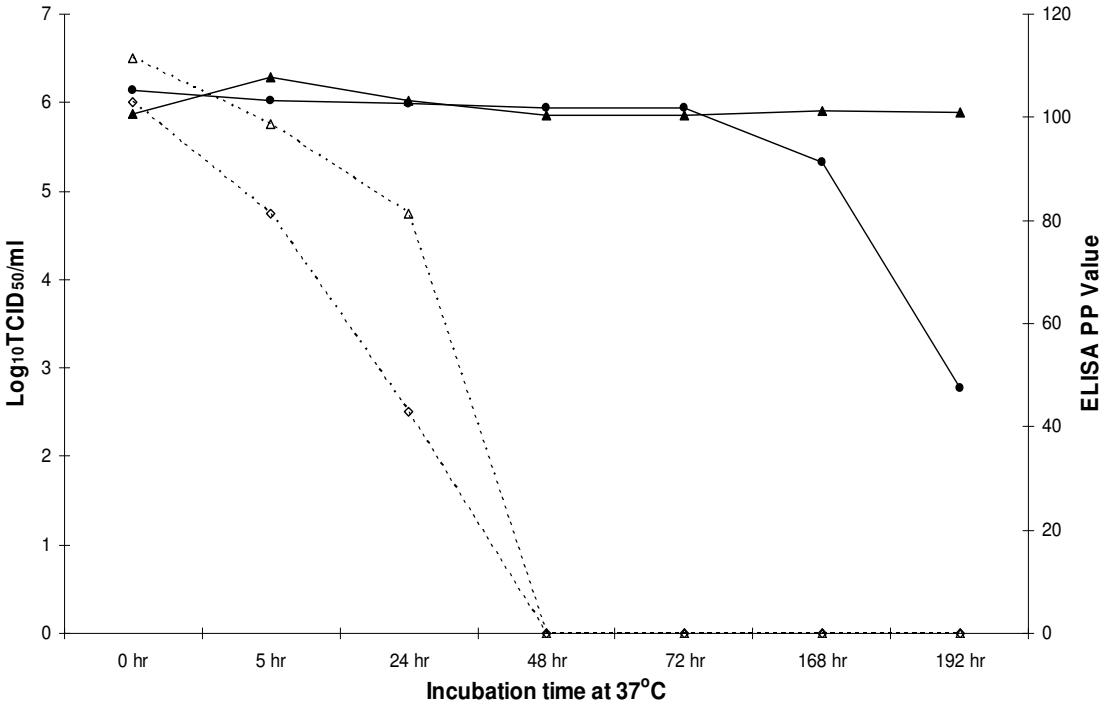


Figure 3.5. Detection of infectious virus and viral nucleocapsid antigen in tissue homogenates spiked with RVFV followed by incubation at 37°C. $TCID_{50}$/ml in bovine (○) and sheep (Δ), and ELISA readings in bovine (●) and sheep (▲) liver homogenates.

Table 3.3. Diagnostic accuracy of the sAg-ELISA for the detection of nucleocapsid protein of RVFV in sheep and human sera

Measure[a]	Human sera – cut-off (PP)[b] V^{+c} = 31, V^{-d} = 99			Sheep sera – cut-off (PP)[b] V^{+c} = 20, V^{-d} = 85		
	1.96[e]	4.11[f]	5.60[g]	0.86[e]	0.91[f]	1.23[g]
D-Se (%)[h]	77.4	67.7	67.7	70.0	70.0	70.0
D-Sp (%)[i]	81.8	95.95	97.97	97.65	97.65	100.0
Efficiency (%)[j]	79.6	81.83	82.84	83.83	83.83	85.0

[a] Sera were categorized according to the results of virus isolation.
[b] Cut-off value expressed as a percentage positivity of high positive antigen control.
[c] Number of sera tested positive for RVFV.
[d] Number of sera tested negative for RVFV.
[e] Cut-off value optimised by the misclassification cost term option of the two-graph receiver operating characteristics analysis at 95% accuracy level.
[f] Cut-off value determined as mean plus two standard deviation derived from PP values in RVFV-negative sera.
[g] Cut-off value determined as mean plus three standard deviation derived from PP values in RVFV-negative sera.
[h] Diagnostic sensitivity.
[i] Diagnostic specificity.
[j] Efficiency.

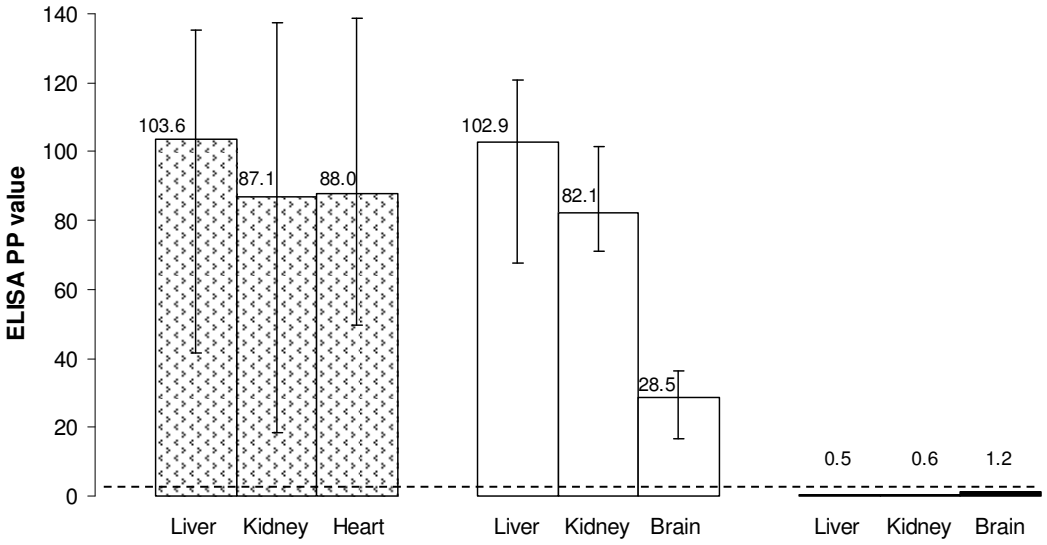


Figure 3.6. Detection of nucleocapsid protein by ELISA in RVFV-infected buffalo foetus (▒) and mouse organ tissues (□). Non-infected mouse organ tissues (■) are included as a control. Mean PP values were determined for the liver, heart and kidney tissues of the three infected buffalo foetuses. Mean PP values were determined for the liver, brain and kidney tissues of three infected mice. Control PP values were obtained from one uninfected mouse. Cut-off of 2.4 PP (---) was determined as the mean plus 2 S.D. of PP values in uninfected mouse and ruminant organ tissues.

3.3.7 Interference on antigen detection in serum by the presence of anti-RVFV antibodies

The ELISA yielded negative results in sera taken from an experimentally infected sheep on day 5 and onwards post infection (p.i.) despite relatively high levels of viremia detected on days 5 and 6 p.i. The negative results coincided with the appearance of the first detectable anti-nucleocapsid IgM and IgG antibody on day 5 p. i. (Figure 3.7). To confirm the blocking effect of RVFV-immune sera in the sAg-ELISA, viremic sheep serum was mixed with an increasing concentration of known sheep immune serum. The inhibitory effect of increasing levels of specific antibodies on the ELISA specific signal in highly viremic sheep serum is shown in Figure 3.8.

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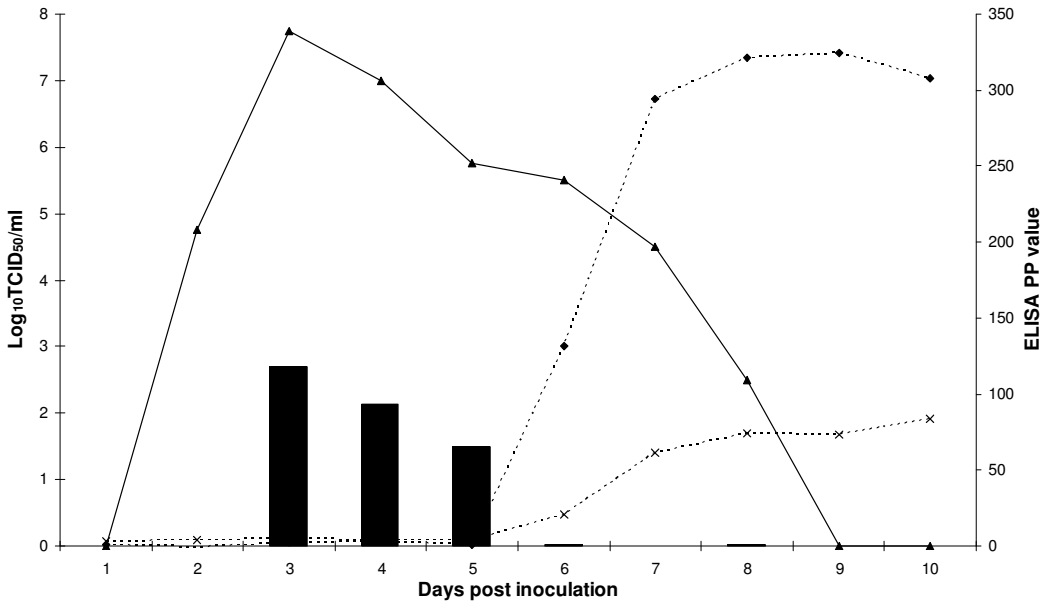


Figure 3.7. Monitoring of viremia (▲), antigenemia (■), anti-nucleocapsid IgM (♦) and IgG (x) responses in a sheep experimentally infected with RVFV.

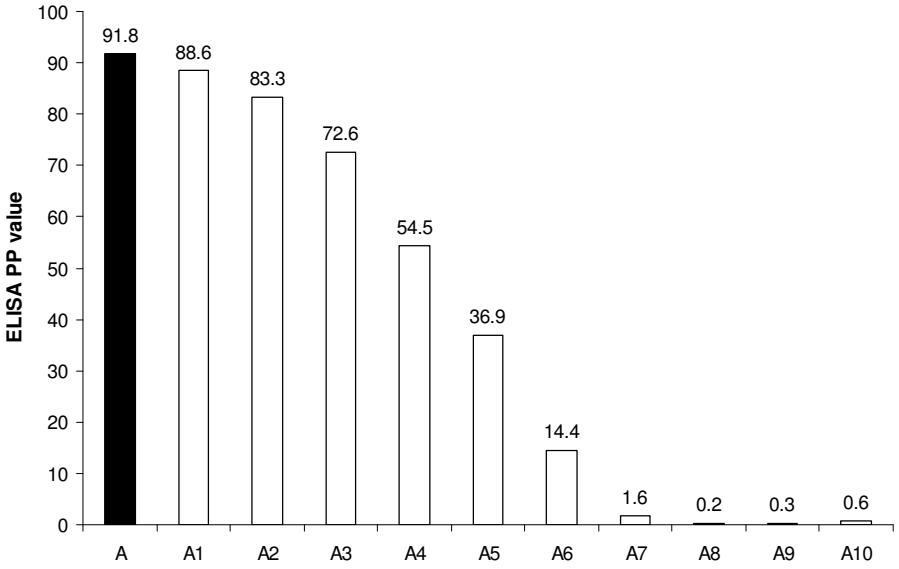


Figure 3.8. Blocking effect of increasing levels of anti-RVFV specific antibody on antigen detection in viremic sheep serum. Viremic serum free of antibody against nucleocapsid of RVFV (A, ■). Mixture (v/v) of viremic serum and immune serum with two-fold increase of anti-RVFV specific antibodies (A1- 1:1024 to A10-1:2, □).

3.4 Discussion

A sandwich ELISA using hyperimmune mouse and rabbit antisera reported by Niklasson et al. (1983) had a sensitivity of 10^5 plaque forming unit (PFU)/ml in detecting RVFV in supernatant fluids from infected Vero cell cultures. However, marked differences between levels of antigen and the virus infectivity in experimentally infected hamsters and rhesus monkeys were noted. While ELISA could reliably detect 10^6 PFU/ml of virus in viremic hamsters, rhesus monkeys with viremia of $10^{3.4}$ PFU/ml tested positive (Niklasson et al., 1983). A sandwich ELISA utilizing a biotin-avidin labelled mouse monoclonal antibody as a detector system had a sensitivity of 29.3% in viremic human sera collected during the 1987 RVF epidemic in West Africa (Meegan et al., 1989). This estimate appears to be rather low compared to 76.9% sensitivity of the assay reported by Niklasson et al. (1983) in sera from experimentally infected rhesus monkeys. Viral titres were demonstrated to be significantly correlated with quantity of viral antigen as measured by ELISA in orally infected Egyptian *Culex pipiens* (Niklasson and Gargan, 1985). Moreover, when comparing results of the infectivity assay with the ELISA, the latter had similar sensitivity (100% vs. 93%) and specificity (94% vs. 94%) in detecting mosquitoes capable of transmitting virus to susceptible hamsters. These early studies clearly demonstrated potential field applications of a sandwich ELISA, however, its wider use for routine laboratory detection of RVFV have been limited due to a number of considerations: testing of RVFV-infected specimens by ELISA pose laboratory biohazards because of intensive pipeting and washing procedures, the use of OD readings for interpretation of ELISA results is not currently recommended, lack of non-infectious and well-characterized internal antigen controls hampered adequate standardization, and evaluation of its performance within and between laboratories. Recently immunofluorescence assays which utilize a pool of mouse IgG monoclonal conjugates reacting with a combination of virus specific antigens (Gs, Gn, N, NSs) were reported (Zaki et al., 2006). Although it was demonstrated to be highly reliable in detecting RVFV in patient sera, its use requires tissue culture amplification and handling of live virus.

ELISA offers an affordable and simple alternative to traditional and molecular techniques for detection of RVFV, but as an open bench system might contribute to laboratory infections when samples containing live virus are analysed. A number of laboratory infections with RVFV were recorded under circumstances which indicate the virus to be highly infectious for man (Findlay, 1932, Kitchen, 1934, Smithburn, 1949, Smithburn et al., 1949b). To address this problem, a sandwich ELISA based on an entirely safe procedure was developed, including a set of internal controls based on recNP protein for monitoring of assay routine performance which increases its utility in surveillance and diagnosis in non-endemic areas.

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RVFV has been shown to be extremely stable when stored in infected plasma at low temperature but is unstable at higher temperatures. For example, infected sheep plasma retained infectivity after 8 years of cold storage (Easterday, 1965) but viremic blood became non-infective after 40 minutes of incubation at 56˚C in phosphate buffer, pH 7.2 (Findlay, 1932), contrary to the results presented in this chapter. The presence of lipid bilayer in the virus envelop makes it highly sensitive to lipid solvents (Bishop et al., 1980). The simple and inexpensive thermo-chemical inactivation procedure used in this study effectively inactivated RVFV present in test specimens as demonstrated by negative virus isolation results *in vivo* and *in vitro* systems. Using a similar procedure West Nile virus can be successfully inactivated at 37˚C for 30 minutes in the presence of 0.05% Tween-20 (Mayo and Beckwith, 2002). In addition to effective virus killing, the inactivation protocol used increases ELISA specific signal compared to that in non-inactivated specimens. This is likely the result of enhanced trapping of nucleocapsid protein by capture antibody in sAg-ELISA after viral envelope had been disrupted by Tween-20. The analytical sensitivity of $\log_{10}10^{3.2}$ $TCID_{50}$/ml determined by testing infective supernatant of Vero cell culture fluid seems to be at least 10-fold higher compared to that reported by Niklasson et al., (1983). Using different concentrations of RVFV we demonstrated that the NP antigen can be detected in supernatant fluids from infected Vero cell cultures as early as 8 h post inoculation and 12 to 30 h before CPE could be microscopically observed. This ability renders the assay very useful for rapid identification of RVFV when primary isolation from clinical specimens is attempted *in vitro*. Live virus became undetectable much earlier than the nucleoprotein by sAg-ELISA when incubated in animal tissues at adverse temperature. This ELISA can therefore be used to diagnose RVF by using decomposing tissues of ruminants that might have been found dead in the field.

Analytical detection limit established in virus-spiked samples which mimicked diagnostically relevant submissions, was the same except in bovine liver homogenate for which it was 10 times less ($\log_{10}10^{4.2}$ $TCID_{50}$/ml). An estimate of ELISA diagnostic sensitivity derived from results in viremic human sera was much higher (67.7%) compared to that (29.3%) reported by Megan et al. (1989) but similar (76.9%) to that in viremic rhesus monkeys sera (Niklasson et al., 1983). Diagnostic accuracy of a sandwich ELISA in viremic human sera but also in other specimens is likely to be dependent on a number of factors, including origin and type-specificity of capture and trapping antibody, their purity (monoclonal vs. polyclonal), titres and spectrum of reactivity to RVFV structural proteins (Meegan et al., 1989).

Serum specimens are commonly used for RVF diagnosis. Viremia titres ranging from $10^{5.6}$ to $10^{9.0}$ of mouse median lethal doses per ml have been recorded in domestic ruminants (Daubney et al., 1931, Barnard and Botha, 1977, Harrington et al., 1980, Swanepoel et al., 1986a, Morrill et al., 1987), $10^{8.6}$ in humans (Peters and Meegan, 1981) and $10^{5.4}$ $TCID_{50}$/ml in adult African buffalo (Davies and

Karstad, 1981). Although viremia in RVFV-infected individuals reaches high titres, it is of short duration which limits the application of viral detection systems for RVF diagnosis when using blood samples. Moreover, results obtained in serial sera from an experimentally infected sheep and ELISA blocking experiments indicate that the appearance of specific antibodies during viremia hampers the assay results despite the presence of relatively high concentrations of the virus. These findings seem to indicate that differences in ELISA diagnostic performance might also be due to variations in immune status among viremic individuals at the time of sampling. Therefore, attempts to detect recent RVFV infection by ELISA should include a combination of assays which target both viral antigens and IgM antibody. It should be noted that high viremia frequently occurs in the absence of severe illness. Consequently, in the absence of noticeable clinical signs and adequate diagnostic procedures, considerable geographic dispersal of RVFV might occur before an outbreak is recognized (McIntosh et al., 1973). On the other hand, the South African outbreaks of 1950-51 and the Egyptian outbreaks of 1977-78 were not recognized as RVF until several months had elapsed with deaths of thousand of animals, and, in the Egyptian outbreaks, many deaths in humans. Delays in recognition of these outbreaks occurred because the disease was previously unknown in those geographical areas and the possibility of RVF was not at first considered.

In this study very high estimates of diagnostic accuracy (100%) where obtained when testing various tissue homogenates of experimentally infected mice and naturally infected African buffalo foetuses. RVFV can persist at high titres for 21 days in ovine brain and liver, and up to 30 days in spleen (Yedloutschnig et al., 1981). High diagnostic accuracy of the sAg-ELISA in detection of RVFV in infected tissues which usually contain virus concentrations at least 10 to 100-fold times above (Easterday et al., 1962, Easterday and Murphy, 1963, Harrington et al., 1980, Morrill et al., 1987) the detection limits determined in this study, indicate that the assay will be highly reliable for testing specimens from aborted fetuses and fatal cases. Massive abortion and high fatality rates in young animals are one of the characteristic features of RVF outbreaks. The ELISA format reported here allows for assaying relatively large numbers of specimens within a short period of time. The assay throughput, if required, could be easily increased by using semi- or fully automated ELISA workstations.

The ability of a diagnostic assay to produce consistent results within the tolerable analytical error limits is one of requirements for any diagnostic device to be accepted for routine applications. While the antigen internal controls based on the recombinant NP protein achieved very high repeatability estimates within the IQC limits, the reproducibility of the sAg-ELISA remains to be addressed for more comprehensive inter-laboratory evaluation. Antibody- and antigen-binding levels should be expressed in relative rather than absolute terms. One of the advantages of converting ELISA

OD data into PP values relative to a known standard is that this method does not assume uniform background activity, and therefore enables inter-laboratory standardisation (Wright et al., 1993).

The polyclonal hyperimmune anti-recNP rabbit and sheep antisera did not cross-react with other members of the *Bunyaviridae* family, including four African *Phleboviruses* tested in this study. No cross-reactivity with other members of the sand fly fever virus group, including sand fly Naples, sand fly Sicilian, Arumowot, Punta Toro, Gordil, Karimabad, Gabek Forest, and Saint Floris, was detected in sandwich ELISA using hyperimmune mouse and rabbit antisera by Niklasson et al. (1983). The nucleocapsid protein appears to be highly conserved among members of the *Bunyaviridae* family (Swanepoel et al., 1986a, Vapalahti et al., 1995, Schwarz, 1996, Magurano and Nicoletti, 1999, Gauliard et al., 2006) and antigenic cross-reactivity studies in animals (Davies, 1975, Swanepoel, 1976, Swanepoel et al., 1986b) and indirect ELISA based on recNP protein (Paweska et al., 2007) failed to provide any evidence that other African phleboviruses could obscure the reliable serodiagnosis of RVF. The sAg-ELISA detected nucleocapsid proteins of a wide range of geographically distinct RVFV isolates collected over 53 years, which represent three major lineages of the virus, namely Egyptian, Western African, and Central, Eastern and Southern African. These results are expected since the RVFV genome, and especially the gene encoding the N protein, is highly conserved (Bird et al., 2007b).

In conclusion the sAg-ELISA procedure developed and evaluated in this study is safe, highly accurate in detection of RVFV NP antigen in diagnostically relevant concentrations, rapid and robust and therefore can be utilized in diagnosis and surveillance in both endemic and non-endemic RVF areas. It offers a less complicated alternative to nucleic acid techniques when large numbers and clinical variety of specimens have to be tested in a short period of time.

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CHAPTER FOUR

EVALUATION OF A RECOMBINANT NUCLEOCAPSID PROTEIN AS AN IMMUNOGEN IN A MOUSE MODEL*

* Partially published as:

Jansen van Vuren, P., Tiemessen, C.T. & Paweska, J.T. (2010). Evaluation of a recombinant Rift Valley fever virus subunit nucleocapsid protein as an immunogen in mice and sheep. *The Open Vaccine Journal* **3**, 114-126

* Partially presented at international conferences as:

Jansen van Vuren, P. & Paweska, J.T. (2009). Preliminary evaluation of a recombinant Rift Valley fever virus nucleocapsid protein as an immunogen in combination with different adjuvants in mice and sheep. *FAO/IAEA International Symposium on Sustainable Improvement of Animal Production and Health*, Vienna, Austria, 8 – 11 June 2009. (Poster presentation).

Jansen van Vuren, P., Tiemessen, C.T. & Paweska, J.T. (2009). Evaluation of a recombinant Rift Valley fever virus nucleocapsid protein as a vaccine immunogen in combination with four adjuvants. *International Meeting on Emerging Diseases and Surveillance*, Vienna, Austria, 13 – 16 February 2009. (Poster presentation).

4.1 Introduction

Until recently there were only two vaccines commercially available for use in livestock: a live attenuated vaccine based on the Smithburn strain and a formalin inactivated vaccine – both available exclusively available from Onderstepoort Biological Products (Pretoria, South-Africa). However, Clone 13 was very recently commercialized by Onderstepoort Biological Products and widely used for the vaccination of livestock in South Africa during the 2010/2011 RVF season (Paweska, J.T., personal communication)(Dungu et al., 2010). There are no commercially available RVF vaccines for human use, but an experimental inactivated vaccine has been used in the past to vaccinate veterinarians, scientists and other personnel at risk of exposure (Bouloy and Flick, 2009). Because limited amounts of this vaccine (TSI-GSD 200) were produced under strict quality controlled conditions at the USAMRID facility, it is currently in short supply and very expensive. As discussed in the literature review (Chapter 1), various vaccine candidates generated by classical virological methods have been evaluated to counter this problem. These, however, are expensive, laborious to produce and not completely safe to use.

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Recently molecular biological methods enabled research into safer, more effective and less expensive RVFV vaccine candidates. These include attenuated viruses generated by reverse genetics (Bird et al., 2008, Habjan et al., 2008b), DNA vaccines (Spik et al., 2006, Lorenzo et al., 2008, Lagerqvist et al., 2009), virus vectored vaccines (Collett et al., 1987, Wallace and Viljoen, 2005, Wallace et al., 2006, Heise et al., 2009, Soi et al., 2010), virus like particles (Habjan et al., 2009a, Mandell et al., 2009, Naslund et al., 2009, Pichlmair et al., 2010) and recombinant subunit vaccines (Collett et al., 1987, Schmaljohn et al., 1989, Wallace et al., 2006). Most of these constructs are aimed at inducing immunity against the glycoproteins that carry neutralizing determinants, with the exception of a vaccine candidate evaluated by Wallace et al. (2006) which consisted of a preliminary experiment with a recombinant RVFV N protein, expressed as an insoluble protein. The nucleocapsid protein is the major antigen of RVFV and strong immune responses against this protein have been shown after natural and experimental infections with the virus (Fafetine et al., 2007, Jansen van Vuren et al., 2007). The 60% protection rate from lethal challenge achieved in mice in the Wallace et al. (2006) preliminary experiment with a recombinant RVFV N protein needed further investigation, especially taking into account good protection rates achieved by using the N proteins of related viruses as immunogens (Schmaljohn et al., 1990, Maes et al., 2006, Maes et al., 2008).

Recombinant protein subunits are generally weak immunogens (O'Hagan et al., 2001, Lautze et al., 2007) and require administration with adjuvants to enhance their immunogenicity (Dasgupta, 2004). Adjuvants promote the uptake of antigens by antigen presenting cells (APC), contribute to the delivery of antigen to lymph nodes, and stimulate cytokine release or expression of co-stimulatory signals on APC which are needed to prime T helper cells for B cell proliferation and induction of cytotoxic T lymphocytes (O'Hagan et al., 2001, O'Hagan and Singh, 2003). Some of the more commonly tested and/or used adjuvants are saponins, alum and water-in-oil adjuvants. Saponin adjuvant, a surface active agent isolated from the Chilean soap bark tree (*Quillaja saponaria*), modulates humoral (Th-2) as well as cellular immunity (Th-1) and biases immune responses towards the Th-1 phenotype and can induce strong CD8+ cytotoxic T-cell responses (Kensil, 1996, Cribbs et al., 2003). $CD8^+$ T cells are able to kill virus-infected cells by inducing apoptosis, and kill infected cells directly in the lymph nodes draining infected sites (Xu et al., 2007). Aluminium hydroxide gel (Alhydrogel), commonly known as alum allows for a depot effect at the inoculation site, and has also been found to promote the release of IL-4 which results in the increased expression of MHC II molecules on monocytes, consequently increasing antigen uptake by APC (Mannhalter et al., 1985, Ulanova et al., 2001, O'Hagan and Singh, 2003). Alum does not induce the cytokines IL-2 and IFN-γ which are involved in the Th-1 type response, but might directly activate NF-kB, that is involved in regulating the cellular response to infections (Ulanova et al., 2001). The NF-kB is required for positive

selection of memory $CD8^+$ T cells (Hettmann and Leiden, 2000, Hettmann et al., 2003). Montanide ISA50 adjuvant is based on a mannide oleate in mineral oil solution, and contributes to the establishment of a depot effect, transportation of emulsified antigen to distant sites through the lymphatic system, and interaction with mononuclear cells such as APC. ISA50 has been shown to direct the immune response against specific antigens towards the Th-2 type response, involved in humoral immunity (O'Hagan et al., 2001). TiterMax Gold (TMG) is a water-in-oil adjuvant that contains a metabolizable oil (squalene), sorbitan monooleate and an immunostimulatory copolymer. It has been shown to induce mixed Th-1/Th-2 responses against specific antigens, but these responses were more directed towards Th-2, indicating humoral immunity (Cribbs et al., 2003).

In this study the immunogenicity of a bacterially expressed recombinant subunit RVFV N protein was evaluated alone, and combined with four different adjuvants. The protection against subsequent viral challenge was studied in a mouse model.

4.2 Immunogenicity of the recombinant nucleocapsid protein alone and in combination with four adjuvants

4.2.1 Materials and methods

4.2.1.1 Mouse immunization

The recombinant RVFV nucleocapsid protein (recNP) was produced as described in section 2.1.2.4. Four-week old female BALB/c inbred mice were used as an experimental animal model. The low dose vaccination group (M-I) consisted of 48 mice divided in 4 sub-groups of 12 mice each which were immunized with a 100µl inoculum containing 35µg RVFV recNP in combination with ISA-50 adjuvant (Seppic, France), TiterMax-Gold adjuvant (TMG)(Sigma, U.S.A.), Alhydrogel (Sigma) or SaponinQ (60µg, Sigma), respectively. The high dose vaccination group (M-II) consisted of 48 mice which were subdivided as the M-I group but immunized with 200µl of inoculum containing 70µg of recNP in combination with the adjuvants as described above. The neat recNP group (M-N) consisted of 12 mice immunized with 70µg recNP in PBS buffer. The adjuvant control group consisted of 36 mice divided in 3 sub-groups of 12 mice each which were respectively inoculated only with ISA-50, Alhydrogel or SaponinQ. The placebo control group consisted of 12 mice which were immunized with PBS buffer.

All mice were inoculated subcutaneously (s.c) and received identical booster immunizations at 14 days after the initial immunization. A mouse from each group was sacrificed and heart-bled every seven days after primary and booster immunizations to monitor immune responses. Adjuvants ISA50, TMG and Alhydrogel were used as suggested by the manufacturers. The dose of SaponinQ adjuvant (Sigma, U.S.A.) was determined by titration in BALB/c mice and by selecting the highest non-toxic

dose at 60µg (results not shown). The selection of recNP doses were determined by recNP concentration and feasible mouse inoculum sizes.

4.2.1.2 Monitoring mouse immune responses

Immunoplates (Maxisorb, Nunc, Denmark) were coated with RVFV recNP antigen at a dilution of 1:2000 in Carbonate-Bicarbonate buffer (pH 9.6) and incubated overnight at 4ºC. After washing three times with a washing buffer consisting of PBS pH7.2 and 0.1% Tween-20, the plates were blocked with 200µl of 10% fat free milk powder ("Elite", Clover SA, Pty, Ltd.) in PBS at 37ºC for 1h and then washed as before. Test sera were diluted 1:400 in diluent buffer consisting of 2% fat free milk powder in PBS, 100µl added to each well and incubated for 1h at 37ºC. Samples were tested in duplicate for each isotype-specific HRPO conjugate used. After washing as before, 100 µl of goat anti-mouse IgG (H+L), goat anti-mouse IgG1 or goat anti-mouse IgG2a HRPO conjugate (Zymed Laboratories, Invitrogen, U.S.A.) at 1:2000 dilution was added to respective plates testing for the same serum specimens in parallel. After 1h incubation at 37ºC plates were washed as before and 100 µl of 2,2'-azinodiethylbenzthiazoline sulfonic acid (ABTS) (KPL Laboratories, Inc., USA) added to each well. After 30 min incubation in the dark the reaction was stopped by the addition of 100 µl of 1% sodium dodecyl sulphate (SDS) to each well. Optical density (OD) was determined at 405nm and the results expressed as the mean OD value for the duplicates tested.

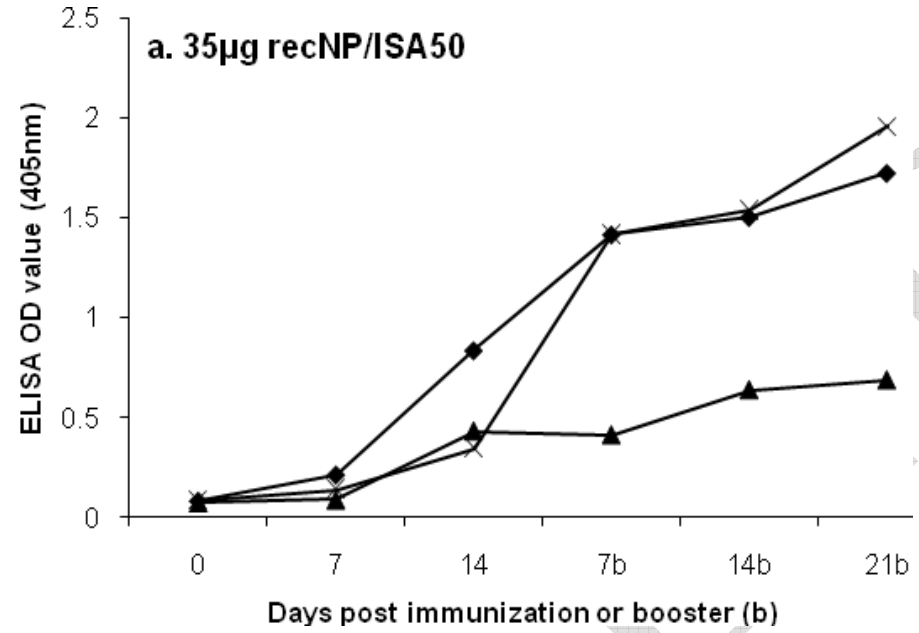
In addition, a virus neutralization test (VNT) was performed on sera collected from mice after immunization. The VNT was performed as described previously (section 2.1.2.3).

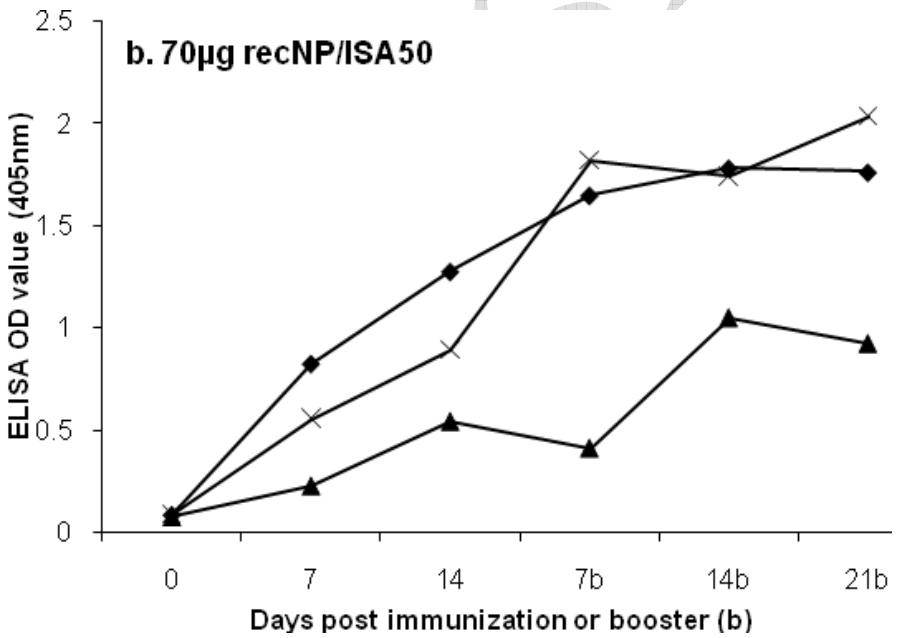
4.2.2 Results

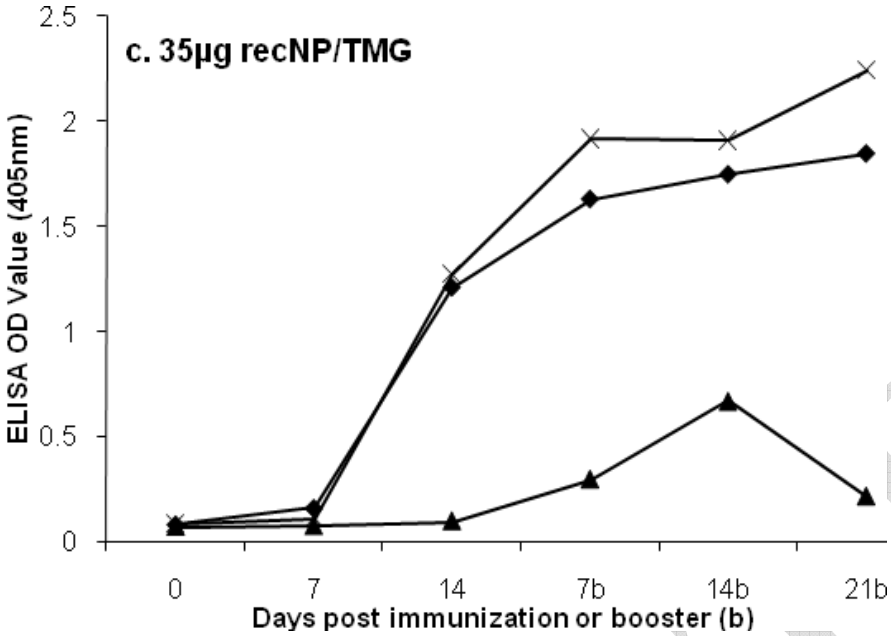
Serial cardiac bleeds taken from one mouse from each group on days 0, 7 and 14 after the primary immunization, and on days 7, 14 and 21 after the booster immunization were analyzed for the presence of total IgG, IgG1 and IgG2A antibodies anti-recNP. The representative mouse from each vaccinated group had produced detectable total IgG anti-N antibodies on day 7 after primary vaccination (Figure 4.1 a-i). The total IgG antibodies increased steadily over the monitoring period in all groups. The IgG1 isotype antibodies developed in a similar fashion to the total IgG in all groups, following the same response kinetics.

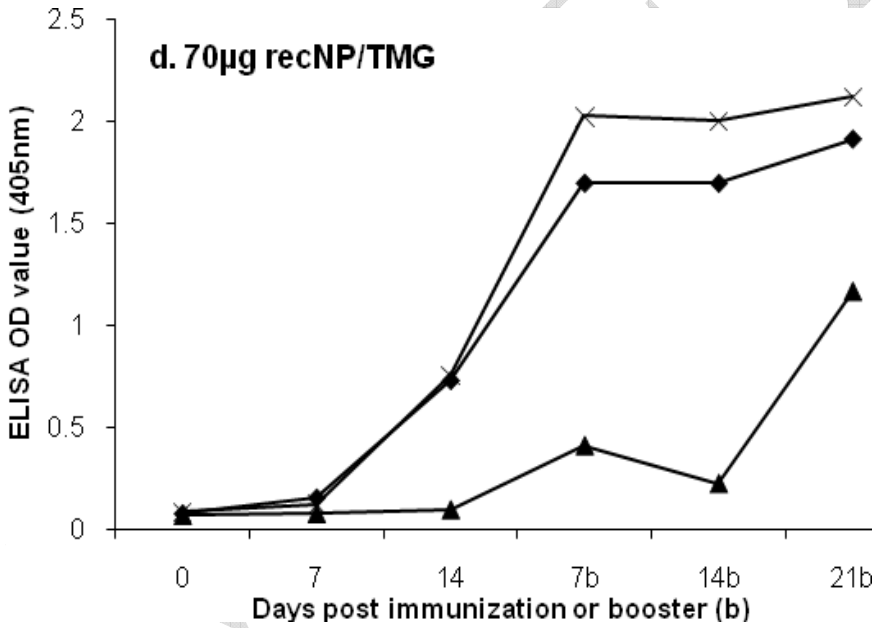
All immunized groups, except those immunized with recNP/SaponinQ combinations, developed weaker IgG2a isotype responses as compared to total IgG and IgG1. The mice from the recNP neat and recNP/Alhydrogel groups developed weak IgG2A responses as compared to IgG1 in the same mice. Only the mice from the recNP/SaponinQ and recNP/ISA50 groups had detectable anti-recNP IgG2A specific antibodies after the first immunization whereas the other groups only developed

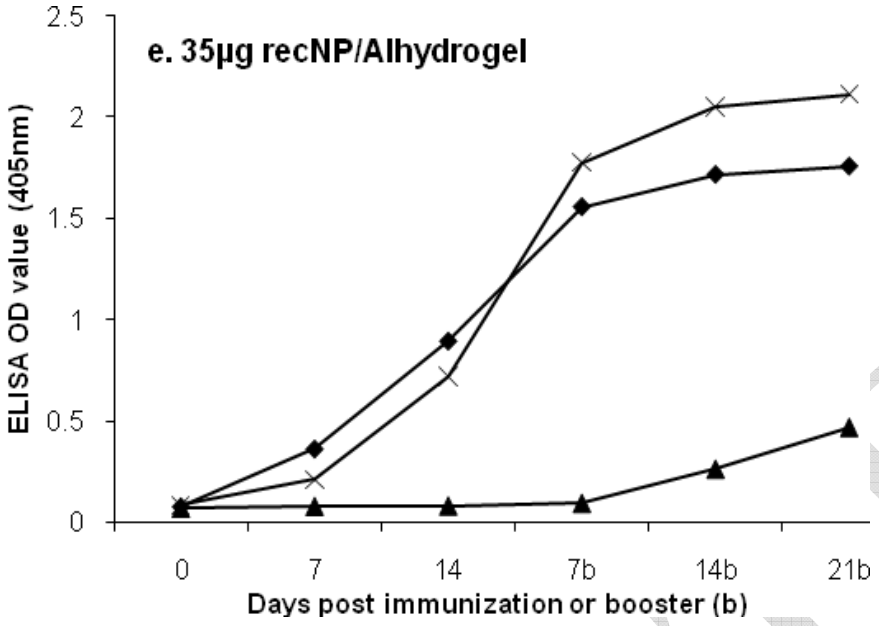
IgG2A after the booster inoculum. As expected mice from the adjuvant and placebo control groups did not develop any anti-recNP responses (results not shown). Anti-recNP immune sera from mice were not able to neutralize the virus *in-vitro* (results not shown).

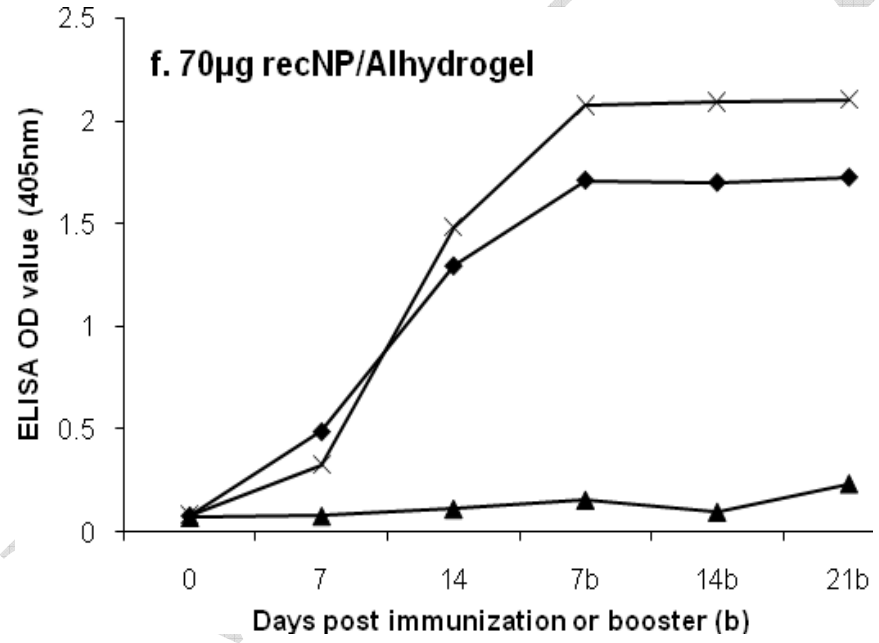


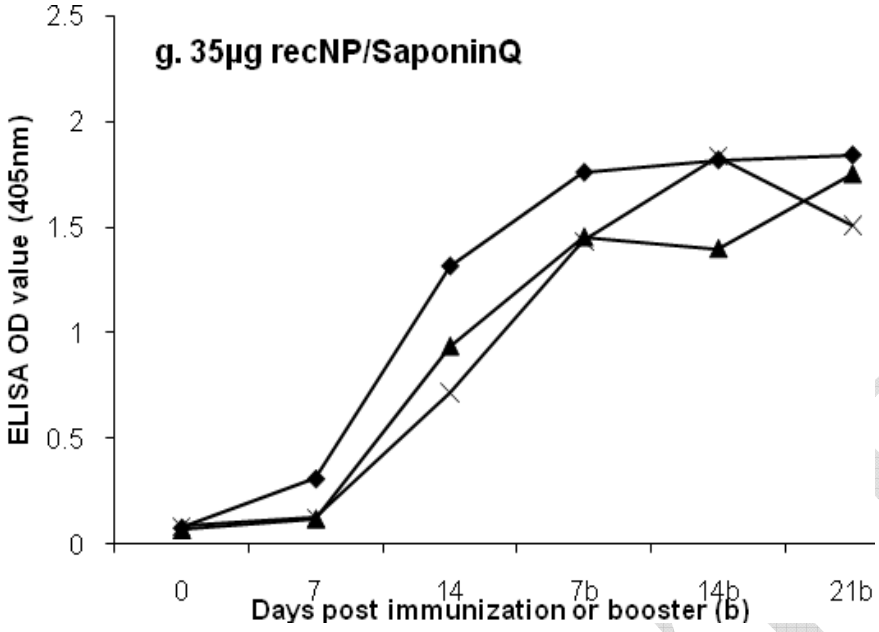


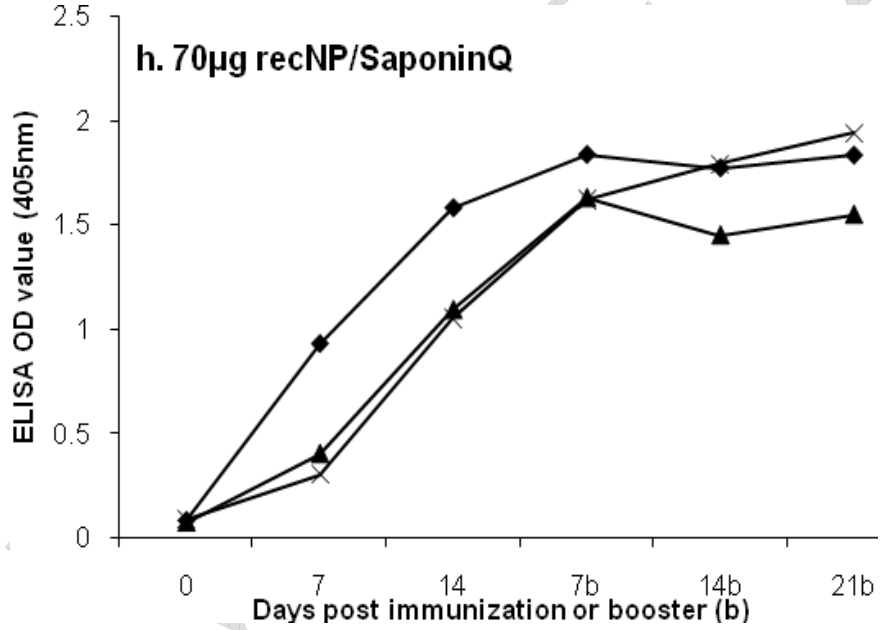

c. 35µg recNP/TMG
ELISA OD Value (405nm)
2.5
2
1.5
1
0.5
0
0
7
14
7b
14b
21b
Days post immunization or booster (b)


d. 70µg recNP/TMG
ELISA OD value (405nm)
2.5
2
1.5
1
0.5
0
0
7
14
7b
14b
21b
Days post immunization or booster (b)


e. 35µg recNP/Alhydrogel
ELISA OD value (405nm)
2.5
2
1.5
1
0.5
0
0
7
14
7b
14b
21b
Days post immunization or booster (b)


f. 70µg recNP/Alhydrogel
ELISA OD value (405nm)
2.5
2
1.5
1
0.5
0
0
7
14
7b
14b
21b
Days post immunization or booster (b)


g. 35µg recNP/SaponinQ
ELISA OD value (405nm)
2.5
2
1.5
1
0.5
0
0
7
14
7b
14b
21b
Days post immunization or booster (b)


h. 70µg recNP/SaponinQ
ELISA OD value (405nm)
2.5
2
1.5
1
0.5
0
0
7
14
7b
14b
21b
Days post immunization or booster (b)

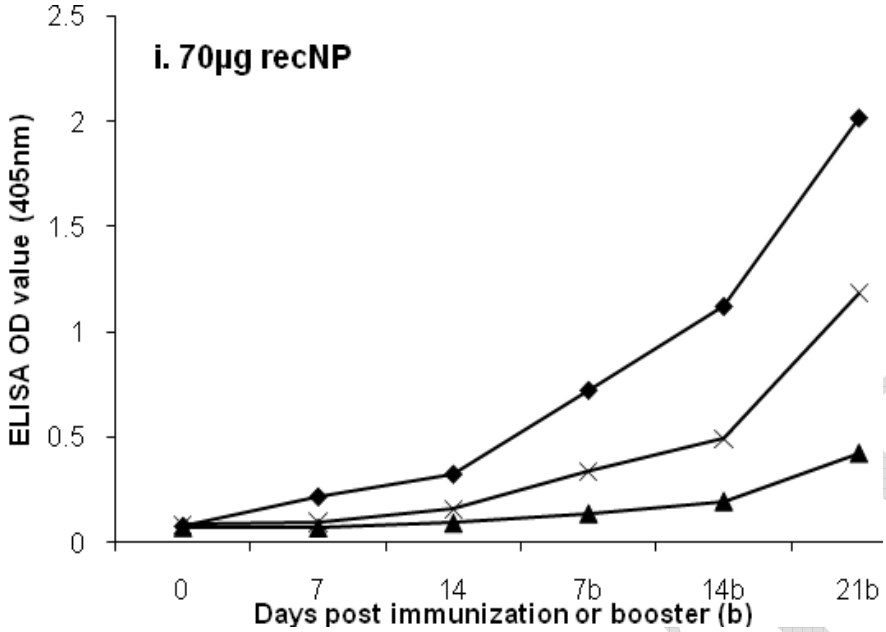


Figure 4.1(a-i). Detection of total IgG (─♦─), IgG1 (─x─) and IgG2A (─Δ─) isotype antibodies against the RVFV recNP in mice after recNP immunization alone or in combination with adjuvants. Individual mice were tested at each time point so means and standard deviations could not be calculated.

4.2.3 Discussion

The nucleocapsid protein induces production of high levels of anti-NP specific IgG and IgM responses in host animals after natural or experimental infection (Fafetine et al., 2007, Jansen van Vuren et al., 2007). The RVFV recNP used in this study easily detects anti-NP antibodies in previously infected individuals (Jansen van Vuren et al., 2007) and it was therefore assumed that it would be immunogenic. The recNP was indeed immunogenic in BALB/c mice, even in the absence of adjuvant, but more immunogenic with adjuvant. With all recNP/adjuvant combinations the total IgG responses to immunization were similar, but delayed and lower without adjuvant. The IgG1 isotype antibodies followed similar kinetics to total IgG in all recNP/adjuvant immunized mice, indicating strong humoral Th-2 response activation by all adjuvants. Only the recNP/SaponinQ combination, irrespective of recNP dose, induced strong IgG2A isotype responses comparable to their IgG and IgG1 responses, indicating strong activation of cellular Th-1 immunity by SaponinQ adjuvant. The combination of recNP with Alhydrogel was unable to generate a strong IgG2A response, indicating that this adjuvant might not induce strong cellular Th-1 immunity.

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Interferon-gamma (IFN-γ) and interleukin-2 (IL-2) are two cytokines known to be involved in the Th-1 type cellular response resulting in increased IgG2A responses. Interferon gamma (IFN-γ) is secreted by natural killer cells (NK) and $CD8^+$ cytotoxic T lymphocytes amongst others and has direct antiviral activity but also acts as an immunoregulatory factor. Interleukin 2 (IL-2) is secreted by T helper cells (Th) and acts by stimulating the growth, differentiation and survival of antigen specific $CD8^+$ cytotoxic T lymphocytes (Kensil, 1996, Cribbs et al., 2003).

The fact that there are no known neutralizing epitopes on the RVFV N protein suggests that a strongly biased humoral response against the N protein would not play a role in protection against viral challenge. Therefore it would seem that only recNP/adjuvant combinations that induced strong cellular Th-1 immunity would confer strong protection against RVF viral challenge. The lack of *in vitro* neutralizing ability of the anti-NP response has been shown before (Lorenzo et al., 2008, Lagerqvist et al., 2009) and confirmed in this study. To evaluate whether humoral antibodies play any role *in vivo* in protection against infection, the *in vivo* neutralizing ability of anti-recNP hyperimmune sera was tested in mice (see section 4.3).

4.3 *In vivo* neutralizing ability of anti-nucleocapsid immune sera in mice

4.3.1 Materials and methods

4.3.1.1 Cells and virus

Vero cells were cultivated in Eagles Minimal Essential Medium (EMEM) (BioWhitaker, MD, USA) containing L-Glutamine, non-essential amino acids, antibiotics (100 IU penicillin, 100 µg streptomycin and 0.25 µg amphotericin B) and 10% foetal bovine serum (Gibco) and maintained at 37ºC in 5% CO_2 incubator. The SPU22/118 KEN 07 strain of RVFV was isolated from a RVF human case during the 2007 Kenyan epidemic. Second passage of the virus, propagated in Vero cells, was used for the challenge.

4.3.1.2 Inoculation of mice with virus/hyperimmune sera mixtures

The ability of anti-recNP antibodies to passively confer immunity was evaluated using polyclonal antisera generated in sheep, rabbits and mice. Mice were immunized with recNP as described 4.2.1.1, and antisera from different recNP/adjuvant experimental groups were respectively pooled before testing. Polyclonal anti-recNP antisera in rabbits and sheep were produced as described previously (Chapter 3, section 3.2.1). All polyclonal sera were mixed to a final dilution of 1:10 with Vero-derived virus preparation containing $10^{7.0}$ $TCID_{50}$/ml of the 2007 Kenya RVFV isolate, and the mixture incubated at 37ºC for 30 min before inoculation. As controls, sera from naïve sheep, rabbits

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and mice were mixed identically with RVFV. To control the effects of non-related compounds in serum, sterile PBS was mixed to a 1:10 with the virus. A total of 42 BALB/c 3-4 weeks old female mice, were divided into groups of 6 animals each and inoculated s.c. with 200 µl of the following mixtures: a) virus and mouse anti-recNP, b) virus and sheep anti-recNP, c) virus and rabbit anti-recNP, d) virus and naive mouse serum, e) virus and naïve sheep serum, f) virus and naïve rabbit serum, and g) virus and PBS. Mice were examined twice daily clinically and those displaying severe signs of illness were euthanized. Surviving mice were monitored for 22 days post infection.

4.3.1.3 Statistical methods

Survival proportions in mice receiving virus/hyperimmune sera mixtures versus control mice after challenge were compared using Fisher's exact test (Soper, 2009).

4.3.2 Results

Anti-recNP immune sera did not neutralize virus *in-vivo* (Figure 4.2). No significant decrease in mortality/morbidity could be shown in any of the groups: a\ virus and mouse anti-recNP (survival 1/6, 17%, p = 0.500), b\ virus and sheep anti-recNP (survival 2/6, 33%, p = 0.227), c\ virus and rabbit anti-recNP (survival 0/6, 0%, p = 1.000), d\ virus and naive mouse serum (survival 0/6, 0%), e\ virus and naïve sheep serum (survival 0/6, 0%) f\ virus and naïve rabbit serum (survival 0/6, 0%), and g\ virus and PBS (survival 0/6, 0%).

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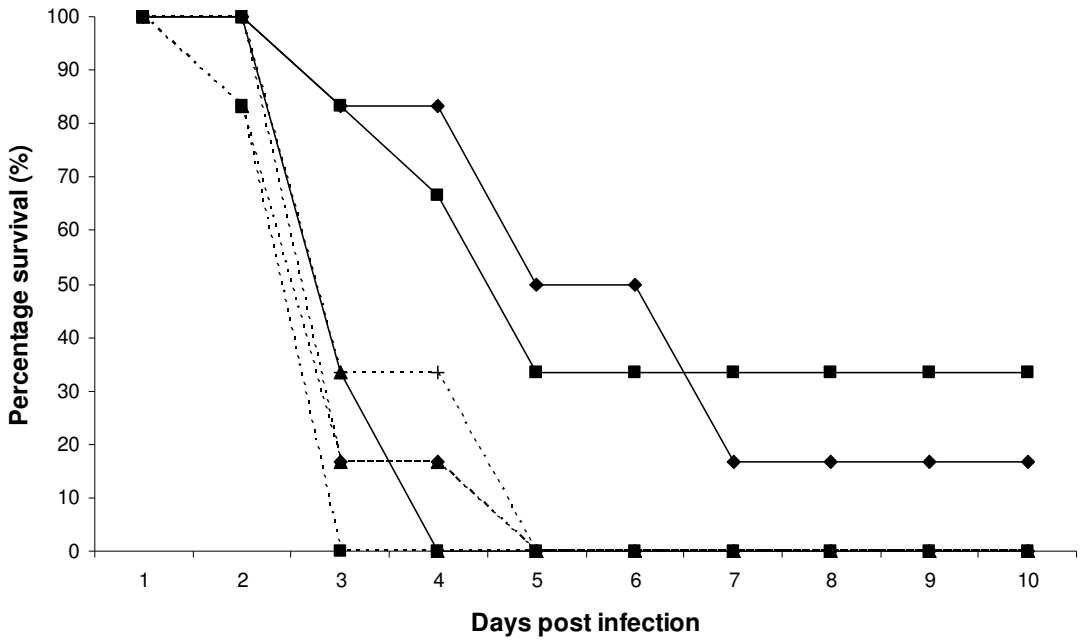


Figure 4.2. In vivo neutralization of RVFV with anti-recNP immune sera in mice. RVFV mixed with immune sera are indicated with solid lines as follows: mouse anti-recNP (♦), sheep anti-recNP (■) and rabbit anti-recNP (▲). Corresponding normal sera from these animals are indicated with dotted line and the same symbols. RVFV mixed with PBS is indicated with a dotted line and (+).

4.3.3 Discussion

Neutralization of viruses can be mediated by various mechanisms including aggregation of virions, virus structure destabilization, inhibition of attachment to cell receptors and blocking the release of virions from infected cells (Reading and Dimmock, 2007). It is widely accepted that the RVFV nucleocapsid protein does not contain neutralizing epitopes (Lorenzo et al., 2008, Lagerqvist et al., 2009) but this has only been evaluated *in vitro* using the cell culture based virus neutralization test. The neutralization of viruses *in vivo* is, however, more complex as it also involves interaction of antibodies with cells and molecules of the innate immune system (Reading and Dimmock, 2007). In this study it was confirmed that the anti-NP antibodies are not neutralizing *in vitro*. However, to evaluate whether anti-NP specific antibodies conferred some form of antibody mediated immunity independent of the various neutralization mechanisms known, an *in vivo* neutralization experiment was conducted in mice.

A recent report shows that human and murine antibodies against the nucleocapsid protein of Toscana virus, a virus of the *Phlebovirus* genus and thus closely related to RVFV, has low neutralizing

ability (Gori Savellini et al., 2008). In this study, however, the high levels of anti-recNP specific antibodies from mice, sheep and rabbits were ineffective in neutralizing RVFV *in vitro* and *in vivo*. No significant decrease in morbidity or mortality could be shown in the experimental groups when compared to the control groups. These results strongly suggest that humoral anti-recNP antibodies do not play a role in protection against viral infection. Cell free immune serum was, however, used for the *in vivo* neutralization experiment and therefore it is not known whether immune cells, and other factors not present in serum, from immunized individuals could play a role in protection. To evaluate the ability of the complete immune response, including innate/cellular/humoral, to protect against viral infection and morbidity/mortality, immunized mice were challenged with RVFV.

4.4 Rift Valley fever virus challenge of mice immunized with the recombinant nucleocapsid protein

4.4.1 Materials and methods

4.4.1.1 Cells and virus

Cells and virus were cultured as described before (section 4.3.1.1).

4.4.1.2 RVFV challenge

The mice remaining in each group after the immunization period (5 to 7 animals depending on group) were challenged with RVFV on day 32 after the booster immunization. Mice were inoculated subcutaneously (s.c.) with a 100 µl inoculum containing $10^{7.0}$ $TCID_{50}$/ml RVF challenge virus, and after challenge examined twice daily for signs of clinical illness. Animals displaying severe illness were euthanized and organs collected. Organs were also collected at regular intervals from healthy, sick and dead mice to monitor challenge virus replication. Surviving mice were monitored for 22 days post infection. A control group was mock inoculated with EMEM free of the virus.

4.4.1.3 Determination of viral loads in mouse tissues

Mouse liver, kidney and brain tissues were homogenized as 10% (w/v) suspensions in EMEM containing L-Glutamine, non-essential amino acids and antibiotics (100 IU penicillin, 100 µg streptomycin and 0.25 µg amphotericin B). After centrifugation at 3000 x g, 4°C for 15 minutes, supernatants were collected and stored at -70ºC until tested.

Virus titrations of mouse tissue homogenates were performed as described before (section 3.2.7). Briefly, four 100µl replicates of 10-fold dilutions (10^{-1} to 10^{-8}) of homogenates were transferred into flat bottomed 96-well cell culture microplates (Nunc, Denmark) and equal volumes of Vero cell suspension in EMEM containing 2 x 10^5 cells/ml, 8% FBS and antibiotics were added. The plates

were incubated at 37ºC in CO_2 and observed microscopically for cytopathic effects (CPE) for 10 days post inoculation. Virus titres, calculated by the Kärber method (Kärber, 1931) were expressed as median tissue culture infectious dose ($TCID_{50}$) per gram of tissue.

4.4.1.4 Real-time reverse transcriptase PCR (qRT-PCR)

Real time PCR was performed only on tissue homogenates that yielded negative results by virus titration. Viral RNA was extracted from 140µl of tissue homogenates using the QIAmp® Viral RNA Kit (QIAgen, Germany) according to the instructions of the manufacturer. The qRT-PCR was performed as described previously (Le Roux et al., 2009). Briefly, amplifications were carried out in 20µl reaction mixtures containing 5µl of the extracted vRNA using the LightCycler RNA Amplification Hybprobe kit (Roche, Germany) and the Roche LightCycler instrument. Primers and a labelled probe targeting the Gn glycoprotein gene of RVFV were used.

4.4.1.5 Statistical methods

Survival proportions in immunized mice versus control mice after challenge were compared using the Fisher exact test (Soper, 2009). Viral load results in mouse organs are based on $TCID_{50}$ titrations of virus in tissues from 3 or more animals and given as means.

4.4.2 Results

All mice in the adjuvant and PBS placebo control groups died or developed severe symptoms by day six after the experimental infection with RVFV, indicating severe challenge. In contrast, mice that were immunized with recNP/adjuvant combinations and challenged identically to the control mice were fully or partially protected from death and severe symptoms, depending on the recNP/adjuvant combination and dose (Figure 4.3). Clinical signs in sick animals included loss of appetite and consequent weight loss, scruffy coat, decreased alertness, decreased mobility, loss of balance, shallow and irregular breathing, and hunched posture. Interestingly, clinical signs in immunized animals that were partially protected were delayed by four to nine days as compared to controls and were more neurological in nature (partial paralysis and loss of balance). Only immunization with 35µg recNP/ISA50, 35 and 70µg recNP/Alhydrogel and 35 and 70µg recNP/SaponinQ yielded significant protection from disease/death (Table 4.1). The best protection from disease/death (100%) was achieved by immunization with 35 and 70µg recNP/Alhydrogel, and 70µg recNP/SaponinQ. The least effective protection achieved was 17% after immunization with 70µg recNP and no adjuvant. The least effective

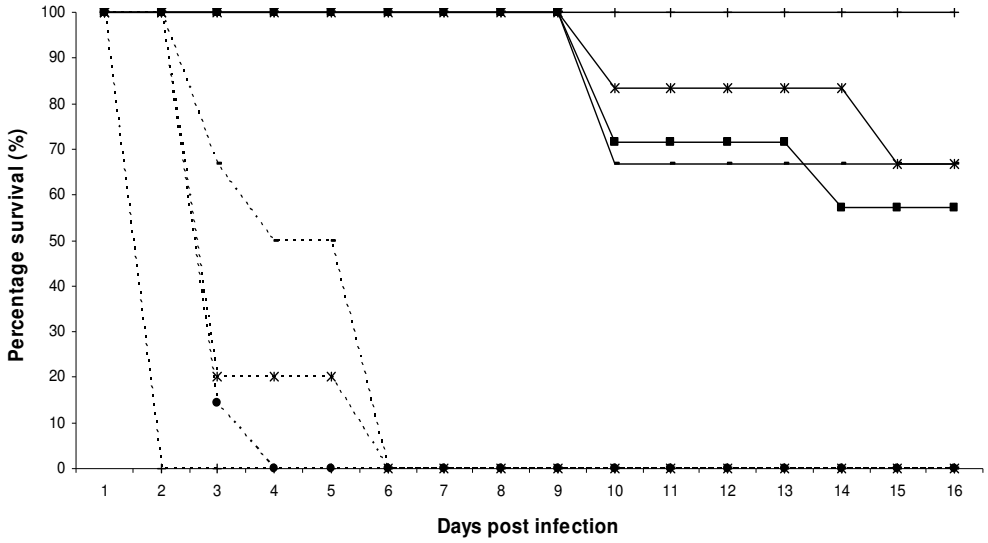
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protection achieved when using adjuvant with the recNP was 40% after immunization with 70µg recNP/ISA50. Mock inoculated mice did not develop any clinical signs during the experiment.

Despite full or partial protection of some immunized mice from disease/death after RVFV challenge, the virus still replicated in immune mice, but to lower levels in liver and kidney tissues when compared to unvaccinated control mice. Generally, however, the amount of virus detected in brain tissues from immunized mice after challenge was higher when compared to control mice, with the exception of mice immunized with 70µg recNP combined with Alhydrogel or SaponinQ (Table 4.2). Mice that developed severe disease or succumbed had higher viral loads in liver, kidney and brain tissues compared to mice that were apparently healthy (Figure 4.4.).

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A



B

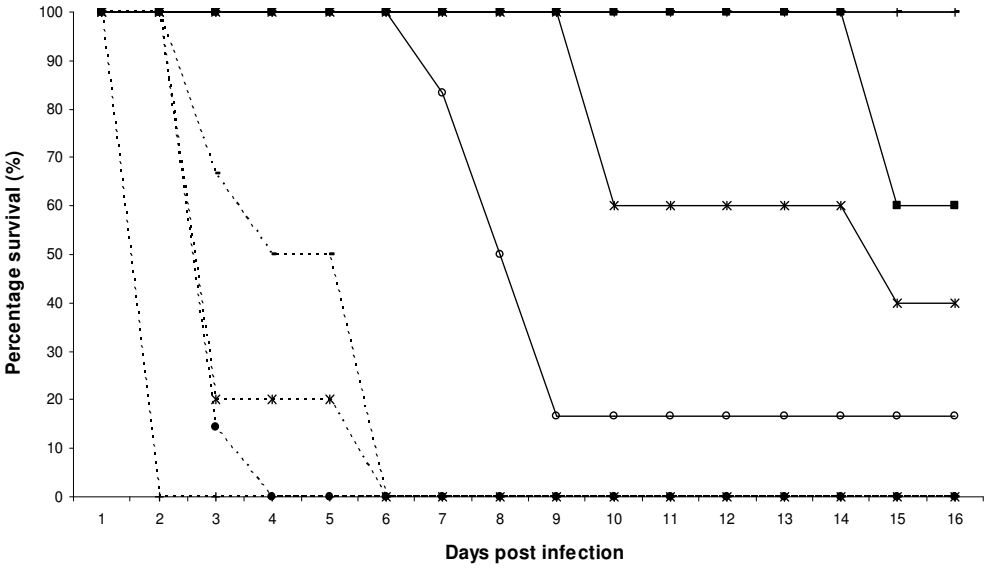


Figure 4.3. Protection of mice immunized with the 35µg (A) and 70µg recNP doses (B) against disease or death after RVFV challenge compared to placebo control mice (A, B). Immunized mice are indicated by solid lines (▬) with different adjuvant/recNP combinations indicated as (*) for ISA50, (■) for TiterMax Gold, (+) for Alhydrogel, (—) for SaponinQ and (o) for 70µg recNP without adjuvant. Placebo control groups are indicated by dashed lines (---) and (*) for ISA50, (+) for Alhydrogel, (—) for SaponinQ and (●) for PBS placebo mice respectively.

Table 4.1. Mouse survival rates after RVFV challenge.

Group	Survivors/Total (% survival)[a]	Significance of protection[b]
35µg recNP/ISA50	4/6 (67%)	p = 0.0303
70µg recNP/ISA50	2/5 (40%)	p = 0.1818
35µg recNP/TMG	4/7 (57%)	p = 0.0489
70µg recNP/TMG	3/5 (60%)	p = 0.0606
35µg recNP/Alhydrogel	6/6 (100%)	p = 0.0011
70µg recNP/Alhydrogel	5/5 (100%)	p = 0.0022
35µg recNP/SaponinQ	4/6 (67%)	p = 0.0303
70µg recNP/SaponinQ	6/6 (100%)	p = 0.0011
70µg recNP	1/6 (17%)	p = 0.500
Adjuvant control group ISA50	0/5 (0%)	N/A
Adjuvant control group Alhydrogel	0/5 (0%)	N/A
Adjuvant control group SaponinQ	0/6 (0%)	N/A
Placebo control group (PBS)	0/6 (0%)	N/A

[a] *Survivors/total number of mice ratio. (%) is percentage survival*

[b] *Significance of protection calculated using Fisher's Exact test. P-values < 0.050 are considered significant.*

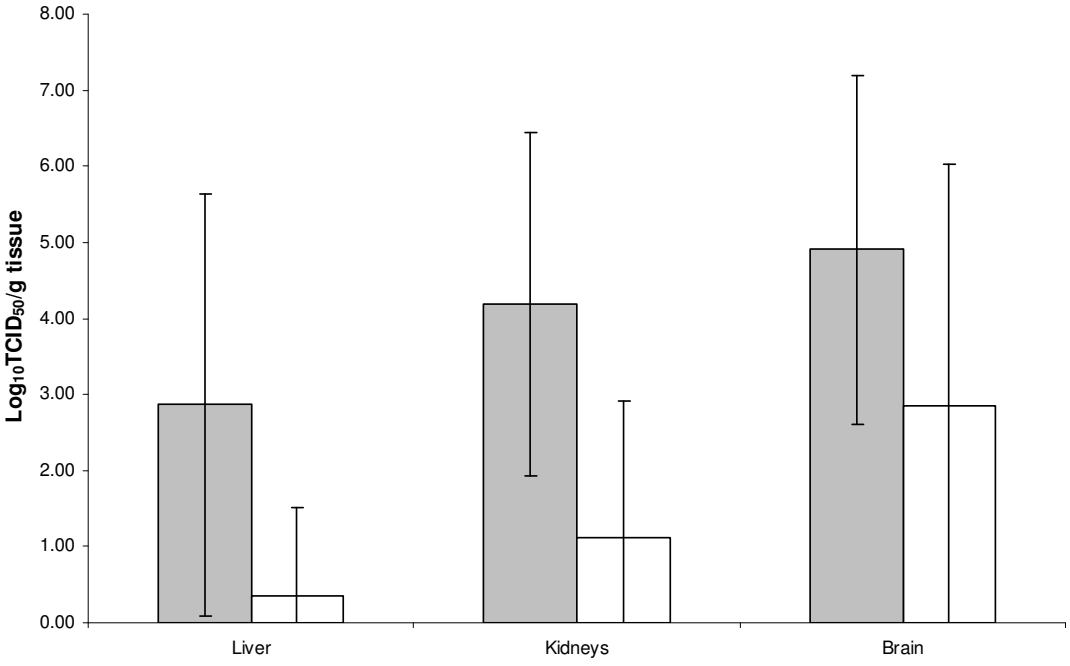


Figure 4.4. Mean RVFV $TCID_{50}$/gram of tissues from dead or sick mice (grey bars) compared to healthy mice (white bars) from all experimental groups. Error bars indicate the standard deviations from the mean values.

Table 4.2. Viral load data in immunized and control mice after RVFV challenge.

Group	Organ tissue	Number of mice	Mean viral load[a]	Range (S.D.)[b]
35µg recNP/ISA50	Liver	3	Negative *	N/A
	Kidney	3	1.3	0.0 – 4.0 (2.3)
	Brain	3	6.6	5.8 – 8.0 (1.2)
70µg recNP/ISA50	Liver	4	Negative	N/A
	Kidney	4	Negative *	N/A
	Brain	4	7.1	6.0 – 8.0 (0.7)
35µg recNP/TMG	Liver	4	Negative	N/A
	Kidney	4	0.9	0.0 – 3.8 (1.9)
	Brain	4	7.4	7.0 – 8.0 (0.5)
70µg recNP/TMG	Liver	3	Negative *	N/A
	Kidney	3	Negative *	N/A
	Brain	3	6.5	0.0 – 7.0 (0.5)
35µg recNP/Alhydrogel	Liver	3	Negative	N/A
	Kidney	3	1.3	0.0 – 4.0 (2.3)
	Brain	3	4.8	3.0 – 7.0 (2.1)
70µg recNP/Alhydrogel	Liver	3	Negative	N/A
	Kidney	3	Negative	N/A
	Brain	3	1.3	0.0 – 4.0 (2.3)
35µg recNP/SaponinQ	Liver	4	1.0	0.0 – 4.0 (2.0)
	Kidney	4	Negative *	N/A
	Brain	4	3.9	0.0 – 8.5 (4.5)
70µg recNP/SaponinQ	Liver	3	Negative *	N/A
	Kidney	3	1.3	0.0 – 3.8 (2.2)
	Brain	3	1.5	0.0 – 4.5 (2.6)
70µg recNP	Liver	6	0.8	0.0 – 4.5 (1.8)
	Kidney	6	3.0	0.0 – 5.3 (2.4)
	Brain	6	6.0	0.0 – 8.5 (3.1)
Adjuvant control group (ISA50)	Liver	5	3.5	0.0 – 5.3 (2.0)
	Kidney	5	5.0	3.8 – 5.3 (0.8)
	Brain	5	2.9	0.0 – 4.3 (1.7)
Adjuvant control group (Alhydrogel)	Liver	5	4.5	3.8 – 5.3 (0.9)
	Kidney	5	5.7	5.0 – 6.5 (0.6)
	Brain	5	4.6	3.0 – 6.5 (1.3)
Adjuvant control group (SaponinQ)	Liver	6	5.3	3.8 – 6.8 (1.3)
	Kidney	6	5.8	4.5 – 6.3 (0.7)
	Brain	6	4.2	3.0 – 5.8 (1.1)
Placebo control group (PBS)	Liver	6	5.9	4.0 – 7.3 (1.3)
	Kidney	6	5.3	4.3 – 6.0 (0.6)
	Brain	6	2.5	0.0 – 3.3 (1.2)

Organs were collected from sick and healthy mice between day 2 and 15 after infection.

[a] *Viral loads are given as mean $log_{10}TCID_{50}$/g tissue*

[b] *Range of $log_{10}TCID_{50}$ values and standard deviation from the mean*

**Indicates where qRT-PCR positives were detected in virus negative tissues*

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4.4.3 Discussion

It was shown in this study that the bacterially expressed recombinant RVFV nucleocapsid protein (recNP) is highly immunogenic in BALB/c mice when used with different adjuvants (section 4.2). These antibodies, however, are not neutralizing *in vitro* or *in vivo* (section 4.3). To evaluate whether immunization with recNP could induce protective immunity in mice by a mechanism other than neutralization of the virus, immunized mice were challenged with virulent RVFV. The recNP/SaponinQ combination was best able to induce a strong IgG2A isotype response. Increased IgG2A isotype antibodies indicate activation of the Th-1 response, which indicates secretion of IFN-γ and IL-2, two cytokines known to be involved in the cellular immune response. Interferon gamma (IFN-γ) is secreted by natural killer cells (NK) and $CD8^+$ cytotoxic T lymphocytes amongst others and has direct antiviral activity but also acts as an immunoregulatory factor. Interleukin 2 (IL-2) is secreted by T helper cells (Th) and acts by stimulating the growth, differentiation and survival of antigen specific $CD8^+$ cytotoxic T lymphocytes. These factors might have played a role in significant protection of recNP/SaponinQ immunized mice from death/disease (67 – 100% protection depending on recNP dose) and in decreasing replication of challenge virus.

Irrespective of the recNP dose used, immunization with recNP/Alhydrogel resulted in 100% protection from morbidity/mortality and the lowest levels of virus replication after challenge. Interestingly, and contrary to findings in recNP/SaponinQ groups, the recNP/Alhydrogel antigen/adjuvant combination induced a low IgG2A response, even though IgG1 and total IgG levels were still comparable to those in other groups. This would indicate that recNP/Alhydrogel was indeed immunogenic but that the response was strongly biased towards the Th-2 humoral response. These results are consistent with previous findings showing no up-regulation of IFN-γ and IL-2, and thus decreased IgG2A isotype antibodies by alum adjuvant (Ulanova et al., 2001). Alhydrogel, however, might directly activate NF-kB, a protein complex found in almost all cell types and that is involved in regulating cellular responses to an infection (Ulanova et al., 2001). Studies have shown that NF-kB is required for the positive selection of memory $CD8^+$ T cells (Hettmann and Leiden, 2000, Hettmann et al., 2003). $CD8^+$ T cells are able to kill virus-infected cells by inducing apoptosis, and have recently been shown to kill infected cells directly in the lymph nodes draining the infected site (Xu et al., 2007). This does not prevent infection but acts by limiting the spread of virus to organs, as it is the case for viruses that cause systemic infections such as RVFV. Likely the combination of recNP/Alhydrogel was able to induce a RVFV N-protein specific memory cellular response independently from the Th1/Th2 pathway by inducing NF-kB transcription factors. This in turn might have resulted in the production of N-protein specific memory $CD8^+$ T cells recognizing specific fragments of the virus' N protein displayed on the surface of APC or other infected cells. Killing of those cells would curb the spread of

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the virus and lower its replication level. This assumption is supported by our observations of drastically lower viral loads in target organs from recNP/Alhydrogel vaccinated mice during the early stages after infection, as compared to virus concentration in placebo control mouse organs. RVFV initially infects the white blood cells in the proximity of the inoculation site and circulates in these cells for 2-3 days, after which large amounts of the virus is released into the bloodstream to infect other organs. Once the complete virus particles are released into the bloodstream the non-neutralizing anti-NP response becomes ineffective since the NP antigen cannot be accessed by cell receptors. However, during the initial stage when white blood cells are infected, these cells would present processed fragments of the N protein antigen on their surface which would make them targets for apoptosis if specific memory cytotoxic T cells are present. It is therefore proposed that recNP/Alhydrogel immunizations were able to protect mice from severe symptoms by inducing the generation of NP-specific memory $CD8^+$ T cells that were able to limit the spread of virus by killing infected white blood cells before they could propagate and release large amounts of virus.

ISA50 have been shown previously to direct the immune response against a specific antigen towards the Th-2 type response, involved in humoral immunity, rather than Th-1 (O'Hagan et al., 2001). We obtained similar results with our antigen, showing that mice immunized with recNP/ISA50 developed very strong IgG1 type responses compared to lower, but still respectable IgG2A responses. The protection rates of 40 – 67% noted in this experimental group is probably due to the fact that Th-1 immunity was induced by the adjuvant, but to a lower level compared to SaponinQ. Mice immunized with both doses of recNP/TiterMax Gold generated very high levels of IgG1, but also intermediate IgG2A responses against recNP, which is consistent with previous findings (Cribbs et al., 2003). The increased Th-2 response over Th-1 pattern was very similar to that found when using ISA50 as adjuvant with recNP, and subsequently also resulted in similar protection rates of 57 – 60%. The distribution and titres of virus in organs from recNP/ISA50 and recNP/TMG immunized mice were also almost identical indicating that these two adjuvants function by very similar mechanisms.

Despite the recNP without adjuvant being able to induce a detectable humoral immune response after a booster immunization, it was not able to protect mice against morbidity/mortality after RVFV challenge. This is most probably due to absence of adjuvant to direct the response against the recNP towards cellular immunity, partly shown by a weak IgG2A response. This result confirms the importance of selecting an adjuvant that directs the immunity towards the correct type of response for the specific antigen used. Interestingly, all recNP/ISA50 or TMG vaccinated mice had similar, or in most cases higher viral loads in brain tissues from day 6 p.i. onwards when compared to placebo control mice, whereas during the first 5 days p.i. placebo control mice had high titres of replicating virus in all tissues tested. This organ specific tropism might explain the clinical observation of some

vaccinated mice developing delayed severe neurological symptoms, as opposed to more general severe symptoms in placebo control mice during the early days after infection. From these results it seems that recNP/ISA50 or TMG vaccinated mice were successful in averting initial disease or death by decreasing viral loads in other target organs, but were not able to prevent RVFV from crossing the blood-brain barrier and viral replication to damaging levels.

4.5 Conclusion

This chapter describes efforts to evaluate whether the response against the nucleocapsid protein of RVFV plays a role in protection against viral infection. To achieve this, different adjuvants with different mechanisms of enhancing immunity was used with a bacterially expressed RVFV nucleocapsid protein to immunize mice. The recNP was highly immunogenic, even in the absence of adjuvant. These anti-recNP humoral antibodies, however, had no neutralizing ability, either *in vitro* or *in vivo*. Despite this, mice immunized with recNP were protected from morbidity/mortality caused by challenge RVFV and depending on adjuvant used, 50 – 100% protection was achieved. Two recNP/adjuvant combinations resulted in significant protection (100%) and reduction of challenge virus replication in organs. The results show that one of these protective antigen/adjuvant combinations (recNP/SaponinQ) induced the strongest IgG2A isotype response compared to other adjuvants in this study, indicating activation of Th-1 cellular immunity. The assays used were not able to indicate activation of cellular immunity by the other protective antigen/adjuvant combination (recNP/Alhydrogel), but this combination might have induced the production of NP-specific memory $CD8^+$ T-cells by a separate pathway, as indicated by results from previous studies with alum adjuvant (Ulanova et al., 2001). The RVFV recNP combined with adjuvants that are known to bias responses towards Th-2 humoral immunity (ISA-50, TiterMax Gold) (O'Hagan et al., 2001, Cribbs et al., 2003) induced weaker IgG2A responses compared to SaponinQ, protected only 40 – 67% of mice from morbidity and mortality, and were not able to sufficiently curb viral replication in important organs.

Results from this study suggest that the anti-NP response play a role in protection of mice against RVFV infection. Even though obtained results indicate that cellular immunity is the major role player further experiments were needed to support this hypothesis. This was attempted and results are presented in chapter 6. Based on the promising results obtained in a mouse model, it was decided to evaluate the recNP as an immunogen in a host animal species. Therefore, additional studies have been undertaken, of which results are presented in chapter 5.

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CHAPTER FIVE

RECOMBINANT NUCLEOCAPSID PROTEIN AS AN IMMUNOGEN IN A RVF HOST ANIMAL SPECIES*

* Partially published as:

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* Partially presented at international conferences as:

Jansen van Vuren, P. & Paweska, J.T. (2009). Preliminary evaluation of a recombinant Rift Valley fever virus nucleocapsid protein as an immunogen in combination with different adjuvants in mice and sheep. *FAO/IAEA International Symposium on Sustainable Improvement of Animal Production and Health*, Vienna, Austria, 8 – 11 June 2009. (Poster presentation).

Jansen van Vuren, P., Tiemessen, C.T. & Paweska, J.T. (2009). Evaluation of a recombinant Rift Valley fever virus nucleocapsid protein as a vaccine immunogen in combination with four adjuvants. *International Meeting on Emerging Diseases and Surveillance*, Vienna, Austria, 13 – 16 February 2009. (Poster presentation).

5.1 Introduction

The advent of molecular biology has enabled development of various novel vaccine candidates for RVF. These next generation vaccines offer advantages over classical vaccines in that they are safe, easy and less expensive to produce. All the recent vaccine candidates have been evaluated in mice or rats, but very few have been further evaluated in a host ruminant animal species.

A recombinant lumpy skin disease virus (LSDV) containing both RVF glycoprotein genes induces strong neutralizing responses and 100% protection from clinical disease in sheep (Wallace et al., 2006). A SINV replicon-based RVF vaccine produces neutralizing responses in sheep but protection against viral challenge was not evaluated (Heise et al., 2009). Immunization of sheep with a DNA construct expressing the RVFV M segment and the nucleocapsid protein was not able to elicit detectable humoral responses but low level antigen-specific cellular responses were induced (Lorenzo et al., 2008). A construct expressing only the nucleocapsid protein, however, is able to induce strong anti-NP IgG1 isotype responses as well as cellular responses in sheep (Lorenzo et al., 2008), although protection against viral challenge was not evaluated.

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The role of the anti-NP response in the protection of a host animal species against RVF viral challenge has not been evaluated before. The recNP used in this study induced protective immune responses in mice; therefore it was decided to evaluate its protective ability against RVFV challenge in sheep using the same recNP/adjuvant combinations as used for mice.

5.2 Immunogenicity of the recombinant nucleocapsid protein alone and in combination with four different adjuvants in sheep

5.2.1 Materials and methods

5.2.1.1 Immunization of sheep

The recombinant RVFV nucleocapsid protein (recNP) was produced as described before (section 2.1.2.4). Sheep were pre-screened for antibodies against RVFV using enzyme linked immunosorbent assay (ELISA). Twenty three adult female Dorper cross sheep, younger than one year, were used. The sheep were divided into groups as described in table 5.1. All sheep were inoculated subcutaneously (s.c.) and received identical booster inoculations as described in table 5.1. Serum was collected at regular intervals, as given in table 5.1, for monitoring of immune responses.

Table 5.1. Group assignments and immunization schedules of sheep.

Group number	Number of sheep	RVFV recNP dose (µg)	Adjuvant/Inoculum	Immunization schedule	Blood collection schedule (day after immunization/booster)
1a	n = 2	175	ISA50	Day 0 (initial) Day 21 (booster)	Day 0, Day 14, Day 26 Day 14 after booster
1b	n = 2	350	ISA50		
2a	n = 2	175	Alhydrogel		
2b	n = 2	350	Alhydrogel		
3a	n = 2	175	TiterMax Gold®		
3b	n = 2	350	TiterMax Gold®		
4a	n = 2	175	SaponinQ		
4b	n = 2	350	SaponinQ		
5	n = 4	0	ISA50, Alhydrogel, TiterMax Gold® or SaponinQ	Day 0 (initial mock) Day 21 (mock booster)	
6	n = 3	0	PBS		

5.2.1.2 Monitoring of immune responses

Immune responses in sheep after immunization was monitored by an indirect ELISA based on the recNP as follows: Immunoplates (Maxisorb, Nunc, Denmark) were coated with RVFV recNP antigen at a dilution of 1:2000 in Carbonate-Bicarbonate buffer (pH 9.6) and incubated overnight at 4ºC. After washing three times with a washing buffer consisting of phosphate buffered saline (PBS) pH7.2 and 0.1% Tween-20, the plates were blocked with 200µl of 10% fat free milk powder (“Elite”,

Clover SA, Pty, Ltd.) in PBS at 37ºC for 1h and then washed as before. Control and test sera were diluted 1:400 in diluent buffer consisting of 2% fat free milk powder in PBS, 100µl added to each well and incubated for 1h at 37ºC. Sheep internal controls were generated as described by Paweska et al. in 2003 [36]. Controls were tested in quadruplicate and test samples in duplicate. After washing as before, 100 µl of rabbit anti-sheep IgG HRPO (Zymed Laboratories, Invitrogen, USA) at 1:6000 dilution was added to plates. After 1h incubation at 37ºC plates were washed as before and 100 µl of 2,2'-azinodiethylbenzthiazoline sulfonic acid (ABTS, KPL Laboratories, Inc.) added to each well. After 30 min incubation in the dark the reaction was stopped by the addition of 100 µl of 1% sodium dodecyl sulphate (SDS) to each well. Optical density (OD) was determined at 405nm and the results expressed as mean OD values. Means and standard deviations from the means were determined based on two animals per group.

5.2.2 Results

All sheep immunized with recNP combined with adjuvants produced detectable anti-NP IgG responses by day 14 after one immunization (Figure 5.1). The anti-NP antibodies in immunized sheep were consistently equal to or higher than the same antibodies in the high positive control serum from an experimentally infected sheep (dotted vertical line in figure 5.1). The second immunization of all sheep had the desired effect of boosting immune responses. The combination of recNP with Alhydrogel (group 2 and 6) was the least immunogenic but still induced strong responses compared to the positive control. The larger recNP dose (350µg) did not induce much stronger responses in any of the immunized groups when compared to the lower dose (175µg). As expected, the adjuvant and PBS control groups did not develop any anti-NP responses during the immunization period.

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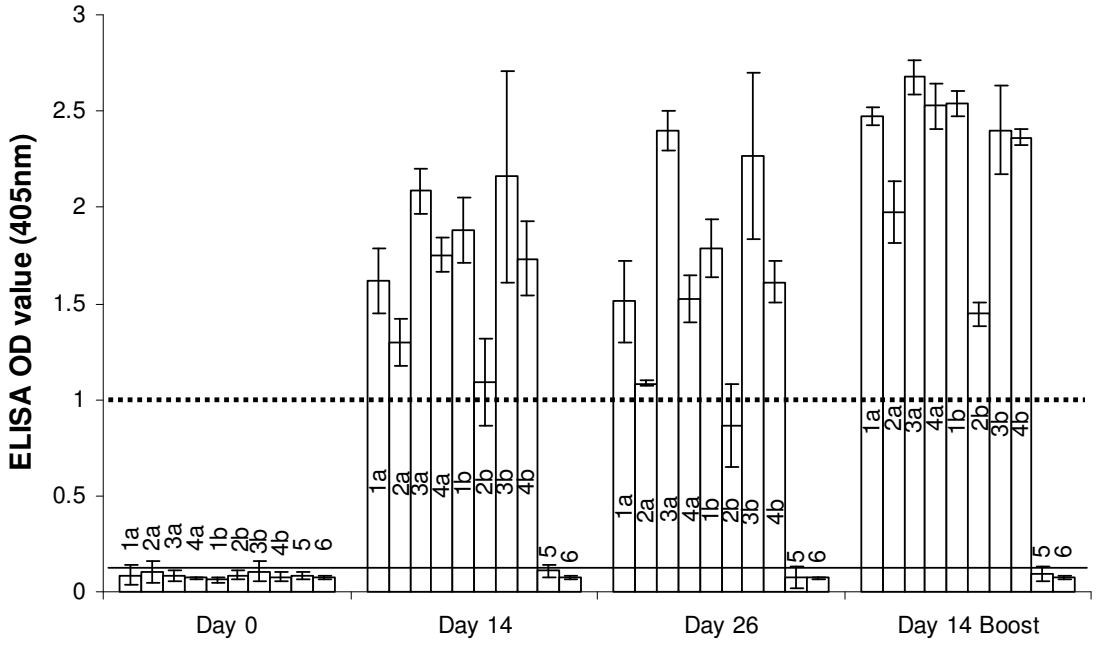


Figure 5.1. Average anti-NP responses in sheep after recNP first and booster immunizations; error bars indicate standard deviations from the means. 1) 175µg recNP/ISA50 (n=2); 2) 175µg recNP/Alhydrogel (n=2); 3) 175µg recNP/TiterMax Gold (n=2); 4) 175µg recNP/SaponinQ (n=2); 5) 350µg recNP/ISA50 (n=2); 6) 350µg recNP/Alhydrogel (n=2); 7) 350µg recNP/TiterMax Gold (n=2); 8) 350µg recNP/SaponinQ; 9) Adjuvant controls (n = 4); 10) PBS controls (n=3); 11) 350µg recNP (n=2). The vertical dotted line indicates the average OD reading for a high positive control serum. The solid vertical line indicates the average OD reading for a negative control serum.

5.2.3 Discussion

The recNP combined with adjuvants was highly immunogenic in sheep, even after a single immunization. The strength of the responses did not depend so much on the dose of recNP used (175 vs. 350µg), but seemed more dependent on the adjuvant used. The highest immunogenicity was achieved when combining recNP with SaponinQ or TiterMax Gold. Alhydrogel seemed to be the least effective of the adjuvants in inducing humoral anti-recNP responses. None of the anti-recNP responses in sheep were neutralizing.

The responses measured were IgG antibodies, and from earlier results in this study it became clear that humoral immunity against the RVFV NP does not play a role in protection against infection. Some of the adjuvants used are known to induce cellular immunity and although the cellular response was not measured in this study, based on the strength of the humoral responses one would think they

were activated. In a recent study it was shown that sheep, immunized with a DNA construct expressing the RVFV NP, developed strong humoral as well as lymphoproliferative responses (IFN-λ) (Lorenzo et al., 2008).

5.3 Rift Valley fever virus challenge of immunized sheep

5.3.1 Materials and methods

5.3.1.1 Cells and virus

Cells and virus were cultured as described before (section 4.3.1.1).

5.3.1.2 RVFV challenge

All sheep were challenged s.c. with 2 ml challenge virus (1 ml on both sides of the neck). Sheep were challenged at different times as follows: one sheep from each sub-group (group 1a,b – 4a,b), all sheep from group five and one sheep from group six were challenged on day 37 after the booster immunization (total = 13 sheep); the remaining sheep were challenged on day 168 after the booster immunization (total = 10 sheep). Sheep were monitored daily for the first two weeks after challenge and blood taken daily for the first seven days, and at regular intervals thereafter to monitor viremia and immune responses until day 70.

5.3.1.3 Immune response monitoring after RVFV challenge

Immune responses in sheep after challenge were monitored by IgM capture ELISA as described previously (Paweska et al., 2003a). A virus neutralization test (VNT) was performed as described before (section 2.1.2.3). Means and standard deviations for IgM ELISA percentage positivity values and VNT titres were based on data from minimum two animals per group.

5.3.1.4 Virus titrations

Virus titrations of sheep sera collected after challenge were performed as described before (section 3.2.7). Means and standard deviations from the means were determined based on two or more animals per group.

5.3.1.5 Statistical methods

The significance of differences between immune responses and viremia in sheep was confirmed using the Fisher F-test giving a two-tailed probability value (Excel, Microsoft Office). P-values lower than 0.01 were considered to be significant. Mean values and standard deviations from the means were calculated using at least two sheep per group.

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5.3.2 Results

5.3.2.1 Immune responses in sheep after RVFV challenge

Because the dose of recNP did not have a significant impact on the strength of the humoral responses, groups that were immunized with the same recNP/adjuvant combination, regardless of dose, were grouped together for the RVFV challenge experiment. All adjuvant control sheep were regarded as one group, and all PBS control sheep were regarded as one group, regardless of when they were challenged with RVFV.

The sheep IgM responses after challenge are shown in figure 5.2a and in figure 5.2b. None of the immunized or control sheep had any detectable RVFV specific IgM antibodies on the day of challenge. High levels of RVFV specific IgG, however, was detected in all immunized sheep on the day of challenge, but as expected not in control sheep (results not shown). Control sheep developed typical IgM responses after RVFV challenge. All immunized sheep developed lower IgM responses after challenge when compared to control sheep, except the recNP/Alhydrogel immunized sheep that were challenged on day 168, which developed elevated IgM responses when compared to other immunized sheep. Incidentally, these recNP/Alhydrogel immunized sheep had decreased RVFV specific IgG on the day 168 when they were challenged (results not shown).

The virus neutralizing antibody responses after challenge are shown in figure 5.3a and figure 5.3b. Immunization did not have significant effect on decreasing the development of virus neutralizing antibodies when compared to PBS control sheep: recNP/ISA50 (day 37, $p = 0.883$; day 168, $p = 0.825$ Fisher F-test), recNP/Alhydrogel (day 37, $p = 0.920$; day 168, $p = 0.850$), recNP/TiterMax Gold (day 37, $p = 0.881$; day 168, $p = 0.975$) and recNP/SaponinQ (day 37, $p = 0.682$; day 168, $p = 0.858$).

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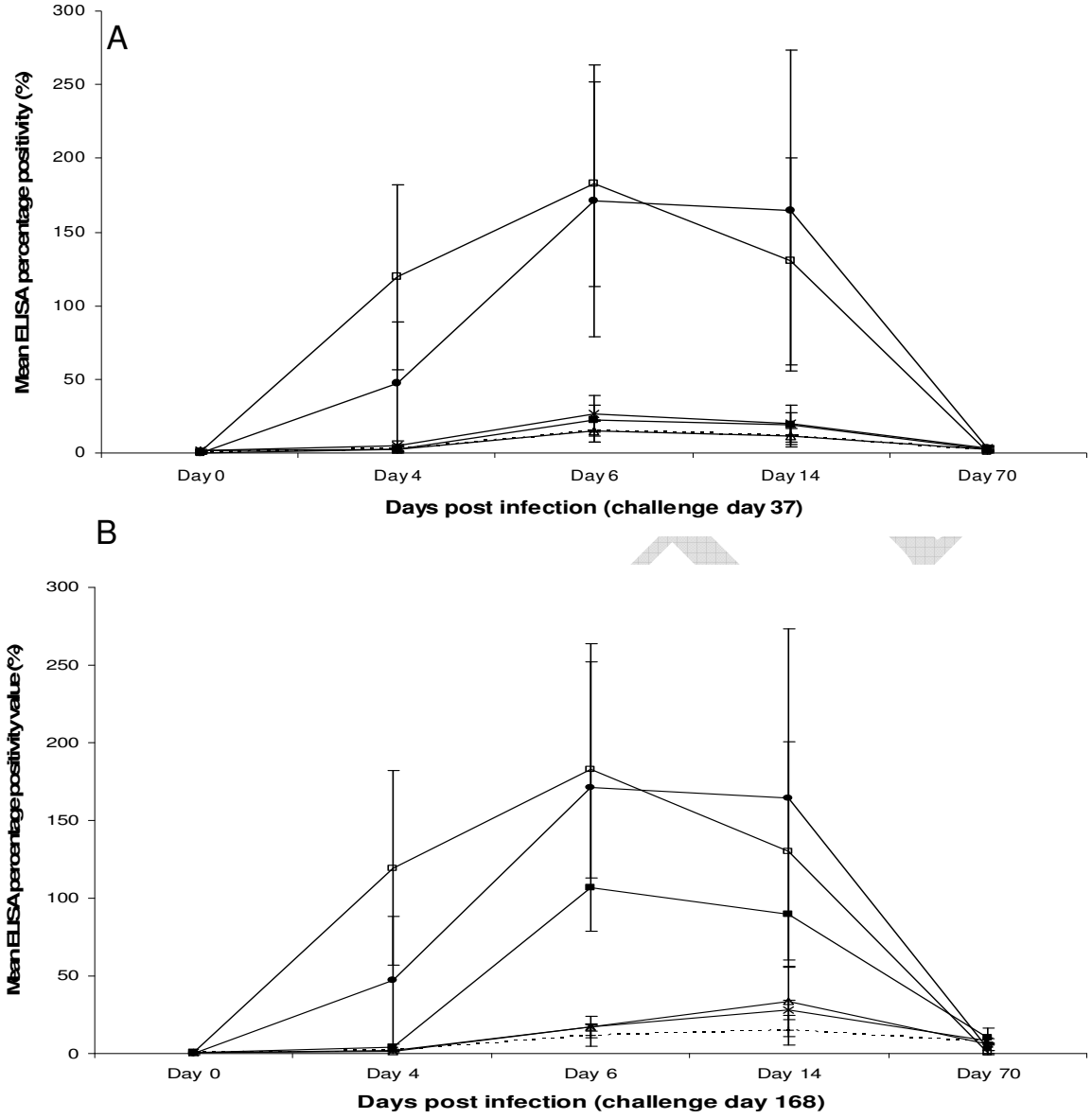


Figure 5.2. Mean IgM responses in sheep after RVFV challenge on day 37 (a) or 168 (b). Sheep groups are indicated as recNP/ISA50 (--+--), recNP/Alhydrogel (—■—), recNP/TiterMax Gold (—Δ—), recNP/SaponinQ (—x—), adjuvant controls (—□—) and PBS controls (—●—). Error bars indicate standard deviations from the means of two or more sheep per group.

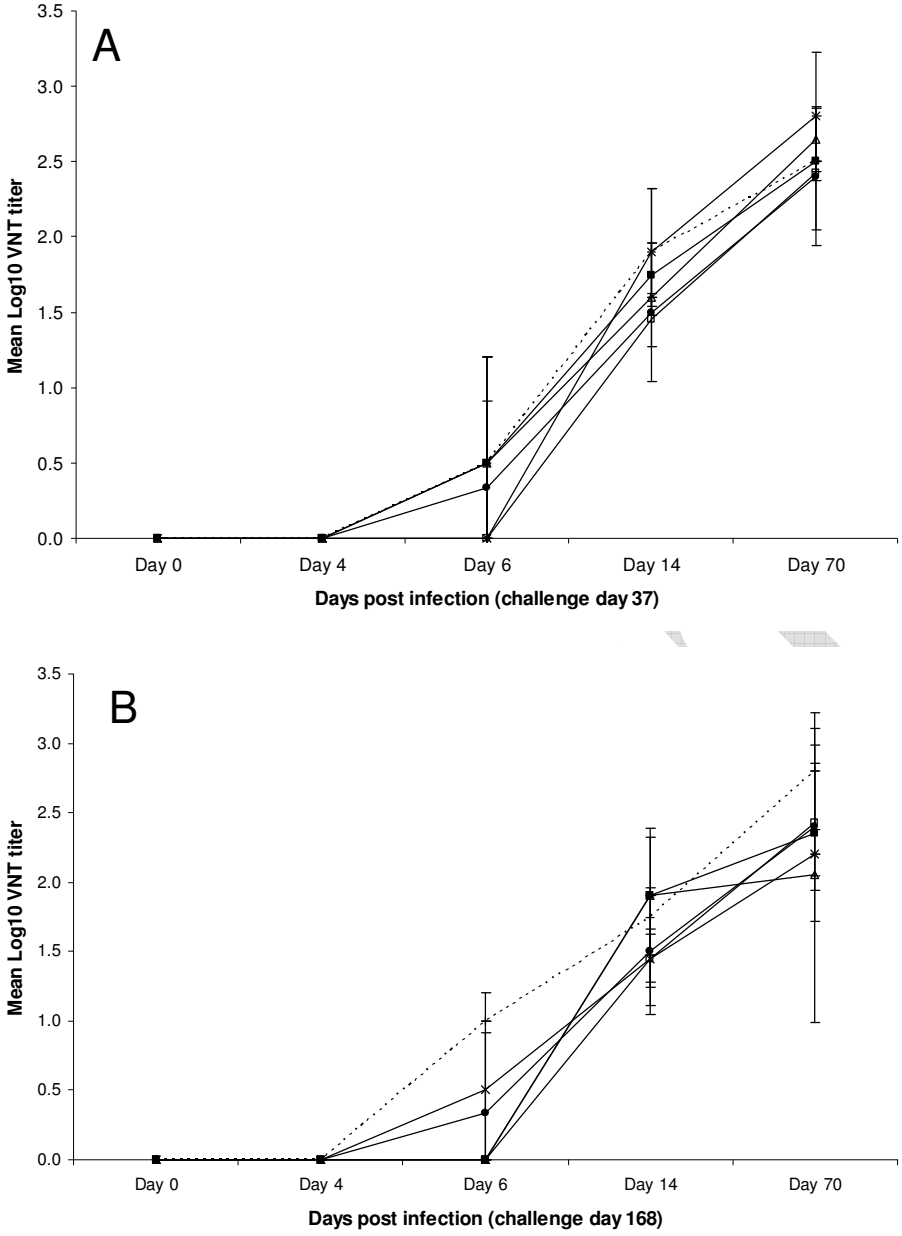


Figure 5.3. Mean virus neutralizing antibody responses in sheep after RVFV challenge on day 37 (a) or 168 (b). Sheep groups are indicated as recNP/ISA50 (--+--), recNP/Alhydrogel (—■—), recNP/TiterMax Gold (—Δ—), recNP/SaponinQ (—x—), adjuvant controls (—□—) and PBS controls (—●—). Error bars indicate standard deviations from the means of two or more sheep per group.

5.3.2.2 Viremia in sheep after RVFV challenge

The viremia in sheep after RVFV challenge is shown in Table 5.2. Immunization of sheep did not result in significant decrease of viral loads in sera when compared to PBS control sheep. Viremia was, however, of two to four days duration whereas one PBS control sheep developed prolonged viremia up to day seven. None of the sheep, including controls, displayed any clinical signs.

5.3.3 Discussion

Despite the recNP being highly immunogenic in sheep when combined with adjuvants (section 5.2), immunity against recNP was not able to decrease the replication of challenge virus in sheep. The IgM responses in immunized sheep after challenge was considerably lower when compared to naïve sheep that were challenged with the same virus. However, this is most probably due to the fact that the IgM ELISA detects antibodies against the whole RVFV, including the glycoproteins. When taking into consideration that virus neutralizing antibody titres, which consists solely of anti-glycoprotein antibodies, were equal in immunized and control sheep, it appears that the decrease in IgM responses in immunized sheep versus control sheep was because anti-NP IgM antibodies were not produced by immunized sheep since they had already been exposed to this antigen during immunization. This is further substantiated by the fact that there was no significant decrease in viremia in serum of immunized sheep after challenge when compared to control sheep.

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Table 5.2. Viremia in immunized and control sheep after RVFV challenge.

Inoculum	Number of sheep	Days post infection	Viremia Mean $Log_{10}TCID_{50}$/ml±Standard deviation (Range)	Significance of decreased viremia[a]
recNP/ISA50 (1a-b) (175 and 350µg combined)	2	1	4.5±0.4 (4.3 to 4.8)	p = 0.55
		2	4.4±0.2 (4.3 to 4.5)	
		3	0.8±1.1 (0.0 to 1.5)	
Challenge day 37		4-7	Negative	
	2	1	3.6±0.5 (3.3 to 4.0)	p = 0.37
		2	6.1±0.5 (5.8 to 6.5)	
Challenge day 168		3-7	2.0±1.8 (0.8 to 3.3)	
recNP/Alhydrogel (2a-b) (175 and 350µg combined)	2	1	2.6±2.7 (0.8 to 4.5)	p = 0.37
		2	2.0±2.8 (0.0 to 4.0)	
		3	1.1±1.6 (0.0 to 2.3)	
Challenge day 37		4-7	Negative	
	2	1	3.4±0.2 (3.3 to 3.5)	p = 0.79
		2	4.4±2.7 (2.5 to 6.3)	
		3	2.4±3.4 (0.0 to 4.8)	
		4	0.5±0.7 (0.0 to 1.0)	
Challenge day 168		5-7	Negative	
recNP/TiterMax Gold (3a-b) (175 and 350µg combined) Challenge day 37	2	1	4.3±1.1 (3.5 to 5.0)	p = 0.88
		2	2.6±1.2 (1.8 to 3.5)	
		3-7	Negative	
	2	1	4.3±0.4 (4.0 to 4.5)	p = 0.49
		2	5.0±1.1 (4.3 to 5.8)	
		3	1.9±1.2 (1.0 to 2.8)	
Challenge day 168		4-7	Negative	
recNP/SaponinQ (4a-b) (175 and 350µg combined)	2	1	4.4±0.5 (4.0 to 4.8)	p = 0.52
		2	4.8±0.4 (4.5 to 5.0)	
		3	1.6±2.3 (0.0 to 3.3)	
Challenge day 37		4-7	Negative	
	2	1	1.9±2.7 (0.0 to 3.8)	p = 0.19
		2	2.0±2.8 (0.0 to 4.0)	
		3	0.4±0.5 (0.0 to 0.8)	
Challenge day 168		4-7	Negative	
Adjuvant control (ISA50, Alhydrogel, TiterMax Gold and SaponinQ combined)	4	1	5.1±0.3 (4.8 to 5.5)	p = 0.40
		2	4.8±1.1 (3.8 to 6.0)	
		3	0.8±1.5 (0.0 to 3.0)	
Challenge day 37		4-7	Negative	
PBS control	3	1	4.5±0.3 (4.3 to 4.8)	Control group
		2	5.3±2.3 (3.3 to 7.8)	
		3	2.3±4.0 (0.0 to 7.0)	
		4	1.9±3.3 (0.0 to 5.8)	
Challenge day 37 and 168		5	1.8±3.2 (0.0 to 5.5)	
		6	1.5±2.6 (0.0 to 4.5)	
		7	0.8±1.4 (0.0 to 2.5)	

[a] Indicates the statistical significance of decrease in viral load as compared to PBS control sheep, as calculated by the Fisher F-test. P-values ≤ 0.01 indicate a statistically significant decrease in viremia.

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5.4 Conclusion

This chapter describes the first evaluation of the recNP, combined with adjuvants, as an immunogen in a RVF host species, sheep, and protection against viral replication after RVFV challenge. Vaccination against arthropod borne viruses should ideally aim to decrease morbidity and mortality, but even more importantly it should stop the spread of the virus by inducing sterilizing immunity. The only study on record where immune responses against specifically the NP were evaluated (Lorenzo et al., 2008) reported on humoral and lymphoproliferative responses induced after immunization with a DNA construct expressing the NP. The protective ability of these responses was not evaluated. The findings from this study highlight some important aspects that should be considered for future research and development of vaccine candidates for RVF. Firstly the anti-nucleocapsid response alone, although protective in mice, does not seem to play a role in the protection of an actual host species against RVFV infection. It must be noted though that in mice protection was evaluated as survival and decrease of viral replication in organs, whereas in sheep protection was based solely on viremia in the blood. The reason for this is that it is almost impossible to produce disease in sheep older than a few months with experimental RVFV infection. For this reason viremia, which is also an important factor in protection since high viremia would lead to subsequent infection of feeding mosquitos, was measured in this study. Therefore it would be more accurate to state that anti-NP responses were not able to curb viremia in the blood of sheep. Secondly, the results show that results in mice cannot necessarily be extrapolated to a host species with the expectation of achieving the same level of protection. It must be remembered that mice are merely used because in some cases the disease caused by viral infection mimicks some of the symptoms seen in actual host species, in addition to their ease of handling and low cost. Therefore RVFV candidate vaccines should always be evaluated in a host species first before conclusions can be drawn about its efficacy. Although the target proteins of choice for RVFV vaccines are glycoproteins because of inducing neutralizing antibody, RVFV vaccine candidates targeting the glycoproteins which were evaluated in mice have also yielded inconsistent protection against challenge (Wallace et al., 2006, Mandell et al., 2009, Naslund et al., 2009). In a recent study it was shown that immunization with VLPs combining the glycoproteins and nucleocapsid protein yielded better protection (Mandell et al., 2009). Therefore it appears that vaccine candidates combining glyco- and nucleocapsid proteins should be further investigated.

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CHAPTER SIX

HOST GENE EXPRESSION IN MICE IMMUNIZED WITH RECOMBINANT NUCLEOCAPSID PROTEIN AND CONTROL MICE AFTER RIFT VALLEY FEVER VIRUS INFECTION*

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6.1 Introduction

As shown in Chapters 4 and 5, the bacterially expressed recombinant RVFV N-protein was highly immunogenic in mice and sheep, and elicited 100% protection against lethal challenge in mice when combined with certain adjuvants. However, the mechanism by which immunization with this protein elicits protection is still not clear, however. A deeper look into these mechanisms might also reveal the discrepant results between mice and sheep in this study. The N-protein is not a viral surface protein and therefore does not play any role in virus entry into host cells. It is therefore not surprising that anti-recNP humoral antibodies from immunized mice and sheep in this study were not able to neutralize the virus *in vitro* or *in vivo* (Chapter 4). The N-protein is the main RVFV immunogen and strong humoral responses have been detected in various species against the RVFV recNP (Jansen van Vuren et al., 2007, Paweska et al., 2007, Paweska et al., 2008b) which has led some to suggest that N-protein acts as a decoy protein (Lorenzo et al., 2008). This theory is, however, not supported by the successful protection of mice by recNP immunization in this study. Low level N-protein specific cellular responses have been noted in sheep after immunization with a DNA vaccine expressing NP (Lorenzo et al., 2008). However, in this study the recNP/adjuvant combination that induced the weakest IgG2a isotype response (indicative of weak Th1 cellular immunity), but still a strong IgG1 isotype response (indicative of activation of Th2 humoral immunity) (Figure 4.1 e-f), in mice after immunization resulted in the best protection against RVFV challenge. This indicates that the activation

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of cellular immunity against NP might not play such a substantial role in protection against RVFV infection.

The host defence against viral infection is a complex response consisting of two categories: innate and adaptive immunity. The innate immune system is the first line of defence against infections but is not pathogen specific and is mainly comprised of the complement system, cytokines, natural killer cells, macrophages and apoptosis (Strauss and Strauss, 2008). Cytokines are a family of proteins that also have a regulatory role in the host adaptive immune system, especially interleukins, whereas interferons also have direct antiviral action. Type I interferons, IFNα and IFNβ, are especially important in vertebrates for controlling viral infections and are produced by almost all cell types in the host. Type I interferons are induced mainly by the detection of double-stranded RNA, an intermediate product in viral replication, by Toll-like receptors on the surface of cells or helicases within the cell (Strauss and Strauss, 2008, Haller and Weber, 2009). Once induced, type I interferons not only stimulate the adaptive immune response by increasing production of class I major histocompatibility complex (MHC I) molecules, but they also create an antiviral state in host cells, thus preventing or decreasing viral replication in those cells. This antiviral state involves antiviral pathways such as protein Kinase R (PKR), 2-5 OAS/RNaseL and the Mx proteins (Strauss and Strauss, 2008, Haller and Weber, 2009) that interferes with viral mRNA translation. RVFV is sensitive to the actions of type I interferons (Anderson and Peters, 1988, Morrill et al., 1990, Sandrock et al., 2001, Peterss et al., 2009) but the virus has developed several mechanisms by which it counteracts the actions of thereof. The NSs protein of RVFV forms filaments in the nuclei of infected cells and interacts with a repressor complex (Sin3A/NCoR/HDAC) inhibiting transcriptional activation of the IFNβ gene (Le May et al., 2008, Bouloy and Weber, 2010). A more general shutdown of cellular gene expression is also caused by interaction of NSs with the p44 subunit of the TFIIH basal transcription factor, resulting in reduced transcriptional activity in RVFV infected cells (Le May et al., 2004). The NSs has also been shown to act on a post-translational level by degrading PKR, a protein responsible for the shutdown of translation of viral proteins (Habjan et al., 2009b). Interferon gamma (IFNγ) is the only type II interferon and is produced by natural killer T cells (NKT) and NK cells as part of the innate immune response, and by cytotoxic T lymphocytes (CTL) and Th-1 cells as a part of the memory cellular response (Strauss and Strauss, 2008). IFNγ is a strong immunoregulator altering the transcription of a number of genes which, amongst others, leads to increased production of MHC I and II molecules, suppression of Th-2 humoral immunity and activation of NK cells. It also has direct antiviral properties. However, the role that IFNγ plays in protection of the host against RVFV infection is debatable. A study in rhesus monkeys showed that prophylactic treatment with recombinant human IFNγ before RVFV infection protected monkeys from clinical disease, and decreased viremia

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significantly (Morrill et al., 1991b). A recent study, however, showed that there was no marked difference in pathogenicity of RVFV MP-12 or Clone-13 in wild type mice compared to mice deficient in IFNγ receptor ($IFNGR^{-/-}$) suggesting that IFNγ only plays a negligible role in RVFV attenuation (Bouloy et al., 2001).

Another non-structural protein of RVFV, encoded by the M segment (NSm), was recently implicated in the pathogenesis of RVF by acting as an anti-apoptotic protein (Won et al., 2007). Apoptosis is a controlled process in the host and a way of eliminating cells that are infected before they can produce a large progeny of virus (Strauss and Strauss, 2008). Another antigen-independent mechanism of killing infected cells is carried out by NK cells. These cytolytic cells express two separate sets of receptors. One of these sets of receptors interacts with MHC I molecules on host cells, which inhibits killing of the host cell. The other set of NK receptors interacts with activating molecules on infected hosts cells, resulting in the stimulation of NK cells to kill the target cell. NK cells also kill any cells that are not expressing MHC I (or low amounts thereof) as a result of the ability of some pathogens to inhibit MHC I expression in infected cells to evade CTL responses (Strauss and Strauss, 2008). NK cells can also play a role in adaptive immunity by means of antibody-dependent cell-mediated cytotoxicity (Weiner and Adams, 2000). This is a process by which antigen specific antibodies form a bridge between a virus infected cell displaying that viral antigen on its surface, and NK cells which bind the Fc portion of antibodies and results in lysis of the target cell.

The adaptive immune response consists of a cellular arm, making use of cytotoxic T lymphocytes (CTL), and a humoral arm, making use of B-lymphocytes (B-cells) that secrete antibodies, with helper T-lymphocytes (Th-1 or Th-2) activating these cells and directing responses (Strauss and Strauss, 2008). Humoral immunity is important against extracellular pathogens, such as for example RVFV circulating in the bloodstream of its host. A B-cell displaying an antibody on its surface would recognize an extracellular viral antigen, leading to activation, followed by a second signal from a Th-2 cell after recognition of a fragment of the antigen displayed by MHC II molecule, causing the activated B-cell to proliferate and produce more cells capable of producing and secreting the same antibody (Strauss and Strauss, 2008). Antibodies play an important role in the control of viral infections by direct inhibition of virus entry into host cells (neutralization), coating of virus for subsequent removal by macrophages and activation of the complement cascade that leads to opsonisation, phagocytosis, chemotaxis or lysis. As discussed earlier, strong humoral responses against the RVFV NP is elicited after RVFV infection but these antibodies are not neutralizing *in vitro* or *in vivo* (Jansen van Vuren et al., 2010, Lorenzo et al., 2010), most probably because the NP plays no role in viral entry into host cells and is not found on the envelope of the virus and thus cannot be coated with antibodies for removal by macrophages. Humoral responses generated against the RVFV

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glycoproteins are, however, neutralizing and a correlate of protection against viral challenge (Wallace et al., 2006, Heise et al., 2009, Kortekaas et al., 2010a, Lorenzo et al., 2010, Mandell et al., 2010a). Cellular immunity is important against intracellular pathogens, such as for example RVFV infecting host cells. Infected cells present peptide fragments of viral proteins in a class I MHC context, which is recognized by T-cell receptors (TCR) on $CD8^+$ T cells (cytotoxic T cells) that consequently become activated when a second co-stimulatory signal is present (Strauss and Strauss, 2008). An activated CTL requires further stimulation by cytokines (i.e. IL-2, IFNγ), supplied by Th-1 cells, to enable it to proliferate and mount a vigorous cellular response against infection. The activation of the cellular arm of the adaptive immune response to RVFV infection has not been shown. A recent study, evaluating RVF virus like particles (VLP) containing both glycoproteins and NP as a vaccine, showed the secretion of some cytokines from RVFV induced spleen cells of mice 31 days post immunization with VLP (Mandell et al., 2010a). These cytokines included those associated with Th1 cellular immunity (IL-2, IFNγ and IL-12) and Th2 humoral immunity (IL-4 and IL-5).

A few recent studies have utilized microarrays or quantitative PCRs to analyze host gene expression in response to arboviral infections (Venter et al., 2005, Calzavara-Silva et al., 2009, Nascimento et al., 2009, do Valle et al., 2010, Momose et al., 2010). One study in particular utilized microarray and quantitative PCR to show a critical role for host innate immunity in resistance to RVF (do Valle et al., 2010). The study showed that a specific strain of mice, BALB/cByJ, was more resistant to RVFV infection when compared to a wild mouse strain, MBT/Pas. The study analyzed the expression of genes involved in the innate immune response by infecting mouse embryonic fibroblasts (MEF) from both mouse strains with RVFV *in vitro*, with results indicating a more significant type I IFN response in the BALB/cByJ MEFs. The results from this study are a further indication of the involvement of innate immunity, especially type I IFN, in the host's fight against RVFV infection. The activation of adaptive immunity after RVFV infection on a gene expression level, and *in vivo* in a known RVFV target organ, has not been shown yet. A better understanding of the activation of memory humoral and cellular immune responses might provide some useful information for future RVFV vaccine developments, or even show some genes that might be targets of gene therapy or antivirals. The role that anti-NP responses play in the protection of vaccinated individuals against RVFV infection is also not well understood.

In an attempt to elucidate the protective mechanism of anti-recNP responses, the regulation of expression of certain genes involved in the activation of T- and B-cell immunity, and innate immunity, were analysed by Real-Time PCR and relative quantification in three organs known to be important in RVFV pathogenesis. Relative quantification is a method by which expression levels of genes in treated subjects are related to expression levels of the same genes in untreated subjects (Livak and Schmittgen,

2001, Schmittgen and Livak, 2008). In this study the three treated groups, consisting of recNP/Alhydrogel immunized mice, Alhydrogel mock-immunized mice and PBS mock-immunized mice, were challenged with RVFV. The untreated group did not receive any immunization and was mock-challenged with tissue culture supernatant instead of RVFV. In this study we report that mice immunized with recNP combined with Alhydrogel adjuvant induced a strong IgG1 immune response, but weak IgG2A, indicating that the response was biased towards Th-2 humoral immunity rather than Th-1 cellular immunity. This humoral immunity still succeeded in protecting mice against clinical disease and decreasing viral load up to 10^4 fold in liver, the main target organ during RVFV infection, compared to control mice, which is consistent with previous results (Jansen van Vuren et al., 2010). The expression of type I IFN is upregulated in the liver of immunized mice shortly after RVFV challenge, compared to an initial downregulation and subsequent delayed upregulation of the same gene in the liver of non-immunized mice. In the acute phase of liver infection, however, a massive upregulation of type I and II interferon occurs in the presence of high viral titres in non-immunized mice, compared to immunized mice. It also shows the up- and downregulation of several genes involved in the activation of B- and T-cells in liver of non-immunized mice at the acute phase of RVFV infection, confirming that both cellular and humoral immunity are activated during RVFV infection in a mouse model. Some of these genes are involved in other immune functions as well. Various genes with pro-apoptotic effects were strongly upregulated, and anti-apoptotic genes downregulated in non-immunized mice. There was also upregulation of several genes involved in pro-inflammatory responses in liver of non-immunized mice.

6.2 Materials and Methods

6.2.1 Immunization and Rift Valley fever virus challenge of mice

6.2.1.1 Mouse immunization

The recombinant RVFV nucleocapsid protein (recNP) was produced as described in section 2.1.2.4. Four-week old female BALB/cOlaHsd (Harlan Laboratories, U.K LTD) mice were used as an experimental animal model. The immunized group (MS-1) consisted of 85 mice each immunized with a 200µl inoculum containing 70µg RVFV recNP in combination with Alhydrogel (Sigma, U.S.A). The adjuvant control group (MS-2) consisted of 40 mice "mock"-immunized with Alhydrogel in PBS. The placebo control group (MS-3) consisted of 40 mice which were "mock"-immunized with PBS buffer. The normal control group (MS-4) consisted of 40 mice and was not inoculated with anything at this point but kept as a control group for the challenge experiment.

All mice (except for MS-4) were inoculated subcutaneously (s.c) and received identical booster immunizations at 14 days after the initial immunization. Three mice from the immunized group MS-1

was sacrificed and heart-bled on the following days to monitor immune responses: day 0, 3, 5, 7, 10 and 12 after the immunization and day 0, 3, 5, 7, 12, 18, 21 and 27 after the booster.

6.2.1.2 Cells and virus

Cells and virus were cultured as described before (section 4.3.1.1).

6.2.1.3 RVFV challenge

The remaining mice in each group (MS-1 = 40 mice; MS-2 = 38 mice and MS-3 = 40 mice) after the immunization period were challenged with RVFV on day 28 after the booster immunization. Mice were inoculated subcutaneously (s.c.) with a 100 µl inoculum containing $10^{6.5}$ $TCID_{50}$/ml RVF challenge virus, and after challenge examined twice daily for signs of clinical illness. The normal control group (MS-4 = 39 mice) was "mock"-infected with tissue culture medium (EMEM) without virus to act as an untreated control group for comparison of up- and downregulation of genes. Animals displaying severe illness were euthanized and organs collected. Three (3 biological replicates) mice were euthanized from each group and liver, spleen and brain tissues collected into RNAlater (QIAgen, Germany), to preserve RNA integrity, at the following time points after infection/"mock"-infection: 3 hours, 6 hours, 12 hours, 24 hours, 72 hours and 120 hours. The same tissues were also collected for virus titration (thus not into RNAlater). All tissue samples were stored at -70°C until RNA extraction (tissues in RNAlater) or virus titration (tissues not in RNAlater).

6.2.1.4 Determination of viral loads in mouse tissues

Mouse liver, spleen and brain tissues were homogenized as 10% (w/v) suspensions in EMEM containing L-Glutamine, non-essential amino acids and antibiotics (100 IU penicillin, 100 µg streptomycin and 0.25 µg amphotericin B). After centrifugation at 3000 x g, 4°C for 15 minutes, supernatants were collected and stored at -70ºC until tested. Three mice per group were analyzed per time point, and average values calculated.

Virus titrations of mouse tissue homogenates were performed as described before (section 3.2.7). Briefly, four 100µl replicates of 10-fold dilutions (10^{-1} to 10^{-8}) of homogenates were transferred into flat bottomed 96-well cell culture microplates (Nunc, Denmark) and equal volumes of Vero cell suspension in EMEM containing 2 x 10^5 cells/ml, 8% FBS and antibiotics were added. The plates were incubated at 37ºC in CO_2 and observed microscopically for cytopathic effects (CPE) for 10 days post inoculation. Virus titres, calculated by the Kärber method (Kärber, 1931) were expressed as median tissue culture infectious dose ($TCID_{50}$) per gram of tissue.

6.2.1.5 Monitoring mouse immune responses after immunization

Mouse immune responses after immunization were measured by a recNP based indirect ELISA. Immunoplates (Maxisorb, Nunc, Denmark) were coated with RVFV recNP antigen at a dilution of 1:2000 in Carbonate-Bicarbonate buffer (pH 9.6) and incubated overnight at 4ºC. After washing three times with a washing buffer consisting of PBS pH7.2 and 0.1% Tween-20, the plates were blocked with 200µl of 10% fat free milk powder ("Elite", Clover SA, Pty, Ltd.) in PBS at 37ºC for 1h and then washed as before. Test sera were diluted 1:400 in diluent buffer consisting of 2% fat free milk powder in PBS, 100µl added to each well and incubated for 1h at 37ºC. Samples were tested in duplicate for each isotype-specific HRPO conjugate used. After washing as before, 100 µl of goat anti-mouse IgG (H+L), goat anti-mouse IgG1 or goat anti-mouse IgG2a HRPO conjugate (Zymed Laboratories, Invitrogen, U.S.A.) at 1:2000 dilution was added to respective plates testing for the same serum specimens in parallel. After 1h incubation at 37ºC plates were washed as before and 100 µl of 2,2'-azinodiethylbenzthiazoline sulfonic acid (ABTS) (KPL Laboratories, Inc., USA) added to each well. After 30 min incubation in the dark the reaction was stopped by the addition of 100 µl of 1% sodium dodecyl sulphate (SDS) to each well. Optical density (OD) was determined at 405nm and the results expressed as the mean OD value for the biological triplicates tested.

6.2.2 Measuring up- and downregulation of genes using qRT-PCR

6.2.2.1 Selection of genes to be analyzed (Quantitect RT-PCR)

A total of 5 genes, involved in the immune response against viral infections, were chosen as target genes for measuring up- and downregulation (Table 6.1). An additional housekeeping gene was included for normalization of data.

WitsETD

Table 6.1. Genes analyzed by Quantitect RT-PCR

Gene	Gene name	Function of transcript	Accession number
Gapdh	glyceraldehyde-3-phosphate dehydrogenase	Housekeeping gene	NM_008084
Nfkb1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1, p105	Transcription factor involved in immunity	NM_008689
Casp3	caspase 3	Involved in cell apoptosis	NM_009810
Il10	interleukin 10 [cytokine synthesis inhibitory factor (CSIF)]	Anti-inflammatory cytokine	NM_010548
Ifng	interferon gamma	Cytokine involved in innate/adaptive immunity against viral infections, direct antiviral activity, activation of macrophages	NM_008337
Ifnb1	Interferon beta 1, fibroblast	Cytokine with antiviral activity involved in innate immunity	NM_010510

Optimized and validated primer sets were ordered specifically for use with the QIAgen SYBR Green-based real-time RT-PCR kit (Quantitect primer assay, QIAgen, Germany) (https://www.qiagen.com/geneglobe/default.aspx).

6.2.2.2 RNA extraction from mouse tissues (Quantitect RT-PCR)

Liver, spleen and brain tissues were transferred from RNAlater directly into 2ml centrifugation tubes containing 800µl buffer RLT (RNeasy Mini kit, QIAgen, Germany) and one 5mm stainless steel bead for tissue disruption and homogenization using the Tissuelyser II (QIAgen, Germany) as recommended by the manufacturer (4 min, 25 Hz). Homogenates were centrifuged for 3 minutes at 13 200 rpm, and the supernatant transferred to a new tube. RNA was extracted from these supernatants using the RNeasy Mini kit (QIAgen, Germany) as suggested by the manufacturer. As suggested, 50% ethanol was used for extraction from liver tissues to increase RNA yield, and 70% ethanol for spleen and brain tissues. The optional on-column DNase digestion was performed using the RNase-free DNase set (QIAgen, Germany) as suggested by the manufacturer, to remove genomic DNA. The RNA was eluted in the supplied RNase-free water, the concentration determined using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, U.S.A) and stored at -70°C until further testing.

WitsETD

6.2.2.3 Determination of RT-PCR efficiency using different primer sets

Determination of RT-PCR efficiencies for the different primer sets was necessary to enable data analysis as described below (6.2.2.5). RNA was extracted as described above (6.2.2.2) from the liver, spleen and brain of a mouse collected before RVFV challenge to use as tissue specific RNA standards. Dilution series were prepared and the following amounts of RNA standard from all organs tested in duplicate using all primer sets: 30 ng; 15 ng, 7.5 ng, 3.75 ng and 1.875 ng.

The real time RT-PCR reactions were performed as described by the manufacturer (Quantifast SYBR Green RT-PCR kit, QIAgen, Germany) using a LightCycler 1.5 (Roche, Germany). Shortly, a reaction mix was prepared by mixing 2 x Quantifast SYBR Green RT-PCR Master Mix (HotStarTaq Plus DNA Polymerase, Quantifast SYBR Green RT-PCR buffer, dNTP mix and ROX passive reference dye), 10 x Quantitect Primer sets (Gapdh, Nfkb1, Casp3, Il10, Ifng or Ifnb1), Quantifast RT-Mix (Omniscript RT and Sensiscript RT), template RNA (30 ng; 15 ng, 7.5 ng, 3.75 ng or 1.875 ng) and RNase-free water to a final volume of 20µl per reaction. This mix was transferred to 20µl LightCycler Capillaries (Roche, Germany) and run on the LightCycler 1.5 using the following cycles: 1 x reverse transcription (10 min, 50°C), 1 x hotstart PCR activation (5 min, 95°C) and 40 x cycles of denaturation (10 sec, 95°C) and annealing/extension (30 sec, 60°C), with fluorescence data collection just after the annealing/extension step. The threshold cycle (C_T) values were determined using the second derivative maximum method (LightCycler Data Analysis Software version 3.5.28, Roche). The C_T values and their corresponding template amount values were then used to determine the PCR reaction efficiencies using the Relative Expression Software Tool (REST, QIAgen, (http://www.qiagen.com/Products/REST2009Software.aspx?r=8042) (Pfaffl et al., 2002). The calculated PCR efficiencies were subsequently used to calculate fold changes in gene expression as described in 6.2.2.5.

6.2.2.4 Quantitect RT-PCR on mouse tissues collected at different time points

Quantifast SYBR Green RT-PCR reactions were performed on RNA extracts from mouse tissues collected at different time points (3, 6, 12, 24, 72 and 120 hours p.i.) as described above, except that 10 ng of RNA was used as template for all reactions. Three mice per group were analyzed per time point, and average values calculated.

6.2.2.5 Data analysis (Quantitect RT-PCR)

Threshold values (Ct) for the five different genes analyzed as determined for the immunized mice, adjuvant control and PBS control mice were firstly normalized to the Ct values of the housekeeping gene in the same samples, and then normalized to the normalized Ct values from the non-infected control mice to determine the relative changes in gene expression compared to age related normal mice. This is called the $2^{-\Delta\Delta Ct}$ method. Fold changes in gene expression of specific tissues at specific collection times were calculated using C_T values and the REST software. Fold change values equal, or close to 1, indicate no change in gene expression. Values > 1 indicate up-regulation, and values < 1 indicate down-regulation. The software determines fold changes by using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001, Pfaffl, 2001). The software also uses randomizations and a hypothesis test, P(H1), to determine the statistical significance of fold changes in gene expression (Pfaffl et al., 2002). Genes were only regarded as statistically up-regulated when the following requirements were met: p-value ≤ 0.05 and fold change ≥ 2.0. For values < 1 the negative inverted value was determined, and genes were only regarded as statistically down-regulated when these values were ≤ -2.0 and the p-value ≤ 0.05. Where fold changes were ≥ 2.0 or ≤ -2.0, but p-values not ≤ 0.05, the results were regarded as indicative of an up- or downregulated trend but not statistically significantly so.

6.2.2.6 RNA extraction (SABiosciences PCR Array)

RNA that was extracted from mouse livers collected at 72 hours (Group MS1,2,3 and 4) after infection as described above (6.2.2.2) were cleaned up further using the RT^2 qPCR-Grade RNA Isolation Kit (SABiosciences, QIAgen, U.S.A) as recommended by the manufacturer. On-column DNase treatment was performed to remove genomic DNA. RNA was eluted in RNase-free H_2O and RNA concentration determined. All RNA extracts were diluted to 150 ng/µl in nuclease-free water.

6.2.2.7 Mouse T- and B-cell activation PCR array (SABiosciences)

Complementary DNA (cDNA) was prepared from the RNA extracted in 6.2.2.6 using the RT^2 First Strand kit (SABiosciences, QIAgen, U.S.A) as described by the manufacturer. A total of 1.2 µg of each RNA preparation was mixed with 5 x genomic DNA Elimination buffer and the reaction incubated at 42°C for 5 minutes (total volume 10 µl). After the incubation the reactions were placed on ice immediately and subsequently an equal volume of RT-cocktail mix added (5 x RT buffer, primers and external control mix, RT-enzyme mix and RNase-free water). These reactions were then incubated at 42°C for 15 minutes and 95°C for 5 minutes. The resultant cDNA of each preparation was then diluted 1:10 with nuclease-free water and stored at -20°C until the assays were run.

The diluted cDNA was then mixed with the master mix (2 x SABiosciences RT^2 qPCR Master Mix) and nuclease-free water, and aliquoted onto the PCR array plates containing primer pairs (25 µl

of reaction mix per well). Plates were run on an ABI 7500 cycler (Applied Biosystems, U.S.A). The following cycling program was used: 1 x 95°C for 10 minutes, 40 x 95°C for 15 seconds and 60°C for 1 minute, followed by the default melting curve program. Fluorescence was measured just after the 1 minute / 60°C step. The cycle threshold (C_T) values were determined using the cycler software and an automatic baseline adjustment (ABI 7500 Software Version 2.0.1, Applied Biosystems, U.S.A). Three mice per group were analyzed per time point, and average values calculated.

6.2.2.8 Data analysis (SABiosciences PCR Array)

Data from the immunized mice, as well as adjuvant control and PBS control mice, were normalized to the data from the non-infected control mice to determine the relative changes in gene expression compared to age related normal mice. For analysis of data from the PCR array plates, the C_T values were exported into Microsoft Excel from the ABI software and subsequently copied into the SABiosciences PCR Array Data Analysis Template Excel Utility, which is freely available on the manufacturer's website (http://sabiosciences.com/pcrarraydataanalysis.php). This template calculates fold changes in gene expression using the $2^{-\Delta\Delta Ct}$ method, as well as the statistical significance (p-value) of results by using a T-test. Fold change values equal, or close to 1, indicate no change in gene expression. Values > 1 indicate up-regulation, and values < 1 indicate down-regulation. Genes were only regarded as up-regulated when the following requirements were met: p-value ≤ 0.05 and fold change ≥ 2.0. For values < 1 the negative inverted value was determined, and genes were only regarded as down-regulated when these values were ≤ -2.0 and the p-value ≤ 0.05. Where fold changes were ≥ 2.0 or ≤ -2.0, but p-values not ≤ 0.05, the results were regarded as indicative of an up- or downregulated trend but not statistically significantly so.

6.3 Results

After recNP/Alhydrogel immunization serum was collected from three mice at each collection point to monitor immune responses. Immunization of mice with recNP combined with Alhydrogel adjuvant yielded an almost identical profile of total IgG, IgG1 and IgG2a responses as shown in Chapter 4 (Figure 4.1 f). Strong total IgG and IgG1 responses were elicited, with a much weaker IgG2a response. High levels of total IgG and IgG1 were still detectable on day 27, a day before RVFV challenge (Figure 6.1).

The expression levels of 5 genes involved in the immune response against viral infections, normalized to a housekeeping gene (Gapdh), were analyzed in liver, spleen and brain tissues of recNP/Alhydrogel immunized mice, adjuvant control mice, PBS control mice and uninfected normal

control mice, by qRT-PCR at 3, 6, 12, 24, 72 and 120 hours post infection. Data from the other three groups were normalized against the data from the uninfected normal control group to show the changes in gene expression relative to age-related normal mice. Viral loads were also determined in the corresponding tissues. The results of the gene expression analysis and virus titration are shown in Table 6.3 (liver), Table 6.4 (spleen) and Table 6.5 (brain) for the following collection times: 3, 6, 12, 24, 72 and 120 hours. Statistically significant results (p-value ≤ 0.05) are indicated by an asterisk.

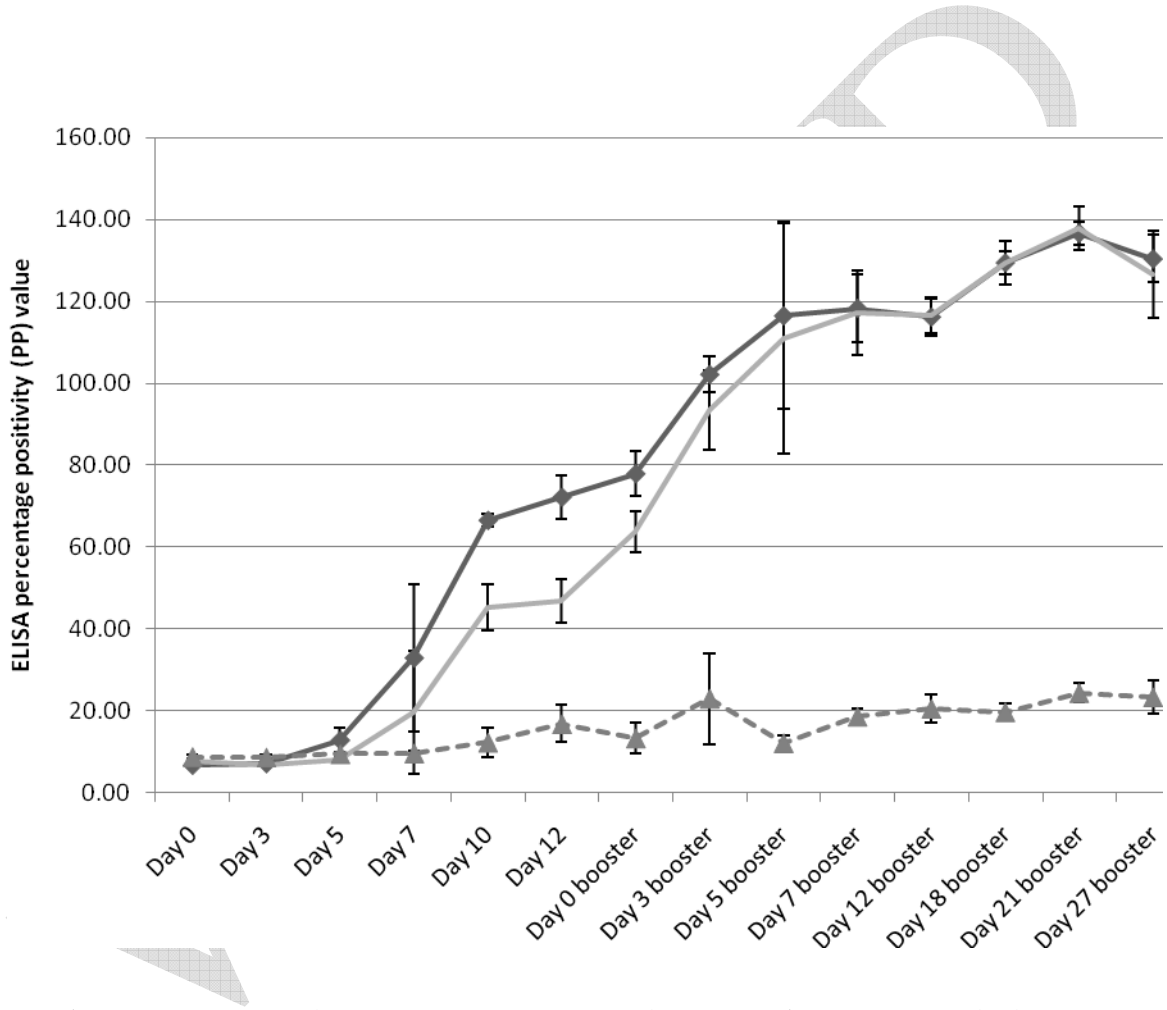


Figure 6.1. Detection of total IgG (—♦—), IgG1 (——) and IgG2A (--Δ--) specific antibodies against the RVFV recNP in mice after recNP immunization with Alhydrogel. Error bars indicate standard deviation from the mean (3 mice per time point).

Table 6.2. Fold changes in gene expression and viral loads in liver specimens from all treated groups at different time points.

	Gene	recNP immunized mice				Alhydrogel control mice				PBS control mice			
		Mean fold change	Standard error	P-value	Up/down regulated	Mean fold change	Standard error	P-value	Up/down regulated	Mean fold change	Standard error	P-value	Up/down regulated
3 hours	NFKB	-1.3	-1.7 to 1.0	0.30	-	1.2	1.0 to 1.5	0.40	-	1.1	-1.1 to 1.3	0.65	-
	CASP3	1.3	1.0 to 1.8	0.20	-	1.1	-1.3 to 1.3	0.56	-	1.0	-1.4 to1.3	0.85	-
	IL10	-1.3	-3.3 to 2.3	0.60	-	4.5	2.1 to 11.2	0.00	**UP***	2.3	-1.4 to7.5	0.38	UP
	IFNγ	-1.85	-2.3 to -1.3	0.07	-	-1.1	-2.5 to 1.8	0.85	-	1.3	1.0 to 1.9	0.20	-
	IFNb1	7.7	5.0 to 10.8	0.03	**UP***	-2.0	-5.0 to 1.3	0.38	DOWN	-2.5	-10.0 to 1.6	0.69	DOWN
6 hours	NFKB	1.2	-1.3 to 1.8	0.71	-	1.3	-1.3 to 2.3	0.40	-	1.0	-1.7 to 1.7	0.78	-
	CASP3	1.9	1.5 to 2.2	0.01	-	1.4	1.1 to 1.7	0.07	-	2.0	1.7 to 2.4	0.02	**UP***
	IL10	-1.3	-2.5 to 1.5	0.58	-	-3.3	-10.0 to 1.5	0.48	DOWN	-3.3	-5.0 to -1.3	0.03	**DOWN***
	IFNγ	17.2	4.9 to 94.2	0.00	**UP***	11.7	3.3 to 76.9	0.00	**UP***	9.3	2.4 to 46.3	0.00	**UP***
	IFNb1	34.9	6.6 to 263.8	0.00	**UP***	4.5	1.2 to 10.9	0.05	**UP***	2.0	-1.7 to 5.1	0.31	UP
	$TCID_{50}$	0	0	-	-	0	0	-	-	0	0	-	-
12 hours	NFKB	1.1	-1.3 to 1.5	0.80	-	-1.1	-1.7 to 1.1	0.46	-	1.2	-1.3 to 1.7	0.42	-
	CASP3	1.1	-1.4 to 1.7	0.65	-	1.0	-1.7 to 1.5	0.90	-	1.3	-1.1 to 1.9	0.31	-
	IL10	-1.1	-1.4 to 1.1	0.73	-	1.6	-1.4 to 3.1	0.33	-	1.0	-2.0 to 1.6	0.94	-
	IFNγ	11.0	6.1 to 25.0	0.06	UP	5.6	3.4 to 10.5	0.03	**UP***	5.8	3.2 to 11.1	0.08	UP
	IFNb1	5.9	2.3 to 12.8	0.07	UP	1.0	-2.0 to 1.8	0.95	-	2.0	1.5 to 2.8	0.04	**UP***
	$TCID_{50}$	0	0	-	-	0	0	-	-	0	0	-	-
24 hours	NFKB	1.2	-1.3 to 1.5	0.55	-	1.1	-1.3 to 1.5	0.67	-	1.4	-1.1 to 1.8	0.34	-
	CASP3	1.0	-1.7 to 1.8	0.97	-	-1.3	-2.0 to 1.7	0.69	-	-1.3	-2.0 to 1.5	0.55	-
	IL10	-1.3	-3.3 to 1.9	0.64	-	1.0	-2.5 to 2.2	0.93	-	3.8	1.7 to 8.6	0.09	UP
	IFNγ	5.3	4.1 to 7.0	0.04	**UP***	-2.0	-5.0 to 1.0	0.10	DOWN	-1.7	-2.5 to -1.3	0.02	-
	IFNb1	1.0	-2.5 to 2.2	0.94	-	1.2	-2.0 to 2.6	0.72	-	-3.3	-10 to -1.4	0.09	DOWN
	$TCID_{50}$	0	0	-	-	$10^{2.1}$	$10^{1.8}$	-	-	0	0	-	-
72 hours	NFKB	1.3	1.1 to 1.6	0.10	-	11.7	5.3 to 20.2	0.00	**UP***	8.5	4.7 to 14.9	0.0	**UP***
	CASP3	-1.1	-1.3 to 1.1	0.44	-	2.5	1.9 to 3.0	0.07	UP	1.3	-1.1 to 1.7	0.37	-
	IL10	24.4	10.6 to 75.0	0.00	**UP***	808.3	275.8 to 2521.9	0.03	**UP***	243.2	107.9 to 844.4	0.0	**UP***
	IFNγ	8.4	3.4 to 30.9	0.03	**UP***	5.8	2.4 to 15.0	0.03	**UP***	4.4	1.5 to 10.0	0.03	**UP***
	IFNb1	-2.0	-3.3 to -1.1	0.16	DOWN	2171.7	1044.3 to 4208	0.03	**UP***	2524.1	1016 to 6214	0.02	**UP***
	$TCID_{50}$	$10^{0.92}$	$10^{1.59}$	-	-	$10^{4.58}$	$10^{0.38}$	-	-	$10^{5.25}$	$10^{0.43}$	-	-
120 hours	NFKB	-1.3	-2.0 to 1.1	0.33	-	1.3	1.0 to 2.3	0.54	-	1.6	1.3 to 2.0	0.02	-
	CASP3	1.3	1.0 to 1.4	0.23	-	1.1	-1.1 to 1.4	0.56	-	1.5	1.2 to 1.9	0.09	-
	IL10	24.3	7.4 to 54.7	0.03	**UP***	54.7	14.1 to 321.6	0.00	**UP***	99.3	29.9 to 244.4	0.03	**UP***
	IFNγ	23.4	10.2 to 65.3	0.03	**UP***	224.0	92.6 to 531.0	0.00	**UP***	237.0	100.3 to 655.4	0.03	**UP***
	IFNb1	-2.0	-3.3 to 1.0	0.24	DOWN	8.7	5.2 to 16.3	0.05	**UP***	9.0	5.1 to 20.3	0.00	**UP***
	$TCID_{50}$	0	0	-	-	$10^{2.5}$	$10^{2.17}$	-	-	$10^{3.75}$	$10^{0.25}$	-	-

Table 6.3. Fold changes in gene expression and viral loads in spleen specimens from all treated groups at different time points.

	Gene	recNP immunized mice				Alhydrogel control mice				PBS control mice			
		Mean fold change	Standard error	P-value	Up/down regulated	Mean fold change	Standard error	P-value	Up/down regulated	Mean fold change	Standard error	P-value	Up/down regulated
3 hours	NFKB	-1.7	-2.5 to 1.0	0.28	-	1.2	1.0 to 1.4	0.38	-	-1.1	-1.4 to 1.1	0.60	-
	CASP3	-1.4	-1.7 to 1.0	0.26	-	1.1	-1.3 to 1.6	0.86	-	1.0	-1.4 to 1.2	1.00	-
	IL10	1.1	-1.3 to 1.7	0.71	-	1.1	-1.3 to 1.5	0.71	-	-1.1	-1.7 to 1.4	0.55	-
	IFNγ	-2.5	-3.3 to 0.9	0.05	**DOWN***	-1.4	-2.0 to 1.0	0.21	-	-1.7	-2.0 to -1.3	0.05	-
	IFNb1	12.0	3.6 to 56.9	0.03	**UP***	1.5	-1.7 to 4.1	0.44	-	1.4	-2.0 to 5.5	0.62	-
6 hours	NFKB	-1.4	-2.0 to -1.1	0.12	-	-1.1	-1.4 to 1.3	0.35	-	-1.4	-1.7 to -1.1	0.08	-
	CASP3	-1.4	-1.7 to -1.1	0.21	-	-1.1	-1.4 to 1.1	0.35	-	1.1	-1.1 to 1.2	0.59	-
	IL10	1.0	-1.7 to 1.4	0.79	-	-1.3	-1.4 to 1.1	0.37	-	-1.1	-1.3 to 1.2	0.39	-
	IFNγ	-1.7	-2.5 to -1.1	0.12	-	-1.3	-1.7 to 1.0	0.09	-	-1.7	-2.5 to 1.1	0.22	-
	IFNb1	5.7	2.5 to 15.9	0.06	UP	10.6	2.1 to 44.6	0.06	UP	9.7	5.0 to 29.3	0.02	**UP***
	$TCID_{50}$	0	0	-	-	0	0	-	-	0	0	-	-
12 hours	NFKB	1.0	-1.7 to 1.4	0.95	-	1.2	-1.1 to 1.8	0.54	-	1.1	-1.3 to 1.5	0.69	-
	CASP3	1.2	1.0 to 1.3	0.10	-	1.1	1.0 to 1.3	0.45	-	1.1	-1.3 to 1.5	0.96	-
	IL10	1.2	-1.3 to 1.8	0.50	-	-1.1	-1.4 to 1.5	0.80	-	1.1	-1.1 to 1.3	0.84	-
	IFNγ	-1.25	-1.4 to 1.0	0.00	-	-1.3	-1.4 to 1.0	0.00	-	1.1	1.0 to 1.3	0.00	-
	IFNb1	1.4	-1.1 to 2.5	0.52	-	2.1	1.0 to 4.0	0.26	UP	1.2	-2.0 to 3.1	0.56	-
	$TCID_{50}$	0	0	-	-	0	0	-	-	0	0	-	-
24 hours	NFKB	-2.0	-2.5 to -1.7	0.01	**DOWN***	-2.0	-2.0 to -1.7	0.02	**DOWN***	-1.1	-1.4 to 1.0	0.00	-
	CASP3	1.3	1.1 to 1.5	0.17	-	1.1	-1.1 to 1.3	0.34	-	1.2	-1.1 to 1.6	0.54	-
	IL10	-1.1	-1.4 to 1.3	0.64	-	1.1	-1.1 to 1.6	0.79	-	1.7	1.1 to 2.3	0.11	-
	IFNγ	-2.0	-3.3 to -1.7	0.07	DOWN	1.0	-1.4 to 1.2	0.92	-	-1.1	-1.7 to 1.4	0.47	-
	IFNb1	-10.0	-10.0 to1.2	0.19	DOWN	-3.3	-10 to -1.7	0.00	**DOWN***	-2.5	-10.0 to 1.5	0.31	DOWN
	$TCID_{50}$	0	0	-	-	$10^{2.33}$	$10^{2.08}$	-	-	0	0	-	-
72 hours	NFKB	-2.0	-10 to -1.4	0.00	**DOWN***	-1.7	-3.3 to 1.6	0.37	-	-2.5	-5.0 to 1.0	0.29	DOWN
	CASP3	1.3	1.1 to 1.6	0.18	-	-3.3	-5.0 to -3.3	0.00	**DOWN***	-2.5	-2.5 to -2.0	0.04	**DOWN***
	IL10	-1.4	-3.3 to 1.8	0.50	-	46.0	25.2 to 119.2	0.00	**UP***	55.0	30.2 to 139.9	0.00	**UP***
	IFNγ	20.0	1.2 to 214.4	0.10	UP	-1.4	-3.3 to 1.8	0.68	-	-1.3	-5.0 to 3.4	0.68	-
	IFNb1	16.1	10.7 to 26.6	0.07	UP	542.3	372.2 to 808.5	0.00	**UP***	510.3	336.7 to 714.3	0.00	**UP***
	$TCID_{50}$	$10^{3.58}$	$10^{0.52}$	-	-	$10^{5.58}$	$10^{0.52}$	-	-	$10^{5.50}$	$10^{0.66}$	-	-
120 hours	NFKB	-10.0	-10 to -3.3	0.00	**DOWN***	-5.0	-10 to -1.7	0.10	DOWN	-5.0	-10 to -1.3	0.13	DOWN
	CASP3	-5.0	-10 to -2.0	0.00	**DOWN***	-2.0	-5.0 to- 1.1	0.10	DOWN	-3.3	-10 to 1.2	0.20	DOWN
	IL10	-5.0	-10 to -3.3	0.03	**DOWN***	-1.4	-3.3 to 1.4	0.55	-	5.2	1.4 to 17.3	0.20	UP
	IFNγ	-1.3	-5.0 to 2.6	0.81	-	2.3	1.2 to 5.6	0.00	**UP***	1.5	-2.0 to 3.0	0.57	-
	IFNb1	4.4	1.5 to 25.4	0.03	**UP***	2.1	-1.4 to 11.8	0.81	UP	2.3	-1.4 to 15.4	0.66	UP
	$TCID_{50}$	0	0	-	-	$10^{5.25}$	$10^{0.25}$	-	-	$10^{5.17}$	$10^{0.29}$	-	-

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Table 6.4. Fold changes in gene expression and viral loads in brain specimens from all treated groups at different time points.

	Gene	recNP immunized mice				Alhydrogel control mice				PBS control mice			
		Mean fold change	Standard error	P-value	Up/down regulated	Mean fold change	Standard error	P-value	Up/down regulated	Mean fold change	Standard error	P-value	Up/down regulated
24 hours	NFKB	1.1	-1.1 to 1.4	0.69	-	-1.1	-1.4 to 1.2	0.82	-	1.1	1.0 to 1.4	0.12	-
	CASP3	1.3	1.0 to 2.0	0.24	-	1.1	-1.1 to 1.3	0.40	-	1.0	-1.3 to 1.3	0.97	-
	IL10	2.2	1.5 to 3.5	0.13	UP	2.7	1.7 to 4.4	0.07	UP	1.3	-2.5 to 3.4	0.71	-
	IFNγ	2.2	1.8 to 2.6	0.05	**UP***	1.4	1.1 to 1.7	0.14	-	1.8	1.5 to 2.1	0.03	-
	IFNb1	6.0	3.4 to 11.1	0.08	UP	4.5	3.2 to 6.7	0.05	**UP***	1.6	-1.4 to 3.6	0.45	-
	$TCID_{50}$	0	0	-	-	$10^{1.08}$	$10^{1.88}$	-	-	0	0	-	-
72 hours	NFKB	-1.1	-1.7 to 1.1	0.61	-	-2.5	-2.5 to -2.0	0.00	**DOWN***	-2.0	-2.5 to -1.7	0.04	**DOWN***
	CASP3	1.1	1.0 to 1.4	0.50	-	-1.3	-1.4 to -1.1	0.10	-	-1.7	-2.0 to -1.4	0.08	-
	IL10	1.3	-1.3 to 2.4	0.79	-	2.7	1.8 to 5.5	0.08	UP	7.0	4.1 to 13.5	0.09	UP
	IFNγ	2.2	1.5 to 2.7	0.03	**UP***	1.3	1.0 to 1.8	0.25	-	1.1	-1.3 to 2.0	0.88	-
	IFNb1	2.4	1.0 to 4.2	0.20	UP	4.6	2.1 to 9.3	0.06	UP	4.9	2.0 to 8.2	0.03	**UP***
	$TCID_{50}$	$10^{0.92}$	$10^{1.59}$	-	-	$10^{3.67}$	$10^{0.14}$	-	-	$10^{3.92}$	$10^{0.52}$	-	-
120 hours	NFKB	-1.7	-2.5 to -1.3	0.12	-	1.0	-1.4 to 1.5	0.82	-	-1.1	-1.7 to 1.4	0.76	-
	CASP3	-1.7	-2.5 to -1.3	0.04	-	-1.7	-2.0 to -1.3	0.06	-	-2.5	-3.3 to -1.7	0.02	**DOWN***
	IL10	1.5	-1.3 to 3.2	0.38	-	1.4	-2.0 to 3.1	0.64	-	1.8	-1.3 to 3.4	0.45	-
	IFNγ	4.0	2.2 to 7.1	0.07	UP	4.1	2.9 to 6.1	0.09	UP	3.9	2.6 to 5.3	0.07	UP
	IFNb1	-3.3	-10 to -1.4	0.13	DOWN	-1.3	-2.0 to 1.2	0.49	-	-2.5	-10.0 to 1.4	0.55	DOWN
	$TCID_{50}$	0	0	-	-	$10^{3.83}$	$10^{1.04}$	-	-	$10^{4.08}$	$10^{0.14}$	-	-

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The virus was detected at the earliest in liver, spleen and brain tissues of Alhydrogel control mice at 24 hours post infection (table 6.3, 6.4 and 6.5)(figure 6.2 a – b). The average titre of virus detected was $10^{2.1}$ $TCID_{50}$/g tissue. Replication of challenge virus was consequently detected in livers of all infected groups at 72 hours (3 days) post infection. Average viral load was drastically lower (±4000 to 20 000 fold lower) in recNP immunized mice ($10^{0.92}$ $TCID_{50}$/g tissue) compared to Alhydrogel control ($10^{4.58}$ $TCID_{50}$/g tissue) and PBS control mice ($10^{5.25}$ $TCID_{50}$/g tissue). At 120 hours (5 days) post infection viremia was undetectable in recNP immunized mice, whereas infectious virus could still be detected in Alhydrogel control ($10^{2.5}$ $TCID_{50}$/g tissue) and PBS control mice ($10^{3.75}$ $TCID_{50}$/g tissue) at lower titres compared to 72 hours.

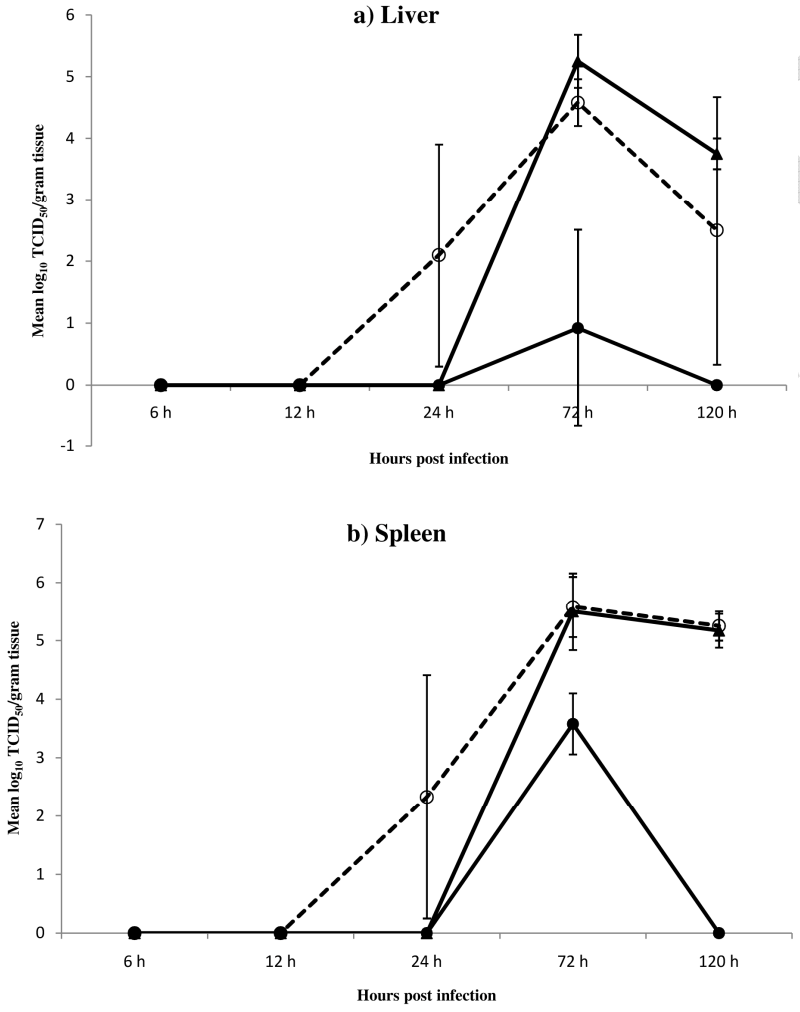


Figure 6.2 (a-b). Mean viral loads in livers and spleens of RVFV infected mice. Groups, consisting of 3 mice per group per time point, are indicated as: RecNP immunized (–●–), adjuvant control (–o–) and PBS control mice (–▲–). Livers are indicated in panel a, and spleens in panel b.

At 24 hours post infection virus to an average titre of $10^{2.33}$ $TCID_{50}$/g tissue was detected in spleens of Alhydrogel control mice. Replication of challenge virus was consequently detected in spleen tissues of all infected groups at 72 hours (3 days) post infection. Average viral load was lower (±100 fold) but still high in recNP immunized mice ($10^{3.58}$ $TCID_{50}$/g tissue) compared to Alhydrogel control ($10^{5.58}$ $TCID_{50}$/g tissue) and PBS control mice ($10^{5.5}$ $TCID_{50}$/g tissue). At 120 hours (5 days) post infection viremia was undetectable in recNP immunized mice, whereas infectious virus could still be detected in Alhydrogel control ($10^{5.25}$ $TCID_{50}$/g tissue) and PBS control mice ($10^{5.17}$ $TCID_{50}$/g tissue) at similar titres compared to 72 hours.

At 24 hours post infection virus to an average titre of $10^{1.08}$ $TCID_{50}$/g tissue was detected in brains of Alhydrogel control mice. Replication of challenge virus was consequently detected in brain tissues of all infected groups at 72 hours (3 days) post infection. Average viral load was drastically lower (± 500 to 1000 fold) in recNP immunized mice ($10^{0.92}$ $TCID_{50}$/g tissue) compared to Alhydrogel control ($10^{3.67}$ $TCID_{50}$/g tissue) and PBS control mice ($10^{3.92}$ $TCID_{50}$/g tissue). At 120 hours (5 days) post infection viremia was undetectable in recNP immunized mice, whereas infectious virus could still be detected in Alhydrogel control ($10^{3.83}$ $TCID_{50}$/g tissue) and PBS control mice ($10^{4.08}$ $TCID_{50}$/g tissue) at similar titres compared to 72 hours.

Survival rates of mice in this specific study could not be compared due to the regular euthanasia of mice for collection of organs. It was noted, however, that remaining mice from the recNP immunized group remained healthy throughout all collection time points. On the other hand mice from the PBS control group displayed illness from day 2 post infection, and 4 mice from the same group were found dead on day 3 post infection, and another on day 4 (these mice were excluded from further gene expression experiments). Three mice from the Alhydrogel control mouse group were also found dead on day 3 post infection. Remaining mice from these two groups continued displaying sings of illness due to RVFV infection until the end of the experiment, but not sick enough to warrant unscheduled euthanasia.

At 3 hours post infection (p.i.) the expression of the IFNβ gene was upregulated with statistical significance (7.7 fold, $p = 0.03$) in liver tissue of recNP immunized mice, whereas expression was decreased in adjuvant control (-2.0 fold, $p = 0.38$) and PBS control (-2.3 fold, $p = 0.69$) groups, but not with statistical significance (figure 6.3). Expression of the gene remained upregulated in immunized mice until 12 hours p.i., after which it waned and subsequently decreased. In adjuvant control mice IFNβ was briefly upregulated at 6 hours p.i. (4.5 fold, $p = 0.048$), but levelled out after that until 72 hours p.i. when it was significantly upregulated (2171.7 fold, $p = 0.03$). In PBS control mice on the other hand expression was increased at 6 hours (2.0 fold, $p = 0.313$) and 12 hours p.i. (2 fold, $p = 0.036$), with a sudden significant decrease in expression at 24 hours p.i. (-3.6 fold, $p = 0.09$), and a

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sudden upregulation at 72 hours post infection (2524.1 fold, $p = 0.02$). At 120 hours p.i. IFNβ expression was still upregulated in control mice (adjuvant control 8.7 fold, $p = 0.032$; PBS control (9 fold, $p < 0.01$).

The expression of IL-10 was upregulated significantly (4.5 fold, $p < 0.01$) in liver tissue of adjuvant control mice, and higher than normal in PBS control mice (2.3 fold, $p = 0.38$), but unaffected in recNP immunized mice (-1.2 fold, $p = 0.6$) at 3 hours p.i (figure 6.3). Just three hours later the same gene was decreased in control mice (adjuvant -3.6 fold, $p = 0.48$; PBS -3.1 fold, $p = 0.03$), returning to normal at 12 hours p.i. At 72 and 120 hours p.i., however, IL-10 expression was upregulated in the adjuvant (72 hours, 808.3 fold, $p = 0.033$; 120 hours, 55.0 fold, $p < 0.01$) and PBS control groups (72 hours, 243.3 fold, $p < 0.01$; 120 hours, 99.3 fold, $p = 0.034$), and upregulated in immunized mice (72 hours, 24.4 fold, $p < 0.01$; 120 hours, 24.3 fold, $p = 0.033$).

The expression of IFNγ was unaffected in liver tissue of all mice at 3 hours, but upregulated in all groups at 6 hours (recNP immunized, 17.2 fold, $p < 0.01$; adjuvant control, 11.7 fold, $p < 0.01$; PBS control, 9.3 fold, $p < 0.01$) and 12 hours post infection (recNP immunized, 11.0 fold, $p = 0.06$; adjuvant control, 5.6 fold, $p = 0.03$; PBS control, 5.8 fold, $p = 0.08$) (figure 6.3). Expression remained upregulated with relative stability in recNP immunized mice until 120 hours p.i., with a slight increase at 120 hours (23.4 fold, $p = 0.03$). In adjuvant and PBS control mice, however, there was a decrease in expression at 24 hours p.i. (adjuvant control, -2.2 fold, $p = 0.1$; PBS control, -1.7 fold, $p = 0.02$), followed by upregulation at 72 hours, and 120 hours (adjuvant control, 224.0 fold, $p < 0.01$; PBS control, 237.0 fold, $p = 0.029$).

The expression of the transcription factor NF-kB was stable in liver tissue of all mice at all time points up to 72 hours. At 72 hours, however, there was a significant upregulation of the gene in adjuvant control (11.7 fold, $p < 0.01$) and PBS control mice (8.5 fold, $p < 0.01$) while expression remained unaffected in immunized mice throughout all collection points. At 120 hours expression returned to constitutive levels in control mice too. The expression of the gene encoding Caspase-3 was relatively normal in liver tissue at all time points in all groups. There was only a brief upregulation in PBS control mice at 6 hours (2.0 fold, $p = 0.02$), and in adjuvant control mice at 72 hours (2.5 fold, $p = 0.07$).

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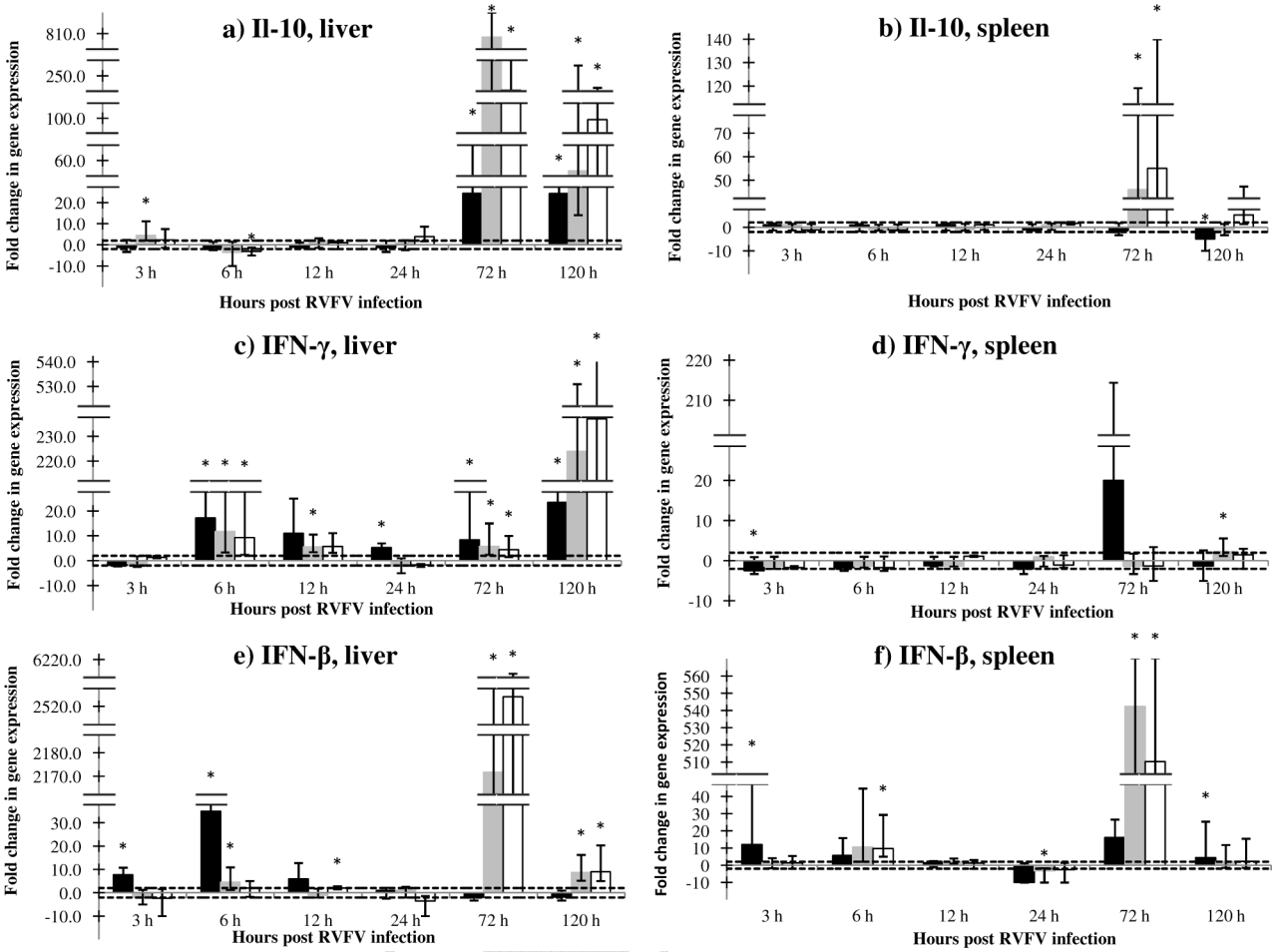


Figure 6.3 (a-f). Fold changes in expression of IL10, IFNγ and IFNβ genes in tissues of mice after RVFV infection. RecNP immunized mice (n = 3 per time point) are indicated by solid black bars, adjuvant control mice (n = 3 per time point) by grey bars and PBS control mice (n = 3 per time point) by white bars. The horizontal dotted lines indicate the cut-off values for upregulation (+2) or downregulation (22). The asterisk (*) indicates where the P-value is smaller than or equal to 0.05 (statistically significant results). Standard error values are indicated by the error bars. Note the differences in the Y-axis scales. The following time points are indicated: 3, 6, 12, 24, 72 and 120 hours.

The expression of IFNβ was also significantly upregulated in spleen tissue of recNP immunized mice at 3 hours p.i. (12.0 fold, p = 0.03), while expression was normal in adjuvant control (1.5 fold, p = 0.44) and PBS control mice (1.4 fold, p = 0.62). By 6 hours p.i. expression of IFNβ was upregulated in control mice as well (recNP immunized, 5.7 fold, p = 0.06; adjuvant control, 10.6 fold, p = 0.06; PBS control, 9.7 fold, p = 0.02). At 24 hours p.i. there was a sudden decrease in IFNβ expression in all groups (recNP immunized, -10.0 fold, p = 0.19; adjuvant control, -3.3 fold, p < 0.01; PBS control, -2.5 fold, p = 0.31), after which the gene was upregulated in recNP immunized mice (16.1

fold, p = 0.07) and upregulated in adjuvant control (542.3 fold, p < 0.01) and PBS control mice (510.3 fold, p < 0.01). Expression was still upregulated at 120 hours p.i., but to much lower levels compared to 72 hours (recNP immunized, 4.4 fold, p = 0.03; adjuvant control, 2.1 fold, p = 0.81; PBS control, 2.3 fold, p = 0.66). The expression of the IL-10 gene remained normal in spleen tissue of all groups until 72 hours p.i. when it was upregulated significantly in adjuvant control (46.0 fold, p < 0.01) and PBS control mice (55.0 fold, p < 0.01). Interestingly its expression was downregulated significantly in recNP immunized mice at 120 hours p.i. (-5.0 fold, p = 0.03).

There was almost no change in the expression of IFNγ in spleen tissue of control mice throughout the experiment, until 120 hours p.i. when it was upregulated in adjuvant control mice (2.3 fold, p < 0.01). In immunized mice, however, its expression was inconsistent, being downregulated shortly after infection (3 hours, -2.5 fold, p = 0.05) and again higher than normal at 72 hours p.i. (20.0 fold, p = 0.1). The expression of NF-kB in spleen tissue was not different between immunized and control groups, being downregulated at 24 hours (recNP immunized, -2.0 fold, p = 0.01, adjuvant control, -2.0 fold, p = 0.02), 72 hours (recNP immunized -2.0 fold, p < 0.01; PBS control, -2.5 fold, p = 0.29) and 120 hours p.i. (recNP immunized, -10.0 fold, p < 0.01; adjuvant control, -5.0 fold, p = 0.1; PBS control, -5.0 fold, p = 0.13). Expression of Caspase-3 remained normal in spleen tissue of all mice until 72 hours p.i. when it was downregulated significantly in adjuvant control (-3.3 fold, p < 0.01) and PBS control mice (-2.5 fold, p = 0.04), but unaffected in recNP immunized mice. At 120 hours p.i. expression was significantly downregulated in recNP immunized mice (-5.0 fold, p < 0.01) and decreased in adjuvant control (-2.0 fold, p = 0.1) and PBS control mice (-3.3 fold, p = 0.2).

The expression of IFNβ was upregulated in brain tissue of recNP immunized mice (6.0 fold, p = 0.08) and adjuvant control mice (4.5 fold, p = 0.05) at 24 hours p.i., but normal in PBS control mice. At 72 hours p.i. its expression was upregulated significantly in PBS control mice (4.9 fold, p = 0.03) and increased in recNP immunized (2.4 fold, p = 0.2) and adjuvant control mice (4.6 fold, p = 0.06). The expression of IFNγ was upregulated in brain tissue of recNP immunized mice at 24 hours (2.2 fold, p = 0.05), 72 hours (2.2 fold, p = 0.03) and 120 hours (4.0 fold, p = 0.07), but only increased at 120 hours p.i. in adjuvant control (4.1 fold, p = 0.49) and PBS control mice (3.9 fold, p = 0.07). The expression of Caspase-3 was only significantly downregulated in brain tissue of PBS control mice at 120 hours p.i. (-2.5 fold, p = 0.02) and normal at all other time points and other groups. The expression of NF-kB was significantly downregulated in brain tissue of adjuvant control (-2.5 fold, p < 0.01) and PBS control mice (-2.0 fold, p = 0.04) at 72 hours p.i. but normal at all other time points and in immunized mice.

The relative expression levels of 84 genes involved in various facets of the immune response against viral infections, normalized to four housekeeping genes, were analyzed in liver of

recNP/Alhydrogel immunized mice, adjuvant control mice, PBS control mice and uninfected normal control mice, by qRT-PCR at 72 hours post infection. Data from the other three groups were normalized against the data from the uninfected normal control group to show the changes in gene expression relative to age-related normal mice. The results of the gene expression analysis of the three different experimental groups are shown in Figures 6.2, 6.3 and 6.4.

Table 6.5. Genes analyzed by SABiosciences PCR array (T- and B-cell activation). Fold change in expression of 84 genes involved in activation of B- and T-cell immunity and other immune functions in immunized mice versus control mice after RVFV challenge at 72 hours in liver, relative to expression in an age-related control group of mice. * = statistically significant result.

Gene	Gene name	Transcript accession number	recNP Immunized Fold change and *P*-value	Adjuvant control Fold change and *P*-value	PBS control Fold change and *P*-value
Ap3b1	Adaptor-related protein complex 3, beta 1 subunit	NM_009680	1.65 p = 0.21	-1.09 p = 0.83	1.13 p = 0.58
Bad	BCL2-associated agonist of cell death	NM_007522	1.06 p = 0.62	**-2.19*** **p = 0.0004**	-1.64* p = 0.001
Cxcr5	Chemochine (C-X-C motif) receptor 5	NM_007551	1.56 p = 0.88	5.53 p = 0.18	5.23 p = 0.23
Cblb	E3 Ubiquitin Ligase Casitas B-lineage lymphoma b	NM_001033238	1.07 p = 0.68	**7.16*** **p = 0.006**	**8.65*** **p = 0.001**
Ccnd3	Cyclin D3	NM_007632	1.02 p = 0.86	1.12 p = 0.29	1.27* p = 0.02
Cd1d1	CD1d1 antigen	NM_007639	1.27 p = 0.41	**-6.27*** **p = 0.005**	**-6.88*** **p = 0.003**
Cd2	CD2 antigen	NM_013486	1.02 p = 0.90	**2.56*** **p = 0.02**	**2.66*** **p = 0.04**
Cd28	CD28 antigen	NM_007642	6.48 p = 0.13	**22.33*** **p = 0.002**	**21.41*** **p = 0.005**
Cd3d	CD3 antigen, delta polypeptide	NM_013487	1.15 p = 0.23	2.39 p = 0.07	1.86 p = 0.06
Cd3e	CD3 antigen, epsilon polypeptide	NM_007648	1.58 p = 0.30	3.44 p = 0.06	**3.28*** **p = 0.02**
Cd3g	CD3 antigen, gamma polypeptide	NM_009850	1.34 p = 0.20	1.14 p = 0.57	-1.47 p = 0.12
Cd4	CD4 antigen	NM_013488	1.06 p = 0.74	-1.89 p = 0.45	-1.01 p = 0.92
Cd40	CD40 antigen	NM_011611	2.91 p = 0.06	**29.40*** **p = 0.002**	**25.87*** **p = 0.003**
Cd40lg	CD40 ligand	NM_011616	1.41 p = 0.48	1.42 p = 0.44	1.83 p = 0.11
Cd74	CD74 antigen (invariant polypeptide of major histocompatibility complex, class II antigen-associated)	NM_010545	2.03 p = 0.12	1.39* p = 0.012	1.36 p = 0.13
Cd81	CD81 antigen	NM_133655	1.04 p = 0.68	**-2.17*** **p = 0.002**	-1.77* p = 0.002
Cd8a	CD8 antigen, alpha chain	NM_001081110	2.33 p = 0.40	4.88 p = 0.13	2.81 p = 0.23
Cd8b1	CD8 antigen, beta chain 1	NM_009858	-1.05 p = 0.99	4.08 p = 0.14	2.39 p = 0.40
Cd93	CD93 antigen	NM_010740	1.14 p = 0.85	83.91 p = 0.06	**144.67*** **p = 0.001**
Cdkn1a	Cyclin-dependent kinase inhibitor 1A (P21)	NM_007669	**9.02*** **p = 0.02**	**127.19*** **p = 0.0000**	**145.01*** **p = 0.0006**

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Clcf1	Cardiotrophin-like cytokine factor 1	NM_019952	1.27 p = 0.89	**33.92*** **p = 0.01**	**52.10*** **p = 0.002**
Cr2	Complement receptor 2	NM_007758	-2.0 p = 0.15	1.07 p = 0.74	1.61 p = 0.10
Csf2	Colony stimulating factor 2 (granulocyte-macrophage)	NM_009969	-1.56 p = 0.66	2.37 p = 0.45	2.09 p = 0.58
Cxcl12	Chemokine (C-X-C motif) ligand 12	NM_021704	1.24* p = 0.03	**-2.21*** **p = 0.02**	-1.64* p = 0.008
Cxcr4	Chemokine (C-X-C motif) receptor 4	NM_009911	1.66* p = 0.006	**22.12*** **p = 0.01**	**22.84*** **p = 0.002**
Dock2	Dedicator of cyto-kinesis 2	NM_033374	1.11 p = 0.43	**4.85*** **p = 0.04**	**3.47*** **p = 0.003**
Egr1	Early growth response 1	NM_007913	**-5.00*** **p = 0.01**	**3.88*** **p = 0.005**	**4.54*** **p = 0.004**
Flt3	FMS-like tyrosine kinase 3	NM_010229	-1.06 p = 0.87	**12.62*** **p = 0.004**	**9.19*** **p = 0.03**
Gadd45g	Growth arrest and DNA-damage-inducible 45 gamma	NM_011817	7.26 p = 0.26	**5.35*** **p = 0.002**	**4.37*** **p = 0.008**
Glmn	Glomulin, FKBP associated protein	NM_133248	1.08 p = 0.59	-1.03 p = 0.82	1.05 p = 0.73
H2-Aa	Histocompatibility 2, class II antigen A, alpha	NM_010378	1.84 p = 0.11	**2.01*** **p = 0.014**	1.56 p = 0.16
H60a	Histocompatibility 60a	NM_010400	-1.51 p = 0.70	**16.50*** **p = 0.046**	**13.99*** **p = 0.036**
Hdac5	Histone deacetylase 5	NM_010412	1.05 p = 0.65	-1.46 p = 0.04	**-2.18*** **p = 0.02**
Hdac7	Histone deacetylase 7	NM_019572	-1.07 p = 0.68	**4.83*** **p = 0.012**	**5.58*** **p = 0.0007**
Hells	Helicase, lymphoid specific	NM_008234	1.34 p = 0.46	2.15 p = 0.08	**2.64*** **p = 0.007**
Hsp90aa1	Heat shock protein 90, alpha (cytosolic), class A member 1	NM_010480	1.08 p = 0.67	1.33 p = 0.13	1.48* p = 0.012
Icosl	Icos ligand	NM_015790	2.33 p = 0.30	3.48 p = 0.25	3.29 p = 0.07
Ifng	Interferon gamma	NM_008337	4.05 p = 0.32	4.90 p = 0.09	3.10 p = 0.12
Igbp1	Immunoglobulin (CD79A) binding protein 1	NM_008784	-1.23 p = 0.15	**-2.39*** **p = 0.002**	**-2.37*** **p = 0.002**
Igbp1b	Immunoglobulin (CD79A) binding protein 1b	NM_015777	1.39 p = 0.37	**7.26*** **p = 0.04**	4.53 p = 0.10
Il10	Interleukin 10	NM_010548	3.99 p = 0.21	**17.64*** **p = 0.04**	**10.65*** **p = 0.02**
Il11	Interleukin 11	NM_008350	1.93 p = 0.20	275.80 p = 0.13	**772.47*** **p = 0.007**
Il12b	Interleukin 12B	NM_008352	2.59 p = 0.25	**11.75*** **p = 0.02**	**20.35*** **p = 0.005**
Il15	Interleukin 15	NM_008357	1.12 p = 0.48	-1.64* p = 0.026	-1.54* p = 0.04
Il18	Interleukin 18	NM_008360	1.21 p = 0.12	**-3.52*** **p = 0.0002**	**-4.91*** **p = 0.0001**
Il27	Interleukin 27	NM_145636	3.25 p = 0.63	5.73 p = 0.32	11.42 p = 0.10
Il2ra	Interleukin 2 receptor, alpha chain	NM_008367	3.18 p = 0.09	**5.66*** **p = 0.04**	4.41 p = 0.06
Il4	Interleukin 4	NM_021283	2.04 p = 0.35	21.42 p = 0.24	**8.73*** **p = 0.03**
Il7	Interleukin 7	NM_008371	**1.64*** **p = 0.05**	-1.70 p = 0.06	**-2.38*** **p = 0.009**
Impdh1	Inosine 5'-phosphate dehydrogenase 1	NM_011829	**2.13*** **p = 0.004**	3.26 p = 0.25	**4.92*** **p = 0.02**
Impdh2	Inosine 5'-phosphate dehydrogenase 2	NM_011830	1.18 p = 0.15	1.00 p = 0.99	1.10 p = 0.48
Inha	Inhibin alpha	NM_010564	-1.16 p = 0.74	4.49 p = 0.06	3.12 p = 0.17
Irf4	Interferon regulatory factor 4	NM_013674	1.51 p = 0.96	2.59 p = 0.39	1.95 p = 0.79
Jag2	Jagged 2	NM_010588	1.67 p = 0.18	**6.79*** **p = 0.02**	**7.46*** **p = 0.02**

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Ms4a1	Membrane-spanning 4-domains, subfamily A, member 1	NM_007641	1.39 p = 0.37	**7.26*** **p = 0.04**	4.53 p = 0.10
Nkx2-3	NK2 transcription factor related, locus 3 (Drosophila)	NM_008699	1.10 p = 0.72	1.56 p = 0.26	1.16 p = 0.59
Nos2	Nitric oxide synthase 2, inducible	NM_010927	1.05 p = 0.97	25.71 p = 0.06	**32.07*** **p = 0.001**
Pawr	PRKC, apoptosis, WT1, regulator	NM_054056	1.06 p = 0.57	**2.68*** **p = 0.0003**	**3.05*** **p = 0.0006**
Pdcd1lg2	Programmed cell death 1 ligand 2	NM_021396	1.90 p = 0.1	**12.42*** **p = 0.0004**	9.47 p = 0.08
Pik3cd	Phosphatidylinositol 3-kinase catalytic delta polypeptide	NM_008840	**3.59*** **p = 0.04**	5.77 p = 0.13	**7.53*** **p = 0.002**
Pik3r1	Phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 1 (p85 alpha)	NM_001024955	1.10 p = 0.63	**-3.80*** **p = 0.007**	**-3.63*** **p = 0.005**
Prkcd	Protein kinase C, delta	NM_011103	1.30 p = 0.1	**3.73*** **p = 0.03**	**4.01*** **p = 0.002**
Prkcq	Protein kinase C, theta	NM_008859	1.92 p = 0.18	4.63 p = 0.09	3.89 p = 0.06
Prlr	Prolactin receptor	NM_011169	1.15 p = 0.42	**-62.79*** **p = 0.0003**	**-59.16*** **p = 0.0003**
Ptprc	Protein tyrosine phosphatase, receptor type, C	NM_011210	2.07 p = 0.57	8.14 p = 0.055	3.69 p = 0.13
Rag1	Recombination activating gene 1	NM_009019	-1.05 p = 0.98	**4.98*** **p = 0.05**	3.11 p = 0.13
Relb	Avian reticuloendotheliosis viral (v-rel) oncogene related B	NM_009046	**2.50*** **p = 0.04**	**12.30*** **p = 0.002**	**15.85*** **p = 0.002**
Rgs1	Regulator of G-protein signalling 1	NM_015811	1.67 p = 0.25	**41.67*** **p = 0.006**	**24.76*** **p = 0.016**
Sftpd	Surfactant associated protein D	NM_009160	1.30 p = 0.41	**20.40*** **p = 0.03**	**26.35*** **p = 0.0006**
Sit1	Suppression inducing transmembrane adaptor 1	NM_019436	1.92 p = 0.24	3.64 p = 0.07	2.96 p = 0.25
Sla2	Src-like-adaptor 2	NM_029983	1.08 p = 0.66	2.38 p = 0.16	**2.30*** **p = 0.007**
Socs5	Suppressor of cytokine signalling 5	NM_019654	-1.01 p = 0.90	**2.55*** **p = 0.006**	**3.63*** **p = 0.0007**
Spp1	Secreted phosphoprotein 1	NM_009263	-1.10 p = 0.62	**29.12*** **p = 0.003**	**26.97*** **p = 0.001**
Tlr1	Toll-like receptor 1	NM_030682	1.82 p = 0.2	8.68 p = 0.07	5.55 p = 0.06
Tlr4	Toll-like receptor 4	NM_021297	3.46 p = 0.06	**14.13*** **p = 0.03**	**8.28*** **p = 0.005**
Tlr6	Toll-like receptor 6	NM_011604	1.30 p = 0.43	2.11 p = 0.14	1.77 p = 0.12
Tnfrsf13b	Tumor necrosis factor receptor superfamily, member 13b	NM_021349	-1.35 p = 0.76	**6.67*** **p = 0.0006**	4.27 p = 0.15
Tnfrsf13c	Tumor necrosis factor receptor superfamily, member 13c	NM_028075	1.23 p = 0.68	2.88 p = 0.12	2.46 p = 0.15
Tnfsf13b	Tumor necrosis factor (ligand) superfamily, member 13b	NM_033622	1.32 p = 0.60	**8.74*** **p = 0.014**	**5.75*** **p = 0.005**
Tnfsf14	Tumor necrosis factor (ligand) superfamily, member 14	NM_019418	2.14 p = 0.08	**24.66*** **p = 0.04**	**16.80*** **p = 0.001**
Traf6	Tnf receptor-associated factor 6	NM_009424	1.53 p = 0.06	1.72* p = 0.03	2.34 p = 0.06
Vav1	Vav 1 oncogene	NM_011691	2.20 p = 0.13	**7.50*** **p = 0.03**	**8.00*** **p = 0.02**
Was	Wiskott-Aldrich syndrome homolog (human)	NM_009515	1.53 p = 0.50	4.33 p = 0.07	**4.64*** **p = 0.004**
Wwp1	WW domain containing E3 ubiquitin protein ligase 1	NM_177327	-1.07 p = 0.38	**-9.16*** **p = 0.0001**	**-11.99*** **p = 0.0001**

Only one gene, Egr1 (early growths response 1 protein) was downregulated with statistical significance in the recNP immunized mouse group relative to the untreated control group (Table 6.2) (upper left quadrant of Figure 6.2). A total of four genes were upregulated with statistical significance

in the same group (Table 6.2) (upper right quadrant of Figure 6.2). A total of nine (9) genes were downregulated with statistical significance in the Alhydrogel control group relative to the untreated control group (Table 6.2) (upper left quadrant of Figure 6.3). A total of 28 genes were upregulated with statistical significance in the Alhydrogel control group relative to the untreated control group (Table 6.2) (upper right quadrant of Figure 6.3). A total of eight (8) genes were downregulated with statistical significance in the PBS control group relative to the untreated control group (Table 6.2) (upper left quadrant of Figure 6.4). A total of 37 genes were upregulated with statistical significance in the PBS control group relative to the untreated control group (Table 6.2) (upper right quadrant of Figure 6.4).

Selected genes that were significantly up- or downregulated in any of the experimental groups and indicating a clear difference or interesting similarity between immunized and control mice were grouped according to known effects on specific immune functions and shown in Figure 6.5 (a-f). Note that some genes are involved in multiple immune functions and are thus present in more than one figure.

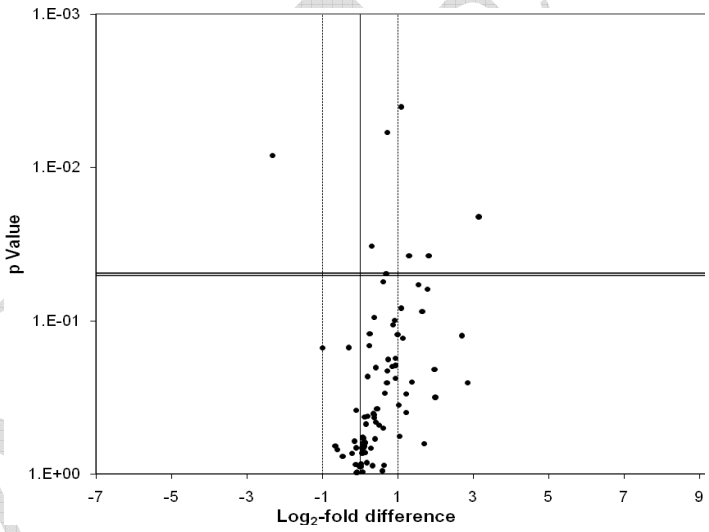


Figure 6.4. Volcano plot displaying average Log_2-fold changes in expression of 84 genes in the livers of three recNP immunized mice at 72 hours post infection, relative to an untreated control group of mice. Different genes are indicated by black dots (●). Black dots above the horizontal double line (representing a p-value of 0.05) indicate statistically significant results. The vertical dotted lines indicate the fold change margins, with dots to the left representing downregulated genes, and dots to the right indicating upregulated genes.

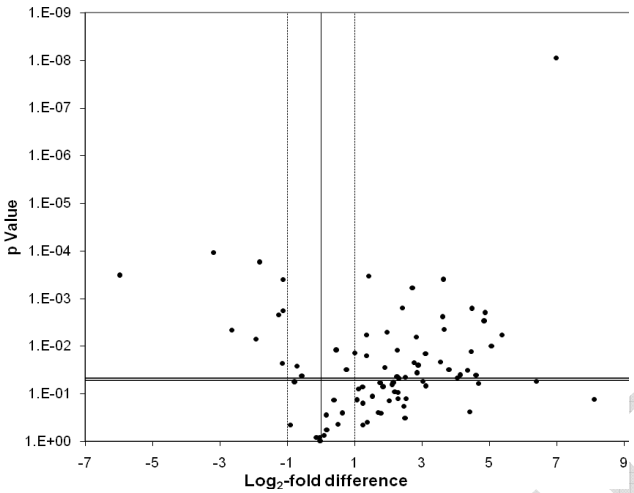


Figure 6.5. Volcano plot displaying average Log_2-fold changes in expression of 84 genes in the livers of three Alhydrogel control mice at 72 hours post infection, relative to an untreated control group of mice. Different genes are indicated by black dots (●). Black dots above the horizontal double line (representing a p-value of 0.05) indicate statistically significant results. The vertical dotted lines indicate the fold change margins, with dots to the left representing downregulated genes, and dots to the right indicating upregulated genes.

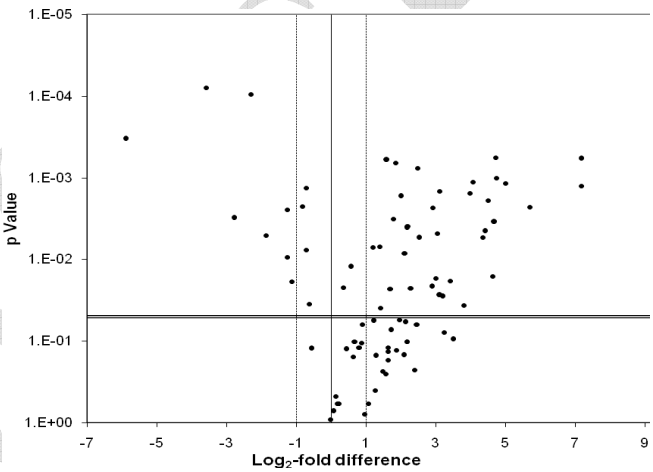


Figure 6.6. Volcano plot displaying average Log_2-fold changes in expression of 84 genes in the livers of three PBS control mice at 72 hours post infection, relative to an untreated control group of mice. Different genes are indicated by black dots (●). Black dots above the horizontal double line (representing a p-value of 0.05) indicate statistically significant results. The vertical dotted lines indicate the fold change margins, with dots to the left representing downregulated genes, and dots to the right indicating upregulated genes.

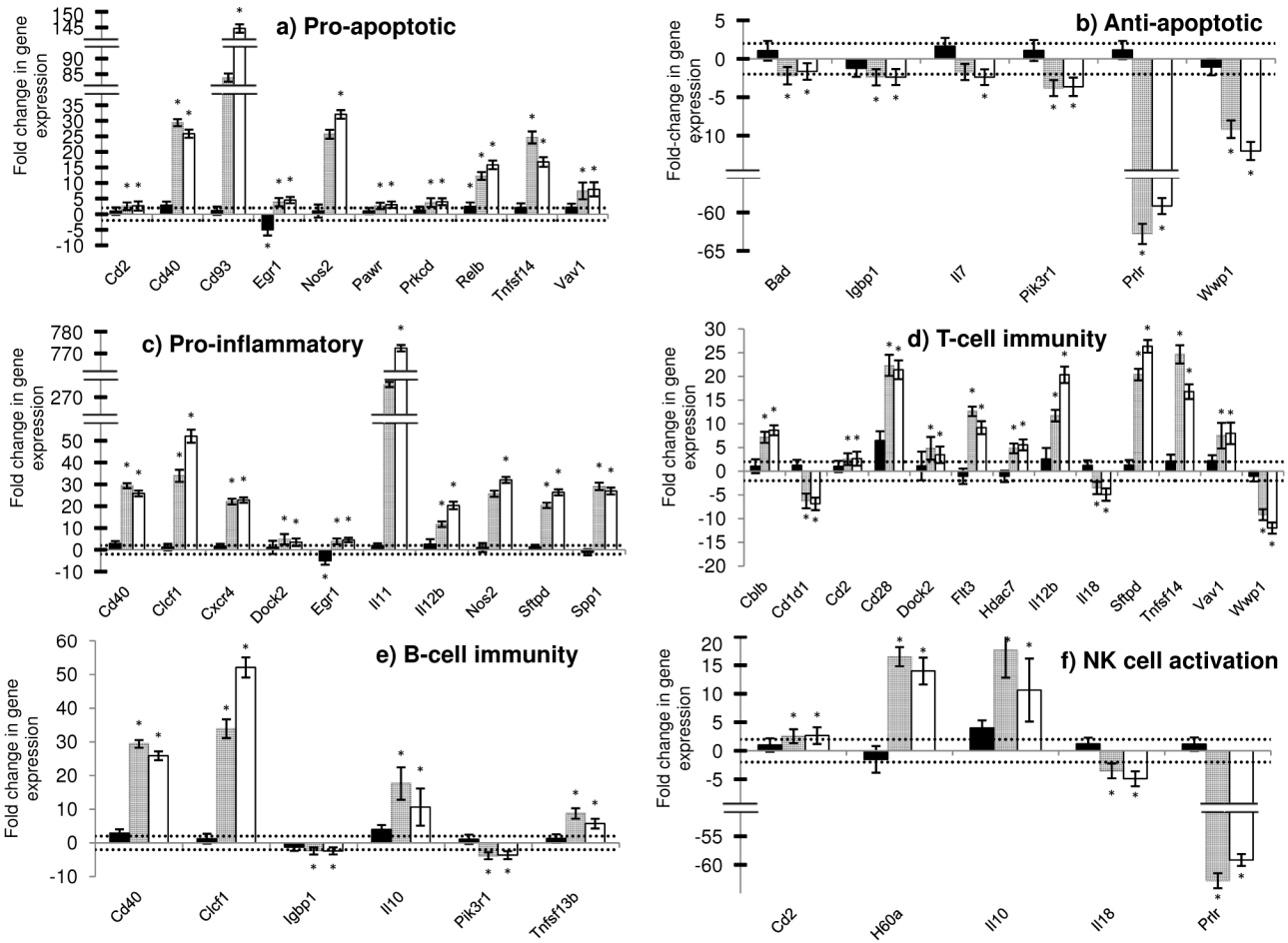


Figure 6.7 (a-f). Changes in expression of gene in the liver of experimental groups at 72 hours after RVFV infection. RecNP immunized mice are indicated by solid black bars, adjuvant control mice by grey bars and PBS control mice by white bars. The horizontal dotted lines indicate the cut-off values for upregulation (+2) or downregulation (-2). The asterisk (*) indicates where the P-value is smaller than or equal to 0.05 (statistically significant results). Standard deviation from the mean fold changes are indicated by the error bars.

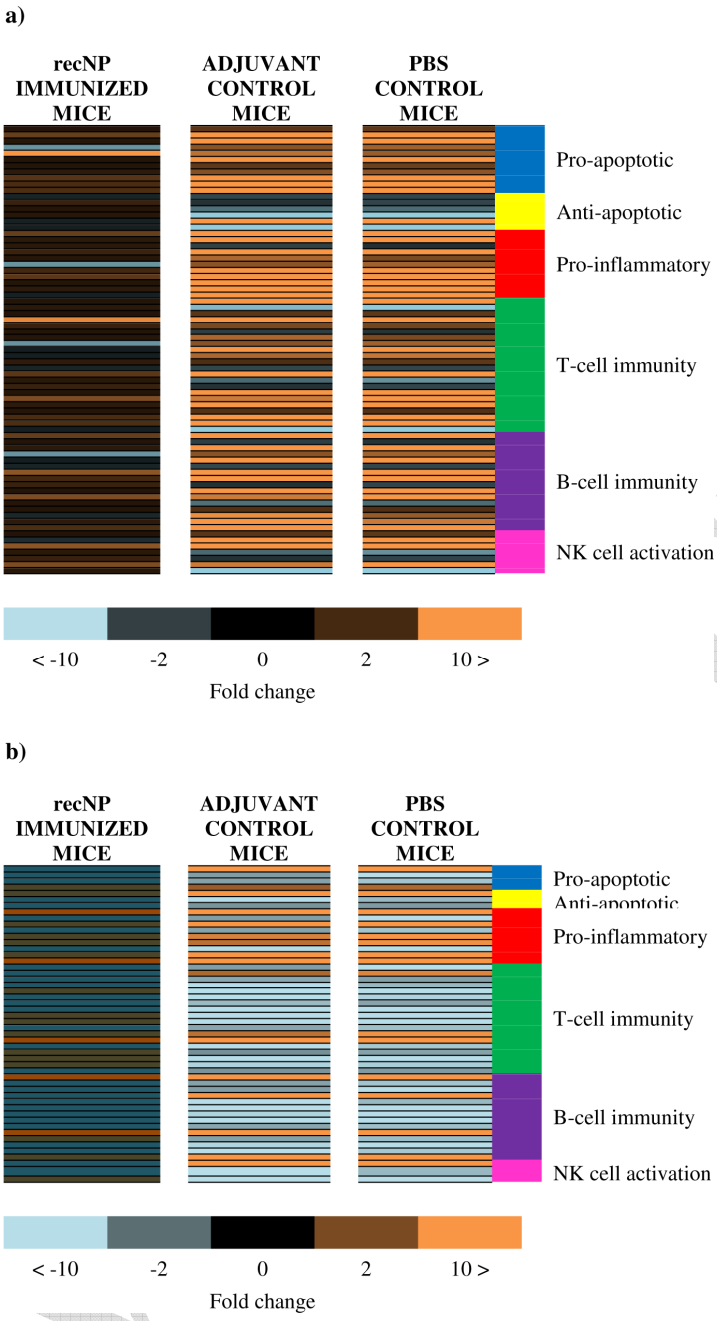


Figure 6.8 (a-b). Heat maps showing fold changes in liver and spleen at 72 hours after RVFV infection. Expression of genes in RecNP immunized, adjuvant control and PBS control mice are organized according to function. Livers are indicated in panel a, and spleens in panel b. The genes shown in orange are upregulated, those in blue are downregulated and those in black or darker shades of orange and blue have fold-change values between -2 and 2 and/or have p-values.0.05.

There was a significant upregulation of several genes that have pro-apoptotic effects in adjuvant and PBS control mice, whereas these genes were normally expressed in recNP immunized

mice (figure 6.7a and 6.8a) (Cho et al., 2006). One gene, encoding the early growth response 1 protein (Egr-1) which is a transcription factor involved in proliferation, differentiation and activation of cell death pathways (Kiebala et al., 2010), that was upregulated in adjuvant (3.9 fold, p = 0.005) and PBS control mice (4.5 fold, p = 0.004) was downregulated in recNP immunized mice (-5.0 fold, p = 0.01). Only one gene encoding the transcription factor RelB, part of the NF-KB family of proteins and responsible for counter-regulating the effects of NF-KB, was upregulated in immunized (2.5 fold, p = 0.04) and control mice (adjuvant control, 12.3 fold, p = 0.002; PBS control, 15.9 fold, p = 0.002) (figure 6.7a) (Yu-Lee, 2002, Kong et al., 2004, Fry and Mackall, 2005, Kittipatarin and Khaled, 2007, He et al., 2008, Jackson et al., 2008, Li et al., 2008, Lomonosova and Chinnadurai, 2008, Ajay et al., 2010). Several genes with anti-apoptotic effects were downregulated in the control mice but normal in recNP immunized mice (figure 6.7b) (Li et al., 2008). Most notably of these were the genes encoding the prolactin receptor (Prlr) (recNP immunized, 1.15 fold, p = 0.42; adjuvant control, -62.8 fold, p = 0.0003; PBS control, -59.2 fold, p = 0.003), an anti-inflammatory protein known to promote proliferation, protect against apoptosis and enhance cell survival, and the WW domain containing E3 ubiquitin protein ligase 1 (Wwp1) (recNP immunized, -1.1 fold, p = 0.38; adjuvant control, -9.2 fold, p = 0.0001; PBS control, -12.0 fold, p = 0.0001), an anti-apoptotic protein playing a role in proliferation (Senaldi et al., 1999, Denhardt et al., 2001, Coleman, 2002, Mazzali et al., 2002, Senaldi et al., 2002, Curnow et al., 2004, Zhou et al., 2004, Dalakas et al., 2005, Leth-Larsen et al., 2005, Cho et al., 2006, Prince et al., 2007, Pritchard et al., 2007, Guo et al., 2008, Lee et al., 2008, Peterss et al., 2009). There was also evidence of severe liver inflammation in adjuvant and PBS control mice, but not in recNP immunized mice (figure 6.7c) (Denhardt et al., 2001, Mazzali et al., 2002). Despite this the gene encoding the anti-inflammatory cytokine interleukin-10 (IL-10) was upregulated (recNP immunized, 4.0 fold, p= 0.21; adjuvant control, 17.6 fold, p = 0.04; PBS control, 10.7 fold, p = 0.02). The expression of osteopontin (gene Spp1), important for tissue damage healing, was upregulated significantly in control mice, compared to normal expression in immunized mice (Table 1) (Choi et al., 2001, Gartel and Radhakrishnan, 2005). The gene expressing the Cyclin-dependent kinase inhibitor P21 (Cdkn1a), a protein with pro- or anti-apoptotic effects and normally upregulated in response to liver injury, was upregulated in immunized (9 fold) and control mice (127 to 145 fold) (Table 1) (Lowenstein and Padalko, 2004). The gene expressing the inducible nitric oxide synthase (Nos2), an effector of the innate immune system targeting viral proteases and inhibiting viral replication, was normal in recNP immunized mice (1.1 fold, p = 0.97), increased in adjuvant control mice (25.7 fold, p = 0.06) and highly upregulated in PBS control mice (32.1 fold, p = 0.001) (Li et al., 1998, Senaldi et al., 1999, Senaldi et al., 2002, Kong et al., 2004, Norsworthy et al., 2004, Bohlson et al., 2005, Ceredig et al., 2006, Kunisaki et al., 2006, Kasler and Verdin, 2007, Qiao et al., 2007, Zhu et al., 2007, Couper

et al., 2008, Qiao et al., 2008, Zhang et al., 2008, Boesteanu and Katsikis, 2009, Duttagupta et al., 2009, Khan, 2009, Miletic et al., 2009, Peterss et al., 2009).

Both arms of the adaptive immune response, humoral (Th2) and cellular (Th1), were activated in control mice, but normal in immunized mice, at 72 hours (Durand et al., 2009). The gene encoding the Phosphatidylinositol 3-kinase catalytic delta polypeptide (Pik3cd), involved in the regulation of B-cells and antibody production, was upregulated in recNP immunized mice (3.6 fold, p = 0.04) and PBS control mice (7.5 fold, p = 0.002), and increased in adjuvant control mice (5.8 fold, p = 0.13) (Renukaradhya et al., 2005, Kunisaki et al., 2006, Kasmar et al., 2009). The genes encoding the Dedicator of cyto-kinesis 2 (Dock2) protein (recNP immunized, 1.1 fold, p = 0.43; adjuvant control, 4.9 fold, p = 0.04; PBS control, 3.5 fold, p = 0.003) and interleukin-12b (recNP immunized, 2.6 fold, p = 0.25; adjuvant control, 11.8 fold, p = 0.02; PBS control, 20.4 fold, p = 0.005), which are involved in the development and induction of NKT cells, were upregulated in control mice. Some important genes were, however, downregulated in control mice (figure 6.7 d-e). The gene encoding the Cd1d1 antigen, which is important for the presentation of antigens to, and activation of NKT cells, was downregulated with statistical significance in control mice (recNP immunized, 1.3 fold, p = 0.42; adjuvant control, -6.3 fold, p = 0.005; PBS control, -6.9 fold, p = 0.003) (Figure 4 d) (Palmer et al., 2008). The expression of the gene encoding interleukin-7, necessary for B- and T-cell and NK cell survival, was downregulated in PBS control mice (-2.4 fold, p = 0.009) (LeVine et al., 2001). The expression of surfactant protein D, a member of the collectin family, important role player in innate immunity and inhibitor of T lymphocyte proliferation, was upregulated in control mice (adjuvant control, 20.4 fold, p = 0.03; PBS control, 26.4 fold, p = 0.0006) but normal in immunized mice (1.3 fold, p = 0.41) (figure 6.7 d) (Qiao et al., 2007, Qiao et al., 2008, Zhang et al., 2008). The expression of the gene encoding the E3 Ubiquitin Ligase Cbl-b, capable of negatively regulating T-cell activation, was upregulated in control mice (adjuvant control, 7.2 fold, p = 0.006; PBS control, 8.7 fold, p = 0.001) but unaffected in immunized mice (1.1 fold, p = 0.68) (Figure 4 d) (Gracie et al., 2003). The expression of the gene encoding interleukin-18, responsible for biasing immunity towards Th-1 cellular immunity and enhancing T-cell cytotoxicity, was downregulated in control mice (adjuvant control, -3.5 fold, p = 0.0002; PBS control, -4.9 fold, p = 0.0001) but unaffected in immunized mice (1.2 fold, p = 0.12) (figure 6.7 d) (Seki et al., 2002). The expression of the suppressor of cytokine signalling 5 (Socs5), part of a family of proteins that negatively regulate cytokine signalling (Kong et al., 2004), was upregulated in control mice (adjuvant control, 2.6 fold, p = 0.006; PBS control, 3.6 fold, p = 0.0007) but normal in immunized mice (-1.01 fold, p = 0.9). The gene encoding the immunoglobulin binding protein 1 (Igbp1), a component of receptor cell signalling in B- and T-cells (Meroni et al., 2007, Palmer et al., 2008), was downregulated in control mice (adjuvant control, -2.4 fold, p = 0.002; PBS control, -2.4

fold, p = 0.002) but unaffected in immunized mice (-1.23 fold, p = 0.15). Other genes (Cd81 and Pik3r1) involved in activation, signalling and differentiation of B- and T-cells were also downregulated in control mice but normal in immunized mice (Table 1) (Li et al., 1998, Mavoungou et al., 2005, O'Connor et al., 2006).

An important role player in innate immunity, NK cells, was also activated in control mice (figure 6.7 f) (O'Connor et al., 2006). The gene encoding the histocompatibility 60 A protein, a ligand for an activating receptor on NK cells (Gracie et al., 2003, Mavoungou et al., 2005), was upregulated in control mice (adjuvant control, 16.5 fold, p = 0.046; PBS control, 14.0 fold, p = 0.036) but unaffected in recNP immunized mice (-1.5 fold, p = 0.7). However, expression of two important genes in NK cell activation and maturation, interleukin-18 (Il-18) and the prolactin receptor (Prlr), were downregulated in control mice but normal in immunized mice (Janssens and Beyaert, 2003). The expression of three genes encoding Toll-like receptors, a component of the innate immune system responsible for recognizing conserved structures, were analyzed with only Tlr4, responsible for recognizing patterns present on viral antigens, being upregulated in control mice (recNP immunized, 3.5 fold, p = 0.06; adjuvant control, 14.1 fold, p = 0.03; PBS control, 8.3 fold, p = 0.005) (Bouloy et al., 2001, Billecocq et al., 2004, Le May et al., 2004).

6.4 Discussion

The immune evasion mechanisms known for RVFV are all directed against the innate immune response, more specifically the type I interferon response (Won et al., 2007) and programmed cell death (apoptosis) (do Valle et al., 2010). Despite the proven involvement of the NSs protein of RVFV in inhibition of the type I interferon response, and thus increase in pathogenicity, it was recently shown *in vitro* that RVFV is not able to completely inhibit the expression of type I interferon (Lorenzo et al., 2008). It was shown that mice displaying an earlier and stronger type I interferon response were less susceptible to RVFV infection than mice with a delayed and partial response. The fact that RVFV does not cause a complete inhibition of the type I interferon response would suggest that the virus must have some additional evasive or regulatory effects, such as other innate immune mechanisms or adaptive immunity, to enable sufficient unhindered replication.

To gain some knowledge on the effects of RVFV on these other immune functions it was decided to test the expression of genes involved mainly in the activation of the B- and T-cell immunity, but also other immune functions, *in vivo* in the liver of mice experimentally infected with RVFV at the time of acute infection. The expression of five genes important in RVFV pathogenicity and involved in innate and adaptive immunity (nuclear factor kappa-beta, caspase-3, interleukin-10 and interferon-

WitsETD

gamma and -beta) was also tested at consecutive time points early and late after RVFV infection in mouse liver, spleen and brain to gain some insight into the dynamics of the expression of these genes during the extent of infection. At the same time the expression of all the genes mentioned above were compared between experimentally infected mice and mice that were vaccinated with a recombinant RVFV nucleocapsid protein (recNP), combined with the adjuvant Alhydrogel, before experimental infection. As shown previously, immunization of mice with recNP combined with Alhydrogel resulted in complete protection against disease and significant reduction in viral replication (Chapter 4). The RVFV NP does not have any neutralizing epitopes and a previous study suggested that a cellular (Th-1) response to the RVFV NP might be responsible for protection (do Valle et al., 2010). However, results shown in Chapter 4 suggested that the response after recNP/Alhydrogel immunization was biased towards Th-2 humoral immunity. The results presented in this chapter shows that recNP immunized mice were able to launch a stronger and earlier, but more controlled later type I interferon response compared to non-immunized mice, most probably contributing to the protection of immunized mice. More importantly the results show activation of several genes with pro-apoptotic and pro-inflammatory effects, but suppression of anti-apoptotic genes, at the time of acute RVFV infection in the liver of infected mice, possibly contributing to hepatic damage which is the main pathological feature of RVF. Also, the expression of several important genes involved in the activation and function of Natural Killer cells (innate immunity) and B- and T-lymphocytes (adaptive immunity) were suppressed in infected mice, indicating possible additional immune evasion tactics of RVFV.

Immunization of mice with recNP combined with Alhydrogel resulted in a strong IgG1 subclass response, compared to a weak IgG2A response, confirming that the immune response was biased towards Th-2 humoral immunity (Figure 6.1). Although tracking of the development of clinical disease in immunized and non-immunized mice after RVFV challenge was already previously shown (Chapter 4) and thus not a priority of the study presented in this chapter, it is worth noting that none of the recNP/Alhydrogel immunized mice developed any clinical illness during the course of the study, compared to non-immunized mice which displayed typical signs from day 3 p.i. to the end of the study (day 5). This corresponds to the viral loads detected in the different experimental groups (Table 6.3, 6.4 and 6.5) which shows that recNP/Immunized mice developed a very short and low viremia on day 3 post infection compared to much higher and extended viremia in non-immunized control mice (day 3 – 5). These results also confirmed that day 3 was indeed the point in acute infection with the highest viral replication in these mice and that analysis of the expression of genes at this point the most applicable.

The induction of expression of IFNβ has been shown to occur *in vitro* at 3 – 6 hours p.i, and it is therefore surprising that the results presented here show induction *in vivo* in liver and spleen tissue

of immunized mice already at 3 hours p.i., taking into account that after host infection the virus is likely conveyed to lymph nodes where it first replicates before it can spread to the liver and other organs (Haller and Weber, 2009). The expression of the same gene was, however, decreased (but not significantly downregulated) in liver tissue, and at constitutive levels in spleen tissue, of adjuvant and PBS control mice at 3 hours p.i. This decrease might already have been a result of the action of NSs on the type I interferon response of the host. The upregulation of IFNβ expression in immunized mice cannot be a direct result of anti-recNP memory since the innate response is general and not antigen specific, and this needs to be further investigated. It might, however, be as a result of some indirect actions. The fact that the anti-recNP response was largely humoral and that these antibodies are not neutralizing might indicate that some other form of antibody dependent mechanism, such as antibody-dependent cell-mediated cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC), was responsible. Antibody-dependent cell-mediated cytotoxicity is an NK cell mediated mechanism making use of specific antibodies, rather than memory cytotoxic T-cells, to form a link between the effector cell and an infected cell presenting an antigen on its surface, leading to lysis of infected cells. Complement-dependent cytotoxicity relies on the interaction of the C1q molecule binding to IgG or IgM already bound to an antigen. The lysis of infected cells that would have otherwise produced progeny virus, because of the inhibitory action of NSs, by these mechanisms might have resulted in the activation of the type I interferon response in neighbouring uninfected cells as a result of the release of dsRNA (Morrill et al., 1990, do Valle et al., 2010). The expression of IFNβ remained upregulated in immunized mice up to 12 hours p.i., compared to a brief and much lower upregulation in control mice between 6 and 12 hours p.i. At 72 and 120 hours p.i. IFNβ expression in liver tissue was decreased but still within the constitutive range in immunized mice compared to over expression in control mice liver and spleen tissue. This over expression of IFNβ in control mice liver and spleen tissue was not able to curb the replication of the virus and was probably more detrimental than valuable, contributing to the pathology of the liver. The sudden downregulation of IFNβ expression in spleen tissue of all mice at 24 hours probably contributed to the inability to control viral replication, as shown by high viral titres at 72 hours p.i. in spleen tissue, and was probably a result of the type I interferon inhibitory effect of the NSs protein. It has been shown previously that an early type I interferon response is protective against RVFV infection (Bouloy et al., 2001) and it is thus highly likely that the early and correctly regulated expression of IFNβ in immunized mice contributed to effective viral clearance and protection from liver pathology. The fact that IFNβ expression was upregulated in brain tissue of adjuvant control mice at 24 hours p.i., yet still the virus replicated to high titres, indicates that IFNβ did not play such an important role in innate immunity against RVFV infection in the brain.

WitsETD

There was not such a striking difference in the expression of type II interferon (IFNγ) between immunized and non-immunized control mice early after infection in liver and spleen tissue. Its expression was more or less similar up to 24 hours after which there was a sudden decline of expression in control mice liver tissue, while expression remained upregulated in immunized mice. This drop preceded the peak of viremia in control mice so it might be that the decreased IFNγ expression at this critical time point during the acute infection might have contributed, or even have been a result of, uncontrolled viral replication. Interestingly the expression of IFNγ in immunized and control mice was again similar at 72 hours, but at 120 hours expression was upregulated in control mice which might have contributed to the pathology of the liver. Interestingly, IFNγ expression was upregulated much earlier in the brain tissue of recNP immunized mice compared to control mice, also corresponding to much less viral replication, which might indicate that IFNγ plays an important role in protection against RVFV infection in brain tissue. It has been suggested that the role of IFNγ in RVFV pathogenesis is negligible (Morrill et al., 1991b) but this suggestion was based on *in vitro* results. It has been shown *in vivo* that IFNγ does indeed play a role in the attenuation of RVFV (Couper et al., 2008). There was a dysregulation of IL-10 expression in liver tissue of control mice very early after infection, with expression being upregulated at 3 hours p.i., and again downregulated at 6 hours. The dysregulated expression of IL-10 during a viral infection might actually contribute to immune escape since IL-10 is an anti-inflammatory cytokine that inhibits the actions of Th-1 cells, NK cells, decreases antigen presentation by cells and limits the production of various important cytokines (i.e. IL-12, IL-18 and TNF-α) (Bai et al., 2009). It has been shown that IL-10 is upregulated *in vivo* and *in vitro* after West Nile virus (WNV) infection, and that IL-10 deficient mice are less susceptible to WNV infection than mice expressing the gene constitutively (Ubol et al., 2010). Dengue virus has also been shown to replicate less efficiently *in vitro* when IL-10 expression is suppressed (Hsu et al., 1990, Spencer, 2007, van Putten et al., 2009). Some viruses even express IL-10 homologs to enable them to modulate the host immune system and escape viral clearance (Couper et al., 2008). At 72 and 120 hours p.i. there was again over expression of IL-10 in control mice, which might have led to immune escape by RVFV, although the anti-inflammatory effects of IL-10 might also have been an attempt by the host to counteract severe inflammation of the liver. On the other hand, expression of IL-10 seemed to follow a constitutive pattern of expression in liver tissue of recNP immunized mice, with the gene only being upregulated at 72 hours p.i. when there was viral replication in the liver, and onwards. The interplay between IL-10 and IFNγ, which are counter regulatory of each other, also seemed to be at constitutive levels in recNP immunized mice, with IFNγ being upregulated early to counter viral infection, and IL-10 only being upregulated later to curb immunopathology of the liver (Afford et al., 2001). The upregulation of IL-10 expression at 72 hours p.i. in spleen tissue of control mice is possibly an

indication of inflammation of the spleen and the hosts attempt counteract inflammation, but might have contributed to the virus being able to escape immune detection and thus replicate efficiently. The downregulation of IL-10 expression in spleen tissue of recNP immunized mice at 120 hours p.i., and the absence of replicating virus at the same time, is an indication that decreased IL-10 expression is advantageous to the host.

The activation of several genes with pro-apoptotic and pro-inflammatory effects, and the suppression of several genes with ant-apoptotic effects, in the liver of control mice 72 hours p.i. most probably contributed to severe hepatic disease. The overexpression of CD40, a member of the TNF receptor superfamily and potent activator of nuclear factor kappa beta, is of particular importance to apoptosis in the liver. CD40 has been shown to induce apoptosis in hepatocytes via a FAS dependent mechanism, the key mechanism for hepatocyte death in the liver (Gold et al., 2003). Mice deficient in the expression of CD40 has been shown to have improved survival during bacterial sepsis as a result of decreased induction of IL-6, IL-10, IL-12 and IFNγ expression (Peterss et al., 2009). Apart from its role in apoptosis of hepatocytes, CD40 plays a very important role in mediating B- and T-cell responses, thus the hosts attempt to launch an adaptive immune response to RVFV might actually contribute to immunopathology (Matsui et al., 2002, Anand et al., 2006). The expression of a TNF receptor superfamily ligand (Tnfsf14) was also upregulated in control mice. This protein is able to block TNFα mediated apoptosis but not FAS mediated apoptosis, and is a co-stimulatory factor that enhances T-cell mediated immunity leading to severe inflammation (Denhardt et al., 2001, Mazzali et al., 2002). The upregulation of the genes expressing osteopontin (Spp1) and the Cyclin-dependent kinase inhibitor P21 (Cdkn1a) is evidence of the host's attempt to counteract the damaging effects of the infection. Osteopontin is a cell survival factor and influences tissue repair at sites of severe inflammation (Gartel and Radhakrishnan, 2005). The Cyclin-dependent kinase inhibitor P21 (Cdkn1a) is a protein that plays a role in cell cycle control, with overexpression of P21 leading to cell cycle arrest (Choi et al., 2001, Gartell and Tyner, 2002). The protein has pro- or anti-apoptotic effects, has the ability to inhibit proliferation of cells and is upregulated in response to tissue injury (Dong et al., 2005). P21 interacts, amongst others, with the growth arrest and DNA damage-inducible gene 45 (Gadd45) (Chung et al., 2003), which was also upregulated in control mice. Gadd45 has been implicated in DNA repair, apoptosis, regulation of signal transduction and cell cycle control (Mansuroglu et al., 2010). The NSs protein of RVFV has been shown to interact with some specific regions of host cell DNA, causing defects in host chromosome structure and segregation (Gracie et al., 2003). Therefore it might be that these DNA damage inducible proteins are upregulated in an attempt to arrest the cell cycle of affected cells and prevent apoptosis. The fact that P21 is upregulated in

healthy immunized mice (9 fold) is probably as a result of the very low level of viral replication in their livers.

Despite several genes involved in the activation, differentiation and proliferation of B- and T-cells being upregulated in control mice and indicating activation of adaptive immunity, several genes with important functions in immune activation were also downregulated. Interleukin-18 (Il-18) is involved in the maturation and cytotoxicity of T-cells and NK cells, which are important cells in cellular and innate immunity respectively, and was downregulated in control mice (Mavoungou et al., 2005). The prolactin receptor is important in the functioning of NK cells but was downregulated in control mice (Kasmar et al., 2009). The expression of Cd1d1 antigen, which activates Natural Killer T-cells, was downregulated in control mice (Palmer et al., 2008). Interleukin-7, which is important for the development and survival of B-cells, T-cells and NK-cells were also downregulated in PBS control mice. The decreased expression of these important genes, and overexpression of other genes that have the ability to negatively regulate T-cell responses (Cblb and Sftpd), might have contributed to the inability of the control mice to clear virus from their liver despite the activation of other genes.

In summary this study shows that expression of IFNβ is upregulated later and to a lesser extent in the liver of non-immunized mice compared to immunized mice after RVFV challenge, but over expressed in control mice during acute infection of the liver. This expression pattern of the type I interferon, which is very important in the pathogenicity of RVF, possibly resulted in immunized mice being able to clear the viral infection much more efficiently than non-immunized mice. The over expression of IFNβ during acute liver infection was probably detrimental to the health of the control mice too. The expression of interleukin-10 was also irregular in the liver of control mice, possibly leading to the virus escaping detection to a certain extent and thus leading to excessive viral replication. The results also indicate activation of apoptosis in infected liver at the acute stage of infection, and severe inflammation which probably contributed to the pathology of the infection. The results for some of the genes also indicate inactivation of the induction of important immune cells which could have contributed to immune evasion. The results from this study will be useful for the evaluation of future candidate vaccines as genes that are regulated in naïve mice in response to RVFV infection have been identified. Host gene responses identified in this study may serve as potential targets for development of therapeutic interventions by suppressing inflammatory and apoptotic effects of RVFV infection in the liver. It would also be interesting to determine whether the same patterns of expressed genes are achieved in a host animal model such as sheep.

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CHAPTER SEVEN

CONCLUSIONS

At the advent of this project, all available techniques for the detection of antibodies against RVFV relied on the use of reagents that were prepared from infectious virus, thus requiring high biocontainment facilities and/or vaccinated personnel which restricts their production to a very limited number of laboratories in the world. In this study a recombinant RVFV nucleocapsid protein was utilized to develop several ELISA-based assays for rapid and safe diagnosis of RVF. An ELISA was developed and validated for the detection of IgG antibodies in wildlife species. There is still some controversy regarding the maintenance of RVFV during long inter-epidemic periods. One school of thought is that the virus is transmitted transovarially to mosquito eggs where it can survive for many years until the next sufficient flooding occurs. However, evidence to support this phenomenon is very limited and, in fact, transovarial transmission has been demonstrated only once and could not be reproduced ever since. The more likely explanation for natural transmission mechanism of RVFV is that there is low level inter-epidemic circulation of the virus between mosquitoes and wildlife that are not as closely monitored by farmers or game wardens as domestic livestock. Exceptional rainfall might then lead to spill-over of RVFV into domestic livestock and humans. This hypothesis seems to be supported by the presence of anti-RVFV antibodies in various wildlife species. The ELISA for IgG detection in wildlife developed in this study will be a valuable tool for facilitating cost-effective, large-scale sero-surveys which data might contribute to better understanding of RVF epidemiology, including natural transmission cycles. Understanding the inter-epidemic transmission of RVFV will contribute to more effective control of the disease. Another useful ELISA that was developed as part of this study is the human anti-RVFV IgM detection test based on a horseradish peroxidase labelled recombinant RVFV nucleocapsid protein. This test is an improvement on the traditional whole RVFV based ELISA, which carries some safety risks and is expensive to produce. An IgM detection ELISA is an essential part of the repertoire of tests necessary for successful diagnosis of a viral hemorrhagic fever, since it is indicative of a recent infection. An ELISA was also developed for the safe detection of RVFV antigen in human and animal specimens, another important addition to the array of tests for RVF diagnosis. The assay is based on a completely safe set of reagents and thus does not have to be prepared in a high containment facility. It enables the detection of antigen in decaying tissue long after infectious virus has been inactivated, which is an important tool for example when dead animals or foetuses are not immediately discovered in the field. A study was done to compare the diagnostic accuracy of the assays developed as part of this study to traditional assays, and it was found that the recombinant NP based ELISAs performed satisfactorily.

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There is no vaccine available for commercial human use, and the vaccines available for animal use are only available from a single facility in Africa, making supply inconsistent, have potential serious drawbacks in terms of adverse effects on animals when live-attenuated vaccines are administered or require multiple immunizations, in case of inactivated vaccines, to enable protection. Recombinant DNA technology and reverse genetics have been used extensively in recent times to develop vaccine candidates. Many of these candidates have shown promising results in preliminary studies but needs further evaluation before they can be used for large-scale vaccination. Most of these candidates have focussed on the glycoproteins of RVFV, because they contain neutralizing epitopes and neutralizing antibodies against RVFV is a known protective correlate. Studies on related viruses from the *Bunyaviridae* family have, however, shown that immunity generated against the nucleocapsid proteins of these viruses also offered some protection against subsequent viral challenge. A preliminary study was also done using a recombinant RVFV nucleocapsid as an immunogen in mice, which resulted in partial protection against lethal RVFV challenge.

Therefore it was decided to do a more in-depth evaluation of a recombinant nucleocapsid protein of RVFV as an immunogen in a mouse model, but also in an actual host animal model, namely sheep. The results from this study pointed out several important findings that could aid future RVFV vaccine development. It was shown that the choice of adjuvant is very important when using subunit immunogens, not only for enhancing immune responses but more importantly to bias immune responses towards a certain branch of the host immune system. Immunization of mice with recNP and certain adjuvants resulted in complete protection from lethal RVFV challenge. Two commercially available adjuvants, Saponin and Aluminium hydroxide gel (alum), yielded the best protection. Saponin is known to activate Th1 and Th2 immunity against antigens, and this was confirmed in this study with strong IgG1 and IgG2A responses against RVFV NP measured in mice. Although mice immunized with a high dose of recNP combined with Saponin yielded 100% protection, a lower dose only partially protected mice against lethal RVFV challenge. When recNP was combined with alum, known to strictly bias responses towards Th2 humoral immunity, even the lower dose resulted in 100% protection against challenge. The fact that recNP combined with alum in this study also activated Th2 humoral rather than Th1 cellular immunity, yet still yielded 100% protection, was surprising since anti-NP responses are not neutralizing and the common hypothesis was that it was cellular responses against the NP that was responsible for protection. There are, however, mechanisms known in addition to physical neutralization of virus by which antibodies can target and destroy pathogens. These are antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). These mechanisms work by using pre-existing antibodies against a foreign antigen to link adaptive immunity to innate immune mechanisms such as the complement cascade and NK cells.

Whereas physical neutralization requires antibodies to bind to surface proteins of the virus, glycoproteins in the case of RVFV, blocking epitopes necessary for entry into host cells by specific receptors, ADCC and CDC utilizes the recognition of antigens presented on the surface of host cells, which would include antigens not necessarily present on the virus surface, and subsequent lysis of those infected cells before virus replication. Therefore it might be that pre-existing anti-NP antibodies in recNP immunized mice resulted in the lysis of infected cells by either of these mechanisms before RVFV could replicate efficiently and cause a systemic infection, resulting in protection. This hypothesis is based on elimination of other possible mechanisms and not on concrete scientific data, and thus needs further investigation. This was the first study showing complete protection in a mouse model using the RVFV NP as vaccine candidate, and it confirmed the importance of including NP in RVFV vaccine candidates.

Although the recNP/adjuvant combinations were highly immunogenic in sheep, this immunity was not able to significantly decrease viral replication. The reason why the same recNP/adjuvant combinations that protected mice from RVFV infection could not protect sheep is unclear, but it is important to point out that mice were considered as protected once they did not develop clinical disease and did not have viral replication in specific organ tissues, whereas in sheep we could only use absence of virus in the blood as an indication of protection. Although it might therefore seem that a proper comparison wasn't done between mice and sheep, this was not the aim of the study. Because RVFV is a mosquito borne virus, the decrease in concentration of infectious virus in the blood is a very important indicator of the effectiveness of a vaccine candidate since the aim of the vaccine would not only be to protect the immunized host animal from development of disease, but more importantly to decrease the risk of transmitting virus amongst susceptible vertebrate hosts and competent mosquito vectors. Based on this information, the specific recNP/adjuvant combinations and concentrations used in this study can therefore not be considered as a potential vaccine candidate in RVF host species. Whether increasing the recNP doses, increasing the number of immunizations or changing the route of immunization would result in better protection is unknown, but the results from this study highlighted the very important fact that any potential RVF vaccine candidate should be properly evaluated in a host species before any conclusions can be made regarding its efficacy.

To enable more efficient development of vaccines and treatments against any infectious disease, one first needs a better understanding of the pathogenesis of that disease. Until recently the pathogenesis of RVF was poorly understood but reverse genetics enabled scientists to determine the RVFV NSs protein as the virulence marker of the virus. This protein acts by counteracting the effects of the very important innate immune system component, type I interferon. Another RVFV protein, NSm, was also recently shown to counteract apoptosis, another important innate immune mechanism.

Not much, however, was known about the activation or repression of other immune functions *in vivo* during RVFV infection. Despite the ability of NSs to counteract type I interferon, it was recently shown that this system is not completely repressed *in vitro* during RVFV infection, and it is therefore logical to assume that there must be some additional mechanisms by which the virus can evade the host immune response to enable sufficient viral replication. Although RVFV is a cytolytic virus that readily causes systemic infections, it was not clear whether immunopathology might also play a role in RVF disease progression and to what extent anti-NP responses might play a role in protection in this regard. In this study the expression of several genes involved in innate and adaptive immunity was evaluated in important target organs of the virus, at several time points after RVFV infection, and compared between recNP/Alhydrogel-immunized mice and two control groups consisting of Alhydrogel and PBS inoculated mice.

The results clearly indicate that recNP immunized mice were able to mount an earlier and stronger innate immune response compared to both control groups where these responses were repressed initially. The immunized mice were also able to control expression of genes with anti-inflammatory effects that might result in immune evasion when incorrectly regulated more appropriately than non-immunized mice early after infection. This resulted in replication being kept to a minimum in immunized mice compared to non-immunized mice that had excessive replication of virus in their target organs. During the acute phase of infection this excessive replication of virus in non-immunized mice was accompanied by massive upregulation of pro-inflammatory responses and genes with pro-apoptotic effects in their livers. These effects that very likely contributed to the pathology of the liver in non-immunized mice, the main target organ of RVFV, were not demonstrable in immunized mice. In addition to these immunopathological effects in non-immunized mice, there was also evidence of up- and downregulation of several important genes that could have translated into dysregulation of the activation of adaptive immunity, which very likely contributed to immune evasion.

In conclusion, this study not only expands the repertoire of safe and validated diagnostic methods for RVF diagnosis, but also contributes to a better understanding of the role of the NP in protection against RVF and the pathogenesis of the disease on a molecular level. There are still, however, many challenging issues regarding this neglected but emerging disease requiring improvements to be made in the serodiagnosis of RVF, for example the development of a point-of-care, or penside tests for rapid detection of antibodies or antigen. This would not only be a valuable diagnostic tool during suspected RVF outbreaks in remote areas where laboratories are not readily available, but also for the rapid screening of livestock at import/export stations to enable safe transportation between endemic and non-endemic regions. The development of ELISAs utilizing other

structural and non-structural recombinant RVFV antigens would also be an important addition to the current array of tests. This might not only further improve sensitivity and specificity of detection, but also enable differentiation between vaccinated and non-vaccinated animals, and better understanding of humoral responses in naturally infected and vaccinated individuals.

Although this study showed earlier activation of innate immunity in recNP immunized mice, the memory mechanism responsible for this protection needs further investigation. The possibility that antibody-dependent cell-mediated cytotoxicity or complement-dependent cytotoxicity is responsible for this protection needs to be elucidated. Because of the bi-phasic nature of RVF in some infected individuals, as a result of virus infection of the brain, it would be interesting to gain insight into the expression of apoptotic and inflammatory genes in this non-regenerative tissue. It will also be important to investigate how the virus crosses the blood-brain barrier, and why only a limited number of individuals develop encephalitis as a result of central nervous system infection. Future RVFV vaccine candidates should include glycoproteins and the nucleocapsid protein to induce the optimal humoral and cellular protection mechanisms against infection with field strains of the virus.

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APPENDIX 1 ETHICS APPROVALS

AESC 3

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2008/16/4

APPLICANT: Mr. PJ van Vuren

SCHOOL: Virology
DEPARTMENT:
LOCATION:

PROJECT TITLE: **Evaluation of a recombinant RVFV nucleocapsid protein as a recombinant vaccine and an immunodiagnostic reagent**

Number and Species

770 female, mice, 25 female sheep

Approval was given for to the use of animals for the project described above at an AESC meeting held on 20080326. This approval remains valid until 20100326

The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application form and to the following additional conditions:

Signed: _____ (Chairperson, AESC) Date: 02/04/08

I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

Signed: _____ (Registered Veterinarian) Date: 02/04/08

cc: Supervisor:
Director: CAS

Works 2000/[illegible]/AESC\Cert.wps

15-MAY-2008 10:24 FROM:WITS-RESEARCH OFFICE 011 717 1265 TO:0086977061 P:1

AH: Mr P Jansen van Vuren

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Janse van Vuren

CLEARANCE CERTIFICATE	**PROTOCOL NUMBER M080448**
PROJECT	Evaluation of a recombinant RVFV necleocapsid protein as a recombinant vacine and an immunodiagnostic reagent
INVESTIGATORS	Mr PJ Jansenvan Vuren
DEPARTMENT	Wits School of Pathology
DATE CONSIDERED	08.04.25
DECISION OF THE COMMITTEE*	Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 08.05.30 **CHAIRPERSON** .....
(Professor P E Cleaton Jones)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor : Dr JT Paweska

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

AESC 2009

Please note that only typewritten applications will be accepted. Should additional space be required for section "I" and/or "j", please use the back of this form.

ANIMAL ETHICS SCREENING COMMITTEE

MODIFICATIONS AND EXTENSIONS TO EXPERIMENTS

a. Name: PETRUS JANSEN VAN VUREN

b. Department: Virology (Division Virology and Communicable Disease Surveillance) NICD-SPU.

c. Experiment to be modified / extended **AESC NO: 2008/16/4**

Original AESC number	2008	16	4
Other M&E's : N/A			

d. Project Title:

Evaluation of a recombinant RVFV nucleocapsid protein as a recombinant vaccine and an immunodiagnostic reagent

e. Number and species of animals **originally approved**:	770 25	BALB/c, female Sheep, female
f. Number of additional animals **previously allocated on M&Es**:	N/A	N/A
g. Total number of animals **allocated** to the experiment to date:	210 25	BALB/c, female Sheep, female
h. Number of animals **used** to date:	210 25	BALB/c, female Sheep, female

i. Specific modification / extension requested:

Phase I of the animal experiments have been carried out (210 BALB/c mice and 25 female sheep), but Phase II which, according to our original application, would have required the additional 560 BALB/c mice has not been carried out. **The current approved method expires on 26 March 2010, but we would like to request an extension until 31 December 2010 to allow sufficient time for completing the project.**

We would also like to apply for a modification of the Phase II experiment of the protocol, based on results achieved so-far (as set out in section J below), which would ultimately result in the use of less experimental animals (**Reduce**) and thus a more streamlined experimental protocol (**Refine**). We would like to replace the originally approved Phase II experiments (as set out in the original application) with the following:

MOUSE IMMUNISATION:

Initial immunization – day 0

RecNP/Alhydrogel group MS-1 (n = 120): All mice from this group will be immunized subcutaneously with a 100 µl inoculum containing 70 µg recNP in an equal volume Alhydrogel adjuvant.

Alhydrogel adjuvant control group MS-2 (n = 75): All mice from this group will be mock immunized subcutaneously with 100 µl containing PBS in an equal volume Alhydrogel adjuvant.

Placebo control group MS-3 (n = 75): All mice from this group will be mock immunized subcutaneously with 100 µl sterile PBS without recNP.

Normal mouse control group MS-4 (n = 75): The mice from this group will not be inoculated with anything, but will be kept with the other mice in order to have a negative control group for the gene expression experiment.

Booster immunization – day 14 after primary immunization
Booster immunization of groups will be carried as descried for primary immunization.

Three mice from the vaccinated group (MS-1) will be sacrificed and bled by cardiac puncture for immune response monitoring on the following days after the primary immunization: Day 0, 3, 5, 7, 9, 11 and 14. Three mice from the vaccinated group (MS-1) will be sacrificed and bled by cardiac puncture on the following days after the booster immunization: Day 3, 5, 7, 9, 12, 15 and 21. A total of 78 mice from the vaccinated group (MS-1) and 75 mice from the other groups will be moved to the BSL-3 animal facility at the SPU-NICD on day 21 after the booster immunization, and accommodated for at least 7 days before challenge with RVFV or mock inoculation with EMEM. Another three mice from the vaccinated group (MS-1) will be pre-bled on the day of the challenge experiment (day 28 after the booster). Bleeding of mice will be done according to NHLS Animal Ethics committee approved methods.

MOUSE CHALLENGE EXPERIMENT

At 3 weeks after booster immunization 78 mice from the vaccinated group (MS-1) and 75 mice from the other groups (MS-2, MS-3, MS-4) will be moved from the NHLS Animal Unit to the BSL-3 animal facility of the SPU and acclimatized to new environment for 7 days before challenge. Three mice from the vaccinated group (MS-1) will be bled directly before challenge.
A total of 75 mice from groups MS-1, MS-2 and MS-3 will be challenged subcutaneously with a 100 µl inoculum containing $10^{7.0}$ TCID50/ml of the 2007 Kenyan isolate of RVFV, passage two on Vero cells. The 75 mice from the normal mouse control group (MS-4) will be inoculated with EMEM.
Five mice from each group will be euthanized and brain, spleen and liver tissues collected 1 hour, 3 h, 6 h, 12 h, 24 h, 48 h, 72 h, 96 h, 120 h, 144 h, 168 h, 216 h, 264 h, 312 h and 360 hours after challenge each. These tissues will be collected into RNAlater and used for the **gene expression study**.
All mice will be clinically monitored twice daily for symptoms which will be recorded.
All mouse carcasses will be autoclaved and discarded in SANUMED biohazard boxes for incineration after collection of organs.

The total number of BALB/c mice needed for the Phase II experiment after the modification will be **345** instead of the originally approved 560, which is a significant **reduction of 215 mice**.

j. Motivation for modification / extension:

We would like to apply for a modification to our current approved project which would actually result in us using less animals than originally planned. This reduction of 215 mice from the Phase II part of the experiment, which still needs to be done, is because of the results obtained in Phase I experiments, which have already been completed. Initially we planned to evaluate three different aspects that could be important in vaccine efficacy studies in the Phase II experiments namely number of immunizations (one or two), determination of lowest antigen dose required for protection (three different doses) and durability of protection (challenge at 4, 12 and 24 weeks after immunization). Combined with this we planned to collect tissues at specific time points to evaluate the effect on gene expression after infection.
Our results from Phase I indicate that although protection against RVFV challenge was induced in some immunized mouse groups, the challenge virus was still able to replicate in organs of apparently healthy mice. Replication of virus in immunized animals is a result which indicates that our antigen would not have great use as a vaccine candidate, and therefore there is no need to evaluate it further as such. Therefore we wish to abandon the determination of number of immunizations required, determination of lowest protective dose and durability of protection. However, there is still no clarity regarding the role of the anti-RVFV nucleocapsid response in protection against disease in animals. For this reason we would like to still carry out the part of the Phase II experiment for the collection of tissues from mice for gene expression analysis, with some minor modifications as set out in section I above. **We would also like to apply for an extension to the time period allowed for the experiments until 31 December 2010, since experimentation was more time-consuming than originally anticipated.**

Date: **2010-02-16** Signature:

RECOMMENDATIONS:

Approved

Date: 10/03/2010 Signature: Chairman, AESC



NATIONAL HEALTH LABORATORY SERVICE
ANIMAL ETHICS COMMITTEE

Chairperson: Dr John Frean	Meeting Convener: Miss. P. Hawkins
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05.03.2007

Dear Mr van Vuren

I have pleasure to inform you that at the AEC meeting held on the 01st of March your application number 109/07 was approved with the providing the following amendments were made.

The above application was approved with the following amendments.

6.1.1 Numbers of mice to be adjusted to accommodate revised sampling procedure
6.1.2 Replace outbred with inbred mice for uniformity of immune response
6.1.3 Accommodate need for deworming sheep flock before commencement
6.1.4 Specify monitoring and amelioration procedure for illness in sheep
6.1.5 Alternative disposal arrangements for rabbits to be investigated, as there is no breeding stock
6.1.6 on site
6.1.7 Details of bleeding procedures (method, volume) in mice, rabbits and sheep to be provided
6.1.8 Diluent control to be changed from sterile water to PBS
6.1.9 Disposal of control mice to be stated
6.1.10 Head of Department declaration to be signed on final version

Once the amendments have been made please forward a sign copy of the application to me so it can be kept on file.

Thanking you

Pauline Hawkins
AEC
Secretary/convener

NATIONAL HEALTH LABORATORY SERVICE

ANIMAL ETHICS CLEARANCE CERTIFICATE.

CLEARANCE CERTIFICATE NUMBER: 118/10

APPLICANT: PETRUS JANSEN VAN VUREN

DEPARTMENT/COMPANY: Special Pathogens Unit, National Institute for Communicable Diseases, Sandringham

PROJECT TITLE: Rift Valley fever host gene expression and pathogenesis study in mice

SPECIES	NUMBER	TYPE OF APPLICATION
BALB/C MICE	345	EXPERIMENTAL

i) Approval is hereby given for the experiment/routine procedure described in the above application.

The use of these animals is subject to the National Code 1990 Guidelines as used by the NHLS AEC. If an application for a routine procedure then the recommended guide lines or SOP must be followed. It is limited to the procedure/s specified in the final version of the application form.

SIGNED---------- DATE: 4 June 2010
(Chairperson: Animal Ethics Committee)

ii) I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23(1) (C) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

SIGNED---------- DATE: 2/6/2010
(Registered Veterinarian)

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Validation of an indirect ELISA based on a recombinant nucleocapsid protein of Rift Valley fever virus for the detection of IgG antibody in humans

Janusz T. Paweska*, Petrus Jansen van Vuren, Robert Swanepoel

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Received 27 February 2007; received in revised form 7 June 2007; accepted 11 June 2007
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Abstract

An indirect enzyme-linked immunoassay (I-ELISA) based on the recombinant nucleocapsid protein (rNp) of Rift Valley fever virus was validated for the detection of specific IgG antibody in human sera. Validation data sets derived from testing sera collected in Africa ($n = 2967$) were categorized according to the results of a virus neutralisation test. The assay had high intra- and inter-plate repeatability in routine runs. No detectable cross-reactions between IgG antibodies generated from mice experimentally infected with viruses representing genus *Phlebovirus*, *Nairovirus*, *Orthobunyavirus* and Bhanja virus of the family *Bunyaviridae* were observed. At a cut-off optimised by the two-graph receiver operating characteristics analysis at 95% accuracy level, the diagnostic sensitivity of the I-ELISA was 99.72% and diagnostic specificity 99.62% while estimates for the Youden's index (*J*) and efficiency (Ef) were 0.993 and 99.62%. When cut-off values determined by mean plus two and by mean plus three standard deviations derived from I-ELISA readings in an uninfected reference population were used, the diagnostic sensitivity was 100% but estimates of Y, Ef and other combined measures of diagnostic accuracy were lower. The I-ELISA based on rNp is highly sensitive, specific and robust and can be applied for diagnosis of infection of Rift Valley fever and disease-surveillance studies in humans.
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Keywords: Rift Valley fever virus; Recombinant nucleocapsid antigen; IgG antibody; Indirect ELISA; Validation; Humans

1. Introduction

Rift Valley fever virus (RVFV) is a zoonotic mosquito-borne member of the genus *Phlebovirus* in the family *Bunyaviridae* (Bishop et al., 1980). The recent occurrence of the first confirmed outbreaks of Rift Valley fever (RVF) in humans and livestock outside the African region, namely in the Kingdom of Saudi Arabia and Yemen in 2000–2001 (Jupp et al., 2002; Shoemaker et al., 2002), re-emergence of the disease in East Africa in 2006–2007 (CDC, 2007) and the fact that RVFV replicates in a wide range of competent mosquito vectors (Turrel et al., 1998) have raised concern that the virus might spread further into non-endemic regions of the world. Moreover, RVFV is considered as a potential bioweapon agent (Peters, 2000; Sidwell and Smee, 2003; Lim et al., 2005). These threats emphasize the need for accurate, robust, safe and validated diagnostic and surveillance tools for RVF.

Highly accurate ELISAs (enzyme-linked immunoassay) based on β-propiolactone inactivated and/or gamma-irradiated, sucrose–acetone-extracted antigens derived from tissue culture or mouse brain have been developed and extensively validated for serodiagnosis of RVF in humans and animals (Paweska et al., 2003a,b, 2005a,b). Unfortunately, current whole antigen preparations have to be manufactured in high bio-containment facilities to limit the risk of exposure for laboratory personnel (Kitchen, 1934; Smithburn et al., 1949), and involve the risk of incomplete inactivation and high production costs.

The indirect ELISA (I-ELISA) based on the recombinant nucleocapsid protein (rNp) of RVFV have been recently reported to have high analytical accuracy for the detection of specific antibodies in humans (Jansen van Vuren et al., 2007), and in experimentally infected and vaccinated sheep (Jansen van Vuren et al., 2007; Fafetine et al., 2007). However, the assay cross-reactivity and other measures of assay performance char-

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acteristics have not been validated. Numerous important reasons for the test validation are well known, including the need for reliable estimates of the diagnostic sensitivity and specificity that are of concern with respect to serological diagnosis, risk assessment and risk factors studies. Hence, in the present study, extensive validation of this assay was undertaken, with special emphasis on test repeatability, cross-reactivity and diagnostic accuracy.

2. Materials and methods

2.1. ELISA serum controls and internal quality control

Freeze-dried, gamma-irradiated serum controls produced previously (Paweska et al., 2005a) were used. Means and standard deviations (S.Ds) of ELISA optical density values and percentage positivity (PP) of internal controls were calculated from their replicates in each plate and each run of the assay to assess intra- and inter-plate variation. Additionally, coefficients of variation [CV = (standard deviation of replicates/mean of replicates) × 100] were calculated for positive serum controls. Data obtained from this analysis were used to estimate the assay repeatability and to establish the upper and lower control limits for each of the internal controls. During routine runs of the ELISA each plate had four replicates of high positive (C++), four of low positive (C+), four of negative serum (C−), and four replicates of conjugate control (C_c).

2.2. Human sera

A total of 2967 sera collected in Kenya ($n = 982$), South Africa ($n = 1255$), Tanzania ($n = 360$), Uganda ($n = 210$) and Zimbabwe ($n = 160$) were used. Sera from South Africa and Zimbabwe represented post RVF outbreak specimens collected in the late 1970s and routine diagnostic submissions to the Special Pathogens Unit of the National Institute for Communicable Diseases (SPU-NICD), Sandringham, South Africa for the period 1999–2005. East African sera were specifically taken to monitor the 1997–1998 outbreak of RVF in the region (Woods et al., 2002). Sera which tested negative in the virus neutralisation test were regarded as reference panel from non-infected individuals, and sera which tested positive as reference panel from infected individuals. ELISA results obtained on the field-collected sera were used for the selection of cut-off values and determination of diagnostic accuracy of IgG I-ELISA.

2.3. Mouse ascetic fluids

Hyperimmune mouse ascetic fluids against viruses representing genus *Phlebovirus*, *Nairovirus*, *Orthobunyavirus* and Bhanja virus of the family *Bunyaviridae* produced as described by Burt et al. (1993) were obtained from reference serum bank of the Arbovirus section of the SPU-NICD. These ascetic fluids were used to evaluate their cross-reactivity with the recombinant nucleocapsid protein of RVFV in I-ELISA.

2.4. Serum neutralisation test

Duplicates of serial twofold dilutions of sera which had been inactivated at 56 °C for 30 min were tested using a microneutralisation procedure as previously described (Paweska et al., 2005a). Titres were expressed as the reciprocal of the serum dilution that inhibited ≥75% of viral cytopathic effect. A serum sample was considered positive when it had a titer of $\geq \log_{10}$ 1.0, equivalent to a serum dilution ≥1:10.

2.5. Antigen and procedure for IgG indirect ELISA

Recombinant nucleocapsid protein of RVFV was prepared and purified, and the ELISA was carried out as described by Jansen van Vuren et al. (2007). Briefly, immunoplates (Maxisorb, Nunc, Denmark) were coated with stock rNp diluted 1:2000 in carbonate–bicarbonate buffer pH 9.6 and incubated overnight at 4 °C. After washing three times with a washing buffer consisting of PBS pH 7.2 and 0.1% Tween 20, the plates were blocked with 200 μl of 10% fat-free milk powder ("Elite", Clover SA Pvt. Ltd.) in PBS and incubated in a moist chamber for 1 h at 37 °C and then washed as described before. Control and test sera were diluted 1:400 in PBS containing 2% milk powder (diluting buffer) and 100 μl volumes were added to the plates. Each serum was tested in duplicate and the control sera and conjugate control were tested in quadruplicate. After incubation in a moist chamber for 1 h at 37 °C, plates were washed three times with the washing buffer and 100 μl of the HRPO conjugated goat anti-human IgG (H + L chain) (Zymed Laboratories, Inc.) or the HRPO conjugated Protein G (Zymed Laboratories, Inc.) diluted 1:5000, was added. Plates were incubated for 1 h at 37 °C, washed three times, and 100 μl of 2,2′-azino di-ethyl-benzothiazoline-sulfonic acid (ABTS) substrate was added to each well. Plates were then incubated in the dark at room temperature for a further 30 min. The reactions were stopped by the addition of 100 μl of 1% sodium dodecyl sulphate (S.Ds) and the optical density (OD) was determined at 405 nm. The results were expressed as percentage of the high-positive control serum (PP) (Paweska et al., 2005a).

2.6. Selection of cut-off values

The cut-off value at 95% accuracy level was optimised using the misclassification cost term option (Greiner, 1996) of the two-graph receiver operating characteristics (TG-ROC) analysis (Greiner, 1995; Greiner et al., 1995). In addition, the cut-off value was determined by mean plus 2 standard deviations (S.Ds) as well as by mean plus 3 S.Ds derived from PP values in sera tested negative in a virus neutralisation test (uninfected reference population).

2.7. Diagnostic accuracy of IgG indirect ELISA

Estimates of diagnostic sensitivity and specificity, Youden's index (J), efficiency (Ef), positive predictive value (PPV) and

Table 1
Internal quality control data and repeatability estimates for Rift Valley fever IgG I-ELISA based on recombinant nucleocapsid antigen

IQC[a] limits	IQC parameters	
	UCL[b]	LCL[c]
OD C++	1.7	0.81
PP[d] C++	117	82
PP C+	39	24
PP C−	9.8	5.4
PP C_c	7.2	3
Repeatability[e]		
Intra-plate variation		
C++	5.98 ± 2.4 S.D. (2.72–9.83)[f]	
C+	6.19 ± 3.25 S.D. (2.73–13.65)	
Inter-plate variation		
C++	6.01 ± 1.54 S.D. (3.35–7.7)	
C+	6.19 ± 2.33 S.D. (3.98–11.03)	

[a] Internal quality control (IQC) data were calculated from the mean ± 2 S.D. of 420 replicates of each control over seven runs each including five plates.
[b] Upper control limit.
[c] Lower control limit.
[d] Percent positivity.
[e] Repeatability estimates for high positive serum control (C++) and low positive serum control (C+) were calculated as the percent coefficient of variation [%CV = (PP S.D. of replicates/PP mean of replicates) × 100].
[f] Range of CV values.

negative predictive value (NPV) were calculated as previously described (Paweska et al., 2005a).

3. Results

3.1. Internal quality control and repeatability

Upper and lower internal quality limits for controls and estimates of repeatability of the assay are given in Table 1. During the routine runs of ELISA, internal controls were within upper and lower limits and there was no evidence for excessive variations within and between routine runs of the assay (Fig. 1).

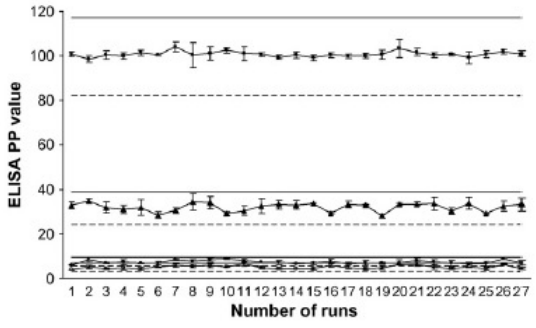


Fig. 1. Upper (—) and lower (– – –) internal quality control limits for PP values of high-positive (■), low-positive (▲), negative (●) serum controls and conjugate control (□) and means ± S.D. for these controls during 27 routine runs of the assays over a period of 12 weeks. Two or three plates were used during each run with four replicates of controls on each plate.

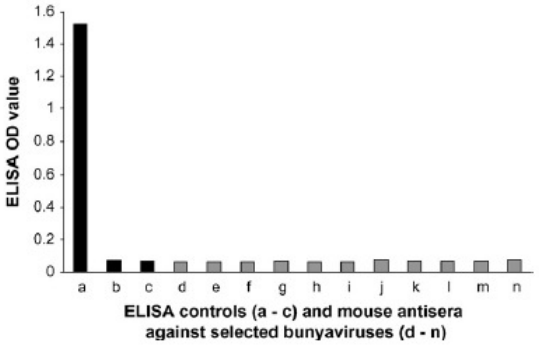


Fig. 2. Cross-reactivity of recombinant nucleocapsid protein of RVFV in indirect ELISA with mouse IgG antibody against selected viruses of the family *Bunyaviridae*. (a) Mouse IgG anti Rift Valley fever virus (*Phlebovirus*), (b) normal mouse serum and (c) conjugate control. Mouse IgG anti: (d) Saint-Floris virus (*Phlebovirus*), (e) Gordil virus (*Phlebovirus*), (f) Arumowot virus (*Phlebovirus*), (g) Gabek Forest virus (*Phlebovirus*), (h) Nairobi sheep disease virus (*Nairovirus*), (i) Hazara virus (*Nairovirus*), (j) Crimean-Congo hemorrhagic fever virus (*Nairovirus*), (k) Akabane virus (*Orthobunyavirus*), (l) Bunyamwera virus (*Orthobunyavirus*), (m) Shuni virus (*Orthobunyavirus*) and (n) Bhanja virus (not assigned to a recognized genera of the family *Bunyaviridae*).

3.2. Cross-reaction with hyperimmune mouse ascetic fluids

The I-ELISA optical density (OD) value of the mouse IgG positive RVFV ascetic fluid was 1.52 while that of normal mouse ascetic fluid and conjugate control was 0.072 and 0.068. The OD readings of hyperimmune ascetic fluids from mice experimentally infected with different viruses of genus *Phlebovirus*, *Nairovirus*, *Orthobunyavirus* and Bhanja virus were within the OD values for negative controls (Fig. 2). These results demonstrate highly specific binding affinity of mouse IgG antibody against RVFV and the rNp of the virus and lack of cross-reaction between the rNp and IgG antibody against other bunyaviruses assayed.

3.3. Cut-off values and diagnostic accuracy

Optimisation of cut-off values using misclassification cost term option of the TG-ROC analysis was based on the non-parametric programme option (Greiner et al., 1995) due to departure from a normal distribution of data sets analysed. At cut-off value of 28.98 PP the overall misclassification costs were minimal under assumption of 50% disease prevalence and equal costs of false-positive and false-negative test results (Fig. 3). Graphical presentation of the effect of three differently determined threshold values on distinguishing between sera which tested negative or positive in the virus neutralisation test is shown in Fig. 4. At a cut-off optimised by the two-graph receiver operating characteristics analysis at 95% accuracy level, the diagnostic sensitivity of the I-ELISA was 99.72% and diagnostic specificity 99.62% while estimates for the J and Ef were 0.993 and 99.62%. When cut-off values determined by traditional statistical approaches were used, the diagnostic sensitivity was 100% but estimates of J, Ef and PPV and NPV values were lower

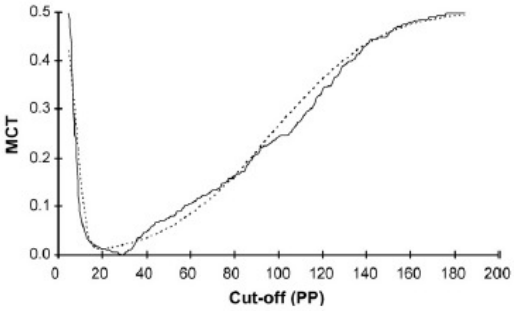


Fig. 3. Optimisation of cut-off for Rift Valley fever IgG I-ELISA in humans using the misclassification cost term (MCT) option of the two-graph receiver operating characteristics analysis. At cut-off value of 28.98 PP the overall misclassification costs become minimal under assumption of 50% disease prevalence and equal costs of false-positive and false-negative test results. The two curves represent MCT values based on non-parametric (dashed line) or parametric (smooth line) estimates of sensitivity and specificity derived from data sets in field-collected sera. Optimisation of cut-off values was based on the non-parametric program option due to departure from a normal distribution of data sets analysed.

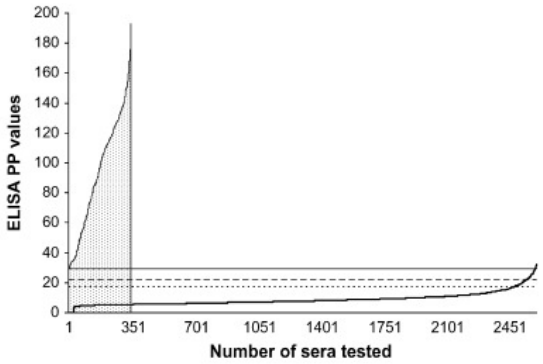


Fig. 4. The effect of different ELISA cut-off values on the discrimination between human sera tested negative or positive in the virus neutralisation test for antibodies against Rift Valley fever virus. Distribution of IgG I-ELISA PP values in human sera tested positive ($n = 350$, ⬚) or negative ($n = 2617$, ___) in the virus neutralisation test. Sera ordered according to ELISA PP values. Horizontal lines: (—) the cut-off value of 28.98 PP determined by the misclassification cost term option of the two-graph receiver operating characteristic analysis, (– – –) the cut-off value of 21.37 PP determined as mean plus three standard deviations, and (- - -) the cut-off value of 17.18 PP determined as mean plus two standard deviations of ELISA PP values observed in sera tested negative in the virus neutralisation test.

compared to those based on the cut-off derived from TG-ROC analysis (Table 2).

4. Discussion

Precise diagnoses can be made when serological tests are used in combination with clinical observations and epidemiological history, and/or when seroconversion is demonstrated. Serodiagnostic techniques are also widely used to demonstrate freedom from a disease, and in epidemiological investigations. The I-ELISA is one of the simplest immunoassay techniques for the detection of antibodies, but its routine application is often impeded by non-specific signals arising from the use of semi- or unpurified antigens (Gravell et al., 1977; Forghani and Schmidt, 1979). Although RVFV replicates to high titres in cell cultures, production of purified and concentrated stocks of the virus for I-ELISA antigen by classical virological methods is expensive and laborious. The results of this study confirm earlier findings (Jansen van Vuren et al., 2007) that the rNp of RVFV binds readily to ELISA plates, generates minimal background activity, and effectively identifies sera with different concentrations of specific RVFV antibodies and therefore constitutes a suitable antigen for use in indirect ELISA format. The I-ELISA used in the present study achieved high repeatability estimates within the statistically predetermined IQC limits.

The performance of a new assay is preferably evaluated by testing samples from individuals of known infectious status relative to the disease of interest. The correctness of the diagnostic discriminator used for initial categorising of subjects as positive or negative, significantly impacts on the selection of the optimal threshold, and subsequently on estimates of diagnostic accuracy. Calculations of diagnostic sensitivity and specificity are most reliable when a gold standard of comparison is available, and may be compromised when a relative standard of comparison is used, because errors in the estimates of diagnostic accuracy are carried over into the estimates for the new assay (Deshpande,

Table 2
Diagnostic accuracy of Rift Valley fever IgG I-ELISA based on recombinant nucleocapsid antigen

Measure[a]	Cut-off 28.98 PP[b]	Cut-off 17.18 PP[c]	Cut-off 21.38 PP[d]
Sensitivity (%) VNT+ = 350[e]	99.72	100	100
Specificity (%) VNT− = 2617[f]	99.62	95.3	97.54
Youden's index	0.993	0.953	0.975
Efficiency (%)	99.62	95.84	97.82
Positive predictive value (%)	97.14	73.23	84.12
Negative predictive value (%)	99.66	100	100

[a] Individuals were categorized according to the results of virus neutralisation test (VNT).
[b] Cut-off value optimised by the misclassification cost term option of the two-graph receiver operating characteristics analysis at 95% accuracy level.
[c] Cut-off value determined by mean plus two standard deviation derived from PP values in uninfected reference population.
[d] Cut-off value determined by mean plus three standard deviation derived from PP values in uninfected reference population.
[e] Number of sera tested positive in virus neutralisation test.
[f] Number of sera tested negative in virus neutralisation test.

1996; Jacobson, 1998, 2000). In this context it is important to note that when sera subjected for testing in the virus neutralisation test are toxic for cell cultures at low dilutions, it may be difficult to determine end-point reading, which would hamper standardizing results at the interface between negative and positive sera (grey-zone samples).

In the present study, we used the virus neutralisation test to dichotomise individuals according to their RVF infection status. It is important to note that infection with RVFV induces life long virus neutralising antibody in humans (Findlay and Howard, 1951), and that there is no evidence of serological subgroups or major antigenic variation between RVFV isolates of disparate chronologic or geographic origins (Swanepoel and Coetzer, 2004). The nucleocapsid protein is one of the most immunodominant viral proteins and appears to be highly conserved among members of the family *Bunyaviridae* (Swanepoel et al., 1986; Vapalahti et al., 1995; Schwarz et al., 1996; Magurano and Nicoletti, 1999; Gauliard et al., 2006). The results of serological cross-reactivity studies presented in this work indicate that infection with African phleboviruses and other members of the family *Bunyaviridae* should not hamper the serodiagnosis of RVF based on the recombinant nucleocapsid protein of the virus. Antigenic cross-reactivity studies in animals (Swanepoel et al., 1986) failed to provide any evidence that other African phleboviruses could obscure the reliable serodiagnosis of RVF. Nevertheless, to allow for possible cross-reactivity with unknown or other phleboviruses not tested in this study, sera in the present work were assayed at relatively high dilution.

There are differences in the antigenic specificities of the antibodies measured by ELISA and the virus neutralisation test (Saunders et al., 1977; Bolton et al., 1981). Thus, it is expected that the ELISA, which detects antibodies against all viral components, would be more sensitive than virus neutralisation which detects only antibodies to neutralizing epitopes. This may explain slightly lower specificity of IgG I-ELISA compared to the virus neutralisation in the study population. It has been shown that the IgG I-ELISA based on the rNp is more sensitive than virus neutralisation test in detecting of the early immune response in experimentally infected sheep (Jansen van Vuren et al., 2007).

The use of a cut-off determined as two or three standard deviations above the mean in uninfected individuals assumes normal distribution of test values in populations targeted by the assay (Jacobson, 1998). Deviations from normality are often observed in serological data and should be addressed in the selection of threshold values (Vizard et al., 1990). In the present study the I-ELISA had higher estimate of diagnostic sensitivity when cut-offs determined by arbitrary statistical approach were used. However, the diagnostic specificity and combined measures of assay performance characteristics were lower compared to those which were based on the cut-off derived from the TG-ROC analysis. The impacts of prevalence and other factors, e.g. biological, nutritional, geographic, stage of infection, on an assay diagnostic performance are mostly unknown and diagnostic sensitivity and specificity estimates are based on average values calculated in non-homogenous populations (Jacobson, 1998; Brenner and Gefeller, 1997; Schrijver and Kramps, 1998). In order to account for the distribution of covariate factors that may impact on the diagnostic sensitivity and specificity relatively large numbers of individuals free from Rift Valley fever were analysed.

In conclusion, the I-ELISA based on rNp is highly accurate and robust for the detection of specific IgG antibody against RVFV in human sera and can be used in the diagnosis of infection and sero-epidemiological studies.

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Recombinant nucleocapsid-based ELISA for detection of IgG antibody to Rift Valley fever virus in African buffalo

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Abstract

Wild ruminants are thought to serve as natural hosts for Rift Valley fever virus (RVFV) but the role of these animals as reservoirs for RVFV during inter-epidemic periods and as amplifiers during epidemics is not well understood. An indirect enzyme-linked immunoassay (I-ELISA) based on the recombinant nucleocapsid protein (rNp) of RVFV was validated for the detection of specific IgG antibodies in African buffalo. Data sets derived from testing buffalo sera from Kenya ($n = 405$) and South Africa ($n = 618$) were dichotomised according to the results of a virus neutralisation test. The assay characteristic performance was analysed using threshold values optimised by the two-graph receiver operating characteristics (TG-ROC) analysis, and by mean plus two, as well as by mean plus three standard deviations derived from I-ELISA PP values in uninfected animals.

Among 1023 buffalo sera tested, 77 (7.5%) had detectable virus neutralising antibodies. The assay had high intra- and inter-plate repeatability in routine runs. At a cut-off optimised by the TG-ROC at 95% accuracy level, the diagnostic sensitivity of the I-ELISA was 98.7% and diagnostic specificity 99.36% while estimates for the Youden's index (J) and efficiency (Ef) were 0.98 and 99.31%. When cut-off values determined by traditional statistical approaches were used, the diagnostic sensitivity was 100% but estimates of J, Ef and other combined measures of diagnostic accuracy were lower compared to those based on cut-off value derived from the TG-ROC. Results of the study indicate that the I-ELISA based on the rNp would be useful for seroepidemiological studies of RVFV infections in African buffalo.
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Keywords: Rift Valley fever virus; Recombinant nucleocapsid ELISA; IgG antibody; Validation; African buffalo

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1. Introduction

Rift Valley fever virus (RVFV) is a zoonotic, mosquito-borne member of the genus *Phlebovirus* in the family *Bunyaviridae* (Bishop et al., 1980) that poses a significant threat to humans and livestock (Swanepoel and Coetzer, 2004). Infections caused by RVFV in livestock are characterized by an acute hepatitis and high abortion and mortality rates, particularly in young animals (Swanepoel and Coetzer, 2004). Although antibodies to RVFV were found in many wildlife species (Davies, 1975; Davies and Karstad, 1981; Anderson and Rowe, 1998; Fischer-Tenhagen et al., 2000; Swanepoel and Coetzer, 2004), the role of particular species in the epidemiology of the disease during epidemic and inter-epidemic periods is not well understood. Experimental RVF infection of African buffalo produced transient viremia, fever, malaise and abortion (Daubney and Hudson, 1932; Davies and Karstad, 1981).

The occurrence of the first confirmed outbreaks of RVF among humans and livestock outside Africa, in the Arabian Peninsula (Jupp et al., 2002), recent re-emergence of the disease in East Africa (CDC, 2007), the ability of RVFV to replicate in a wide range of competent mosquito vectors (Turrel et al., 1998) and the effects of global warming which facilitate spread of arthropod-born viruses (Purse et al., 2005), have raised concerns that other regions of the world could be receptive to the virus. Moreover, RVFV is considered as a potential bioweapon agent (Peters, 2000; Sidwell and Smee, 2003; Lim et al., 2005). These threats prompt an increasing international demand for accurate, robust and safe tools for rapid RVF diagnosis in different host vertebrates.

Various traditional methods, including haemagglutination-inhibition, complement fixation, indirect immunofluorescence, and virus neutralisation test, have been used for detecting antibodies against RVFV (Swanepoel et al., 1986a). Disadvantages of these techniques include lack of standardization and unsuitability for automation and screening of large numbers of sera (Wright et al., 1993). Different forms of enzyme-linked immunoassay (ELISA) have been recently evaluated for serodiagnosis of RVF in domestic and wild ruminants (Paweska et al., 2003a,b, 2005b). Unfortunately, high bio-containment facilities are required for preparation of antigens for these ELISAs to limit the risk of exposure for laboratory personnel (Kitchen, 1934; Smithburn et al., 1949; McIntosh et al., 1980). Other disadvantages include high production costs of whole antigen and the risk of its incomplete inactivation.

The indirect ELISA (I-ELISA) based on the recombinant nucleocapsid protein (rNp) of RVFV have been recently reported to have high analytical accuracy for the detection of specific antibodies in humans (Jansen van Vuren et al., 2007), and in experimentally infected and vaccinated sheep (Jansen van Vuren et al., 2007; Fafetine et al., 2007). However, the assay performance characteristics have not been validated in any animal wild species. Numerous important reasons for the test validation are well known, including the need for reliable estimates of the diagnostic sensitivity and specificity that are of concern with respect to serological diagnosis, risk assessment and risk factors studies (Jacobson, 1998, 2000). Hence, in the present study, the I-ELISA based on the recNp of RVFV was validated for the detection of specific IgG antibody in African buffalo sera, with special emphasis on test diagnostic accuracy.

2. Materials and methods

2.1. ELISA serum controls and internal quality control

Freeze-dried, gamma-irradiated sheep serum controls produced previously (Paweska et al., 2003a) were used.

During routine runs of the ELISA, each plate had four replicates of each high-positive (C++), low-positive (C+), negative serum (C−), and conjugate (Cc) controls. The upper and lower control limits for each of the internal controls were established as described previously (Paweska et al., 2003a). Means and standard deviations (S.D.) of ELISA optical density (OD) readings and percentage positivity (PP) of internal controls were calculated from the replicates on each plate and from each run of the assay to assess intra- and inter-plate variation. Additionally, coefficients of variation (CV = standard deviation of replicates/mean of replicates × 100) were calculated for positive serum controls. Data obtained from this analysis were used to estimate assay repeatability.

2.2. Serum specimens

A total of 1023 individual buffalo sera collected in 2002–2007 in Kenya ($n = 405$) and in 2005 in South Africa ($n = 618$) were used. Sera which tested negative ($n = 77$) in the virus neutralisation test (VNT) were regarded as a reference panel from non-infected animals, and sera which tested positive ($n = 946$) as a reference panel from animals infected with RVFV.

2.3. Serum neutralisation test

Duplicates of serial twofold dilutions of sera, inactivated at 56 °C for 30 min, were tested as previously described (Paweska et al., 2003a). Titres were expressed as the reciprocal of the serum dilution that inhibited ≥75% of viral cytopathic effect. A serum sample was considered positive when it had a titer of $\geq \log_{10} 1.0$, equivalent to a serum dilution ≥1:10.

2.4. ELISA antigen and procedure

Production of rNp antigen and the assay procedure was carried out as described previously (Jansen van Vuren et al., 2007) with minor modification. Maxisorb immunoplates (Nunc, Denmark) were coated with stock antigen, diluted 1:2000 in carbonate–bicarbonate buffer pH 9.6 and incubated overnight at 4 °C. After washing three times with a washing buffer consisting of phosphate-buffered saline (PBS) pH 7.2 and 0.1% Tween 20, the plates were blocked with 200 μl of 10% fat-free milk powder in PBS and incubated in a moist chamber for 1 h at 37 °C and then washed as described before (Jansen van Vuren et al., 2007). Control and test sera were diluted 1:400 in PBS containing 2% milk powder (diluting buffer) and 100 μl of diluted sera was added to the plates. Each test serum was assayed in duplicate and each internal control was tested in quadruplicate. After incubation in a moist chamber for 1 h at 37 °C, plates were washed three times with the washing buffer and 100 μl of a 1:5000 dilution of the HRPO conjugated Protein G (Zymed Laboratories, Inc.) was added. Plates were incubated for 1 h at 37 °C, washed three times, and 100 μl of 2,2′-azino di-ethyl-benzothiazoline-sulfonic acid substrate was added to each well. Plates were then incubated in the dark at room temperature for 30 min. The reactions were stopped by the addition of 100 μl of 1% sodium dodecyl sulphate and ODs were determined at 405 nm. The results were expressed as percentages of the high-positive control serum (PP) (Paweska et al., 2003a).

2.5. Selection of cut-off values and determination of ELISA diagnostic accuracy

Cut-off values at the 95% accuracy level were optimised using the misclassification cost term option (Greiner, 1996) of the two-graph receiver operating characteristics (TG-ROC) analysis (Greiner, 1995; Greiner et al., 1995). Optimisation of cut-off values was based on the following equation: misclassification cost term $= (1 - p)\ (1 - \mathrm{Sp}) + rp\ (1 - \mathrm{Se})$, where p (prevalence) = 0.5 and r (costs of false-positive and false-negative results) = 1.0 (Greiner, 1996). In addition, cut-off value was determined by mean plus 2S.D. and by mean plus 3S.D. derived from PP values in uninfected animals.

Estimates of diagnostic sensitivity and specificity, Youden's index, efficiency (Ef), positive predictive value (PPV) and negative predictive value were calculated as described previously (Paweska et al., 2005b).

3. Results

3.1. Internal quality control and assay repeatability

The rNp-based IgG I-ELISA generated minimal background activity and clearly differentiated between all the internal controls used. There was no evidence for excessive variations within and between routine runs of the assay (Fig. 1).

3.2. Antibody dilution curves

Dose response curves using different dilutions of sera known to be positive in the VNT had the required characteristic analytical slope. The I-ELISA clearly differentiated between different levels of specific IgG antibody against RVFV in buffalo sera (Fig. 2).

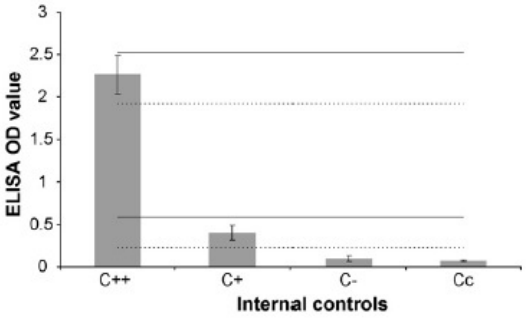


Fig. 1. Mean ± S.D. for OD values of high-positive (C++), low-positive (C+), negative serum (C−) and conjugate control (Cc) controls in rNp-based IgG I-ELISA on 40 plates during routine runs of the assay over a period of 3 months. Each plate includes four replicates of each of the internal controls. Horizontal lines indicate: upper (—) and lower (- - -) internal quality limits for OD values of high-positive (C++) and low-positive (C+) serum controls.

Table 1
Diagnostic accuracy of Rift Valley fever recombinant nucleocapsid-based I-ELISA.

Cut-off (PP)[a]	26.94[b]	22.23[c]	17.73[d]
Diagnostic sensitivity (%)	98.70	100	100
Diagnostic specificity (%)	99.36	97.78	94.18
Youden's index	0.980	0.977	0.941
Efficiency (%)	99.31	97.94	94.57
Positive predictive value (%)	92.45	77.95	55.01
Negative predictive value (%)	99.89	100	100

Animals were categorized according to the results of virus neutralisation test (VNT).

[a] Cut-off value expressed as a percentage positivity (PP) of an internal high-positive serum control.

[b] Cut-off value optimised by TG-ROC analysis.

[c] Cut-off value based on mean + 3S.D. of ELISA PP values in VNT-negative population.

[d] Cut-off value based on mean + 2S.D. of ELISA PP values in VNT-negative population.

3.3. Comparison of virus neutralizing titres and IgG I-ELISA PP values

Using the Spearman test, a high correlation ($R^2 = 0.882$) between VNT titres and the I-ELISA PP values was demonstrated. At the I-ELISA cut-offs of 22.23 PP and 17.73 (mean + 3S.D. and mean + 2S.D. derived from PP readings in VNT-positive sera), all 77 sera, which tested positive in the virus neutralisation test were also positive in the I-ELISA. At the I-ELISA cut-off of 26.94 PP optimised by TG-ROC analysis, all but one serum which tested positive in the virus neutralisation test were also positive in the I-ELISA (Table 1). Compared to the VNT, there were more false-positive results when using ELISA cut-offs determined by classical statistical approach.

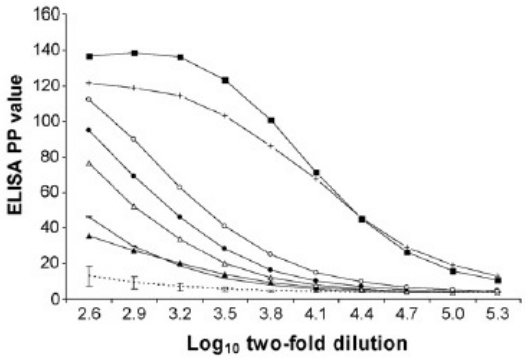


Fig. 2. Dose response curves of individual African buffalo sera in I-ELISA. Sera were collected from 15 animals in RVF endemic areas in Kenya and South Africa of which 7 tested positive and 8 negative in virus neutralization test. Positive sera (—) represent different levels of virus neutralizing antibody ranging in titres from $\log_{10} 10^{1.6}$ (▲) to $\log_{10} 10^{3.1}$ (■). Mean ± S.D. of 8 negative sera (- - -) with VNT titres $<\log_{10} 10^{1}$.

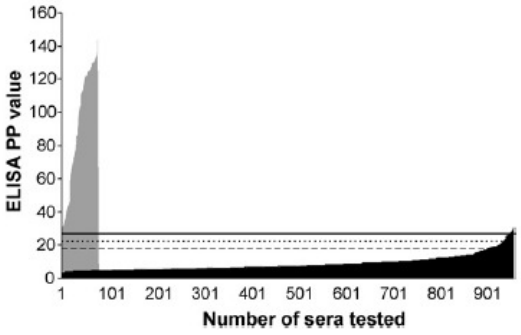


Fig. 3. The effect of different ELISA cut-off values on the discrimination between buffalo sera tested negative or positive in the virus neutralisation test for antibodies against Rift Valley fever virus. Distribution of IgG I-ELISA PP values in sera tested positive ($n = 77$, gray area) and sera tested negative ($n = 946$, black area) in the virus neutralisation test. Sera ordered according to ELISA PP values. Vertical lines indicate the ELISA cut-off values determined by the TG-ROC analysis (—), and as a mean plus three (- - -) and two (– – –) standard deviations of the ELISA PP values observed in the VNT-negative population.

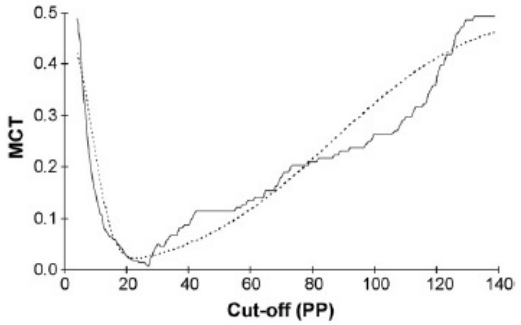


Fig. 4. Optimisation of cut-off for Rift Valley fever I-ELISA in African buffalo using the misclassification cost term (MCT) option of the two-graph receiver operating characteristic analysis. At cut-off value of 26.94 PP, the overall misclassification costs become minimal under assumption of 50% disease prevalence and equal costs of false-positive and false-negative test results. The two curves represent MCT values based on non-parametric (—) or parametric (- - -) estimates of sensitivity and specificity derived from data sets in field-collected sera. Optimisation of cut-off value was based on the non-parametric program option due to departure from a normal distribution of data sets analysed.

3.4. Cut-off values and diagnostic accuracy

The effect of three differently determined threshold values on distinguishing between sera, which tested negative or positive in this assay is shown in Fig. 3. Estimates of sensitivity, specificity, and other estimates of combined measures of diagnostic accuracy (J, Ef, PPV, NPV) derived from the study data sets are given in Table 1.

Optimisation of cut-off values using misclassification cost term option of the TG-ROC analysis was based on the non-parametric programme option (Greiner et al., 1995) due to departure from a normal distribution of the data sets. In the I-ELISA validated in this study, the correlation coefficients were $r = 0.9683$ and $r = 0.9781$ for linear correlations of the non-parametric diagnostic sensitivity and specificity versus parametric diagnostic sensitivity and specificity parameters, respectively. Graphical presentation of the TG-ROC analysis is shown in Fig. 4.

4. Discussion

Of the various classical serological methods used for the detection of antibodies to RVFV, the virus neutralisation test has the highest sensitivity and specificity (Swanepoel et al., 1986a). However, it is laborious, expensive, and it can be carried out only when stocks of live virus and tissue cultures are available. Consequently, it is rarely used, and then only in highly specialized reference laboratories housed in high level bio-containment facilities. ELISA techniques for the detection of virus-specific antibodies are less expensive and time consuming. (Wright et al., 1993). Other advantages of ELISA techniques, with special reference to RVFV rNp-based I-ELISA, have recently been discussed (Fafetine et al., 2007; Jansen van Vuren et al., 2007).

The diagnostic performance of a new assay is preferably determined by testing samples from individuals of known infection status relative to the disease of interest. The correctness of a diagnostic discriminator used for initially categorising subjects as positive or negative, impacts on the selection of the optimal threshold and, consequently, on estimates of diagnostic sensitivity and specificity (Jacobson, 1998; Deshpande, 1996). The virus neutralisation test has been either validated in domestic or wildlife animal for the detection of antibodies to RVFV, according to the current international guidelines (Jacobson, 2000).

Calculations of diagnostic sensitivity and specificity are most reliable when a gold standard is available for comparison (Jacobson, 2000). When a relative standard of comparison is used, such as the VNT, estimates of diagnostic accuracy may be compromised because the error in the estimates of diagnostic sensitivity and specificity for relative standards is carried over into those estimates for the new assay (Jacobson, 1998). In this context it is important to note that when sera subjected for testing in the virus neutralisation test are toxic for cell cultures at low dilutions, it may be difficult to determine end-point reading, which would hamper standardizing results at the interface between negative and positive sera (grey-zone samples). Unfortunately, in practice there is no better standard available than the VNT and the true disease state is rarely known in practice, because perfect test results may be difficult or impossible to obtain (Tyler and Cullor, 1989).

Infection with RVFV induces life long virus neutralising antibodies in animals (Barnard, 1979), and there is no evidence of serological subgroups or major antigenic variation between virus isolates of

disparate chronologic or geographic origins (Swanepoel and Coetzer, 2004). The nucleocapsid protein appears to be the immunodominant viral protein and this characteristic is highly conserved among members of the *Bunyaviridae* family (Swanepoel et al., 1986b; Vapalahti et al., 1995; Schwarz et al., 1996; Magurano and Nicoletti, 1999; Gauliard et al., 2006). Antigenic cross-reactivity studies in animals (Davies, 1975; Swanepoel, 1976; Swanepoel et al., 1986b) failed to provide any evidence that other African phleboviruses could obscure the reliable serodiagnosis of RVF. Nevertheless, to allow for possible cross-reactivity with unknown phleboviruses, sera in the present study were tested at relatively high dilution. There are differences in the antigenic specificities of the antibodies being measured by ELISA and the virus neutralization test (Saunders et al., 1977). It is expected that the ELISA, which detects antibodies against all viral components, would be more sensitive than the virus neutralization test, which detects only antibodies to viral neutralising epitopes. This may explain the slightly lower diagnostic specificity of IgG I-ELISA compared to the virus neutralisation in the study population. It has been shown that the IgG I-ELISA based on the rNp was more sensitive than the VNT in detection of the early immune response in sheep experimentally infected with wild type RVFV and in sheep immunized with the live-attenuated Smithburn strain of the virus (Jansen van Vuren et al., 2007).

A cut-off determined as two or three standard deviations above the mean in uninfected individuals is commonly used for interpretation of serodiagnostic assays. However, this assumes a normal distribution of test values in the population targeted by the assay, and provides only an estimate of diagnostic specificity but not sensitivity (Jacobson, 2000). Deviations from normality are often observed in serological data and should be addressed in the selection of threshold values (Vizard et al., 1990). In the present study the I-ELISA had a higher estimate of diagnostic sensitivity when cut-offs determined by traditional statistical approach were used. However, the diagnostic specificity and combined measures of assay accuracy were lower compared to those, which were based on the cut-off derived from the TG-ROC analysis. Estimates of diagnostic accuracy vary with disease prevalence (Brenner and Gefeller, 1997). The prevalence assumed in the study sample may not be representative of the prevalence in the target populations and this should be borne in mind in applying the estimates of diagnostic accuracy reported for the assays in the present work. Biological variables contribute more heavily to false positive than to false negative results (Jacobson, 1998; Schrijver and Kramps, 1998). In order to account for the distribution of covariate factors that may impact on the diagnostic sensitivity and specificity, relatively large numbers of buffalo sera free from antibodies to RVFV were analysed. One of the advantages of converting ELISA OD raw data into PP values relative to a known standard is that this method does not assume uniform background activity, and therefore enables inter-laboratory standardisation (Wright et al., 1993).

The results of the present study confirm previous findings (Jansen van Vuren et al., 2007) that the rNp of RVFV binds readily to ELISA plates, generates minimal background activity, and effectively identifies sera with different concentrations of specific RVFV antibodies. We conclude that the rNp-based IgG I-ELISA is robust, has high diagnostic accuracy, and therefore can be used for diagnosis, import–export veterinary certification and for seroepidemiological studies of RVFV infections in African buffalo.

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Laboratory safe detection of nucleocapsid protein of Rift Valley fever virus in human and animal specimens by a sandwich ELISA

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A B S T R A C T

A safe laboratory procedure, based on a sandwich ELISA (sAg-ELISA), was developed and evaluated for the detection of nucleocapsid protein (NP) of Rift Valley fever virus (RVFV) in specimens inactivated at 56 °C for 1 h in the presence of 0.5% Tween-20 (v/v) before testing. Polyclonal capture and detection immune sera were generated respectively in sheep and rabbits immunized with recombinant NP antigen. The assay was highly repeatable and specific; it detected strains of RVFV from the entire distributional range of the disease, isolated over a period of 53 years; no cross-reactivity with genetically related African phleboviruses or other members of the family *Bunyaviridae* was observed. In specimens spiked with RVFV, including human and animal sera, homogenates of liver and spleen tissues of domestic ruminants, and *Anopheles* mosquito homogenates, the sAg-ELISA detection limit ranged from $\log_{10}10^{2.2}$ to $10^{3.2}$ $TCID_{50}$/reaction volume. The ELISA detected NP antigen in spiked bovine and sheep liver homogenates up to at least 8 days of incubation at 37 °C whereas infectious virus could not be detected at 48 h incubation in these adverse conditions. Compared to virus isolation from sera from RVF patients and sheep infected experimentally, the ELISA had 67.7% and 70% sensitivity, and 97.97% and 100% specificity, respectively. The assay was 100% accurate when testing tissues of various organs from mice infected experimentally and buffalo foetuses infected naturally. The assay was able to detect NP antigen in infective culture supernatants 16–24 h before cytopathic effects were observed microscopically and as early as 8 h after inoculation with $10^{5.8}$ $TCID_{50}$/ml of RVFV. This ability renders the assay for rapid identification of the virus when its primary isolation is attempted *in vitro*. As a highly specific, safe and simple assay format, the sAg-ELISA represents a valuable diagnostic tool for use in less equipped laboratories in Africa, and for routine differential diagnosis of viral hemorrhagic fevers.

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1. Introduction

Rift Valley fever virus (RVFV), a member of the *Phlebovirus* genus of the *Bunyaviridae* family (Bishop et al., 1980), is the causative agent of Rift Valley fever (RVF), a mosquito-borne, viral, zoonotic disease that poses a significant health threat to domestic ruminants and humans in Africa. Infection with RVFV in livestock is characterized by an acute hepatitis, abortion and high mortality rates in new born animals. Humans infected with RVFV typically develop a mild self-limited febrile illness, but retinal degeneration, severe encephalitis, fatal hepatitis and hemorrhagic manifestations occur in a small proportion of patients (Swanepoel and Coetzer, 2004). Historically, RVF was restricted to sub-Saharan Africa but in 1977 spread to northern Africa (Meegan, 1979), in 1987 to West Africa (Jouan et al., 1988), and in 2000 to the Arabian Peninsula (Shoemaker et al., 2002). The ability of RVFV to utilise a wide range of mosquito vectors (Turrel et al., 2008) and cause extensive outbreaks with severe socio-economic losses, as well as global climate change that facilitates spread of vector-borne diseases outside their traditional geographical boundaries, are of great international concern. There is an increasing demand for diagnostic tools for rapid, accurate and safe laboratory diagnosis of RVF. This demand is challenged by the fact that work with RVFV requires high biocontainment facilities and also that the virus is regarded as a potential bioweapon agent. For these reasons, the current capacity for laboratory diagnosis of RVF is restricted to a limited number of reference laboratories worldwide. Diagnosis of RVF is achieved by different methods, including virus isolation (Shope and Sather, 1979; Anderson et al., 1989), nucleic acid techniques (Ibrahim et al., 1997; Garcia et al., 2001; Drosten et al., 2002) and detection of specific antibodies (Swanepoel et al., 1986; Paweska et al., 2003a). Virus isolation is lengthy, expensive and involves propagation of live virus. Nucleic

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acid techniques require highly specialized laboratory equipment, sophisticated reagents, and well-trained laboratory personnel, the availability of which could be problematic when outbreaks occur in remote regions and rapid diagnosis is necessary. Highly sensitive and specific ELISAs based on inactivated whole RVFV antigen (Paweska et al., 2003a,b, 2005a,b) and recombinant nucleocapsid antigen (Jansen van Vuren et al., 2007; Paweska et al., 2007, 2008b) have been validated extensively and shown to be highly accurate for detection of anti-RVFV antibody in humans and animals. An optical fiber immunosensor based on a sandwich ELISA has recently been reported for the detection of IgG antibody to RVFV in humans (Sobarzo et al., 2007). ELISAs for the detection of RVF viral antigen have been reported (Niklasson et al., 1983; Meegan et al., 1989; Zaki et al., 2006) but most of these assays are based on reagents that are difficult and expensive to produce, pose a biohazard risk to laboratory personnel, and were not validated according to current recommendations.

This paper describes the first development and evaluation of a sandwich ELISA for antigen detection (sAg-ELISA) based on an anti-nucleocapsid antibody capture and detection system and the use of thermo-chemically inactivated specimens to ensure safety of laboratory staff involved in the diagnosis of RVF.

2. Materials and methods

2.1. Specimens

2.1.1. Human and sheep sera

A total of 130 human sera submitted to the Special Pathogens Unit of the National Institute for Communicable Diseases, Sandringham, South Africa (SPU-NICD) for routine testing were used; 70 specimens were from suspected RVF cases sampled during the 2006–2008 disease outbreaks in Southern Africa.

A total of 105 sheep sera were used of which 20 were from sheep inoculated subcutaneously with the SPU22/118 KEN 07 strain of RVFV, isolated from a RVF human case during the 2007 Kenyan epidemic (CDC, 2007), and the remaining 85 were from naïve sheep.

2.1.2. Animal tissues

Heart, lung, liver, kidney and brain tissues were harvested from three female Balb/c mice on day 2 after subcutaneous inoculation with the SPU22/118 KEN 07 strain of RVFV. The same tissues were harvested from a mock inoculated Balb/c mouse.

Diagnostic submissions of liver, heart, kidney, lung, and brain tissues from three aborted buffalo foetuses during the 2008 RVF outbreak in South Africa (Paweska et al., 2008a) were used. Animal tissues were homogenized as 10% (w/v) suspensions in Eagles Minimal Essential Medium (EMEM) (BioWhitaker, MD, USA) containing L-glutamine, non-essential amino acids and antibiotics (100 IU penicillin, 100 μg streptomycin, and 0.25 μg amphotericin B). After centrifugation at 3000 × *g*, supernatants were harvested and stored at −70 °C.

2.1.3. Animal tissue homogenates and sera spiked with RVFV

Homogenates (10%, w/v) of uninfected ovine and bovine liver and spleen tissues, prepared as described in Section 2.1.2, and uninfected human, sheep and cattle sera were spiked with the Ar20368 RSA 81 strain of RVFV to a final virus concentration of $\log_{10}10^{6.5}$ $TCID_{50}$/ml. Half $\log_{10}$ dilutions of these preparations were used to determine the sAg-ELISA analytical detection limit in tissues and sera. As controls, the unspiked homogenates and sera were used with EMEM in place of virus solution.

2.1.4. Mosquito pools homogenates spiked with RVFV

Homogenates (10%, w/v) of mosquito pools, each containing 100 individuals of *Anopheles arabiensis*, *A. gambiae* and *A. funestus* obtained from laboratory mosquito colonies at Vector Control Unit of the NICD, were prepared in EMEM and spiked with the Ar20368 RSA 81 strain of RVFV as described in Section 2.1.3.

2.1.5. Viruses

Seventeen RVFV isolates recovered over a period of 53 years (1955–2008) in African countries, Madagascar and Saudi Arabia (Table 2), four African phleboviruses (Arumowot, Gabek Forest, Gordil and Saint-Floris) and two other members of the family *Bunyaviridae* (Akabane and Bunyamwera viruses) were used to evaluate the analytical sensitivity and specificity of the sAg-ELISA.

2.2. Virus titration

Virus titrations of clinical and laboratory generated specimens were performed as described previously (Swanepoel et al., 1986). Briefly, four 100 μl replicates of 10-fold dilutions (10^{-1} to 10^{-7}) of specimens in EMEM were transferred into flat bottomed 96-well cell culture microplates (Nunc) and equal volumes of Vero cell suspension in EMEM, containing 2×10^5 cells/ml, 8% foetal bovine serum/ml (Gibco) and antibiotics, were added. The inoculated microplates were incubated at 37 °C in a CO_2 incubator and observed under microscope for CPE for 10 days post-infection (p.i.). Virus concentrations, calculated by the method of Kärber (1931), were expressed as median tissue culture infectious dose ($TCID_{50}$) per ml of serum.

2.3. Monitoring of RVFV growth in vitro

Tenfold dilutions of the Ar20368 RSA 81 strain of RVFV in EMEM (from $10^{5.8}$ $TCID_{50}$/ml to $10^{0.8}$ $TCID_{50}$/ml) were used for inoculation of 25 cm^2 tissue culture flasks containing 48 h confluent Vero cell monolayers. Inoculated flasks were incubated on a rotating platform for 1 h at 37 °C. Two mock inoculated flasks were included as controls. After 1 h of incubation, inoculated flasks were removed, cells were washed with PBS and supplemented with 10 ml of EMEM containing 1% foetal calf serum and antibiotics. Inoculated cells were maintained at 37 °C in a CO_2 incubator. 1 ml aliquots of tissue culture medium were collected hourly for the first 8 h, and thereafter at 12, 24, 30, 48, 54, 72, 78, 96 and 102 h after inoculation. The collected aliquots of tissue culture medium were replaced each time with the same volume of fresh medium. Appearance of cytopathic effect (CPE) was documented at each collection time.

2.4. Detection of RVFV nucleoprotein in tissue homogenates incubated at adverse temperature

To mimic clearance of NP protein in decomposing tissues from RVFV-infected animals, homogenates (5%, w/v) of fresh normal ovine and bovine liver tissues prepared as described in Section 2.1.2 were mixed with an equal volume of tissue culture supernatant containing $10^{7.9}$ $TCID_{50}$/ml of RVFV Zim688/78 strain, and then incubated at 37 °C for a period of 8 days during which aliquots were taken for testing immediately after mixing, and 5, 24, 48, 72, 168 and 192 h thereafter. Supernatants were collected after centrifugation at 3000 × *g* at 4 °C and tested by sAg-ELISA and virus titration.

2.5. Production of recombinant N-protein and pET32 control antigen

Bacterial expression and purification of the recombinant RVFV nucleocapsid protein was done as described previously (Jansen van Vuren et al., 2007). Briefly, the recombinant protein was expressed in Origami (DE3) cells (Novagen) using a recombinant

pET32(a)+ (Novagen) vector. After induction with isopropyl β-D-1-thiogalactopyranoside (Roche, Germany) for 4 h, the bacterial cells were collected by centrifugation and lysed using the Bugbuster reagent (Novagen) combined with Lysonase (Novagen). Purification was from the soluble phase utilizing the 6×His tag, fused to the target protein, using Protino Ni–chelate columns (Machery-Nagel). The pET32 control antigen was produced in the same way but using the wild-type pET32(a)+ vector without the insert of the nucleocapsid gene.

2.6. Generation of hyperimmune rabbit and sheep anti-nucleocapsid of RVFV anti-sera

Three New Zealand white rabbits were inoculated subcutaneously (s.c.) with 140 μg of RVFV recNP emulsified in an equal volume of TiterMax Gold adjuvant according to the manufacturer's instructions (Sigma–Aldrich). Rabbits received an identical booster immunization 14 days later, followed by a second booster of 375 μg RVFV recNP without adjuvant on day 33 after the first booster. Two Dorper cross-sheep were immunized s.c. with 350 μg recNP emulsified in the same adjuvant as used for immunization of rabbits, and subsequently received an identical booster on day 21 after the primary immunization. Animals were bled regularly to monitor immune responses. When their anti-recNP antibody responses reached a high level, defined as an ELISA optical density reading ≥2.0, they were bled to obtain bulk serum. Bulk bleeds from individual animals were pooled to obtain homogenous unpurified preparations of hyperimmune polyclonal anti-recNP sera.

2.7. Inactivation of specimens and safety testing

Specimens were inactivated by adding an equal volume of 1% Tween-20 in PBS followed by incubation at 56 °C for 1 h. Sera and tissue culture homogenates spiked with the Ar20368 RSA 81 strain of RVFV to a final virus concentration of $\log_{10}10^{6.5}$ $TCID_{50}$/ml were tested after inactivation for the presence of live virus in 2–3 days old suckling mice and 24–48 h Vero cell monolayers. Mice were monitored for clinical symptoms until day 10 p.i. and Vero cells were monitored for CPE for a period of 14 days p.i.

2.8. Procedure for the antigen detection sandwich ELISA

A sandwich ELISA (sAg-ELISA) format was evaluated for specific detection of nucleocapsid protein of RVFV. The top half of each immunoplate (Maxisorb, Nunc, Denmark) was coated with sheep anti-recNP serum (capture antibody) and the bottom half with normal sheep serum, both at a dilution of 1:400 in phosphate-buffered saline (PBS) pH 7.2 and incubated overnight at 4 °C. After washing three times with a washing buffer consisting of PBS pH 7.2 and 0.1% Tween-20, the plates were blocked with 200 μl of 10% fat free milk powder ("Elite", Clover SA, Pty, Ltd.) in PBS, incubated in a moist chamber for 1 h at 37 °C and then washed as described before. RVFV recNP stock antigen diluted 1:3000 in 2% milk powder (diluting buffer) was used as high-positive control; 100 μl of the antigen was added in quadruplicate to the top and to the bottom half of the plates. RVFV recNP stock antigen diluted 1:30,000 was used as a low-positive control antigen and pET32 diluted 1:3000 was used as a negative control antigen; 100 μl of each was added in duplicate to the top as well as the bottom half of the plates. A volume of 100 μl of each specimen, inactivated as described in 1.7, was added undiluted in duplicate to the top and bottom halves of each plate. The plates with their internal controls and specimens were incubated at 37 °C in a moist chamber for 1 h, washed as before and 100 μl of hyperimmune rabbit anti-recNP serum (detecting antibody), diluted 1:3000 in diluting buffer, was added to each well. After incubation for 1 h at 37 °C in a moist chamber, plates were washed as before and 100 μl of goat anti-rabbit IgG (H+L) HRPO conjugate (Zymed Laboratories, Inc.) diluted 1:8000 was added to each well. After 1 h incubation at 37 °C in a moist chamber, plates were washed three times and 100 μl of 2,2′-azinodiethylbenzthiazoline sulfonic acid (ABTS, KPL Laboratories, Inc.) substrate was added to each well. Plates were incubated for 30 min in the dark at room temperature and the reaction was stopped by the addition of 100 μl of 1% sodium dodecyl sulfate (SDS) to each well. Optical density (OD) was determined at 405 nm and results expressed as a percentage of the mean high-positive control antigen (PP) using the formula: (mean net OD of duplicate test specimen/mean net OD of high-positive control) × 100.

2.9. ELISA internal quality control (IQC)

The internal quality control data were generated as described previously (Paweska et al., 2003b). Means and standard deviations (S.D.) of the ELISA optical density readings and the percentage positivity (PP) of high-positive antigen control were calculated from replicates of all internal controls in each plate and each run of the assay to assess intra- and inter-plate variation. Additionally, coefficients of variation (CV = standard deviation of replicates/mean of replicates × 100) were calculated for positive antigen controls. Data obtained from this analysis were used to estimate the assay repeatability and to establish the upper and lower control limits for each of the internal controls. Upper and lower control limits together with CV values (≤10 for high-positive serum and ≤15 for low-positive serum) were applied as IQC rules for further analysis. During routine runs of the ELISA each plate had four replicates of high-positive antigen control (Ag^{2+}) and two replicates each of low-positive antigen (Ag^{+}) and negative control antigen (Ag^{-}).

2.10. Dose–response curves of nucleocapsid antigen versus whole antigen of RVFV

For comparison of the dose–response curves of nucleocapsid versus the whole antigen of RVFV, recNP and Vero-derived strain Ar20368 RSA of the virus, inactivated as described in Section 2.3, were diluted serially and tested in sAg-ELISA ELISA.

2.11. Selection of cut-off values and determination of ELISA diagnostic accuracy

Cut-off values at the 95% accuracy level were optimized using the misclassification cost term option (Greiner, 1996) of the two-graph receiver operating characteristics (TG-ROC) analysis (Greiner, 1995; Greiner et al., 1995). Additionally, cut-off values were determined by mean plus 2 standard deviations (S.D.s) and mean plus 3 S.D.s derived from PP values in known RVFV-negative human and sheep sera. Estimates of diagnostic sensitivity, specificity, and efficiency were calculated as described previously (Paweska et al., 2005b).

2.12. Indirect IgM and IgG ELISA

Experimental sheep sera were tested for anti-nucleocapsid IgG and IgM antibody by indirect ELISA as described previously (Jansen van Vuren et al., 2007).

3. Results

3.1. Internal quality control and repeatability

The sAg-ELISA generated minimal background noise and discriminated clearly between all the internal controls used.

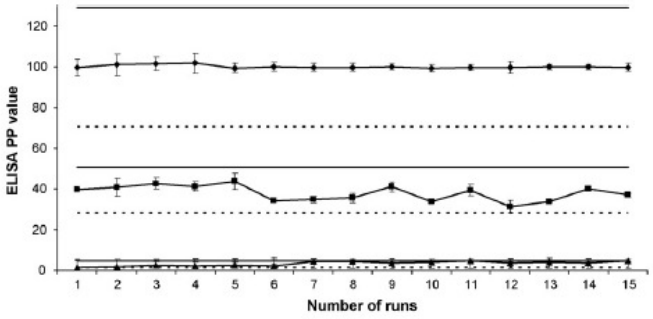


Fig. 1. Upper (–) and lower (- - -) internal quality control limits for PP values of high-positive (♦), low-positive (■), negative (▲) antigen controls and means ±2 S.D. for these controls during 15 routine runs of the assays over a period of 16 weeks. Two to six plates were used during each run with four replicates of high-positive, two replicates of low-positive and negative controls on each plate.

Table 1
Internal quality control limits and repeatability estimates of RVF nucleocapsid protein detection ELISA.

Internal controls	IQC[a] limits		Repeatability[e]	
	UCL[b]	LCL[c]	Intra-plate variation	Inter-plate variation
OD Ag^{2+}	2.0	1.1		
PP[d] Ag^{2+}	129	71	1.04 ± 0.9 S.D.	1.28 ± 0.63 S.D.
PP Ag^{+}	51	28	1.85 ± 1.83 S.D.	2.55 ± 1.72 S.D.
PP Ag^{-}	4.9	1.4		

[a] Internal quality control: data were calculated from the mean ±2 S.D.s of 180 replicates of high-positive antigen control (Ag^{2+}), 90 replicates of low-positive (Ag^{+}) and negative (Ag^{-}) antigen controls over five runs each including three plates.
[b] Upper control limit.
[c] Lower control limit.
[d] Percentage positivity of high-positive antigen control.
[e] Repeatability estimates for high (Ag^{2+}) and low (Ag^{+}) positive antigen controls were calculated as the percentage coefficient of variation [%CV = (PP S.D. of replicates/PP mean of replicates) × 100]

During routine runs of the assay there was no evidence for excessive variations within the upper and lower control limits (Fig. 1). CV values for intra- and inter-plate runs were below 5% (Table 1) demonstrating high repeatability of the assay.

3.2. Biological safety of inactivated specimens

Mice inoculated with inactivated samples of sera and tissue culture homogenates, which were spiked with a high concentration of RVFV ($\log_{10} 10^{6.5}$ $TCID_{50}$/ml), were clinically normal for a period of 10 days and no CPE was observed in Vero cell cultures for a period of 14 days after inoculation. These results demonstrate that the thermo-chemical inactivation procedure used in this study effectively kills RVFV in specimens such as those which are commonly submitted for routine diagnosis of RVF. The inactivation protocol not only renders the clinical specimens safe for testing when using an ELISA workstation but also enhances specific ELISA readings compared to those in non-inactivated samples (results not shown).

3.3. Nucleocapsid and whole virus dilution curves

The dose–response curves in tested samples had the expected analytical slopes. The sAg-ELISA differentiated clearly between different concentrations of recNP and infectivity of the virus in relevant clinical samples (Figs. 2 and 3).

3.4. Analytical detection limit, specificity and sensitivity

The sAg-ELISA was able to detect as little as 110 pg of recNP, corresponding to $10^{2.2}$ $TCID_{50}$ per 100 µl of Vero-derived whole

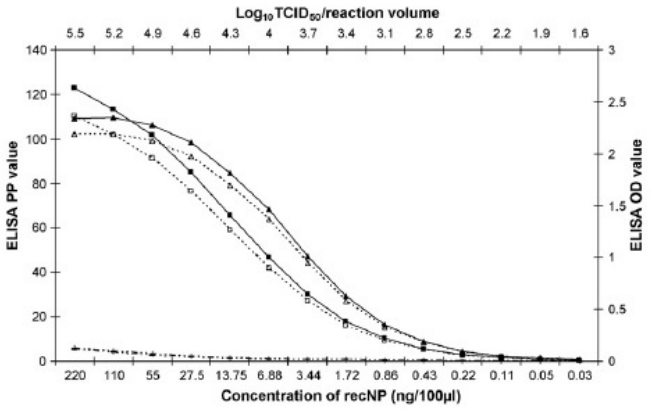


Fig. 2. Dose–response curves of recNP (PP (■), OD (□)), RVFV Ar20368 RSA 81 (PP (▲), OD (△)), and control antigen (PP+, OD–).

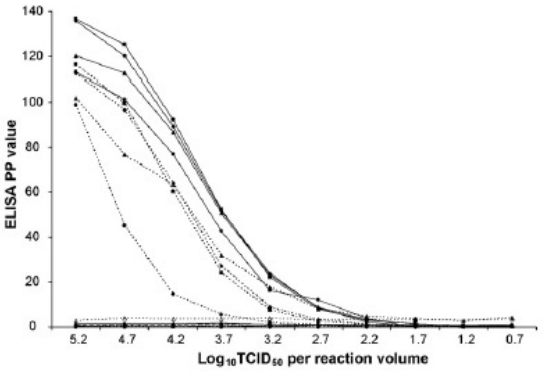


Fig. 3. Dose–response curves of human and animal sera, animal tissue and mosquito homogenates spiked with RVFV and their corresponding non-spiked controls. Virus-spiked samples: human (–●–), sheep (- -♦- -), bovine (- -■- -) serum; sheep (–♦–), bovine (–■–) spleen; sheep (- -▲- -), bovine (- -●- -) liver; *Anopheles* mosquito (–▲–). Uninfected samples: human (–○–), sheep (- -◊- -), bovine (- -□- -) serum; sheep (–◊–), bovine (–□–) spleen; sheep (- -△- -), bovine (- -○- -) liver; *Anopheles* mosquito (–△–).

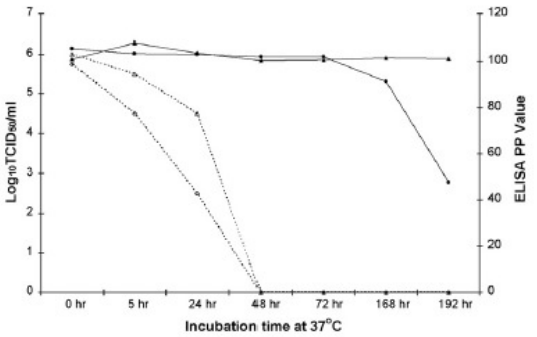


Fig. 4. Detection of infectious virus and viral nucleoprotein in tissue homogenates spiked with RVFV followed by incubation at 37 °C. $TCID_{50}$/ml in bovine (○) and sheep (△), and ELISA readings in bovine (●) and sheep (▲) liver homogenates.

antigen of RVFV (Fig. 2). Analysis of the ELISA readings for sera and tissue homogenates spiked with different concentrations of RVFV shows that the detection limit in most assayed samples was approximately $\log_{10} 10^{2.2}$ $TCID_{50}$ per ELISA reaction volume (100 μl) except for bovine liver where the detection limit was 10 times lower (Fig. 3). When testing infectious tissue culture supernatant containing related African phleboviruses (Arumowot, Gabek Forest, Gordil and Saint-Floris) and two other members of the family *Bunyaviridae* (Akabane and Bunyamwera viruses), ELISA readings ranged from 0 to 0.63 PP (mean 0.41 ± 0.26) (results not shown) whereas non-specific background noise of normal tissue culture fluid was 0.44 PP. These results demonstrate the highly specific binding affinity of anti-recNP RVFV polyclonal hyperimmune sheep and rabbit sera and the absence of detectable cross-reactions between these anti-sera and nucleocapsid proteins of the other bunyaviruses assayed. All of the 17 RVFV strains were easily detected by ELISA (Table 2).

3.5. Monitoring of viral growth in vitro

RVFV replication kinetics in Vero cells inoculated with different concentrations of the virus as measured by antigen detection ELISA is shown in Fig. 5. Irrespective of the inoculum used, the sAg-ELISA yielded positive results earlier than CPE was observed. For example, in the flasks inoculated with $10^{5.8}$ and $10^{0.8}$ $TCID_{50}$/ml RVFV the ELISA detected antigen 8 and 48 h after inoculation respectively, whereas CPE could only be observed 16 and 24 h later.

3.6. Detection of viral antigen in decomposing tissues

The sAg-ELISA detected nucleocapsid protein at equal levels in sheep liver homogenate at all time points where samples were taken, whereas a steady decline of detectable levels of antigen occurred in bovine liver homogenate from 168 h onwards. In contrast, incubation of spiked homogenates at 37 °C resulted in relatively rapid clearance of infectious virus, as indicated by negative results obtained at 48 h of incubation (Fig. 4).

Table 2
Identification, year of isolation, origin and concentration of RVFV strains used in this study.

Strain	Year of isolation	Source	Country of origin	Concentration $Log_{10}TCID_{50}$/ml	ELISA PP value[a]
Lunyo UGA 55	1955	Mosquito	Uganda	$10^{6.3}$	112.0
ZH 501	1977	Human	Egypt	$10^{5.8}$	121.9
Zim 688/78	1978	Bovine	Zimbabwe	$10^{8.5}$	137.8
VRL-825-ZIM79	1979	Bovine foetus	Zimbabwe	$10^{7.0}$	123.3
Ar20368 RSA 81	1981	Mosquito	South Africa	$10^{6.8}$	110.3
Ank-6087	1984	Bat	Guinea	$10^{7.0}$	108.1
ArD38661 SEN 83	1983	Mosquito	Senegal	$10^{7.3}$	134.2
ArD38388 BF 83	1983	Mosquito	Burkina Faso	$10^{7.5}$	126.1
CAR R1662	1985	Human	Central African Republic	$10^{6.5}$	131.5
SPU-204-ANGL85	1985	Human	Angola	$10^{6.0}$	115.0
900085MAU88	1988	Human	Mauritania	$10^{7.3}$	125.3
An991-MAD91	1991	Bovine	Madagascar	$10^{7.5}$	121.7
SPU12002-SOM98	1998	Caprine	Somalia	$10^{8.0}$	124.6
AR21229-SA00	2000	Mosquito	Saudi Arabia	$10^{7.3}$	117.2
SPU 77/04	2004	Human	Namibia	$10^{7.0}$	132.3
SPU22.118 KEN 07	2007	Human	Kenya	$10^{6.8}$	131.2
AR 52/08	2008	Human	South Africa	$10^{6.8}$	127.5

[a] Percentage positivity of ELISA high-positive antigen control.

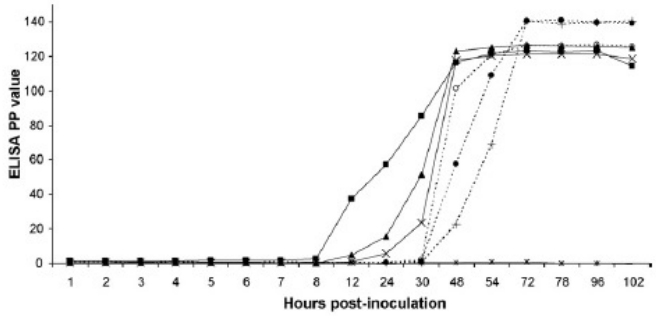


Fig. 5. RVFV replication kinetics in Vero cells inoculated with different concentrations of the virus measured by antigen detection ELISA. $Log_{10}TCID_{50}$ virus concentrations in 1 ml of inoculum were $10^{5.8}$ (■), $10^{4.8}$ (▲), $10^{3.8}$ (×), $10^{2.8}$ (○), $10^{1.8}$ (●) and $10^{0.8}$ (+), mock control (*).

Table 3
Diagnostic accuracy of a sandwich ELISA for the detection of nucleocapsid protein of RVFV in sheep and human sera.

Measure[a]	Human sera–cut-off (PP)[b] $V^{+c} = 31$, $V^{-d} = 99$			Sheep sera–cut-off (PP)[b] $V^{+c} = 20$, $V^{-d} = 85$		
	1.96[e]	4.11[f]	5.60[g]	0.86[e]	0.91[f]	1.23[g]
D-Se (%)[h]	77.4	67.7	67.7	70.0	70.0	70.0
D-Sp (%)[i]	81.8	95.95	97.97	97.65	97.65	100.0
Efficiency (%)[j]	79.6	81.83	82.84	83.83	83.83	85.0

[a] Sera were categorized according to the results of virus isolation.
[b] Cut-off value expressed as a percentage positivity of high-positive antigen control.
[c] Number of sera tested positive for RVFV.
[d] Number of sera tested negative for RVFV.
[e] Cut-off value optimised by the misclassification cost term option of the two-graph receiver operating characteristics analysis at 95% accuracy level.
[f] Cut-off value determined as mean plus two standard deviation derived from PP values in RVFV-negative sera.
[g] Cut-off value determined as mean plus three standard deviation derived from PP values in RVFV-negative sera.
[h] Diagnostic sensitivity.
[i] Diagnostic specificity.
[j] Efficiency.

3.7. *Diagnostic cut-off values and accuracy*

The effect of three differently determined cut-off values on the estimates of diagnostic sensitivity, specificity, and efficiency of the sAg-ELISA in human and sheep sera are given in Table 3. The highest diagnostic accuracy for human and sheep serum data sets was achieved when threshold values (5.6 and 1.23 PP, respectively) determined as mean plus 3 S.D. were used. However, estimates of diagnostic performance based on a cut-off determined as the mean plus 2 S.D. or derived from the TG-ROC analysis were similar (Table 3). In ELISA-positive human sera at the optimal cut-off, mean $TCID_{50}$/ml of the virus was 5.6 ± 0.83, and in ELISA-negative sera, it was 3.7 ± 1.61. At the cut-off determined as mean plus 2 S.D. derived from PP values of normal mice and ruminant tissues (2.4 PP), the sAg-ELISA had 100% sensitivity and specificity in detecting the nucleocapsid protein of RVFV in various tissues of mice infected experimentally and buffalo foetuses infected naturally (Fig. 6).

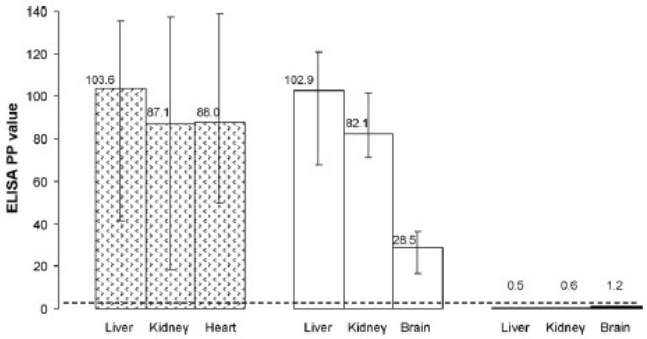


Fig. 6. Detection of nucleocapsid protein by ELISA in RVFV-infected buffalo foetus (▒) and mouse organ tissues (□). Non-infected mouse organ tissues (■) are included as a control. Mean PP values were determined for the liver, heart and kidney tissues of the three infected buffalo foetuses. Mean PP values were determined for the liver, brain and kidney tissues of three infected mice. Control PP values were obtained from one uninfected mouse. Cut-off of 2.4 PP (- - -) was determined as the mean plus 2 S.D. of PP values in uninfected mouse and ruminant organ tissues.

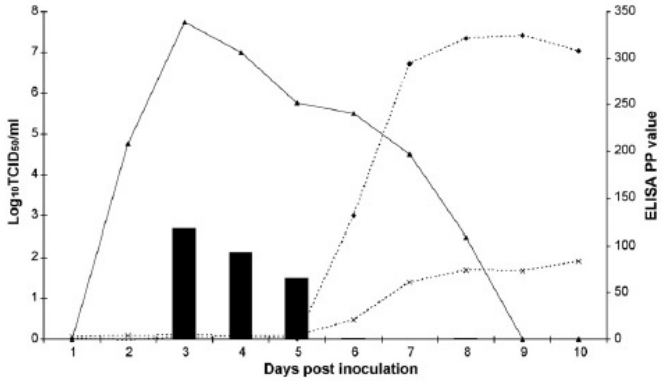


Fig. 7. Monitoring of viremia (▲), antigenemia (■), anti-nucleocapsid IgM (♦) and IgG (×) responses in a sheep experimentally infected with RVFV.

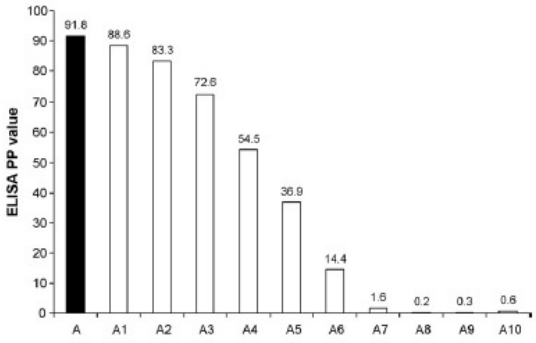


Fig. 8. Blocking effect of increasing levels of anti-RVFV specific antibody on antigen detection in viremic sheep serum. Viremic serum free of antibody against nucleocapsid of RVFV (A, ■). Mixture (v/v) of viremic serum and immune serum with 2-fold increase of anti-RVFV specific antibodies (A1-1:1024 to A10-1:2, □).

3.8. The effect of anti-RVFV NP antibody on the results of antigen detection ELISA

The ELISA yielded negative results in sera taken from a sheep infected experimentally on day 5 p.i. and later despite relatively high levels of viremia detected on days 5 and 6 p.i. The negative results coincided with the appearance of the first detectable anti-nucleocapsid IgM and IgG antibody on day 5 p.i. (Fig. 7). To confirm the blocking effect of RVFV-immune sera in the sAg-ELISA, viremic sheep serum was mixed with increasing concentrations of known immune sheep serum. The inhibitory effect of increasing levels of specific antibodies on the ELISA-specific signal in highly viremic sheep serum is shown in Fig. 8.

4. Discussion

The recent epidemics of RVF in the Arabian Peninsula have demonstrated the potential for RVFV to extend its traditional boundaries and cause severe outbreaks in naïve populations of humans and livestock. There is an increased need internationally for rapid diagnostic tools to detect RVFV in humans and animals both in endemic regions and potentially receptive regions.

A sandwich ELISA reported by Niklasson et al. (1983), using hyperimmune mouse and rabbit anti-sera, had a sensitivity of 10^5 plaque forming units (PFU)/ml for detecting RVFV in supernatant fluid from infected Vero cell culture. However, marked differences were noted between antigen titers and virus infectivity in experimentally infected hamsters and rhesus monkeys. While ELISA could only reliably detect 10^6 PFU/ml of virus in viremic hamsters, rhesus monkeys with viremia of $10^{3.4}$ PFU/ml tested positive (Niklasson et al., 1983). A sandwich ELISA utilizing biotin–avidin-labelled mouse monoclonal antibody had a sensitivity of only 29.3% in viremic human sera from the 1987 RVF epidemic in West Africa (Meegan et al., 1989), compared to 76.9% in experimentally infected rhesus monkeys with the assay reported by Niklasson et al. (1983). Niklasson and Gargan (1985) demonstrated significant correlation between viral titer and quantity of viral antigen as measured by ELISA in orally infected Egyptian *Culex pipiens*. Moreover, the ELISA had similar sensitivity (100% vs. 93%) and specificity (94% vs. 94%) to the infectivity assay for detecting mosquitoes capable of transmitting virus to susceptible hamsters. These early studies clearly demonstrated the potential of the sandwich ELISA for field application. However, its use as a routine diagnostic technique for RVFV in the laboratory has been limited for a number of reasons: testing of RVFV-infected specimens by ELISA pose a biohazard risk because of pipetting and washing procedures, the use of OD readings for interpretation of ELISA results is no longer recommended, and inter-laboratory standardization has been impractical due to the unavailability of non-infectious, well-characterized internal antigen controls. Recently, Zaki et al. (2006) reported immunofluorescence assays using pools of mouse IgG monoclonal conjugates capable of reacting with a combination of virus-specific antigens (Gs, Gn, N, NSs). Although it was demonstrated to be highly reliable in detecting RVFV in patient sera, its use requires tissue culture amplification and handling of live virus.

The nucleocapsid protein (NP) is the most abundant viral component in the RVFV virion and viral protein in infected cells. It is associated closely with viral genomic RNA along with the L-polymerase to form a helical ribonucleoprotein structure (Le May et al., 2005). The fact that large amounts of RVFV NP could be purified from the media of Sf9 cells infected with recombinant baculovirus suggests, however, that NP might have a pathway for its release independent of the viral proteins Gn, Gc or the viral genome (Liu et al., 2008). It has been shown that nucleoproteins of other virus groups can be released from host cells even when expressed in the absence of other viral proteins (Betenbaugh et al., 1995; Jiang et al., 1992; Kirnbauer et al., 1992; Laurent et al., 1994). The abundance of NP and its extra-cellular release are likely the major factors which contribute to the rapid production of detectable antibody against this protein of RVFV (Fafetine et al., 2007; Jansen van Vuren et al., 2007; Paweska et al., 2007, 2008a,b). As the most abundantly

expressed and immunogenic viral component in the RVFV virion, NP is an obvious choice for development of immunoreagents for antigen detection assays.

ELISA offers an affordable and simple alternative to traditional and molecular techniques for detection of RVFV, but as an open bench system might contribute to laboratory infections when samples containing live virus are analysed. A number of laboratory infections with RVFV were recorded under circumstances which indicate the virus to be highly infectious for man (Findlay, 1932; Kitchen, 1934; Smithburn et al., 1949). To address this problem, we developed a sandwich ELISA based on an entirely safe procedure, including a set of internal controls based on a recombinant nucleocapsid protein for monitoring assay performance, which increases its utility in surveillance and diagnosis in non-endemic areas.

RVFV has been shown to be extremely stable when stored in infected plasma at low temperature. For example, infected sheep plasma was shown to retain infectivity after 8 years of cold storage (Easterday, 1965) but viremic blood became non-infective after 40 min of incubation at 56 °C in phosphate buffer, pH 7.2 (Findlay, 1932). The presence of a lipid bilayer in the virus envelope makes RVFV highly sensitive to lipid solvents (Bishop et al., 1980). The simple and inexpensive thermo-chemical inactivation procedure used in this study effectively inactivated RVFV present in test specimens, as demonstrated by negative virus isolation results *in vivo* and *in vitro*. Using a similar procedure, Mayo and Beckwith (2002) inactivated West Nile virus successfully at 37 °C for 30 min in the presence of 0.05% Tween-20. In addition, the inactivation protocol increased the specific ELISA signal, probably due to increased availability of nucleocapsid protein after viral envelope disruption by Tween-20. The analytical sensitivity of $\log_{10}10^{3.2}$ $TCID_{50}$/ml, as determined by testing infective Vero cell culture fluid, seems to be at least 10-fold higher compared to that reported by Niklasson et al. (1983). Using different concentrations of RVFV, we demonstrated that the NP antigen can be detected in supernatants from infected Vero cell cultures as early as 8 h post-inoculation and 16–24 h before CPE could be observed. This ability renders the assay very useful for rapid identification of RVFV when primary isolation from clinical specimens is attempted *in vitro*. Live virus was cleared much faster than its nucleoprotein in virus-spiked homogenates of liver incubated at adverse temperature. These results indicate that the ELISA can be used for diagnosis of RVF in the decomposing tissues of ruminants that have been found dead in the field.

The analytical detection limit established in virus-spiked samples designed to mimic diagnostic specimens, was the same for all specimen types, except in bovine liver homogenate for which it was 10 times less ($\log_{10}10^{4.2}$ $TCID_{50}$/ml). An estimate of the diagnostic sensitivity of the sAg-ELISA derived from results in viremic human sera was much higher (67.7%) compared to that (29.3%) reported by Meegan et al. (1989) but similar (76.9%) to that in viremic rhesus monkey sera (Niklasson et al., 1983). The diagnostic accuracy of a sandwich ELISA in viremic human sera and other specimens is likely to be dependent on a number of factors, including origin, purity (monoclonal vs. polyclonal) and type-specificity of capture and trapping antibody, antigen titer and spectrum of reactivity to RVFV structural proteins (Meegan et al., 1989).

Serum specimens are commonly submitted for RVF diagnosis. Viremia titers ranging from $10^{5.6}$ to $10^{9.0}$ of mouse median lethal doses per ml have been recorded in domestic ruminants (Daubney et al., 1931; Barnard and Botha, 1977; Harrigton et al., 1980; Swanepoel et al., 1986; Morril et al., 1987), up to $10^{8.6}$ in humans (Peters and Meegan, 1981) and $10^{5.4}$ $TCID_{50}$/ml in adult African buffalo (Davies and Karstad, 1981). Despite such high RVFV titres, viremia is of short duration, with obvious implications for diagnosis. Results obtained in serial sera from an experimentally infected sheep with a blocking ELISA indicate that the appearance of specific antibodies during viremia hampers the assay results, despite the presence of relatively high concentrations of the virus. These findings seem to indicate that differences in ELISA diagnostic performance might also be due to variations in immune status among viremic individuals at the time of sampling. Therefore, attempts to detect recent RVFV infection by ELISA should include a combination of assays which target both viral antigens and IgM antibody. It should be noted that high viremia frequently occurs in the absence of severe illness. Consequently, in the absence of noticeable clinical signs and adequate diagnostic procedures, considerable geographic dispersal of RVFV might occur before an outbreak is recognized (McIntosh et al., 1973). On the other hand, the South African outbreaks of 1950–1951 and the Egyptian outbreaks of 1977–1978 were not recognized as RVF until several months had elapsed with deaths of thousands of animals, and, in the Egyptian outbreaks, many deaths in humans. Delays in recognition of these outbreaks occurred because the disease was previously unknown in those geographical areas and the possibility of RVF was not considered at first (Porterfield and Della-Porta, 1981).

In this study very high estimates of diagnostic accuracy (100%) were obtained when testing various tissue homogenates of mice infected experimentally and of buffalo foetuses infected naturally. RVFV can persist at high titers for 21 days in ovine brain and liver, and up to 30 days in spleen (Yedloutschnig et al., 1981). The high diagnostic accuracy of the sAg-ELISA for detecting RVFV in infected tissues, which usually contain virus concentrations at least 10- to 100-fold above the detection limits determined in this study (Easterday et al., 1962; Easterday and Murphy, 1963; Harrigton et al., 1980; Morril et al., 1987), indicate that the assay will be highly reliable for testing specimens from aborted foetuses and fatal cases. The ELISA format reported here allows for assaying relatively large numbers of specimens within a short period of time. The assay throughput, if required, could easily be increased by using semi-or fully automated ELISA workstations.

The ability of a diagnostic assay to produce consistent results within tolerable limits of analytical error is one of the requirements for any diagnostic technique to be accepted for routine applications. While the internal antigen controls based on the recombinant NP protein achieved very high repeatability estimates within the IQC limits, the reproducibility of the sAg-ELISA remains to be addressed for more comprehensive inter-laboratory evaluation. Antibody- and antigen-binding levels should be expressed in relative rather than absolute terms. One of the advantages of converting ELISA OD readings to PP values relative to a known standard is that this method does not assume uniform background activity, and therefore enables inter-laboratory standardisation (Wright et al., 1993).

The polyclonal hyperimmune anti-recNP rabbit and sheep antisera did not cross-react with other members of the *Bunyaviridae* family, including four African *Phleboviruses*, in this study. No cross-reactivity with other members of the sand fly fever virus group, including sand fly Naples, sand fly Sicilian, Arumowat, Punta Toro, Gordil, Karimabad, Gabek Forest, and Saint Floris viruses, was detected in a sandwich ELISA, using hyperimmune mouse and rabbit anti-sera, by Niklasson et al. (1983). The nucleocapsid protein appears to be highly conserved in members of the *Bunyaviridae* family (Swanepoel et al., 1986; Vapalahti et al., 1995; Schwarz et al., 1996; Magurano and Nicoletti, 1999; Gauliard et al., 2006) and antigenic cross-reactivity studies in animals (Davies, 1975; Swanepoel, 1976; Swanepoel et al., 1986) and an indirect ELISA based on the recNP protein (Paweska et al., 2007) failed to provide any evidence that other African phleboviruses would cross-react with RVFV. The sAg-ELISA detected nucleocapsid proteins of a wide range of geographically distinct RVFV isolates collected over 53 years, representing three major lineages of the virus, namely Egyptian, West African, and the Central–Eastern–Southern African lineage. These results are to be expected since the RVFV genome and the gene

encoding the N-protein, specifically, are highly conserved (Bird et al., 2007).

In conclusion the sAg-ELISA procedure developed and evaluated in this study is safe, highly accurate in detecting RVFV NP antigen in diagnostically relevant concentrations, rapid and robust, and can therefore be utilized in diagnosis and surveillance in both endemic and non-endemic RVF areas. It offers a less complicated alternative to nucleic acid techniques when large numbers and a variety of clinical specimens have to be tested in a short period of time. The assay could also be useful for confirmation of severe forms of RVF infections since positive ELISA results correlate with high levels of viremia.

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A Recombinant Nucleocapsid-based Indirect ELISA for Serodiagnosis of Rift Valley Fever in African Wildlife

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ABSTRACT

An indirect ELISA (I-ELISA) based on the recombinant nucleocapsid protein (rNp) of Rift Valley fever virus (RVFV) was evaluated for the detection of specific serum IgG antibody in African wildlife. Data sets derived from field-collected sera (n = 918) in Africa (antelopes = 570, black rhinoceros = 43, common zebra = 24, elephant = 73, giraffe = 81, grevy zebra = 78, warthog = 49) were categorised according to the results of a virus neutralisation test (VNT). At cut-offs optimised by the two-graph receiver operating characteristics analysis, the diagnostic sensitivity of the I-ELISA was 100% and diagnostic specificity ranged from 99.8% to 100% while estimates for the Youden's index (J) and efficiency (Ef) ranged from 0.99 to 1 and from 99.7% to 100%, respectively. The rNp-based I-ELISA is highly accurate, safe, and offers a single assay format for rapid detection of IgG antibody to RVFV in sera of different wildlife species.

Key words: *Rift Valley fever virus, recombinant nucleocapsid protein, IgG antibody, indirect ELISA, African wildlife.*

INTRODUCTION

The recent occurrence of the first confirmed outbreaks of Rift Valley fever (RVF) outside Africa (Jupp et al., 2002), together with the ability of RVF virus to replicate in a wide range of mosquito vectors (Turrel et al., 2008) and the effects of global warming which facilitate spread of arthropod-borne viruses (Purse et al., 2005) into non-endemic regions of the world are of medical and veterinary concern. Antibodies to RVF virus have been found in many wildlife species (Davies, 1975; Anderson and Rowe, 1998; Fischer-Tenhagen et al., 2000; Paweska et al., 2005; Evans et al., 2008; Paweska et al., 2008) but their importance in the epidemiology of the disease during the inter-epidemic and epidemic periods has yet to be elucidated.

Various forms of enzyme-linked immunoassays (ELISA) for serodiagnosis of RVF in different host vertebrates have recently been validated. Whilst these assays were shown to be highly sensitive and specific, they are based on β-propiolactone inactivated and/or gamma-irradiated, sucrose-acetone-extracted whole antigens (Paweska et al., 2003a; Paweska et al., 2003b; Paweska et al., 1995). The production of such antigens requires high bio-containment facilities to limit the risk of exposure of laboratory personnel while culturing the virus prior to inactivation. Other disadvantages include high production costs and the risk of incomplete inactivation. An indirect ELISA (I-ELISA) based on recombinant nucleocapsid protein (rNp) of RVF virus was reported to have high analytical accuracy for the detection of IgG antibody in experimentally infected and vaccinated sheep (Jansen van Vuren et al., 2007; Fafetine et al., 2007). The test was also shown to have high diagnostic performance characteristics in testing African buffalo sera (Evans et al., 2008; Paweska et al., 2008).

This paper describes evaluation of the rNp I-ELISA as a single test format for rapid detection of IgG antibody to RVF virus in different wildlife species.

Table 1. Number of field-collected wildlife sera tested in the VNT.

Species	Total Tested	VNT-[a]	VNT+[b]
Black rhinoceros	43	29	14
Common zebra	24	24	0
Elephant	73	69	4
Giraffe	81	81	0
Grevy zebra	78	77	1
Warthog	49	47	2
Eland	66	63	3
Gerenuk	6	1	5
Hartebeest	10	10	0
Impala	339	330	9
Kudu	73	66	7
Waterbuck	42	40	2
Thomson gazelle	8	1	7
Grand Total	918	864	54

[a] = number of sera tested negative in VNT; [b] = number of sera tested positive in VNT.

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MATERIALS AND METHODS

Serum Specimens

A total of 918 wildlife sera collected between 1978 and 2008 in Kenya, South Africa and Zimbabwe were used. Sera which tested negative in the virus neutralisation test (VNT) were regarded as reference panel from non-infected animals, and sera which tested positive as reference panel from animals infected with RVF virus (**Table 1**).

Virus Neutralisation Test

Duplicates of serial two-fold dilutions of sera inactivated at 56 °C for 30 min were tested as previously described (Paweska et al., 2003b). Titres were expressed as the reciprocal of the serum dilution that inhibited ≥ 75% of viral cytopathic effect. A serum sample was considered positive when it had a titre of ≥ $\log_{10}$ 1.0, equivalent to a serum dilution ≥ 1:10.

ELISA Antigen and Procedure

The assay procedure was carried out as previously described (Paweska et al., 2008), and ELISA results were expressed as a percentage of the high-positive control serum (PP) (Paweska et al., 2003b). The assay runs were accepted within the upper and lower control limits for the internal controls as previously statistically determined (Paweska et al., 2008).

Selection of Cut-off Values and Determination of ELISA Diagnostic Accuracy

Cut-off values at 95% accuracy level were optimised using the misclassification cost term option of the two-graph receiver operating characteristics (TG-ROC) analysis (Greiner, 1996). In addition, cut-off values were determined by mean + 2 standard deviations (SD). and by mean + 3SDs derived from PP values in uninfected animals. Estimates of diagnostic sensitivity and specificity and other measures of combined diagnostic accuracy were calculated as previously described (Paweska et al., 2008).

RESULTS

Antibody Dilution Curves

Dose response curves using different dilutions of sera known to be positive or negative in the VNT had the expected analytical slope and the I-ELISA clearly differentiated between different levels of specific IgG antibody against RVFV in African wildlife (**Figure 1**).

Cut-off Values and Diagnostic Accuracy

Threshold values for IgG I-ELISA were derived from data sets dichotomised according to the results of the VNT (**Table 1**). The effect of differently determined cut-off values on distinguishing between sera which tested negative or positive in this assay, and consequently on estimates of sensitivity, specificity, and other estimates of diagnostic accuracy is given in **Table 2**. Optimisation of cut-off values using the misclassification cost term option of the TG-ROC analysis was based on the non-parametric programme option (Greiner, 1996) due to departure from a normal distribution of data sets analysed. Graphical presentation of the TG-ROC analysis is shown in **Figure 2**.

At a cut-off value of 20.4 PP the overall misclassification costs become minimal under the assumption of a 50% disease prevalence and equal costs of false-positive and false-negative test results. The two curves represent MCT values based on non-parametric (smooth line) or parametric (dashed line) estimates of sensitivity and specificity derived from data sets in field-collected sera. Optimisation of cut-off values was based on the non-parametric program option due to departure from a normal distribution of data sets analysed.

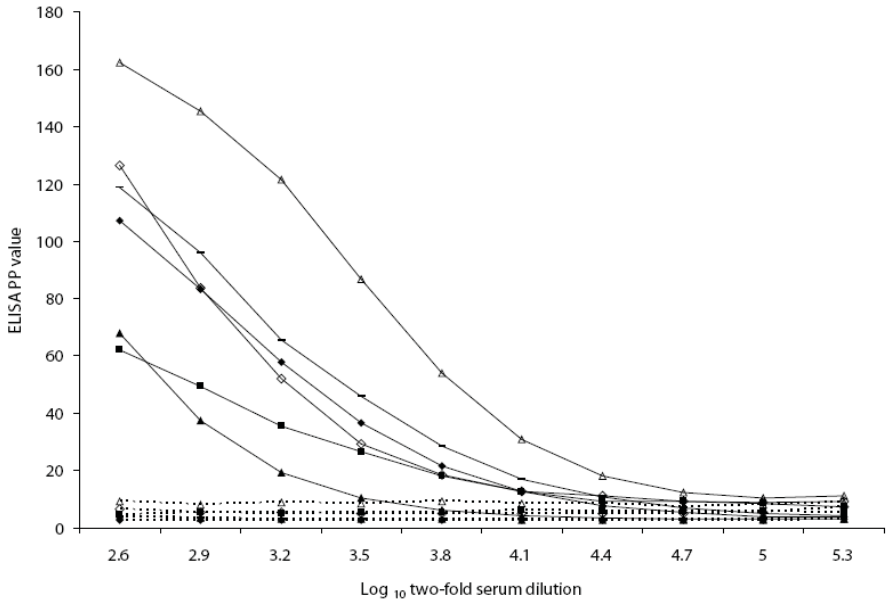


Figure 1. Dose response curves of wildlife sera in IgG I-ELISA tested positive (□) or negative (---) in a VNT: black rhinoceros (♦), eland (▲), gerenuk (■), kudu (◊), impala (Δ), Thomson gazelle (—). VNT titres in positive sera ranging from $\log_{10}10^{1.9}$ (■) to $\log_{10}10^{3.1}$ (Δ).

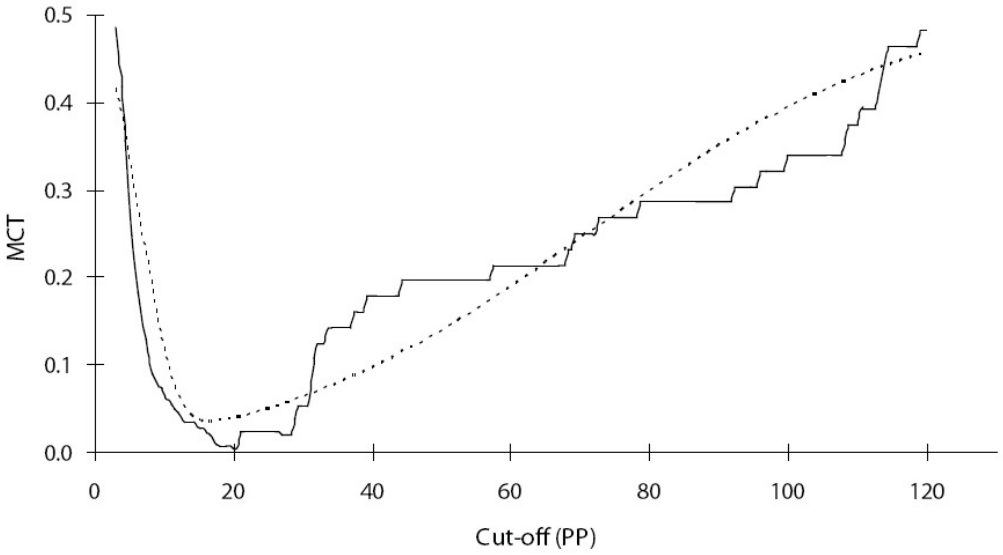


Figure 2. Optimisation of cut-off value for Rift Valley fever nucleocapsid-based I-ELISA in African antelopes using the misclassification cost term (MCT) option of the two-graph receiver operating characteristic analysis.

Table 2. Diagnostic accuracy of Rift Valley fever recombinant nucleocapsid–based I–ELISA in African wildlife.

Species	Cut–off[a]	D–Sn[b]	D–Sp[c]	Y[d]	Ef[e]	PPV[f]	NPV[g]
Black rhinoceros	33.6[h]	100	100	1	100	100	00
	27.5[i]	100	91.3	0.91	93.7	81.5	00
	35.5[j]	100	100	1	100	100	00
Common zebra	–	–	–	–	–	–	–
	13.9	–	100	–	–	–	–
	17.9	–	100	–	–	–	–
Elephant	28	100	100	1	100	100	100
	10.6	100	95.8	0.96	99.7	99.7	100
	13.6	100	97.2	0.97	99.8	99.8	100
Giraffe	–	–	–	–	–	–	–
	11.7	–	100	–	–	–	–
	14.3	–	100	–	–	–	–
Grevy zebra	–	–	100	–	–	–	–
	17.3	–	100	–	–	–	–
	22.5	–	100	–	–	–	–
Warthog	27.7	100	100	1	100	100	100
	13.5	100	95.9	0.96	96	49.7	100
	17.5	100	97.9	0.98	98	66.4	100
Antelopes[l]	20.4	100	99.8	0.99	99.7	95.6	100
	8.4	100	88.1	0.88	88.7	32.4	100
	14.4	100	97.0	0.97	97.1	67.9	100

Animals were categorised according to the results of the VNT.

[a] — cut–off value expressed as a percentage positivity (PP) of an internal high–positive serum control; [b] — diagnostic sensitivity (%); [c] —diagnostic specificity (%); [d] —Youden's index; [e] — efficiency (%); [f] — positive predictive value (%); [g] — negative predictive value (%); [h] — cut–off value optimised by TG–ROC analysis; [i] — cut–off value based on mean + 2 SD of ELISA PP values in VNT–negative population; [j] — cut–off value based on mean + 3 SD of ELISA PP values in VNT–negative population; [k] — not determined due to unavailability or very limited number of VNT–positive sera; [l] — eland, gerenuk, hartebeest, impala, kudu, Thomson gazelle, waterbuck.

DISCUSSION

Traditional methods for detecting antibodies to RVF virus include haemagglutination-inhibition, complement fixation, indirect immunofluorescence, and virus neutralisation assays. The last of these is regarded as a gold standard but is laborious, expensive and dependent on the availability of live virus and tissue cultures. Therefore, it is only used in specialised reference laboratories housing high biocontainment facilities. Laboratory safety and other advantages of the rNp-based I-ELISA compared with the VNT have been discussed recently (Paweska et al., 2008).

Antigenic cross-reactivity studies in animals (Swanepoel et al., 1986) and recent results in the I-ELISA failed to provide any evidence that other African phleboviruses could hamper reliable serodiagnosis of RVF (Paweska et al., 2007). However, to account for possible cross-reactivity with unknown phleboviruses, sera in this study were tested at relatively high dilution. The I-ELISA had high estimates of diagnostic sensitivity when cut-offs determined by traditional statistical approach were used but the diagnostic specificity and combined measures of assay accuracy were lower compared with those which were based on the cut-offs derived from the TG-ROC analysis. A cut-off determined as two or three SDs above the mean in uninfected individuals which is still commonly used for interpretation of serodiagnostic assays, assumes a normal distribution of test values in targeted populations, and therefore provides only an estimate of diagnostic specificity but not sensitivity (Jacobson, 1998). Deviations from normality are often observed in serological data and should be addressed in the selection of threshold values (Vizard et al., 1990). The prevalence assumed in the study sample may not be representative of the prevalence in the target populations and this should be borne in mind in applying the estimates of diagnostic accuracies reported for the rNp-based I-ELISA in the present work.

This study confirm previous findings (Paweska et al., 2008) that the rNp-based I-ELISA accurately identifies sera with different concentrations of specific IgG antibodies to RVF virus, and compared with the VNT it has very high diagnostic performance in various wildlife animal species. As a single and safe test format, it provides a useful tool for seroepidemiological studies of RVF virus infections in African wildlife species. Such investigations might help to elucidate their specific role in the epidemiology of the disease during the inter-epidemic and epidemic periods, and including enigmatic mechanisms of the virus cryptic maintenance within the host-vector natural cycle.

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Evaluation of a Recombinant Rift Valley Fever Virus Subunit Nucleocapsid Protein as an Immunogen in Mice and Sheep

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Abstract: The possible role of the most abundant structural protein of Rift Valley fever virus (RVFV), the nucleocapsid protein (NP), in inducing protective immune responses has only been evaluated preliminarily in mice but not in any natural host species. In this study we demonstrate that a soluble recombinant RVFV subunit NP in combination with adjuvants (ISA50, Alhydrogel, TiterMax Gold or SaponinQ) is highly immunogenic in mice and sheep but the level of clinical protection and virus replication in mice after lethal challenge was dependent on the adjuvant used. Immunization with NP in combination with Alhydrogel conferred 100% protection against morbidity, mortality and viral replication in mice, but sterilizing immunity could not be achieved in sheep with any NP/adjuvant combinations used. Although this is the first study showing that sterilizing immunity can be elicited in mice immunized with a RVFV subunit nucleocapsid protein, our findings seem to suggest that mice might not be the best animal model for studying the protective ability of RVF subunit vaccines. The results of our study also emphasize the importance of adjuvant selection when evaluating subunit RVF vaccines.

Keywords: Rift Valley fever virus, nucleocapsid, adjuvants, mice, sheep, immunization.

INTRODUCTION

Rift Valley fever (RVF) is an emerging mosquito-borne zoonosis with severe health and socio-economic impacts [1, 2] caused by RVF virus (RVFV), a member of the *Phlebovirus* genus in the *Bunyaviridae* family [3]. Competent RVFV mosquito vectors are widespread outside the virus' current geographic range causing fears that it might spread to previously RVFV naïve regions [4-7]. The RVFV genome comprises three segments (large, medium, and small) of single-stranded RNA, and is in the negative-sense, except for the small (S) segment which consists of ambisense RNA. The latter codes for the nucleocapsid (N) protein and a non-structural protein (NSs) [8, 9]. The N protein is highly conserved and the most immunodominant viral protein in the *Bunyaviridae* family [10-13] inducing rapid production of high levels of anti-NP humoral antibodies in infected animals and humans [14, 15].

Vaccination of livestock in RVF endemic areas would be the most practical way of preventing the disease in animals and its spread to humans. The modified live Smithburn strain was shown to induce lifelong protective immunity but its use is limited due to abortogenic and teratogenic properties in pregnant ewes, inadequate immunogenicity in cattle, and potential safety problems related to risks of incomplete inactivation, and reversion to virulence. The formalin-inactivated vaccines do not induce durable immunity thus necessitating annual booster vaccinations [16-18] of which administration during long inter-epizootic periods, but also due to unpredictable occurrence of RVF outbreaks, might be difficult to implement. Recent advances include development of attenuated recombinant RVFV strains by reverse genetics [19], RVF virus-like particles [20, 21], virus-vectors expressing RVFV genes [22-25], DNA plasmid vaccines [26, 27] and recombinant subunit immunogens [22, 24, 28]. Recombinant RVF viruses with deletions in the non-structural genes of the small (S) and medium (M) segments were highly attenuated and conferred complete protection against lethal challenge in the rat model [19]. Modified RVFV strains, however, carry the risk of being spread by mosquitoes and the possibility of recombination and reversion to virulence. Immunization of mice with RVF virus-like particles (VLP) resulted in virus-neutralizing antibody responses and protection from subsequent lethal RVFV-challenge [20]. Gene gun vaccination with DNA plasmids expressing RVFV glycoproteins induced full [27] or partial protection [26], whereas vaccination with a DNA plasmid expressing the nucleocapsid protein gave only partial protection. A recombinant vaccinia virus containing glycoprotein genes [22], a recombinant lumpy skin

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disease virus vector expressing RVFV glycoproteins [24, 25] and a Sindbis virus replicon vector expressing Gn, Gc and NSm proteins [23] induced protection against RVFV challenge in mice, or mice and sheep. The use of other viruses as vaccine-vectors in regions where the wild-type viruses are endemic, however, might be problematic because of possible background immunity. Bacterially expressed recombinant subunit glycoproteins [22, 24, 28] were shown to induce varying levels of protection against RVFV challenge.

Recombinant protein subunits are generally weak immunogens [29, 30] and require administration with adjuvants to enhance their immunogenicity [31]. Adjuvants promote the uptake of antigens by antigen presenting cells (APC), contribute to the delivery of antigen to lymph nodes, and stimulate cytokine release or expression of co-stimulatory signals on APC which are needed to prime T helper cells for B cell proliferation and induction of cytotoxic T lymphocytes [29, 32]. Some of the more commonly tested and/or used adjuvants are saponins, alum and water-in-oil adjuvants. Saponin adjuvant, a surface active agent isolated from the Chilean Soap bark tree (*Quillaja saponaria*), modulates humoral (Th-2) as well as cellular immunity (Th-1) and bias immune responses towards the Th-1 phenotype and can induce strong CD8+ cytotoxic T-cell responses [29, 33, 34]. $CD8^+$ T cells are able to kill virus-infected cells by inducing apoptosis, and kill infected cells directly in the lymph nodes draining infected sites [35]. Aluminium hydroxide gel (Alhydrogel), commonly known as alum allows for a depot effect at the inoculation site [29], and has also been found to promote the release of IL-4 which results in the increased expression of major histocompatibility class II (MHC II) molecules on monocytes, consequently increasing antigen uptake by APC [36, 37]. Alum does not induce the cytokines interleukin-2 (IL-2) and interferon-gamma (IFN-γ) which are involved in the Th-1 type response, but might directly activate nuclear factor kappa-beta (NF-κB), that is involved in regulating the cellular response to infections [37]. The NF-κB is required for positive selection of memory $CD8^+$ T cells [38, 39]. Montanide ISA50 adjuvant is based on a mannide oleate in mineral oil solution, and contributes to the establishment of a depot effect, transportation of emulsified antigen to distant sites through the lymphatic system, and interaction with mononuclear cells such as APC. ISA50 has been shown to direct the immune response against specific antigens towards the Th-2 type response, involved in humoral immunity [29]. TiterMax Gold (TMG) is a water-in-oil adjuvant that contains a metabolizable oil (squalene), sorbitan monooleate and an immunostimulatory copolymer. It has been shown to induce mixed Th-1/Th-2 responses against specific antigens, but these responses were more directed towards Th-2, indicating humoral immunity [33]. The different mechanisms by which adjuvants enhance different types of immune responses stress the importance of adjuvant selection for vaccine formulations.

In a recent study, a bacterially expressed RVFV recombinant nucleocapsid protein, together with Quil-A adjuvant, was used to immunize BALB/c mice which were subsequently challenged with a lethal dose of RVFV. A protection rate of 60% was achieved but replication of virus, or lack thereof, was not evaluated in surviving or dead mice [24]. Immunization of animals with recombinant subunit N proteins from related viruses in the *Bunyaviridae* family, Dobrova and Hantaan did, however, result in complete protection against viral challenge [40-42]. Recently we expressed a recombinant RVFV nucleocapsid protein, using a bacterial system, in a completely soluble form which was subsequently shown to efficiently bind antibodies from various species [15, 43-46].

In this study we expanded the earlier observations [24] by using a soluble RVFV recNP [15] together with four adjuvants to immunize mice, evaluation of its ability to induce protective immune responses not only by measuring survival ratios but also reduction of viral replication in mouse organs. Further we evaluated the immunogenicity of the recNP antigen, combined with the same adjuvants, in a host animal model and the ability of anti-recNP responses to limit viral replication in sheep after RVFV challenge. To our knowledge this is the first study showing the immunogenicity of a recombinant subunit RVFV NP in a host animal model.

MATERIALS AND METHODOLOGY

Cells and Virus

Vero cells were cultivated in Eagles Minimal Essential Medium (EMEM) (BioWhitaker, MD, USA) containing L-Glutamine, non-essential amino acids, antibiotics (100 IU penicillin, 100 µg streptomycin and 0.25 µg amphotericin B) and 10% foetal bovine serum (Gibco) and maintained at 37ºC in 5% CO_2 incubator. The SPU22/118 KEN 07 strain of RVFV was isolated from a RVF human case during the 2007 Kenyan epidemic [47]. Second passage of the virus, propagated in Vero cells, was used for the challenge.

Bacterial Expression of Recombinant RVFV N Protein

Bacterial expression and purification of the recombinant N protein was carried out as described previously [15]. Briefly, the N gene was cloned into the pET32(a)+ expression vector (Novagen, Germany) from RNA of the RVFV Zim688/78 strain, and subsequently expressed in Origami(DE3) cells (Novagen) after induction with isopropyl β-D-1-thiogalactopyranoside (IPTG, Roche, Germany) for 4 h at 37ºC. Cells were lysed with Bugbuster reagent and Lysonase (Novagen), and recombinant fusion protein containing a 6xHis tag subsequently purified from the soluble fraction using Protino Ni-chelate columns (Machery-Nagel). *E. coli* background proteins were not detectable in the purified end product [results shown in 15].

Mouse Immunization

Four-week old female BALB/c inbred mice were used. The low dose vaccination group (M-I) consisted of 48 mice divided in 4 sub-groups of 12 mice each which were immunized with a 100µl inoculum containing 35µg RVFV recNP in combination with ISA-50 adjuvant (Seppic, France), TiterMax-Gold adjuvant (TMG)(Sigma, U.S.A.), Alhydrogel (Sigma) or SaponinQ (60µg, Sigma), respectively. The high dose vaccination group (M-II) consisted of 48 mice which were subdivided as the M-I group but immunized with 200µl of inoculum containing 70µg of recNP in combination with the adjuvants as described above. The neat recNP group (M-N) consisted of 12 mice immunized with 70µg recNP in PBS buffer. The adjuvant control group consisted of 36 mice di-

vided in 3 sub-groups of 12 mice each which were respectively inoculated only with ISA-50, Alhydrogel or SaponinQ. Note: because of immunization error a fourth adjuvant control subgroup (TiterMax Gold) was excluded from the study. The placebo control group consisted of 12 mice which were inoculated with PBS buffer. All mice were inoculated subcutaneously (s.c) using 1 ml syringes and 25 gauge needles to mimic the natural route of RVFV infection. All animals received identical booster immunizations at 14 days after the initial immunization. A mouse from each group was sacrificed and heart-bled every seven days after primary and booster immunizations to monitor immune responses. Adjuvants ISA50, TMG and Alhydrogel were used as suggested by the manufacturers. The dose of SaponinQ adjuvant (Sigma, U.S.A.) was determined by titration in BALB/c mice and by selecting the highest non-toxic dose at 60µg (results not shown). The selection of recNP doses were determined by recNP concentration and feasible mouse inoculum sizes.

Sheep Immunization

Sheep were pre-screened for antibodies against RVFV using enzyme linked immunosorbent assay (ELISA). Twenty three adult female Dorper cross sheep, younger than one year, were used. The sheep were divided into groups as described in Table **1**. All sheep were inoculated subcutaneously (s.c.) using 1 ml syringes and 25 gauge needles to mimic the natural route of RVFV infection. All animals received identical booster inoculations as described in Table **1**. Serum was collected at regular intervals, as indicated in Table **1**, for immune response monitoring.

IMMUNE RESPONSE MONITORING AFTER IMMUNIZATION

Indirect IgG ELISA

The indirect ELISA was done as described previously [15]. Briefly, immunoplates (Maxisorb, Nunc, Denmark) were coated with RVFV recNP antigen at a dilution of 1:2000 (0.5µg/ml) in Carbonate-Bicarbonate buffer (pH 9.6) and incubated overnight at 4ºC. After washing three times with a washing buffer consisting of phosphate buffered saline (PBS) pH7.2 and 0.1% Tween-20, the plates were blocked with 200µl of 10% fat free milk powder ("Elite", Clover SA, Pty, Ltd.) in PBS at 37ºC for 1h and then washed as before. Test sera were diluted 1:400 in diluent buffer consisting of 2% fat free milk powder in PBS, 100µl added to each well and incubated for 1h at 37ºC. Samples were tested in duplicate. After washing as before, 100 µl of goat anti-mouse IgG horseradish peroxidase (HRPO)(H+L) at 1:2000 dilution (for mice) or recombinant Protein G HRPO (Zymed Laboratories, Invitrogen, U.S.A.) at 1:6000 dilution (for sheep) was added to the plates. After 1h incubation at 37ºC plates were washed as before and 100 µl of 2,2'-azinodiethylbenzthiazoline sulfonic acid (ABTS) (KPL Laboratories, Inc., USA) added to each well. After 30 min incubation in the dark the reaction was stopped by the addition of 100 µl of 1% sodium dodecyl sulfate (SDS) to each well. Optical density (OD) was determined at 405nm and the results expressed as the mean OD value for the duplicates tested. The positive control serum was generated as described previously [48] by infecting eight sheep with the AR 20368 RVFV strain and pooling highly reactive sera from collections made between day 1 and 72 post-infection. The negative control serum was serum pooled from six animals shown to be negative in the virus neutralization test as described previously [48].

Virus Neutralization Test

In addition, a virus neutralization test (VNT) was performed on sera collected from mice and sheep after immunization to measure neutralizing ability of these sera. The VNT was performed as described previously [48] by titrating sera from a 1:10 dilution in two-fold dilutions. The titer is expressed as the reciprocal of the serum dilution that inhibited ≥ 75% of the viral cytopathic effect (CPE).

Table 1. Group Assignments, Inoculums and Sheep Immunization and Sampling Schedule

Group Number	Number of Sheep	RVFV recNP Dose (µg)	Adjuvant/Inoculum	Immunization Schedule	Blood Collection Schedule (Day After Immunization/Booster)
1a	n = 2	175	ISA50	Day 0 (initial) Day 21 (booster)	Day 0, Day 14, Day 26 Day 14 after booster
1b	n = 2	350	ISA50		
2a	n = 2	175	Alhydrogel		
2b	n = 2	350	Alhydrogel		
3a	n = 2	175	TiterMax Gold®		
3b	n = 2	350	TiterMax Gold®		
4a	n = 2	175	SaponinQ		
4b	n = 2	350	SaponinQ		
5	n = 4	0	ISA50, Alhydrogel, TiterMax Gold® or SaponinQ	Day 0 (initial mock) Day 21 (mock booster)	
6	n = 3	0	PBS		

RVFV CHALLENGE

Mice

After the immunization period, 5 to 7 animals from each group were challenged with RVFV on day 32 after the booster immunization. Mice were inoculated s.c. with a 100 µl inoculum containing $10^{7.0}$ $TCID_{50}$/ml RVF challenge virus, and after challenge examined twice daily for signs of clinical illness including loss of appetite and consequent weight loss, scruffy coat, decreased alertness, decreased mobility, loss of balance, shallow and irregular breathing, and hunched posture. Animals displaying severe illness were euthanized and organs collected. Organs were also collected at regular intervals from healthy, sick and dead mice to monitor viremia. Surviving mice were monitored for 22 days post infection. A control group was mock inoculated with Eagle's minimum essential medium (EMEM) free of the virus.

Sheep

All sheep were challenged s.c. with 2 ml challenge virus (1 ml on both sides of the neck). Sheep were challenged at different times as follows: one sheep from each sub-group (group 1a,b - 4a,b), all sheep from group five and one sheep from group six were challenged on day 37 after the booster immunization (total = 13 sheep); the remaining sheep were challenged on day 168 after the booster immunization (total = 10 sheep). Sheep were monitored daily for the first two weeks after challenge and blood taken daily for the first seven days, and at regular intervals thereafter to monitor viremia and immune responses until day 70.

IMMUNE RESPONSE MONITORING AFTER RVFV CHALLENGE

Immune responses in sheep after challenge were monitored by IgM capture ELISAs as described previously [48]. A virus neutralization test (VNT) was performed as described above. Means and standard deviations for IgM ELISA readings and VNT titres were based on data from minimum two animals per group.

PASSIVE IMMUNIZATION OF MICE WITH ANTI-RECNP IMMUNE SERA

The ability of anti-N antibodies to passively confer immunity in mice was evaluated using polyclonal antisera generated in sheep, rabbits and mice. Mice were immunized with recNP as described earlier, and antisera from different recNP/adjuvant experimental groups were respectively pooled before testing. Polyclonal anti-N antisera in rabbits and sheep were produced as described previously [43]. All polyclonal sera were mixed to final dilution of 1:10 with Vero-derived virus preparation containing $10^{7.0}$ $TCID_{50}$/ml of the 2007 Kenya RVFV isolate, and the mixture incubated at 37ºC for 30 min before inoculation. As controls, sera from naïve sheep, rabbits and mice were mixed identically with RVFV. To control the effects of non-related compounds in serum, sterile PBS was mixed to a 1:10 with the virus. A total of 42 BALB/c 3-4 weeks old female mice, were divided into groups of 6 animals each and inoculated s.c. with 200 µl of the following mixtures: a) virus and mouse anti-recNP, b) virus and sheep anti-recNP, c) virus and rabbit anti-recNP, d) virus and naive mouse serum, e) virus and naïve sheep serum, f) virus and naïve rabbit serum, and g) virus and PBS. Mice were examined twice daily clinically and those displaying severe signs of illness were euthanized. Surviving mice were monitored for 22 days post infection.

VIRUS TITRATIONS

Mouse liver and kidney tissues were homogenized as 10% (w/v) suspensions in EMEM containing L-Glutamine, non-essential amino acids, antibiotics (100 IU penicillin, 100 µg streptomycin and 0.25 µg amphotericin B). After centrifugation at 3000 x g, 4°C for 15 minutes, supernatants were collected and stored at -70ºC until tested.

Virus titrations of mouse tissue homogenates and sheep sera collected after challenge were performed as described previously [49]. Briefly, four 100µl replicates of 10-fold dilutions (10^{-1} to 10^{-8}) of homogenates or sera were transferred into flat bottomed 96-well cell culture microplates (Nunc) and equal volumes of Vero cell suspension in EMEM containing 2 x 10^5 cells/ml, 8% FBS and antibiotics were added. The plates were incubated at 37ºC in CO_2 and observed microscopically for cytopathic effects (CPE) for 10 days post inoculation. Virus titers, calculated by the Kärber method [50] were expressed as median tissue culture infectious dose ($TCID_{50}$) per ml of serum or gram of tissue. Means and standard deviations from the means were determined based on two or more animals per group.

REAL TIME REVERSE TRANSCRIPTASE PCR (QRT-PCR)

Real time PCR was performed only on mouse tissue homogenates that yielded negative results by virus titration. Viral RNA was extracted from 140µl of tissue homogenates using the QIAmp® Viral RNA Kit (QIAgen, Germany) according to the instructions of the manufacturer. The qRT-PCR was performed as described previously [49]. Briefly, amplifications were carried out in 20µl reaction mixtures containing 5µl of the extracted vRNA using the LightCycler RNA Amplification Hybprobe kit (Roche, Germany) and the Roche LightCycler instrument. Primers and a labeled probe targeting the G2 glycoprotein gene of RVFV were used.

STATISTICAL METHODS

Survival proportions in immunized mice versus control mice after challenge were compared using the Fisher exact test (Soper online statistics calculator) [51]. Viral load results in mouse organs are based on $TCID_{50}$ titrations of virus in tissues from three or more animals and given as means. P-values lower than 0.01 were considered to be significant.

The significance of differences between immune responses and viremia in sheep was confirmed using the Fisher F-test giving a two-tailed probability value (Excel, Microsoft Office). P-values lower than 0.01 were considered to be significant. Mean values and standard deviations from the means were calculated using at least two sheep per group.

RESULTS

Immunogenicity of the recNP

All recNP/adjuvant combinations induced detectable anti-recNP IgG responses by day seven after a single immu-

nization of mice (Fig. **1**) with similarly increasing levels over time. The recNP without adjuvant induced a weaker but still detectable IgG response. None of the control mice (PBS or adjuvant controls) developed any antibodies against the recNP. Anti-recNP immune sera from mice were not able to neutralize the virus *in-vitro* (results not shown).

All sheep immunized with recNP combined with adjuvants produced detectable anti-NP IgG responses by day 14 after the first immunization (Fig. **2**). The anti-NP antibody levels of immunized sheep were consistently equal to or higher than that in experimentally infected sheep (dotted vertical line in Fig. **2**). The second immunization of all sheep

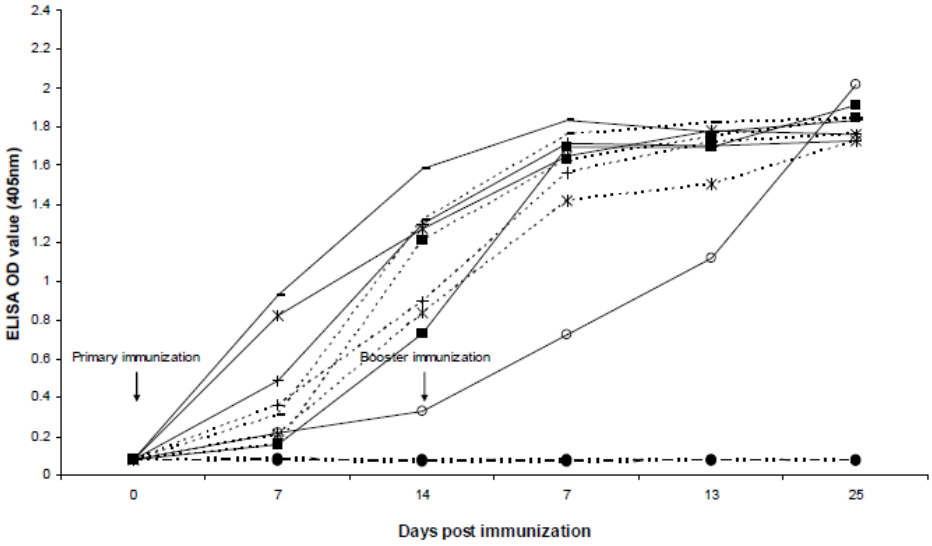


Fig. (1). Anti-RVFV recNP IgG responses in immunized and control mice. Low dose (35µg recNP) mice are indicated by dotted lines (---) and high dose (70µg recNP) mice by solid lines (—). Adjuvant combinations are indicated as ISA50 (x), TiterMax Gold (■), Alhydrogel (+), SaponinQ (-) and recNP without adjuvant (o). Adjuvant and PBS placebo mice are indicated by dotted lines and ●.

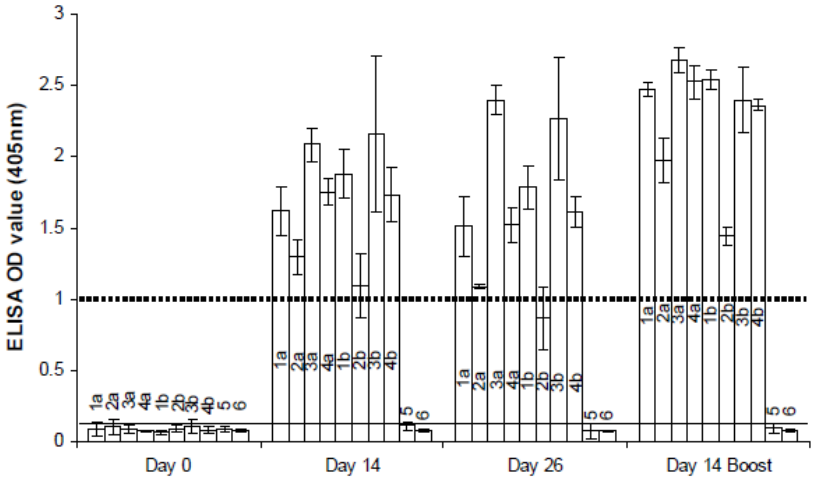


Fig. (2). Average anti-NP responses in sheep after recNP first and booster immunizations; error bars indicate standard deviations from the means. 1a) 175µg recNP/ISA50 (n=2); 2a) 175µg recNP/Alhydrogel (n=2); 3a) 175µg recNP/TiterMax Gold (n=2); 4a) 175µg recNP/SaponinQ (n=2); 1b) 350µg recNP/ISA50 (n=2); 2b) 350µg recNP/Alhydrogel (n=2); 3b) 350µg recNP/TiterMax Gold (n=2); 4b) 350µg recNP/SaponinQ; 5) Adjuvant controls (n = 4) and 6) PBS controls (n=3). The vertical dotted line indicates the average OD reading for a high positive control serum. The solid vertical line indicates the average OD reading for a negative control serum.

had the desired effect of boosting humoral immune responses. The combination of recNP with Alhydrogel (group 2a-b) was the least immunogenic but still induced strong responses compared to the positive control. The larger recNP dose (350µg) did not induce significantly stronger responses in any of the immunized groups when compared to the lower dose (175µg) with the same adjuvants: recNP/ISA50 (Group 1a-b, Fischer F-test p = 0.915), recNP/Alhydrogel (Group 2a-b, p = 0.638), recNP/TiterMax Gold (Group 3a-b, p = 0.902) and recNP/SaponinQ (Group 4a-b, p = 0.930). Because the dose of recNP did not have a significant impact on the strength of the humoral responses, groups that were immunized with the same recNP/adjuvant combination, regardless of dose, were grouped together for the RVFV challenge experiment. As expected, the adjuvant and PBS control groups did not develop any anti-NP responses during the immunization period. The immune responses elicited by recNP immunization were not neutralizing (results not shown).

PROTECTION OF MICE AGAINST RVFV CHALLENGE

All mice in the adjuvant and PBS placebo control groups died or developed severe symptoms by day six post-infection. In contrast, clinical signs in all unprotected immunized animals were delayed by four to nine days as compared to controls (Table **2**). Clinical signs in sick animals included loss of appetite and consequent weight loss, scruffy coat, decreased alertness, decreased mobility, loss of balance, shallow and irregular breathing, and hunched posture. Only immunization with 35 and 70µg recNP/Alhydrogel, and 70µg recNP/SaponinQ yielded significant protection from disease/death ($p \leq 0.01$, Fischer exact test) (Table **2**). The best protection (100%) was achieved after immunization with both doses of recNP combined with Alhydrogel, as well as immunization with 70µg recNP/SaponinQ. The mock inoculated mice (with EMEM free of virus) did not develop any clinical signs during the experiment. Despite full or partial clinical protection resulting from immunization, the challenge virus replicated in most immunized mice, but to lower levels than in adjuvant and PBS control mice. Replication of virus could not be shown in liver and kidney tissues from mice immunized with 70µg recNP/Alhydrogel, either by virus isolation or qRT-PCR. Table **3** summarizes viral load data in mice during the acute period of infection (day 1 - 6).

PASSIVE IMMUNITY BY ANTI-RECNP IMMUNE SERA

Anti-recNP immune sera did not neutralize virus *in-vivo*. No significant decrease in mortality/morbidity could be shown in any of the groups: a\ virus and mouse anti-recNP

Table 2. Mouse Survival Rates

Group	Survivors/Total (% Survival)[a]	Morbidity / Mortality (Day Post Infection)[b]	Significance of Protection[c]
35µg recNP/ISA50	4/6 (67%)	1 (Day 10) 1 (Day 15)	p = 0.0303
70µg recNP/ISA50	2/5 (40%)	2 (Day 10) 1 (Day 15)	p = 0.1818
35µg recNP/TMG	4/7 (57%)	2 (Day 10) 1 (Day 14)	p = 0.0489
70µg recNP/TMG	3/5 (60%)	2 (Day 15)	p = 0.0606
35µg recNP/Alhydrogel	6/6 (100%)	No morbidity / mortality	p = 0.0011
70µg recNP/Alhydrogel	5/5 (100%)	No morbidity / mortality	p = 0.0022
35µg recNP/SaponinQ	4/6 (67%)	2 (Day 10)	p = 0.0303
70µg recNP/SaponinQ	6/6 (100%)	No morbidity / mortality	p = 0.0011
70µg recNP	1/6 (17%)	1 (Day 7) 2 (Day 8) 2 (Day 9)	p = 0.500
Adjuvant control group ISA50	0/5 (0%)	4 (Day 3) 1 (Day 6)	No protection
Adjuvant control group Alhydrogel	0/5 (0%)	5 (Day 2)	No protection
Adjuvant control group SaponinQ	0/6 (0%)	2 (Day 3) 1 (Day 4) 3 (Day 6)	No protection
Placebo control group (PBS)	0/6 (0%)	5 (Day 3) 1 (Day 4)	No protection

[a] Survivors/total number of mice ratio. (%) indicates percentage survival.
[b] Indicates the number of mice that died or were euthanized because of severe morbidity, with the days post infection indicated in brackets.
[c] Significance of protection calculated using Fisher's Exact test. P-values ≤ 0.01 were considered significant.

(survival 1/6, 17%, Fischer's exact test p = 0.500), b\ virus and sheep anti-recNP (survival 2/6, 33%, p = 0.227), c\ virus and rabbit anti-recNP (survival 0/6, 0%, p = 1.000), d\ virus and naive mouse serum (survival 0/6, 0%), e\ virus and naïve sheep serum (survival 0/6, 0%) f\ virus and naïve rabbit serum (survival 0/6, 0%), and g\ virus and PBS (survival 0/6, 0%).

IMMUNE RESPONSES IN SHEEP AFTER RVFV CHALLENGE

Because the dose of recNP did not have a significant impact on the strength of the humoral responses, groups that were immunized with the same recNP/adjuvant combination, regardless of dose, were grouped together for the RVFV challenge experiment. All adjuvant control sheep were regarded as one group, and all PBS control sheep were regarded as one group, regardless of when they were challenged with RVFV.

The sheep IgM responses after challenge are shown in Fig. (**3A**) and in Fig. (**3B**). None of the immunized or control sheep had any detectable RVFV specific IgM antibodies on the days they were challenged. High levels of RVFV specific IgG, however, was detected in all immunized sheep on the days they were challenged, but as expected not in control

Table 3. Viral Load Data in Mice During the Acute Period of Infection (day 1 - 6)

Group	Organ Tissue	Number of Mice Tested	Mean Viral Load[a]	Range (StDev)[b]
idne recNP/ISA50	Liver	3	Negative *	No virus detected*
	Kidney	3	1.3	0.0 - 4.0 (2.3)
70µg recNP/ISA50	Liver	4	Negative	No virus detected
	Kidney	4	Negative *	No virus detected*
35µg recNP/TMG	Liver	4	Negative	No virus detected
	Kidney	4	0.9	0.0 - 3.8 (1.9)
70µg recNP/TMG	Liver	3	Negative *	No virus detected*
	Kidney	3	Negative *	No virus detected*
35µg recNP/Alhydrogel	Liver	3	Negative	No virus detected
	Kidney	3	1.3	0.0 - 4.0 (2.3)
70µg recNP/Alhydrogel	Liver	3	Negative	No virus detected
	Kidney	3	Negative	No virus detected
35µg recNP/SaponinQ	Liver	4	1.0	0.0 - 4.0 (2.0)
	Kidney	4	Negative *	No virus detected*
70µg recNP/SaponinQ	Liver	3	Negative *	No virus detected*
	Kidney	3	1.3	0.0 - 3.8 (2.2)
70µg recNP	Liver	6	0.8	0.0 - 4.5 (1.8)
	Kidney	6	3.0	0.0 - 5.3 (2.4)
Adjuvant control group (ISA50)	Liver	5	3.5	0.0 - 5.3 (2.0)
	Kidney	5	5.0	3.8 - 5.3 (0.8)
Adjuvant control group (Alhydrogel)	Liver	5	4.5	3.8 - 5.3 (0.9)
	Kidney	5	5.7	5.0 - 6.5 (0.6)
Adjuvant control group (SaponinQ)	Liver	6	5.3	3.8 - 6.8 (1.3)
	Kidney	6	5.8	4.5 - 6.3 (0.7)
Placebo control group (PBS)	Liver	6	5.9	4.0 - 7.3 (1.3)
	Kidney	6	5.3	4.3 - 6.0 (0.6)

Organs were collected from sick and healthy mice after infection.
[a] Viral loads are indicated as mean $\log_{10}TCID_{50}$/g tissue.
[b] Range of $\log_{10}TCID_{50}$ values and standard deviation from the mean.
*Indicates where viral RNA was detected by qRT-PCR in virus negative tissues.

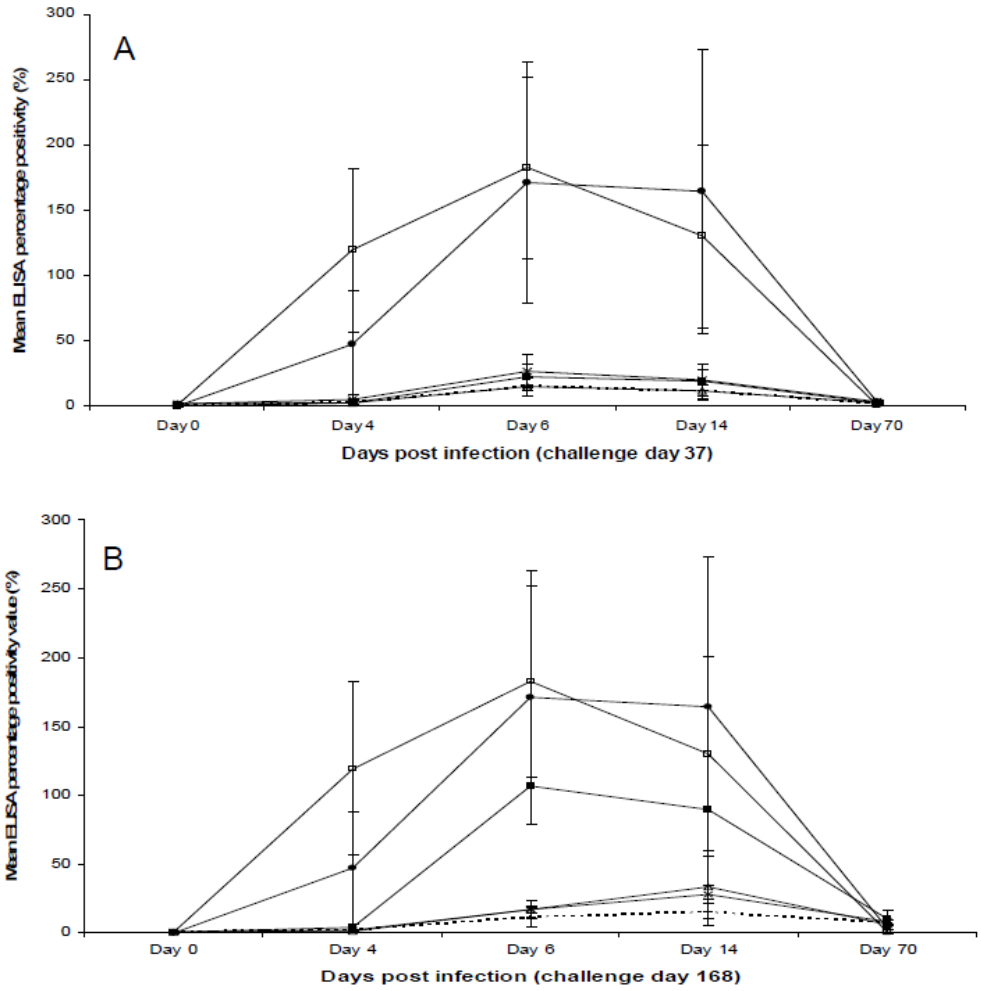


Fig. (3). Mean IgM responses in sheep after RVFV challenge on day 37 (**A**) or 168 (**B**). Sheep groups are indicated as recNP/ISA50 (--+--), recNP/Alhydrogel (—■—), recNP/TiterMax Gold (—Δ—), recNP/SaponinQ (—x—), adjuvant controls (—□—) and PBS controls (—●—). Error bars indicate standard deviations from the means of two or more sheep per group.

sheep (results not shown). Control sheep developed typical transient IgM responses after RVFV challenge. All immunized sheep developed lower IgM responses after challenge when compared to control sheep, except the recNP/Alhydrogel immunized sheep that were challenged on day 168, which developed elevated IgM responses when compared to other immunized sheep. Incidentally, these recNP/Alhydrogel immunized sheep had decreased RVFV specific IgG on the day 168 when they were challenged (results not shown).

The virus neutralizing antibody responses after challenge are shown in Fig. (**4A**, **4B**). Immunization did not have significant effect on decreasing the development of virus neutralizing antibodies when compared to PBS control sheep: recNP/ISA50 (day 37, p = 0.883; day 168, p = 0.825 Fisher F-test), recNP/Alhydrogel (day 37, p = 0.920; day 168, p = 0.850), recNP/TiterMax Gold (day 37, p = 0.881; day 168, p = 0.975) and recNP/SaponinQ (day 37, p = 0.682; day 168, p = 0.858).

VIREMIA IN SHEEP AFTER RVFV CHALLENGE

The viremia in sheep after RVFV challenge is shown in Table **4**. Immunization of sheep did not result in significant decrease of viral loads in sera when compared to PBS control sheep. Viremia was, however, of two to four days duration whereas one PBS control sheep developed pro-

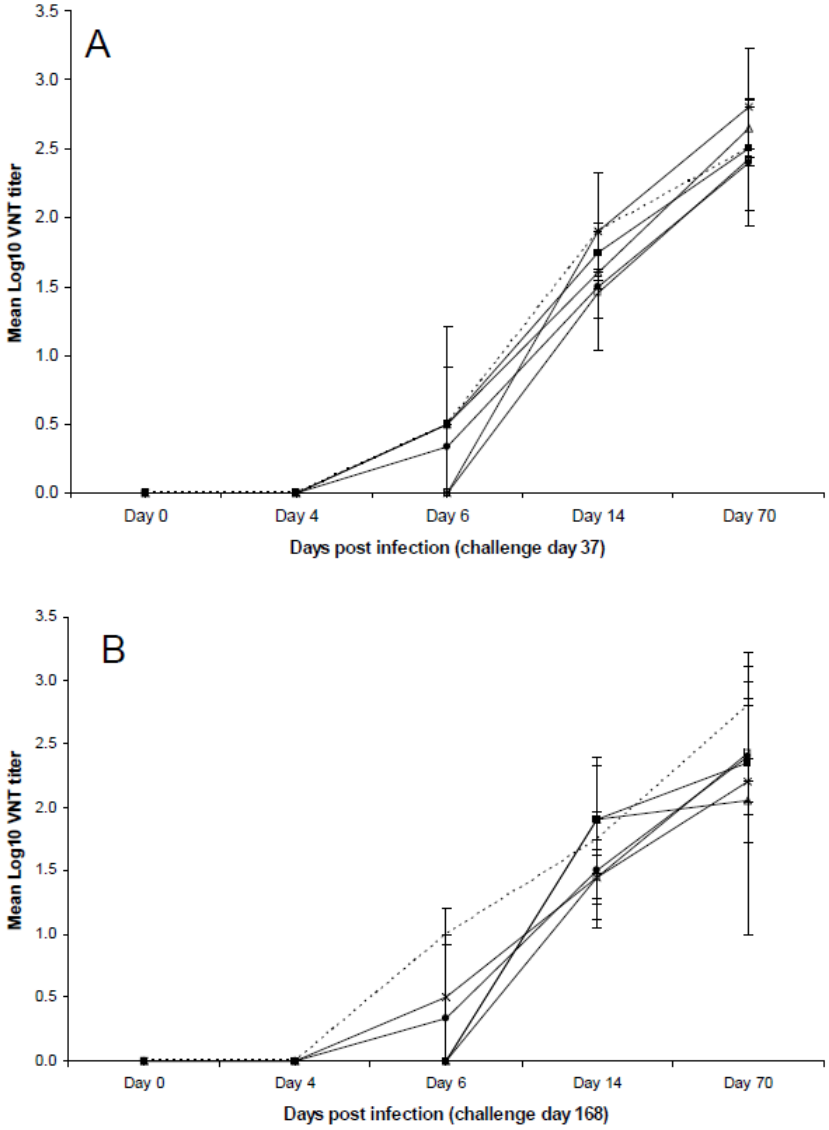


Fig. (4). Mean virus neutralizing antibody responses in sheep after RVFV challenge on day 37 (**A**) or 168 (**B**). Sheep groups are indicated as recNP/ISA50 (--+--), recNP/Alhydrogel (—■—), recNP/TiterMax Gold (—Δ—), recNP/SaponinQ (—x—), adjuvant controls (—□—) and PBS controls (—●—). Error bars indicate standard deviations from the means of two or more sheep per group.

longed viremia up to day seven. Despite severe challenge none of the sheep, including controls, displayed any clinical signs.

DISCUSSION

There is no available RVF vaccine for humans and the currently in use live-attenuated and inactivated animal vaccines are expensive to produce and pose safety problems. Recombinant DNA technology has proven to be a useful tool for the development of alternative vaccine candidates for RVF, including recombinant subunit vaccines, recombinant attenuated virus prepared by reverse genetics, virus like particles and recombinant viruses using a non-related virus as backbone for RVF antigens. These new generation vaccines have been shown to have their distinct advantages and disadvantages, but have mostly targeted the glycoproteins of the virus which are known to induce protective neutralizing antibodies [22, 25, 27, 28].

Table 4. Viremia in Sheep After RVFV Challenge

Inoculum	Number of Sheep	Days Post Infection	Viremia Mean $Log_{10}TCID_{50}$/ml±Standard Deviation (Range)	Significance of Decreased Viremia*
recNP/ISA50 (1a-b) (175 and 350µg combined) Challenge day 37 Challenge day 168	2	1 2 3 4-7	4.5±0.4 (4.3 to 4.8) 4.4±0.2 (4.3 to 4.5) 0.8±1.1 (0.0 to 1.5) Negative	p = 0.55
	2	1 2 3-7	3.6±0.5 (3.3 to 4.0) 6.1±0.5 (5.8 to 6.5) 2.0±1.8 (0.8 to 3.3)	p = 0.37
recNP/Alhydrogel (2a-b) (175 and 350µg combined) Challenge day 37 Challenge day 168	2	1 2 3 4-7	2.6±2.7 (0.8 to 4.5) 2.0±2.8 (0.0 to 4.0) 1.1±1.6 (0.0 to 2.3) Negative	p = 0.37
	2	1 2 3 4 5-7	3.4±0.2 (3.3 to 3.5) 4.4±2.7 (2.5 to 6.3) 2.4±3.4 (0.0 to 4.8) 0.5±0.7 (0.0 to 1.0) Negative	p = 0.79
recNP/TiterMax Gold (3a-b) (175 and 350µg combined) Challenge day 37 Challenge day 168	2	1 2 3-7	4.3±1.1 (3.5 to 5.0) 2.6±1.2 (1.8 to 3.5) Negative	p = 0.88
	2	1 2 3 4-7	4.3±0.4 (4.0 to 4.5) 5.0±1.1 (4.3 to 5.8) 1.9±1.2 (1.0 to 2.8) Negative	p = 0.49
recNP/SaponinQ (4a-b) (175 and 350µg combined) Challenge day 37 Challenge day 168	2	1 2 3 4-7	4.4±0.5 (4.0 to 4.8) 4.8±0.4 (4.5 to 5.0) 1.6±2.3 (0.0 to 3.3) Negative	p = 0.52
	2	1 2 3 4-7	1.9±2.7 (0.0 to 3.8) 2.0±2.8 (0.0 to 4.0) 0.4±0.5 (0.0 to 0.8) Negative	p = 0.19
Adjuvant control (ISA50, Alhydrogel, TiterMax Gold and SaponinQ combined) Challenge day 37	4	1 2 3 4-7	5.1±0.3 (4.8 to 5.5) 4.8±1.1 (3.8 to 6.0) 0.8±1.5 (0.0 to 3.0) Negative	p = 0.40
PBS control Challenge day 37 and 168	3	1 2 3 4 5 6 7	4.5±0.3 (4.3 to 4.8) 5.3±2.3 (3.3 to 7.8) 2.3±4.0 (0.0 to 7.0) 1.9±3.3 (0.0 to 5.8) 1.8±3.2 (0.0 to 5.5) 1.5±2.6 (0.0 to 4.5) 0.8±1.4 (0.0 to 2.5)	Control group

* Indicates the statistical significance of decrease in viral load as compared to PBS control sheep, as calculated by the Fisher F-test. P-values ≤ 0.01 indicate a statistically significant decrease in viremia.

The nucleocapsid protein induces production of high levels of anti-NP specific IgG and IgM responses in host animals [14, 15]. It has been postulated that the strongly biased humoral antibody response to N protein might be a part of

virus strategy to direct the host immune response towards viral antigenic determinants not relevant in protection [52]. On the other hand, immunization of animals with recombinant subunit N proteins from related bunyaviruses resulted in complete protection against viral challenge [40-42]. Immunization with a recombinant RVFV nucleocapsid protein with Quil-A adjuvant protected 60% of mice from lethal challenge [24]. Immunization of mice with cDNA encoding the N protein resulted in 50% protection from clinical signs after the induction of NP-specific lymphoproliferative responses [26] but the replication of challenge virus in immunized mice was not evaluated and there was 86% recovering rate in naïve mice indicating low challenge. Immunization of mice with virus like particles containing both glycoproteins and the N protein resulted in 92% protection [20]. This protection was most probably due to the presence of glycoproteins in the VLP since anti-NP responses could not be detected after immunization. Immunization of mice with VLPs containing the glycoproteins and NP resulted in better protection against lethal challenge when compared to those immunized with VLPs without NP [21]. Immunization of sheep with a DNA construct expressing the RVFV N protein resulted production of anti-NP antibodies but protection against RVFV challenge was never evaluated [52]. From these earlier reports it seems that the anti-NP response does play a role in protection of mice against morbidity and mortality from RVFV infection. The decrease of viral replication in these immunized mice have, however, not been evaluated yet. Vaccination against arthropod borne viruses should ideally aim to decrease morbidity and mortality, but even more importantly it should stop the spread of the virus by inducing sterilizing immunity. From the previous studies it is unclear whether NP immunization was able to decrease viral replication in challenged animals. The protective ability of the anti-NP response in a host animal species, such as sheep, has never been evaluated. In this study we evaluated a recombinant subunit N protein of RVFV as an immunogen, in combination with four different adjuvants, in mice and measured its protective ability against RVFV challenge by using survival rates as well as decrease of viral replication after severe RVFV challenge. We also evaluated for the first time a recombinant subunit RVFV NP as an immunogen in a host animal model, sheep, and its ability to decrease viral replication in sheep after severe RVFV challenge. To our knowledge this is the first study evaluating the effect of anti-NP immunity on viral replication in both mice and a host animal species.

Four different adjuvants with differing mechanisms of enhancing immune responses were used in this study to evaluate whether this could have an effect on the level of protection against severe RVFV challenge. The RVFV recNP used was not only highly immunogenic in mice and sheep in combination with all adjuvants, but also induced a measurable response in the absence of adjuvant in mice. Even though in the limited number of mice tested at each time point there wasn't drastic differences in the strength of humoral responses in mice after immunization, there were significant differences in the level of protection against challenge. The best protection against morbidity and mortality (100%) was achieved with 35 or 70µg of the recNP when combined with Alhydrogel adjuvant, or 70µg recNP with SaponinQ. Additionally no virus or viral RNA could be detected in the liver or kidney tissues from mice immunized with 70µg recNP/Alhydrogel after RVFV challenge, indicating possible sterilizing immunity. Alhydrogel, more commonly known as alum, promote the release of IL-4 which results in the increased expression of MHC-II molecules on monocytes, consequently increasing antigen uptake by antigen presenting cells (APC) [36, 37]. Alum also activates NF-κB, a protein complex found in almost all cell types and that is involved in regulating the cellular response to infections by effecting positive selection of memory $CD8^+$ T cells [37-39]. This is the first study showing 100% protection from morbidity and mortality and viral replication in mice immunized with a RVFV subunit nucleocapsid protein after severe RVFV challenge, and confirms previous findings that the anti-NP response does play a role in protecting mice from RVFV challenge [20, 21, 24, 26]

Other recNP/adjuvant combinations were not as effective as Alhydrogel in inducing protective and sterilizing immunity. The mice immunized with 70µg recNP/SaponinQ was also 100% protected from morbidity/mortality but replicating virus or viral RNA was found in the organ tissues of some of the mice in this group. The lower dose of the recNP/SaponinQ combination was less effective and resulted in protection of only 67% of mice from morbidity/mortality while virus still replicated in their organs. Saponins modulate humoral (Th-2) as well as cellular immunity, but seem to be more effective *via* the Th-1 cellular route and can induce strong CD8+ cytotoxic T-cell responses [34]. The two adjuvants that enhance immune responses by very similar mechanisms, ISA50 and TiterMax Gold, were equally ineffective in inducing significant protective and sterilizing immunity in mice after RVFV challenge. These two adjuvants induce mixed Th-1/Th-2 responses, but responses are usually biased towards Th-2 which is indicative of a stronger humoral response [29, 33, 53]. The worst protected immunized mice were those in the group immunized only with recNP and no adjuvant. Our findings emphasize the importance of an adjuvant in modulating a desired protective immune response to a specific antigen.

Irrespective of the dose used all recNP/adjuvant combinations were highly immunogenic in sheep even after a single immunization. Anti-NP IgG responses in immunized sheep were equal to or higher than the level of anti-NP antibodies in experimentally infected sheep. After RVFV challenge the control sheep developed strong IgM response, which in immunized sheep was much weaker, but still detectable after challenge. Although these results might have indicated lower virus replication in immunized sheep after challenge; surprisingly there were no significant differences in the virus neutralizing titres in immunized sheep when compared to control sheep. Also, humoral immunity against the NP was not able to significantly decrease viremia when compared to control sheep.

The results of the study demonstrate that sterilizing immunity could be induced with a recombinant subunit RVFV nucleocapsid protein in a mouse model when used with specific adjuvants, but the same recNP/adjuvant combinations were not able to induce the same level of immunity in a ruminant host species. Our findings highlight also some important aspects that should be considered for future research and development of vaccine candidates for RVF. Firstly the anti-

nucleocapsid response alone, although protective in mice, does not seem to play a role in protection of an actual host species against RVFV infection. Secondly, our results show that mice might not be the best animal model for studying protective ability of RVF vaccines. Although the target proteins of choice for RVFV vaccines are glycoproteins because of inducing neutralizing antibody, RVFV vaccine candidates targeting the glycoproteins which were evaluated in mice have also yielded inconsistent protection against challenge [20, 21, 23]. In a recent study it was shown that immunization with VLPs combining the glycoproteins and nucleocapsid protein yielded better protection [21]. Therefore it appears that vaccine candidates combining glyco- and nucleocapsid proteins should be further investigated.

ETHICS CLEARANCES

Ethics clearances for the use of animals were obtained from the National Health Laboratory Service Animal Ethics Committee (NHLS AEC 109/07) and the University of the Witwatersrand Animal Ethics Screening Committee (AESC 2008/16/4).

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Comparison of Enzyme-Linked Immunosorbent Assay–Based Techniques for the Detection of Antibody to Rift Valley Fever Virus in Thermochemically Inactivated Sheep Sera

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Abstract

Different enzyme-linked immunosorbent assay (ELISA)–based techniques for the detection of antibodies to Rift Valley fever virus (RVFV) have been developed in recent years, but their diagnostic sensitivity was not directly compared. In addition, their use might still be restricted to high biocontainment facilities when sera to be tested are collected from viremic individuals. In this study, we report on direct comparison of various ELISA forms for the detection of anti-RVFV antibody in preinactivated sera using a simple thermochemical treatment. Results in naive and treated sera from experimentally infected sheep demonstrate that inactivation method used had no adverse effect on ELISA readings, but the assays analyzed differ in their ability to detect the early humoral responses to infection with RVFV. The IgM-capture ELISA was slightly more sensitive than the IgG-sandwich ELISA to detect early humoral response after infection. The indirect IgG ELISA, using Protein G Horseradish Peroxidase, was less sensitive in detecting seroconversion than the IgG-sandwich ELISA, but this problem was alleviated when using anti-sheep IgG conjugated with Horseradish Peroxidase. The high concentration of viral antigen in sheep sera collected shortly after infection might contribute to false-positive results in the inhibition ELISA, but its ability to detect seroconversion was comparable to that of IgM-capture ELISA.

Key Words: ELISA—Inactivation—Rift Valley fever.

Introduction

Historically, Rift Valley fever (RVF) was endemic to sub-Saharan Africa, but in the last four decades it spread to northern Africa (1977/1978), West Africa (1987), and most recently to the Arabian Peninsula (2000/2001) (reviewed in Swanepoel and Coetzer 2004). The potential for further northward spread of the disease is of great international medical and veterinary concern. The spread of the disease might be facilitated by the ubiquitous presence of competent mosquito vector populations (Turrel et al. 2008) in potentially receptive RVF-free regions, changing weather patterns, world globalization, and uncontrolled livestock trade from endemic countries. There is an increasing demand for rapid, accurate, and safe diagnostic tools for RVF serodiagnosis. This might be hampered because of strict requirements for handling and processing of RVF specimens under high biocontainment facilities. In this study we report on direct comparison of various enzyme-linked immunosorbent assay (ELISA) forms for the detection of specific antibody in preinactivated sera using a simple thermochemical treatment that allows for complete inactivation of RVF virus.

Materials and Methods

Enzyme-linked immunosorbent assays

The following ELISAs were directly compared using serial bleeds from experimentally infected sheep: IgG-sandwich ELISA based on gamma-irradiated, sucrose-acetone-extracted mouse liver RVF virus (RVFV) whole antigen (Paweska et al. 2003), IgM-capture ELISA based on the same antigen

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(Paweska et al. 2003), an inhibition ELISA based on gamma-irradiated tissue culture RVFV whole antigen (Paweska et al. 2005), and an indirect ELISA based on recombinant RVFV N protein (Fafetine et al. 2007).

Experimental sheep sera

Serial sera were obtained from three sheep experimentally infected with wild-type RVF virus as described previously (Le Roux et al. 2009).

Thermochemical inactivation

Sheep sera were inactivated as described by Jansen van Vuren and Paweska (2009). Briefly, an equal volume of 1% Tween20 in phosphate-buffered saline was added to each serum and incubated at 56°C for 1 h. Inactivated sera were tested for complete inactivation on 24–48-h-old Vero cell monolayers and in 2–3-day-old suckling mice. Cells were monitored for cytopathic effect until 14 days after inoculation and mice until 10 days postinfection (p.i.).

Results

The immune responses in sheep after experimental infection with wild-type RVFV were monitored using the IgG sandwich, indirect, IgM capture, and inhibition ELISAs. There was no significant difference in detection of antibodies between naive versus inactivated serum using any of the ELISAs, but these assays differed in their ability to detect the early humoral responses to infection with RVFV (Fig. 1). The IgM-capture ELISA was able to detect seroconversion on day 4 p.i. compared with day 5 p.i. with the IgG-sandwich ELISA. The inhibition ELISA yielded false-positive results on days 2 and 3 p.i. as a result of the capturing of viral antigen in highly viremic sera on days 2 and 3 p.i. (results not shown). The recombinant N protein–based IgG ELISA, using Protein G Horseradish Peroxidase, was less sensitive in detecting seroconversion (day 9 p.i.) than the IgG-sandwich ELISA (day 5 p.i.). This problem was alleviated when replacing Protein G with anti-sheep IgG Horseradish Peroxidase (Fig. 1).

Conclusion

A simple thermochemical method for inactivation of RVF virus in serum specimens had no adverse effect on the level of detectable IgG and IgM antibodies in various forms of ELISA directly compared in this study; however, they differ in their ability to detect early humoral responses. This might be of practical importance for early recognition of infections and outbreaks.

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The authors would like to thank the International Atomic Energy Agency (IAEA) (Technical contract no. 15274/R0) and the Poliomyelitis Research Foundation (PRF grant number 08/14) for supporting the study.

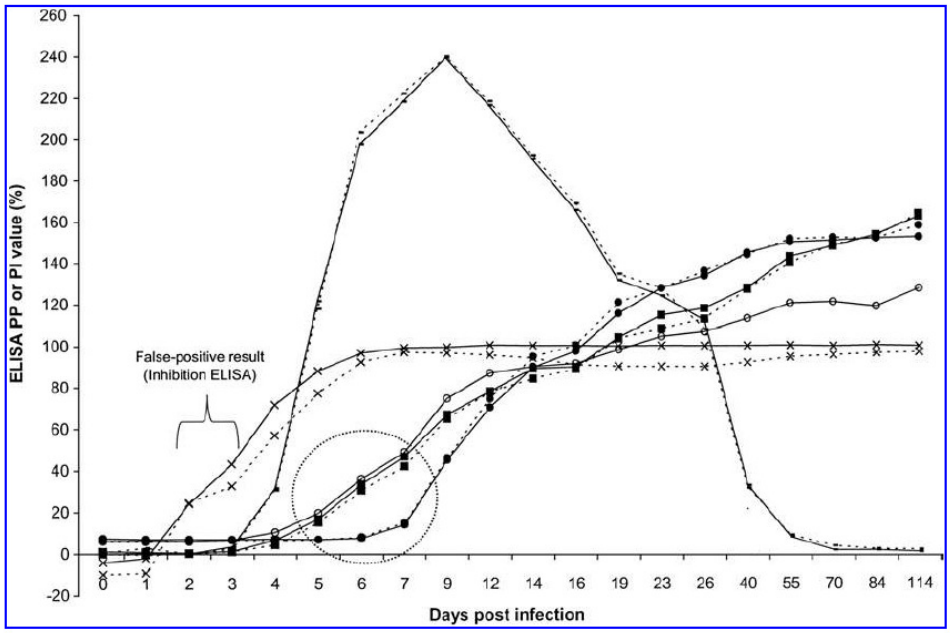


FIG. 1. Comparison of immune responses in three experimentally infected sheep as measured by testing naive (solid lines) versus thermochemically inactivated (dotted lines) sera by IgM-capture ELISA (−), inhibition ELISA (×), indirect ELISA Protein G Horseradish Peroxidase (●), indirect ELISA anti-sheep IgG Horseradish Peroxidase (○), and IgG-sandwich ELISA (■). Note the level of sensitivity of indirect ELISA when using different HRPO conjugates, as indicated by the dotted circle. ELISA, enzyme-linked immunosorbent assay.

Disclosure Statement

No competing financial interests exist.

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Anti-Nucleocapsid Protein Immune Responses Counteract Pathogenic Effects of Rift Valley Fever Virus Infection in Mice

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Abstract

The known virulence factor of Rift Valley fever virus (RVFV), the NSs protein, counteracts the antiviral effects of the type I interferon response. In this study we evaluated the expression of several genes in the liver and spleen involved in innate and adaptive immunity of mice immunized with a RVFV recombinant nucleocapsid protein (recNP) combined with Alhydrogel adjuvant and control animals after challenge with wild type RVFV. Mice immunized with recNP elicited an earlier IFNβ response after challenge compared to non-immunized controls. In the acute phase of liver infection in non-immunized mice there was a massive upregulation of type I and II interferon, accompanied by high viral titers, and the up- and downregulation of several genes involved in the activation of B- and T-cells, indicating that both humoral and cellular immunity is modulated during RVFV infection. Various genes involved in pro-inflammatory responses and with pro-apoptotic effects were strongly upregulated and anti-apoptotic genes were downregulated in liver of non-immunized mice. Expression of many genes involved in B- and T-cell immunity were downregulated in spleen of non-immunized mice but normal in immunized mice. A strong bias towards apoptosis and inflammation in non-immunized mice at an acute stage of liver infection associated with suppression of several genes involved in activation of humoral and cellular immunity in spleen, suggests that RVFV evades the host immune response in more ways than only by inhibition of type I interferon, and that immunopathology of the liver plays a crucial role in RVF disease progression.

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Introduction

Rift Valley fever virus (RVFV), a mosquito borne *Phlebovirus* of the *Bunyaviridae* family, causes irregular but large outbreaks characterized by high fatality rates in young domestic ruminants and abortion storms in pregnant animals [1–3]. The RVFV RNA genome consists of large (L) and medium (M) segments which are in the negative sense, and a small (S) segment utilizing an ambisense coding strategy. The L segment encodes a viral RNA-dependent RNA polymerase, the M segment encodes two structural glycoproteins, two non-structural proteins, and the S segment encodes the nucleocapsid protein (NP) and a non-structural protein (NSs) [4,5]. The NP is highly conserved and constitutes the most immunodominant viral protein in the *Bunyaviridae* family [6–11]. The NP associates with the viral RNA-dependent RNA polymerase and viral RNA to form the ribonucleoproteins (RNP) necessary for transcription and replication. The known virulence factor of RVFV, the NSs protein, counteracts the antiviral effects of the type I interferon response. Not much is known, however, about other molecular aspects of RVFV pathogenicity. Although the nucleocapsid protein (NP) of the virus does not induce production of neutralizing antibodies, anti-NP antibodies have been shown to be protective in mice [12–17]. Humoral responses against the RVFV glycoproteins effectively neutralize the virus and are believed to solely protect against challenge [14,16,18–20].

RVFV is sensitive to the actions of type I interferons [21–24] but it has developed several counteracting mechanisms. The NSs protein of RVFV interacts with a repressor complex (Sin3A/NCoR/HDAC) to inhibit transcriptional activation of the IFNβ gene [25,26]. A more general shutdown of cellular gene expression is caused by interaction of NSs with the p44 subunit of the basal transcription factor II H (TFIIH), resulting in reduced transcriptional activity in RVFV infected cells [27]. The NSs has also been shown to act on a post-translational level by degrading Protein Kinase R (PKR) [28]. Interferon gamma (IFNγ) is a strong immunoregulator and has direct antiviral properties, but its role in the protection of the host against RVFV infection is debatable [29]. A study in rhesus monkeys showed that prophylactic treatment with recombinant human IFNγ before RVFV infection protected monkeys from clinical disease and decreased viremia significantly [30]. However, there was no marked difference in pathogenicity of RVFV MP-12 or Clone-13 in wild type mice compared to mice deficient in IFNγ receptor ($IFNGR^{-/-}$), suggesting that NSs does not have the same inhibitory effect on the type II interferon response (IFNγ) as it has on the type I

interferon response [31]. The RVFV M segment non-structural protein (NSm) was implicated in the pathogenesis of RVF by acting as an anti-apoptotic protein [32].

Do Valle et al. [33] utilizing microarray and quantitative PCR showed that a specific strain of mice, BALB/cByJ, was more resistant to RVFV infection when compared to a wild mouse strain, MBT/Pas. The study analyzed the expression of genes involved in the innate immune response by infecting mouse embryonic fibroblasts (MEF) isolated from both strains with RVFV *in vitro*, with results indicating a more significant type I IFN response in the BALB/cByJ MEFs.

The activation of adaptive immunity after RVFV infection at gene expression level in the main target organ for RVFV replication, the liver, as well as in the spleen which plays a very important role in host immunity, has not been investigated. A better understanding of the activation of memory humoral and cellular immune responses in these organs might aid the development of improved RVFV vaccines and identification of genes and their products as useful targets for development of gene therapy and antivirals. The role that anti-NP responses play in the protection of vaccinated individuals against RVFV infection is also not clearly understood.

The results from this study suggest that an earlier type I interferon response in recNP/Alhydrogel immunized mice contribute to decreased challenge virus replication, whereas upregulation of genes with the ability to result in immunopathology, combined with uncontrolled challenge virus replication, in non-immunized mice probably contributed to liver damage and morbidity, and downregulation of humoral and cellular immunity in the spleen possibly contributed to immune evasion.

Results

Immune response elicited by recNP/Alhydrogel immunization

The activation of either Th-1 cellular immunity, indicated by IgG2A subclass expression, or Th-2 humoral immunity, indicated by IgG1 subclass expression, was evaluated by testing serial bleeds after recNP/Alhydrogel immunization. Strong total IgG and IgG1 subclass responses were detectable by day 7 after the first immunization, with strong responses still detectable on the day before the booster inoculation (day 12) and RVFV challenge (day 27 after the booster)(IgG: average = 72.2 PP, standard deviation = 5.5 PP, $OD_{405\ nm}$ = 0.79; IgG1: average = 46.7 PP, standard deviation = 5.3 PP, $OD_{405\ nm}$ = 0.47 on day 12 after the initial immunization; IgG: average = 129.9 PP, standard deviation = 4.7 PP, $OD_{405\ nm}$ = 1.42; IgG1: average = 131.3 PP, standard deviation = 12.0 PP, $OD_{405\ nm}$ = 1.31 on day 27 after the booster, Figure 1). The IgG2A subclass response (IgG2A: average = 16.8 PP, standard deviation = 4.5 PP, $OD_{405\ nm}$ = 0.16) was weak

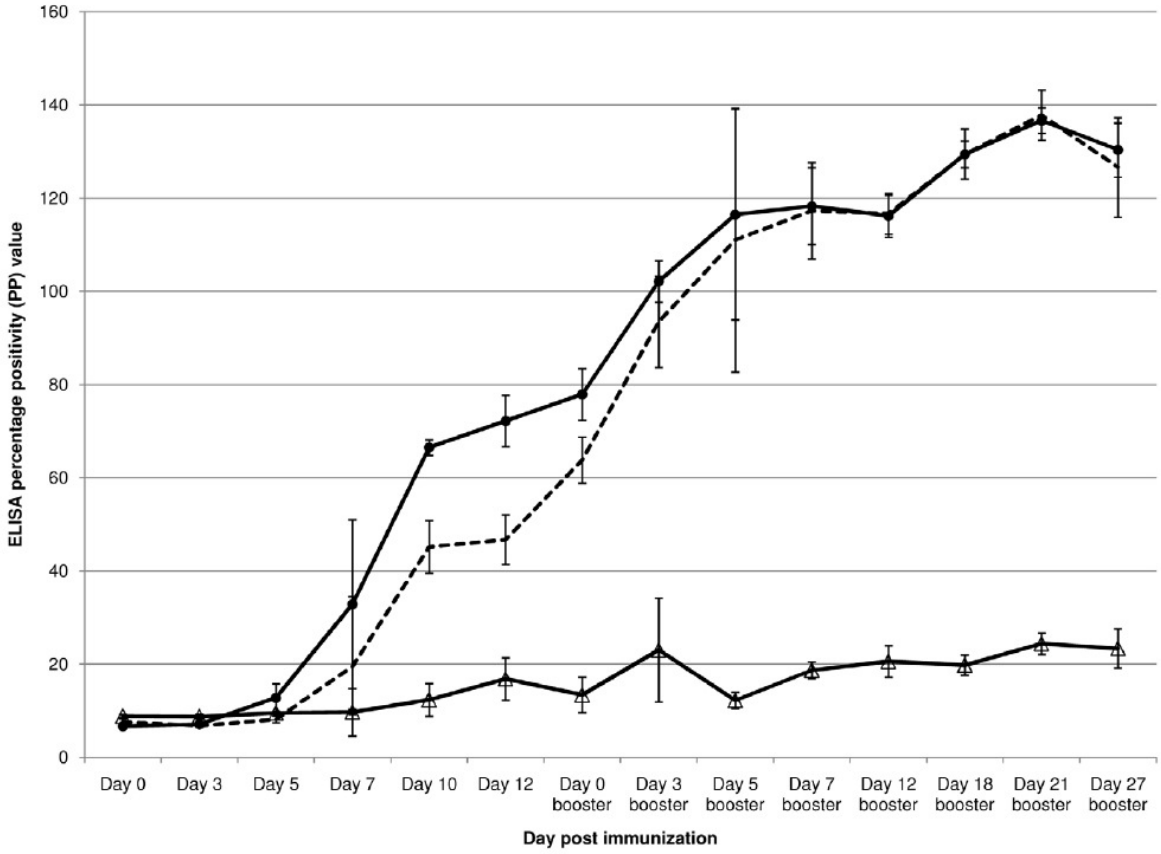


Figure 1. Anti-RVFV recNP response in recNP/Alhydrogel immunized mice. The total IgG (–●–), IgG1 subclass (—) and IgG2A subclass (–Δ–) responses are shown, with error bars indicating the standard deviations from the mean PP values of three biological replicates per time point.
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compared to IgG1 and only detectable on day 12 after the first immunization (PP ratio of IgG1:IgG2A = 2.8:1). On the day before RVFV challenge (IgG2A: average = 23.4 PP, standard deviation = 4.2 PP, $OD_{405\ nm}$ = 0.22), a weak IgG2A response was still detectable(PP ratio of IgG1:IgG2A = 5.6:1) (Figure 1).

Dynamics of gene expression and virus load in livers and spleens of immunized and control mice

The normalized expression of IL-10, IFNγ and IFNβ was analyzed at 3, 6, 12, 24, 72 and 120 hours post RVFV infection in liver and spleen tissue of recNP immunized, and non-immunized adjuvant and PBS control mice. The results are shown in Figure 2a–f.

The expression of IL-10 was upregulated with statistical significance (4.5 fold, $p<0.01$) in adjuvant control mice but normal in recNP immunized mice at 3 hours p.i. in the liver (Figure 2a). At 72 and 120 hours p.i., however, IL-10 expression was massively upregulated in the adjuvant (72 hours, 808.3 fold, $p = 0.033$; 120 hours, 55.0 fold, $p<0.01$) and PBS control groups (72 hours, 243.3 fold, $p<0.01$; 120 hours, 99.3 fold, $p = 0.034$), and upregulated to a lesser extent in immunized mice (72 hours, 24.4 fold, $p<0.01$; 120 hours, 24.3 fold, $p = 0.033$) in the liver (Figure 2a). In the spleen expression of IL-10 was only upregulated significantly at 72 hours p.i. in control mice (adjuvant 46.0 fold, $p<0.01$; PBS 55.0 fold, $p<0.01$) (Figure 2b).

The expression of IFNγ was normal in all mice livers at 3 hours, but upregulated in all groups at 6 hours (recNP immunized, 17.2 fold, $p<0.01$; adjuvant control, 11.7 fold, $p<0.01$; PBS control, 9.3 fold, $p<0.01$), 72 hours (recNP immunized, 8.4 fold, $p = 0.03$; adjuvant control, 5.8 fold, $p = 0.03$; PBS control, 4.4 fold, $p = 0.03$) and 120 hours p.i. (recNP immunized, 23.4 fold, $p = 0.03$; adjuvant control 224.0 fold, $p<0.01$; PBS control 237.0 fold, $p = 0.03$) (Figure 2c). Expression of IFNγ remained unaltered in spleen tissue (Figure 2d).

At 3 hours post infection (p.i.) the expression of the IFNβ gene was significantly upregulated in the livers (7.7 fold, $p = 0.03$) and spleens (12.0 fold, $p = 0.03$) of recNP immunized mice, whereas expression was normal in adjuvant control and PBS control groups (Figure 2e–f). Expression of the gene remained upregulated in liver of immunized mice until 12 hours p.i., after which it waned (Figure 2e–f). In adjuvant control mice, IFNβ was massively upregulated at 72 hours p.i. in liver (2171.7 fold, $p = 0.03$) and spleen (542.3 fold, $p<0.01$), with similar results obtained in PBS control mice liver (2524.1 fold, $p = 0.02$) and spleen (510.3 fold,

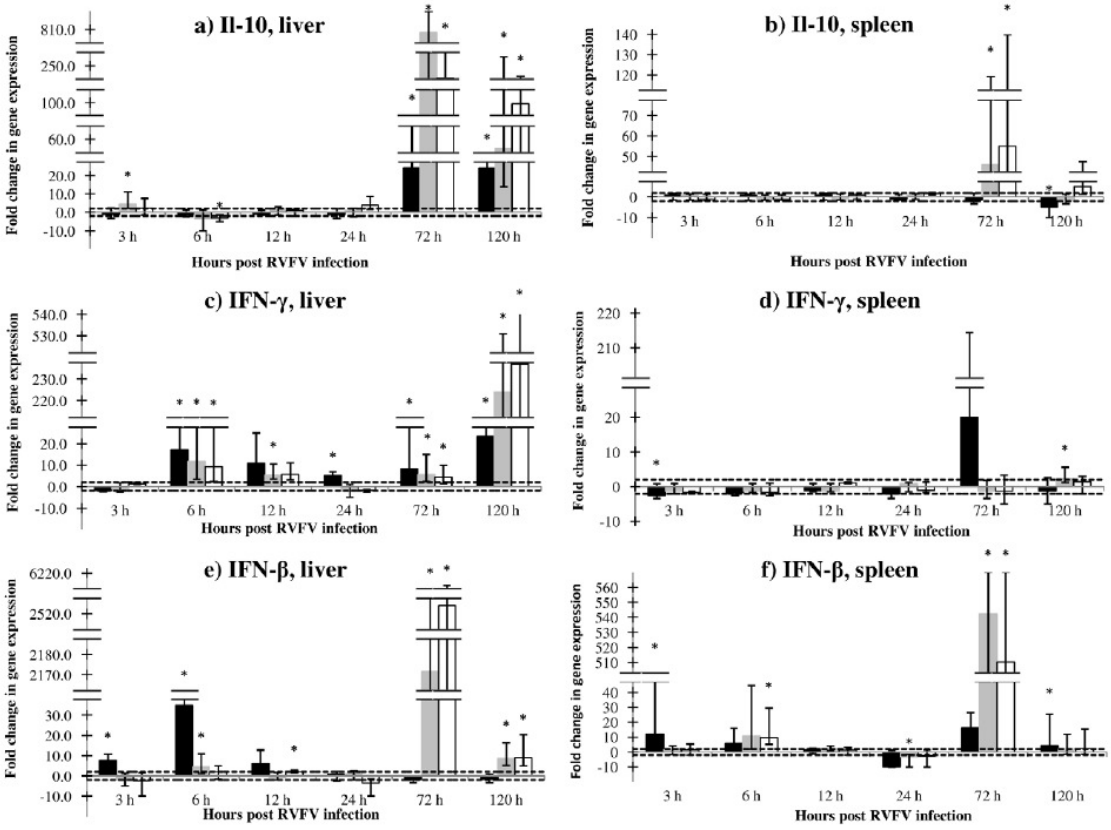


Figure 2. (a–f). Fold changes in expression of IL10, IFNγ and IFNβ genes in tissues of mice after RVFV infection. RecNP immunized mice (n = 3 per time point) are indicated by solid black bars, adjuvant control mice (n = 3 per time point) by grey bars and PBS control mice (n = 3 per time point) by white bars. The horizontal dotted lines indicate the cut-off values for upregulation (+2) or downregulation (−2). The asterisk (*) indicates where the *P*-value is smaller than or equal to 0.05 (statistically significant results). Standard error values are indicated by the error bars. Note the differences in the Y-axis scales. The following time points are indicated: 3, 6, 12, 24, 72 and 120 hours.
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$p<0.01$) (Figure 2e–f). At 120 hours p.i. IFNβ expression remained upregulated in control mice liver.

The challenge virus was first detected in the tissues of adjuvant control mice at 24 hours p.i. and, subsequently, at high titers in adjuvant and PBS controls at 72 and 120 hours p.i. Low titer of the virus was detected in liver of recNP-immunized mice only at 72 hours p.i. (10,000 fold lower than control mice). Surprisingly the virus replicated to relatively higher titers in the spleen of recNP-immunized mice, but only at 72 hours and still 100 fold lower compared to control mice (Figure 3a–b).

The expression of genes involved in B- and T-cell immunity and other immune functions at 72 hours

The expression of an array of 84 genes involved in B- and T-cell immunity, and other immune functions, were analyzed in the liver and spleen tissues of recNP-immunized, and non-immunized adjuvant and PBS control mice at 72 hours post infection relative to an age related normal control group. The list of genes tested, fold change values for different groups and *P*-values are shown in Table S1.

Only five genes were significantly upregulated and one downregulated in the liver of recNP-immunized mice, compared to 34 upregulated and nine downregulated in non-immunized adjuvant control mice, and 37 upregulated and eight downregulated in non-immunized PBS control mice (Table S1). There was an almost 100% overlap between the up- or downregulated genes in liver of non-immunized adjuvant and PBS control mice, with only a few genes not consistent between these two groups, most likely as a result of the fold change in some of these genes being on the borderline of the cut-off fold-change value, indicating that mock

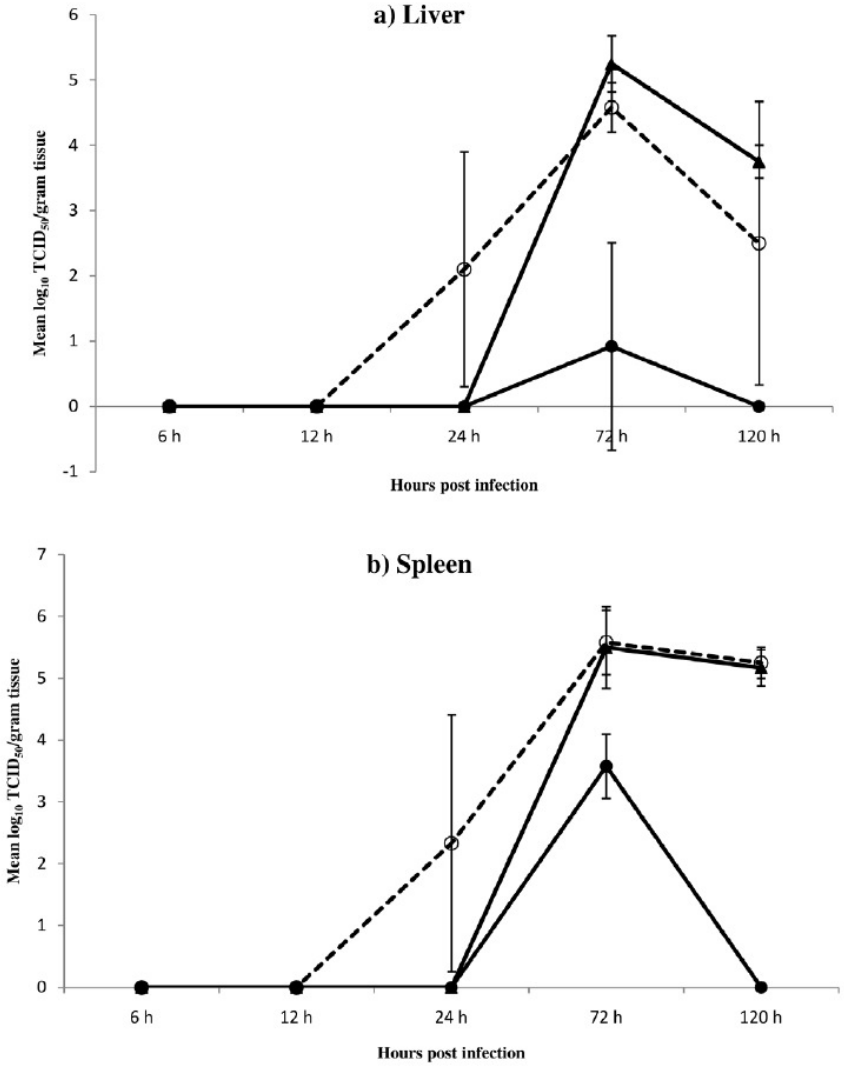


Figure 3. (a–b). Mean viral loads in livers and spleens of RVFV infected mice. Groups, consisting of 3 mice per group per time point, are indicated as: RecNP immunized (–●–), adjuvant control (–o–) and PBS control mice (–▲–). Livers are indicated in panel a, and spleens in panel b.
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immunization with the adjuvant alone likely had no effect on immune responses after RVFV challenge. The expression of all 84 genes remained unaltered in the spleen of recNP immunized mice compared to normal mice, whereas nine genes were significantly upregulated and 20 downregulated in adjuvant control mice spleens, compared to 11 upregulated and 20 downregulated in PBS control mice spleens. Comparing tissues, only 10 genes were similarly up- or downregulated in liver and spleen of adjuvant control mice, whereas 45 genes yielded contrasting results between tissues. Only 13 genes were similarly expressed in liver and spleen of PBS control mice, compared to 44 genes which yielded contrasting results between tissues (Table S1), indicating that there was a tissue specific modulation of host gene expression in response to RVFV infection. Selected genes that were significantly up- or downregulated in any of the experimental groups and indicating a clear difference or interesting similarity between immunized and control mice were grouped according to known effects on specific immune functions and shown in Figure 4 (a–b), Figure 5 (a–f) and Figure 6 (a–d).

There was a significant upregulation of several genes in the liver that have pro-apoptotic effects [34–42] in non-immunized adjuvant and PBS control mice, whereas these genes were normally expressed in recNP-immunized mice (Figure 4a, Figure 5a). One gene in particular, encoding the early growth response 1 protein (Egr-1) which is a transcription factor involved in proliferation, differentiation and activation of cell death pathways [43], that was upregulated in non-immunized mice was downregulated in recNP-immunized mice. Only one gene encoding the transcription factor RelB, part of the NF-KB family of proteins and responsible for counter-regulating the effects of NF-KB, was upregulated in liver of immunized and non-immunized mice (Figure 5a) [40]. Several genes with anti-apoptotic effects [44–49] were downregulated in the liver of non-immunized control mice but normal in recNP-immunized mice (Figure 5b). Most notable of these were the genes encoding the prolactin receptor (Prlr), an anti-inflammatory protein known to promote proliferation, protect against apoptosis and enhance cell survival, and the WW domain containing E3 ubiquitin protein ligase 1 (Wwp1), an anti-apoptotic protein playing a role in proliferation [49]. There was also evidence of severe liver inflammation [43,50–58] in non-immunized adjuvant and PBS control mice, but not in recNP immunized mice (Figure 5c). Despite this the gene encoding the anti-inflammatory cytokine IL-10 was upregulated in all mice. The expression of osteopontin (gene Spp1), important for wound healing, was upregulated significantly in liver of non-immunized control mice, compared to normal expression in immunized mice (Table S1) [58,59]. The gene expressing the Cyclin-dependent kinase inhibitor P21 (Cdkn1a), a protein with pro- or anti-apoptotic effects and normally upregulated in response to liver injury, was upregulated in liver of all mice but more pronounced in non-immunized mice (Table S1) [60]. The gene expressing the inducible nitric oxide synthase (Nos2), an effector of the innate immune system targeting viral proteases and inhibiting viral replication, was normal in liver of recNP-immunized mice but upregulated in non-immunized mice. Nitric oxide, a product of the Nos2 gene, is a radical molecule that can become toxic under oxidative stress conditions (Table S1) [61].

Both arms of the adaptive immune response, humoral (Th2) and cellular (Th1) [45,50,52,62–74], were activated in liver of non-immunized control mice, but normal in immunized mice, at 72 hours. The gene encoding the Phosphatidylinositol 3-kinase catalytic delta polypeptide (Pik3cd), involved in the regulation of B-cells and antibody production, was also upregulated in liver of

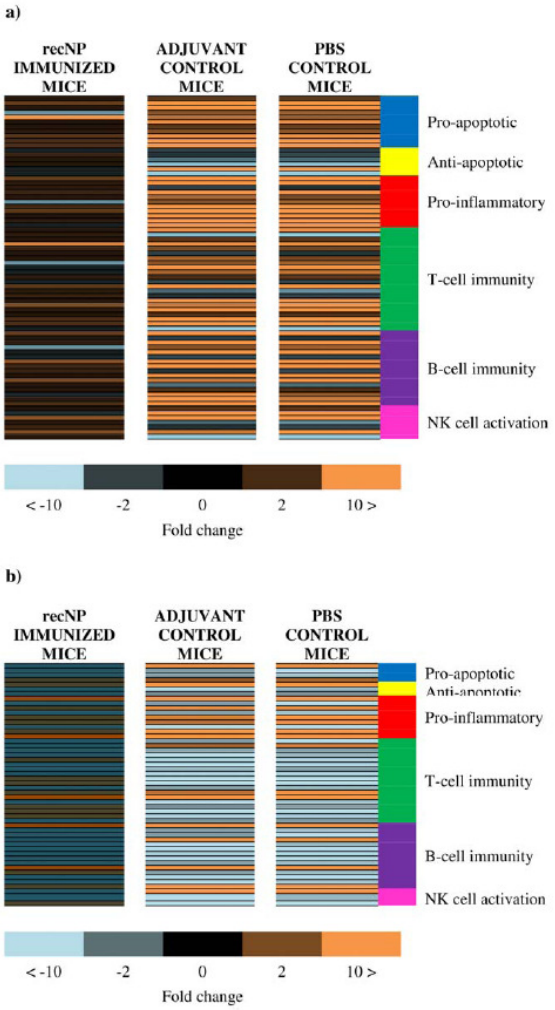


Figure 4. (a–b). Heat maps showing fold changes in liver and spleen at 72 hours after RVFV infection. Expression of genes in RecNP immunized, adjuvant control and PBS control mice are organized according to function. Livers are indicated in panel a, and spleens in panel b. The genes shown in orange are upregulated, those in blue are downregulated and those in black or darker shades of orange and blue have fold-change values between −2 and 2 and/or have p-values>0.05.
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recNP-immunized mice indicating re-activation of humoral immunity in mice recognizing one of the viral proteins as one it has encountered before (Table S1) [75]. The genes encoding the Dedicator of cyto-kinesis 2 (Dock2) protein and interleukin-12b, which are involved in the development and induction of NKT cells, were upregulated in liver of non-immunized control mice. Some important genes were, however, downregulated in liver of non-immunized control mice (Figure 5 d and Table S1). The gene encoding the Cd1d1 antigen, which is important for the presentation of antigens to, and activation of NKT cells, was downregulated with statistical significance in non-immunized control mice (Figure 5 d) [65,76,77]. The expression of the gene encoding interleukin-7, necessary for B- and T-cell and NK cell

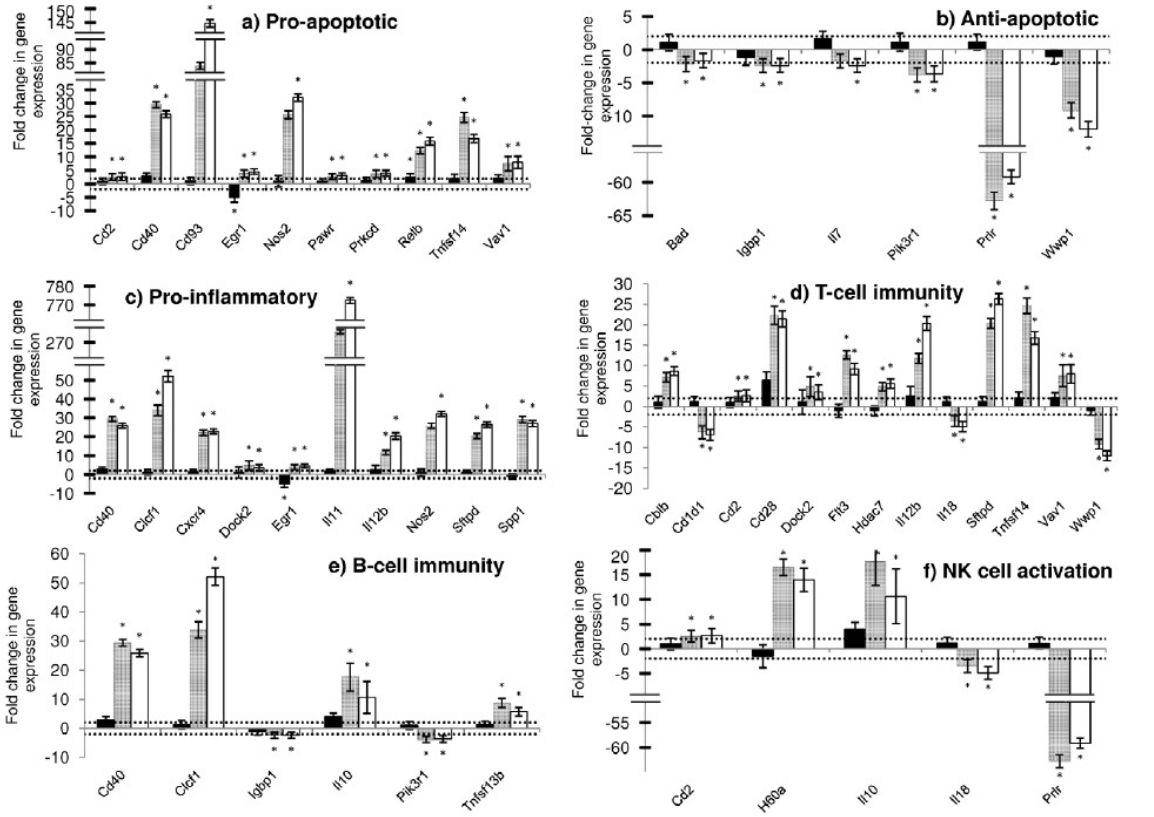


Figure 5. (a–f). Fold changes in expression of genes in the liver of experimental groups at 72 hours after RVFV infection. RecNP-immunized mice (n = 3) are indicated by solid black bars, adjuvant control mice (n = 3) by grey bars and PBS control mice (n = 3) by white bars. The horizontal dotted lines indicate the cut-off values for upregulation (+2) or downregulation (−2). The asterisk (*) indicates where the *P*-value is smaller than or equal to 0.05 (statistically significant results). Standard deviation from the mean fold changes are indicated by the error bars.
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survival, was downregulated in liver of non-immunized PBS control mice (Figure 5b) [78]. The expression of surfactant protein D (Sftpd), a member of the collectin family, important role player in innate immunity and inhibitor of T lymphocyte proliferation, was upregulated in liver of non-immunized control mice (Figure 5 d) [79]. The expression of the gene encoding the E3 Ubiquitin Ligase Cbl-b, capable of negatively regulating T-cell activation, was upregulated in liver of non-immunized control mice (Figure 5 d) [67,68,80]. The expression of the gene encoding interleukin-18, responsible for biasing immunity towards Th-1 cellular immunity and enhancing T-cell cytotoxicity, was downregulated in liver of non-immunized control mice (Figure 5 d) [81]. The expression of the suppressor of cytokine signaling 5 (Socs5), part of a family of proteins that negatively regulate cytokine signaling [82], was upregulated in liver of non-immunized control mice and the gene encoding the immunoglobulin binding protein 1 (Igbp1), a component of receptor cell signaling in B- and T-cells [45], was downregulated in liver of non-immunized control mice. Other genes (Cd81 and Pik3r1) involved in activation, signaling and differentiation of B- and T-cells were also downregulated in liver of non-immunized control mice (Table S1) [78,83].

An important role player in innate immunity, NK cells, was also activated in liver of non-immunized control mice (Figure 5 f) [64,84,85]. The gene encoding the histocompatibility 60 A protein, a ligand for an activating receptor on NK cells [85], was upregulated in non-immunized control mice. However, expression of two important genes in NK cell activation and maturation, IL-18 and the prolactin receptor (Prlr), were downregulated in liver of non-immunized control mice [81,84]. The expression of three genes encoding Toll-like receptors, a component of the innate immune system responsible for recognizing conserved structures of pathogens, were analyzed with only Tlr4 being upregulated in liver of non-immunized control mice (Table S1) [86].

In contrast to the liver, there was not such a clear bias towards apoptosis in the spleens of adjuvant and PBS control mice (Figure 4b, Figure 6a). Only two genes with pro-apoptotic effects were upregulated, and two genes with anti-apoptotic effects downregulated in spleen of control mice. Interestingly expression of Nos2 and Cd40, both with known pro-apoptotic effects and upregulated in liver of control mice, were downregulated in spleen of control mice. Similarly to liver, some genes with pro-inflammatory effects were upregulated in the spleens of adjuvant and PBS control mice (Figure 6b), except for the genes encoding Nos2, Cd40 and Cd40 ligand. The expression of various genes involved in the activation of B- and T-cell immunity were,

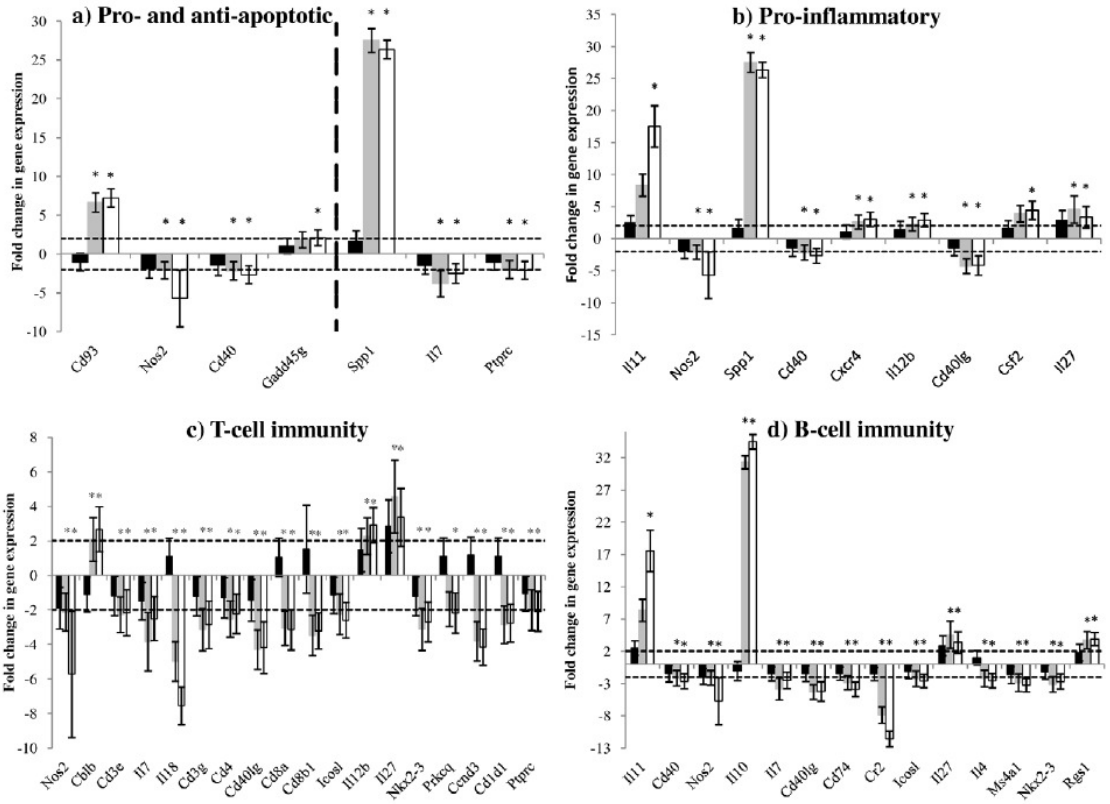


Figure 6. (a–d). Fold changes in expression of genes in the spleen of experimental groups at 72 hours after RVFV infection. RecNP-immunized mice (n = 3) are indicated by solid black bars, adjuvant control mice (n = 3) by grey bars and PBS control mice (n = 3) by white bars. The horizontal dotted lines indicate the cut-off values for upregulation (+2) or downregulation (−2). The asterisk (*) indicates where the *P*-value is smaller than or equal to 0.05 (statistically significant results). Standard deviation from the mean fold changes are indicated by the error bars.
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however, downregulated in the spleens of adjuvant and PBS control mice (Figure 6c–d). The most notable of these were Cd40 and Cd40 ligand which are responsible for inducing effective CD8+ cytotoxic T-lymphocyte (CTL) responses and activation of B-cells for antibody production [51], and Cd8a and Cd8b1 which are responsible for CTL activation (Figure 6c) [87]. Expression of Complement receptor 2 (Cr2), which plays an important role in B-cell activation and maturation, was also downregulated in spleen of non-immunized control mice (Figure 6d).

Discussion

The immune evasion mechanisms known for RVFV are directed against the type I interferon response [24,28,88] and programmed cell death [29]. However, it was recently shown that mice displaying an earlier and stronger type I interferon response were less susceptible to RVFV infection than mice with a delayed and partial response which suggests some additional evasive or regulatory effects on innate or adaptive immune mechanisms enabling its replication [33].

Immunization of mice with recNP combined with Alhydrogel protected against disease and significantly reduced viral replication [17]. A study by Lorenzo et al (2008) implicated a cellular (Th-1) response to the RVFV NP as a protective mechanism [12], however, our present data shows that immunization with recNP/Alhydrogel induces Th-2 humoral immunity. The discrepant outcome of the two studies might be explained by the fact that Lorenzo et al (2008) used DNA vaccination [12], whereas in our study a subunit antigen combined with adjuvant known to favour Th-2 immunity, was used. Immunized mice launched an earlier and stronger type I interferon response compared to non-immunized mice which showed activation of several genes with pro-apoptotic and pro-inflammatory effects, and suppression of anti-apoptotic genes during acute phase of RVFV infection in the liver. This possibly contributed to hepatic damage, which is the main pathological feature of RVF [89]. The expression of several genes involved in the activation and function of NK cells and B- and T-lymphocytes were suppressed in infected mice, indicating additional immune evasion tactics of RVFV.

The induction of expression of IFNβ has been shown to occur *in vitro* at 3–6 hours p.i [33], while in the livers and spleens of immunized mice in this study it occurred at 3 hours p.i. Taking into account that after host infection, the virus is likely conveyed to the lymph nodes where it first replicates before it can spread to the liver and other organs [2], our results show the ability of RVFV to rapidly spread in the infected host. The expression of the same gene was, however, not upregulated in the tissues of adjuvant and PBS control mice early after infection. Type I interferon could not

be upregulated as a direct result of recNP immunization since it is not memory dependent. The fact, however, that the protective anti-recNP responses shown here was largely humoral, but without neutralizing effects, suggests that some other form of antibody dependent mechanism, such as antibody-dependent cell-mediated cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC), might be responsible. It appears that the RVFV nucleocapsid protein might be released from infected cells independently from other viral proteins [13], and/or processed by the infected host cell proteasome into peptides that are displayed on their surface by MHC-1. Consequently these cells can be coated with anti-NP IgG antibodies complexed with presented NP or peptides, and become a target for ADCC or CDC. It has been shown for influenza virus, with highly variable envelope glycoprotein antigens but a conserved internal nucleoprotein, that previous infection causes heterosubtypic immunity and that this is due to antibodies against the NP involving CD8+ cells in an antibody dependent manner [90]. The resultant lysis of RVFV infected cells, that would otherwise have evaded the host innate immune response and produced progeny virus because of the inhibitory action of NSs on type I interferon might have resulted in the activation of the type I interferon response in neighbouring uninfected cells. The lysed cells would release dsRNA, a by-product of viral replication and activator of type I interferon, helping close-by uninfected cells to remain uninfected and thus decrease virus spread [91]. It has been shown that an early type I interferon response is protective against RVFV infection [18,33] and it is thus highly likely that the early expression of IFNβ in immunized mice contributed to effective viral clearance and protection. The excessive expression of IFNβ, however, in control mice later after infection (72–120 h) was not able to curb the replication of the virus, as evidenced by high viral titers, and probably contributed to the pathology in the liver and spleen. It has been suggested that the role of IFNγ in RVFV pathogenesis is negligible [28] yet it has been shown *in vivo* that IFNγ does act as an antiviral against RVFV [27]. In our study expression of IFNγ was upregulated in all mice after infection, and there was only a decrease in viral replication in immunized mice indicating that IFNγ did not play a significant role in protection. Also, the massive expression of IFNγ in control mice late after infection did not contain viral replication in the liver and likely rather contributed to liver pathology.

The improper expression of IL-10 during a viral infection might contribute to immune escape events since IL-10 is an anti-inflammatory and immunosuppressive cytokine that inhibits the actions of Th-1 and NK cells, decreases antigen presentation and limits the production of various important cytokines (i.e. IL-12, IL-18 and TNF-α) [73]. It has been shown that IL-10 is upregulated after West Nile virus (WNV) infection, and that IL-10 deficient mice are less susceptible to WNV infection than mice expressing the gene normally [92]. Dengue virus has also been shown to replicate less efficiently when IL-10 expression is suppressed [93]. Some viruses even express IL-10 homologs to enable them to modulate the host immune system and escape viral clearance [94–96]. There was an early upregulation of IL-10 expression in liver of adjuvant control mice when an anti-inflammatory response would seem unnecessary. The early detection of replicating RVFV in liver of adjuvant control mice might be contributed to immune escape because of this early activation of IL-10. During the later stages of infection when high level virus replication was noted in non-immunized mice, the host upregulated expression of IL-10 to counteract severe inflammation of tissues. A consequence of this, however, is the suppression of various immune responses as described above which possibly contributed to inefficient clearance of virus in control mice or immune escape. The cytokines IL-10 and IFNγ are counter-regulatory of each other with IFNγ being responsible for pathogen clearance and IL-10 minimizing pathology [73], and therefore their relative expression levels can be an indication of host immune response bias towards virus clearance or minimizing immunopathology. At the acute phase of infection and height of viral replication in non-immunized mice the IL-10 to IFNγ ratio was massively favoured towards IL-10, indicating that these host responses were biased towards decreasing inflammation and pathology rather than clearing the virus, which most likely lead to ineffective viral clearance and high virus titers still being detectable at 120 hours p.i. It was interesting to note replication of virus to a much higher titer in spleen of recNP immunized mice compared to liver. This, however, did not result in any form of clinical disease in immunized mice which is further evidence that early disease progression is proportional to viral replication in the host liver and not necessarily other organs.

The activation of genes with pro-apoptotic and pro-inflammatory effects and the suppression of genes with anti-apoptotic effects in the liver of control mice as a result of uncontrolled viral replication, most probably contributed to severe hepatic disease. In the spleen activation of apoptosis and inflammation was not as pronounced as in the liver of non-immunized mice at 72 hours p.i. The liver is, however, the main target organ for viral replication, the primary site for lesions and rapid severe hepatic damage is probably responsible for the early clinical signs of RVF [89]. The overexpression of CD40, a member of the TNF receptor superfamily and potent activator of nuclear factor kappa beta, in the liver of non-immunized mice is of particular importance to apoptosis and inflammation. Mice deficient in the expression of CD40 have been shown to have improved survival during bacterial sepsis as a result of decreased induction of IL-6, IL-10, IL-12 and IFNγ expression [97]. Contrary to upregulation in liver the expression of CD40, as well as its ligand CD40L (CD154), was downregulated in spleen of non-immunized mice during acute infection. Apart from its role in apoptosis of hepatocytes, CD40 plays a very important role in mediating B- and T-cell responses by controlling cytokine secretion, proliferation and differentiation of B- and T-cells [51]. In addition to downregulation of CD40, various other genes involved in B- and T-cell immunity were also downregulated in the spleens of non-immunized mice. These include NOS2, CD3e, IL7, IL18, CD8a,CD1D1, CD74, CR2 and IL4. The spleen is a very important organ in the host immune system and these results indicate that infection with RVFV has a marked effect on the regulation of the host immune response on a molecular level. It is possible that this broad downregulation of humoral and cellular immunity could have resulted in uncontrolled replication of RVFV, since the virus was still detectable at high titers 2 days later in non-immunized mice. The expression of a TNF receptor superfamily ligand (Tnfsf14) was also upregulated in liver of control mice, but normal in their spleens. This protein is able to block TNFα-mediated apoptosis but not FAS-mediated apoptosis, and is a co-stimulatory factor that enhances T-cell-mediated immunity, leading to severe inflammation [41,98]. The upregulation of the genes expressing the cell survival factor osteopontin (Spp1) and the Cyclin-dependent kinase inhibitor P21 (Cdkn1a), which is upregulated in response to tissue injury [99,100], is evidence of the host's attempt to counteract the damaging effects of the infection. P21 interacts, amongst others, with the growth arrest and DNA damage-inducible gene 45 (Gadd45) [101], which was also upregulated in control mice. Gadd45 has been implicated in DNA repair, apoptosis, regulation of signal transduction and cell cycle control [102]. The NSs protein of RVFV has been shown to interact with some specific

regions of host cell DNA, causing defects in host chromosome structure and segregation [103]. Therefore it might be that these DNA damage inducible proteins are upregulated in an attempt to arrest the cell cycle of affected cells and prevent apoptosis. The fact that P21 was upregulated in healthy recNP-immunized mice is probably as a result of the very low level of viral replication in their livers.

Although the expression of some genes were similarly regulated during acute infection in the livers and spleens of non-immunized mice, a number of genes were quite distinctly only up- or downregulated in either liver or spleen, with some even being upregulated in one tissue and downregulated in the other. This distinct expression pattern between tissues is very likely a result of the different cellular composition of the liver and spleen. The liver consists mostly of hepatocytes, whereas the spleen consists of a red pulp where red blood cells are filtered and monocytes are stored, and a white pulp which consists of B- and T-lymphocytes. It seems like the specific pattern of genes regulated during acute infection in the liver actually contributed to liver pathology by enhancing apoptosis and inflammation without being able to decrease viral replication to less virulent levels. On the other hand in the spleen the expression of genes involved in activation and modulation of B- and T-cell immunity were altered during acute infection, probably contributing to persistence of the virus in the host up to 120 hours. It was very interesting to note that, despite viral replication to a relatively high level in spleen of recNP immunized mice at 72 hours p.i., none of the 84 genes analyzed were up- or downregulated in their spleens. The mechanism by which recNP immunized mice can, 72 hours after infection, maintain expression levels of genes involved in immune activation similar to expression levels in spleens of uninfected mice, despite viral replication close to that in non-immunized mice where several genes were altered, needs to be further investigated. It is, however, fair to assume that this normal expression of genes involved in immune activation at 72 hours p.i. contributed to efficient viral clearance in immunized mice spleens, as is evidenced by the absence of replicating virus at the next sampling point (120 h). Further it is also fair to assume that the downregulation of B- and T-cell immunity in non-immunized mice at 72 hours p.i. contributed to the virus still replicating in their spleens at 120 hours p.i. Therefore, although not the main target organ of RVFV and not as important for disease progression as the liver, the spleen seems to be very important in regulating immune responses and decreasing the replication of the virus with dysregulation resulting in immune evasion. Further evidence of the dysregulation of host immune responses by RVFV infection is the fact that up- or downregulation of several genes (i.e. IL-18, IL-12b, CD40 and SOCS5) indicate bias towards both Th1 and Th2 immunity in the same host.

In conclusion, the expression of type I IFN was upregulated in the liver and spleen of immunized mice shortly after RVFV challenge, compared to a delayed upregulation of the same gene in non-immunized mice. In the acute phase of liver infection, however, there was a massive upregulation of type I and II interferon in the presence of high viral titers in non-immunized mice associated with downregulation of several genes involved in the activation of B- and T-cells in the spleen, compared to normal expression in immunized mice. Furthermore various genes with pro-apoptotic and pro-inflammatory effects were strongly upregulated, and anti-apoptotic genes downregulated in liver of non-immunized mice.

Host gene responses identified in this study may be useful targets for the development of therapeutic interventions, e.g. suppressing inflammatory and apoptotic effects of RVFV infection in the liver, or limiting the suppression of B- and T-cell activation in the spleen, and further aid the evaluation of subunit candidate vaccines.

Materials and Methods

Ethics statement

This study was carried out in strict accordance with the recommendations of the South African National Standards for the Care and Use of Animals for Scientific Purposes (SANS 10386:2008) and the Guidelines for the Use and Care of Animals in Experimental Education and other Scientific Purposes of the University of the Witwatersrand, South Africa. The protocol was approved by the University of the Witwatersrand Animal Ethics Screening Committee (clearance certificate number AESC 2008/16/4). Blood collection after immunization was done under Ketamine/Xylazine anaesthesia, organ tissue collection after viral challenge was done post-mortem after euthanasia with carbon dioxide (CO_2) asphyxiation, and all efforts were made to minimize suffering.

Bacterial expression of recombinant RVFV N protein

Bacterial expression and purification of the recombinant N protein was carried out as described previously [10].

Mouse immunization

Four-week old female BALB/cOlaHsd (Harlan Laboratories, U.K LTD) mice were housed in groups of six in standard plastic mouse cages with wood shaving bedding in a specific pathogen free (SPF) facility during the immunization part of the experiment. Group sizes were calculated so as to have three mice per group per time point for each collection to enable statistical relevance. The immunized group consisted of 60 mice each immunized with a 200 µl inoculum containing 70 µg RVFV recNP in combination with Alhydrogel (Sigma, U.S.A). The adjuvant control group consisted of 18 mice inoculated with Alhydrogel in PBS. The placebo control group consisted of 18 mice which were inoculated with PBS buffer. The normal control group consisted of 18 mice that did not receive any inoculation during the immunization experiment, but were included as an age related control group for the gene expression experiment. All mice, except the age-related normal control group, were inoculated subcutaneously (s.c.) and received identical booster inoculums on day 14 after the initial inoculation. Three mice from the recNP-immunized group were anaesthetized by intramuscular inoculation with a combination of Ketamine (35 mg/kg body mass) and Xylazine (5 mg/kg body mass) and bled by cardiac puncture on each of the following days post-immunization to monitor development of immune responses: day 0, 3, 5, 7, 10 and 12 after the first immunization, and day 0, 3, 5, 7, 12, 18, 21 and 27 after the booster immunization.

Monitoring of immune response

Anti-recNP humoral responses were monitored by the indirect ELISA which was done as described previously [17]. A mouse serum with high levels of both IgG1 and IgG2A subclass antibodies, generated by immunization with RVFV recNP combined with SaponinQ adjuvant (Sigma, U.S.A) in a previous study [17], was used as a positive control. The positive control serum yielded similar optical density values at 405 nm for IgG (1.094), IgG1 (1.01) and IgG2A (0.94) subclass antibodies at the same dilution used for experimental sera (1:400). Optical density (OD) was determined at 405 nm for each IgG subclass and means calculated for duplicate measurements of each sample or control. The mean OD values of samples for different IgG subclass

antibodies were converted to percentage positivity (PP) values by the following calculation: PP = Mean OD sample/Mean OD positive control ×100, using the OD values described above for IgG, IgG1 and IgG2A to calculate PP values for those specific subclasses. The PP values determined for each of the three biological replicates at each time point (three mice sampled per time point) were then used to calculate the biological mean PP values and standard deviations (S.D.) at each sampling point for the different IgG subclasses.

RVFV challenge

During the RVFV challenge phase of the experiment the mice were housed as during the immunization part except for being moved to a Biosafety level 3 animal facility.

Vero cells were cultivated in Eagles Minimal Essential Medium (EMEM) (BioWhitaker, MD, U.S.A) containing L-Glutamine, non-essential amino acids, antibiotics (100 IU penicillin, 100 µg streptomycin and 0.25 µg amphotericin B) and 10% foetal bovine serum (Gibco) and maintained at 37°C in 5% CO_2 incubator. The SPU22/118 KEN 07 strain of RVFV was isolated from a RVF human case during the 2007 Kenyan epidemic [104].

Second passage of the virus, propagated in Vero cells, was used for the challenge of mice on day 28 after the booster immunization. The recNP immunized, adjuvant control and PBS control mice were inoculated s.c. with 100 µl of $10^{7.0}$ $TCID_{50}$/ml RVFV, whereas the age related control group of mice were "mock" challenged with 100 µl uninfected Vero cell culture supernatant. Mice were monitored twice daily for clinical signs.

Three mice were randomly collected from each group, euthanized by CO_2 asphyxiation and liver and spleen tissues collected at the following time points after RVFV challenge: 3, 6, 12, 24, 72 and 120 hours. A piece of approximately 50 mg of each tissue was collected into an RNA stabilization reagent (RNAlater, Qiagen, Germany) according to the instructions of the manufacturer, and stored at −70°C until further processing. The remaining tissue was stored in cryotubes at −70°C for viral load determination.

Virus titrations

Liver and spleen tissues were homogenized as 10% (w/v) suspensions in EMEM containing L-glutamine, non-essential amino acids and antibiotics (100 IU penicillin, 100 µg streptomycin and 0.25 µg amphotericin B) using a Tissuelyser II and 5 mm stainless steel beads (Qiagen, Germany) according to the manufacturer's instructions (4 min, 25 Hz). After centrifugation at 10,000× g for 3 minutes the supernatants were collected and stored at −70°C until tested. Titrations were performed as described previously [17]. Virus titers, calculated by the Kärber method [105], were expressed as median tissue culture infectious dose ($TCID_{50}$) per gram of tissue. Means and standard deviations from the means were determined based on three animals per group per each time point tested.

RNA extraction

The tissues were moved from RNAlater into lysis buffer (Buffer RLT, Qiagen, Germany) and lysis-homogenization performed by using a Tissuelyser II as described above. Lysates were centrifuged for 3 minutes at 13,200 rpm at room temperature and supernatants collected. Extraction of RNA from the supernatants was performed by using the RNeasy Mini Kit (Qiagen, Germany). A 50% ethanol was used to increase RNA yield from livers and 70% ethanol for spleens as suggested by the manufacturer, followed by on-column Dnase digestion using the Rnase-free Dnase set (Qiagen, Germany), to remove genomic DNA. The RNA was eluted in Rnase-free water, the concentration determined using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, U.S.A) and stored at −70°C until further testing.

For testing with the SABiosciences quantitative PCR array (as described below), further RNA cleanup was performed on RNA already extracted from livers and spleens collected at 72 hours post infection, as per the manufacturer's instructions. This cleanup, which introduces an additional Dnase digestion, was performed using the RT^2 qPCR-Grade RNA Isolation Kit (SABiosciences, Qiagen, U.S.A) as per the manufacturer's instructions. The RNA was eluted in Rnase-free water, the concentration determined using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, U.S.A) and stored at −70°C until further testing.

Quantitect qRT-PCR

The RNA extracted from all experimental mice at all time points after RVFV challenge were diluted to 10 ng/µl and used in the qRT-PCR reactions. The Quantitect qRT-PCR reactions were performed as described by the manufacturer (Quantifast SYBR Green RT-PCR kit, Qiagen, Germany) using a Lightcycler 1.5 (Roche, Germany). Briefly, a reaction mix was prepared by mixing 2× Quantifast SYBR Green RT-PCR Master Mix (HotStarTaq Plus DNA Polymerase, Quantifast SYBR Green RT-PCR buffer, dNTP mix and ROX passive reference dye), 10× Quantitect Primer set (for detection of the genes for Gapdh, Il10, Ifng and Ifnb1), Quantifast RT-Mix (Omniscript RT and Sensiscript RT), template RNA (10 ng) and RNase-free water to a final volume of 20 µl per reaction. This mix was transferred to 20 µl Lightcycler Capillaries (Roche, Germany) and run on the Lightcycler 1.5 using the following cycles: 1× reverse transcription (10 min, 50°C), 1× hotstart PCR activation (5 min, 95°C) and 40× cycles of denaturation (10 sec, 95°C) and annealing/extension (30 sec, 60°C), with fluorescence data collection just after the annealing/extension step. The threshold cycle (C_T) values were determined using the second derivative maximum method (Lightcycler Data Analysis Software version 3.5.28, Roche). The C_T values were then used for relative quantification (as described below).

RNA was extracted from the liver and spleen of a normal BALB/cOlaHsd mouse to generate standard curves in order to determine PCR reaction efficiencies (necessary for the relative quantification calculations) with primer sets for different genes (GAPDH, IL-10, IFNã and IFNâ1). Dilution series were prepared and the following amounts of RNA tested in duplicate using all primer sets: 30 ng; 15 ng, 7.5 ng, 3.75 ng and 1.875 ng. The C_T values and their corresponding template amount values were then used to determine the PCR reaction efficiencies using the Relative Expression Software Tool (REST, Qiagen, Germany) [106] as described by the manufacturer (results not shown).

Relative quantification data analysis (Quantitect qRT-PCR)

Threshold values (C_T) from biological triplicates for the different genes analyzed as determined for the immunized mice, adjuvant control and PBS control mice were first normalized to the C_T values of the housekeeping gene (Gapdh) analyzed in the same samples, and further normalized to the C_T values from the non-infected control mice to determine the relative changes in gene expression compared to age related normal mice. This is the so-called $2^{-\Delta\Delta Ct}$ and the result is a fold change value that is an indication of the expression level of a gene in the experimental group being higher or lower than the expression level in the normal age-related control group [107]. These calculations were done using the REST software (Qiagen, Germany) which uses the PCR efficiencies for different primer sets (as determined above,

results not shown) and the C_T values of the biological triplicates. The negative inverted values were determined (i.e. $0.5 = {}^{1}/_{-0.5} = -2.0$) for fold change values smaller than one (<1). Genes were only regarded as upregulated with fold changes ≥2.0, and downregulated with fold changes ≤−2.0, based on the same cut-off values used in a recent study [33].

SABioscienes quantitative PCR array

The RNA extracted with the RT^2 qPCR-Grade RNA Isolation Kit from liver and spleen collections of all mice at 72 hours post infection were diluted to 150 ng/µl in nuclease free water. Complementary DNA (cDNA) was prepared from the RNA using the RT^2 First Strand kit (SABiosciences, Qiagen, U.S.A) as described by the manufacturer. A total of 1.2 µg of each RNA preparation was mixed with 5× genomic DNA Elimination buffer and the reaction incubated at 42°C for 5 minutes (total volume 10 µl). After the incubation, the reactions were immediately moved to ice and, subsequently, an equal volume of RT-coctail mix added (5× RT buffer, primers and external control mix, RT-enzyme mix and RNase-free water). These reactions were then incubated at 42°C for 15 minutes and 95°C for 5 minutes. The resultant cDNA of each preparation was then diluted 1:10 with nuclease-free water and stored at −20°C until tested.

The 1:10 diluted cDNA was mixed with the master mix (2× SABiosciences RT^2 qPCR Master Mix) and nuclease-free water, and aliquoted onto the PCR array plates containing primer sets (listed in Table S1)(25 µl of reaction mix per well)(PAMM-053, SABiosciences, Qiagen, Germany). Plates were run on an ABI 7500 cycler (Applied Biosystems, U.S.A). The following cycling program was used: 1×95°C for 10 minutes, 40×95°C for 15 seconds and 60°C for 1 minute, followed by the default melting curve program. Fluorescence was measured just after the 1 minute/60°C step. The cycle threshold (C_T) values were determined using the cycler software and an automatic baseline adjustment (ABI 7500 Software Version 2.0.1, Applied Biosystems, U.S.A). Three mice per group were analyzed and average values calculated.

Relative quantification data analysis (SABioscienes quantitative PCR array)

Calculation of fold change values from the results of the SABiosciences quantitative PCR array relies essentially on the same principles as for the Quantitect qRT-PCR, making use of the $2^{-\Delta\Delta Ct}$ method, except for the use of up to 5 housekeeping genes (Gusb, Hprt1, Hsp90ab1, Gapdh and Actb) for normalization of data. These calculations were done using the SABiosciences PCR Array Data Analysis Template Excel Utility (http://sabiosciences.com/pcrarraydataanalysis.php)(Qiagen, Germany). The negative inverted values were determined (i.e. $0.5 = {}^{1}/_{-0.5} = -2.0$) for fold change values smaller than one (<1). Genes were only regarded as upregulated with fold changes ≥2.0, and downregulated with fold changes ≤−2.0.

Statistical analysis

The REST software uses a Pair-Wise Fixed Reallocation Randomization Test© [106] with 2000 randomizations to determine a *P*-value which gives an indication of the statistical significance of fold changes. The SABiosciences PCR Array Data Analysis Template Excel Utility incorporates the calculation of a *P*-value using a T-test. Fold changes with *P*-values smaller or equal to 0.05 (≤0.05) were taken as statistically significant. Three biological replicates were used at all experimental time points in all groups to enable statistical reliability of results.

Supporting Information

Table S1 Fold change in expression of 84 genes involved in activation of B- and T-cell immunity. Fold changes are shown for immunized mice versus non-immunized control mice (n = 3 per group) after RVFV challenge at 72 hours in liver, relative to expression in an age-related control group of mice (n = 3).
(DOC)

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Author Contributions

Conceived and designed the experiments: PJvV CTT JTP. Performed the experiments: PJvV. Analyzed the data: PJvV CTT JTP. Contributed reagents/materials/analysis tools: PJvV CTT JTP. Wrote the paper: PJvV CTT JTP.

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Table 1S

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Gene	Gene name	Transcript accession number	recNP Immunized Fold change and *P*-value	Adjuvant control Fold change and *P*-value	PBS control Fold change and *P*-value
Ap3b1	Adaptor-related protein complex 3, beta 1 subunit	NM_009680	1.65 p = 0.21	-1.09 p = 0.83	1.13 p = 0.58
Bad	BCL2-associated agonist of cell death	NM_007522	1.06 p = 0.62	**-2.19*** **p = 0.0004**	-1.64* p = 0.001
Cxcr5	Chemochine (C-X-C motif) receptor 5	NM_007551	1.56 p = 0.88	5.53 p = 0.18	5.23 p = 0.23
Cblb	E3 Ubiquitin Ligase Casitas B-lineage lymphoma b	NM_001033238	1.07 p = 0.68	**7.16*** **p = 0.006**	**8.65*** **p = 0.001**
Ccnd3	Cyclin D3	NM_007632	1.02 p = 0.86	1.12 p = 0.29	1.27* p = 0.02
Cd1d1	CD1d1 antigen	NM_007639	1.27 p = 0.41	**-6.27*** **p = 0.005**	**-6.88*** **p = 0.003**
Cd2	CD2 antigen	NM_013486	1.02 p = 0.90	**2.56*** **p = 0.02**	**2.66*** **p = 0.04**
Cd28	CD28 antigen	NM_007642	6.48 p = 0.13	**22.33*** **p = 0.002**	**21.41*** **p = 0.005**
Cd3d	CD3 antigen, delta polypeptide	NM_013487	1.15 p = 0.23	2.39 p = 0.07	1.86 p = 0.06
Cd3e	CD3 antigen, epsilon polypeptide	NM_007648	1.58 p = 0.30	3.44 p = 0.06	**3.28*** **p = 0.02**
Cd3g	CD3 antigen, gamma polypeptide	NM_009850	1.34 p = 0.20	1.14 p = 0.57	-1.47 p = 0.12
Cd4	CD4 antigen	NM_013488	1.06 p = 0.74	-1.89 p = 0.45	-1.01 p = 0.92
Cd40	CD40 antigen	NM_011611	2.91 p = 0.06	**29.40*** **p = 0.002**	**25.87*** **p = 0.003**
Cd40lg	CD40 ligand	NM_011616	1.41 p = 0.48	1.42 p = 0.44	1.83 p = 0.11
Cd74	CD74 antigen (invariant polypeptide of major histocompatibility complex, class II antigen-associated)	NM_010545	2.03 p = 0.12	1.39* p = 0.012	1.36 p = 0.13
Cd81	CD81 antigen	NM_133655	1.04 p = 0.68	**-2.17*** **p = 0.002**	-1.77* p = 0.002
Cd8a	CD8 antigen, alpha chain	NM_001081110	2.33 p = 0.40	4.88 p = 0.13	2.81 p = 0.23
Cd8b1	CD8 antigen, beta chain 1	NM_009858	-1.05 p = 0.99	4.08 p = 0.14	2.39 p = 0.40
Cd93	CD93 antigen	NM_010740	1.14 p = 0.85	83.91 p = 0.06	**144.67*** **p = 0.001**
Cdkn1a	Cyclin-dependent kinase inhibitor 1A (P21)	NM_007669	**9.02*** **p = 0.02**	**127.19*** **p = 0.0000**	**145.01*** **p = 0.0006**
Clcf1	Cardiotrophin-like cytokine factor 1	NM_019952	1.27 p = 0.89	**33.92*** **p = 0.01**	**52.10*** **p = 0.002**
Cr2	Complement receptor 2	NM_007758	-2.0 p = 0.15	1.07 p = 0.74	1.61 p = 0.10
Csf2	Colony stimulating factor 2 (granulocyte-macrophage)	NM_009969	-1.56 p = 0.66	2.37 p = 0.45	2.09 p = 0.58
Cxcl12	Chemokine (C-X-C motif) ligand 12	NM_021704	1.24* p = 0.03	**-2.21*** **p = 0.02**	-1.64* p = 0.008
Cxcr4	Chemokine (C-X-C motif) receptor 4	NM_009911	1.66* p = 0.006	**22.12*** **p = 0.01**	**22.84*** **p = 0.002**
Dock2	Dedicator of cyto-kinesis 2	NM_033374	1.11 p = 0.43	**4.85*** **p = 0.04**	**3.47*** **p = 0.003**
Egr1	Early growth response 1	NM_007913	**-5.00*** **p = 0.01**	**3.88*** **p = 0.005**	**4.54*** **p = 0.004**

WITHDRAWN

Flt3	FMS-like tyrosine kinase 3	NM_010229	-1.06 p = 0.87	**12.62*** **p = 0.004**	**9.19*** **p = 0.03**
Gadd45g	Growth arrest and DNA-damage-inducible 45 gamma	NM_011817	7.26 p = 0.26	**5.35*** **p = 0.002**	**4.37*** **p = 0.008**
Glmn	Glomulin, FKBP associated protein	NM_133248	1.08 p = 0.59	-1.03 p = 0.82	1.05 p = 0.73
H2-Aa	Histocompatibility 2, class II antigen A, alpha	NM_010378	1.84 p = 0.11	**2.01*** **p = 0.014**	1.56 p = 0.16
H60a	Histocompatibility 60a	NM_010400	-1.51 p = 0.70	**16.50*** **p = 0.046**	**13.99*** **p = 0.036**
Hdac5	Histone deacetylase 5	NM_010412	1.05 p = 0.65	-1.46 p = 0.04	**-2.18*** **p = 0.02**
Hdac7	Histone deacetylase 7	NM_019572	-1.07 p = 0.68	**4.83*** **p = 0.012**	**5.58*** **p = 0.0007**
Hells	Helicase, lymphoid specific	NM_008234	1.34 p = 0.46	2.15 p = 0.08	**2.64*** **p = 0.007**
Hsp90aa1	Heat shock protein 90, alpha (cytosolic), class A member 1	NM_010480	1.08 p = 0.67	1.33 p = 0.13	1.48* p = 0.012
Icosl	Icos ligand	NM_015790	2.33 p = 0.30	3.48 p = 0.25	3.29 p = 0.07
Ifng	Interferon gamma	NM_008337	4.05 p = 0.32	4.90 p = 0.09	3.10 p = 0.12
Igbp1	Immunoglobulin (CD79A) binding protein 1	NM_008784	-1.23 p = 0.15	**-2.39*** **p = 0.002**	**-2.37*** **p = 0.002**
Igbp1b	Immunoglobulin (CD79A) binding protein 1b	NM_015777	1.39 p = 0.37	**7.26*** **p = 0.04**	4.53 p = 0.10
Il10	Interleukin 10	NM_010548	3.99 p = 0.21	**17.64*** **p = 0.04**	**10.65*** **p = 0.02**
Il11	Interleukin 11	NM_008350	1.93 p = 0.20	275.80 p = 0.13	**772.47*** **p = 0.007**
Il12b	Interleukin 12B	NM_008352	2.59 p = 0.25	**11.75*** **p = 0.02**	**20.35*** **p = 0.005**
Il15	Interleukin 15	NM_008357	1.12 p = 0.48	-1.64* p = 0.026	-1.54* p = 0.04
Il18	Interleukin 18	NM_008360	1.21 p = 0.12	**-3.52*** **p = 0.0002**	**-4.91*** **p = 0.0001**
Il27	Interleukin 27	NM_145636	3.25 p = 0.63	5.73 p = 0.32	11.42 p = 0.10
Il2ra	Interleukin 2 receptor, alpha chain	NM_008367	3.18 p = 0.09	**5.66*** **p = 0.04**	4.41 p = 0.06
Il4	Interleukin 4	NM_021283	2.04 p = 0.35	21.42 p = 0.24	**8.73*** **p = 0.03**
Il7	Interleukin 7	NM_008371	**1.64*** **p = 0.05**	-1.70 p = 0.06	**-2.38*** **p = 0.009**
Impdh1	Inosine 5'-phosphate dehydrogenase 1	NM_011829	**2.13*** **p = 0.004**	3.26 p = 0.25	**4.92*** **p = 0.02**
Impdh2	Inosine 5'-phosphate dehydrogenase 2	NM_011830	1.18 p = 0.15	1.00 p = 0.99	1.10 p = 0.48
Inha	Inhibin alpha	NM_010564	-1.16 p = 0.74	4.49 p = 0.06	3.12 p = 0.17
Irf4	Interferon regulatory factor 4	NM_013674	1.51 p = 0.96	2.59 p = 0.39	1.95 p = 0.79
Jag2	Jagged 2	NM_010588	1.67 p = 0.18	**6.79*** **p = 0.02**	**7.46*** **p = 0.02**
Ms4a1	Membrane-spanning 4-domains, subfamily A, member 1	NM_007641	1.39 p = 0.37	**7.26*** **p = 0.04**	4.53 p = 0.10
Nkx2-3	NK2 transcription factor related, locus 3 (Drosophila)	NM_008699	1.10 p = 0.72	1.56 p = 0.26	1.16 p = 0.59
Nos2	Nitric oxide synthase 2, inducible	NM_010927	1.05 p = 0.97	25.71 p = 0.06	**32.07*** **p = 0.001**
Pawr	PRKC, apoptosis, WT1, regulator	NM_054056	1.06 p = 0.57	**2.68*** **p = 0.0003**	**3.05*** **p = 0.0006**
Pdcd1lg2	Programmed cell death 1 ligand 2	NM_021396	1.90 p = 0.1	**12.42*** **p = 0.0004**	9.47 p = 0.08
Pik3cd	Phosphatidylinositol 3-kinase catalytic delta polypeptide	NM_008840	**3.59*** **p = 0.04**	5.77 p = 0.13	**7.53*** **p = 0.002**
Pik3r1	Phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 1 (p85 alpha)	NM_001024955	1.10 p = 0.63	**-3.80*** **p = 0.007**	**-3.63*** **p = 0.005**

WITSETD

Prkcd	Protein kinase C, delta	NM_011103	1.30 p = 0.1	**3.73*** **p = 0.03**	**4.01*** **p = 0.002**
Prkcq	Protein kinase C, theta	NM_008859	1.92 p = 0.18	4.63 p = 0.09	3.89 p = 0.06
Prlr	Prolactin receptor	NM_011169	1.15 p = 0.42	**-62.79*** **p = 0.0003**	**-59.16*** **p = 0.0003**
Ptprc	Protein tyrosine phosphatase, receptor type, C	NM_011210	2.07 p = 0.57	8.14 p = 0.055	3.69 p = 0.13
Rag1	Recombination activating gene 1	NM_009019	-1.05 p = 0.98	**4.98*** **p = 0.05**	3.11 p = 0.13
Relb	Avian reticuloendotheliosis viral (v-rel) oncogene related B	NM_009046	**2.50*** **p = 0.04**	**12.30*** **p = 0.002**	**15.85*** **p = 0.002**
Rgs1	Regulator of G-protein signaling 1	NM_015811	1.67 p = 0.25	**41.67*** **p = 0.006**	**24.76*** **p = 0.016**
Sftpd	Surfactant associated protein D	NM_009160	1.30 p = 0.41	**20.40*** **p = 0.03**	**26.35*** **p = 0.0006**
Sit1	Suppression inducing transmembrane adaptor 1	NM_019436	1.92 p = 0.24	3.64 p = 0.07	2.96 p = 0.25
Sla2	Src-like-adaptor 2	NM_029983	1.08 p = 0.66	2.38 p = 0.16	**2.30*** **p = 0.007**
Socs5	Suppressor of cytokine signaling 5	NM_019654	-1.01 p = 0.90	**2.55*** **p = 0.006**	**3.63*** **p = 0.0007**
Spp1	Secreted phosphoprotein 1	NM_009263	-1.10 p = 0.62	**29.12*** **p = 0.003**	**26.97*** **p = 0.001**
Tlr1	Toll-like receptor 1	NM_030682	1.82 p = 0.2	8.68 p = 0.07	5.55 p = 0.06
Tlr4	Toll-like receptor 4	NM_021297	3.46 p = 0.06	**14.13*** **p = 0.03**	**8.28*** **p = 0.005**
Tlr6	Toll-like receptor 6	NM_011604	1.30 p = 0.43	2.11 p = 0.14	1.77 p = 0.12
Tnfrsf13b	Tumor necrosis factor receptor superfamily, member 13b	NM_021349	-1.35 p = 0.76	**6.67*** **p = 0.0006**	4.27 p = 0.15
Tnfrsf13c	Tumor necrosis factor receptor superfamily, member 13c	NM_028075	1.23 p = 0.68	2.88 p = 0.12	2.46 p = 0.15
Tnfsf13b	Tumor necrosis factor (ligand) superfamily, member 13b	NM_033622	1.32 p = 0.60	**8.74*** **p = 0.014**	**5.75*** **p = 0.005**
Tnfsf14	Tumor necrosis factor (ligand) superfamily, member 14	NM_019418	2.14 p = 0.08	**24.66*** **p = 0.04**	**16.80*** **p = 0.001**
Traf6	Tnf receptor-associated factor 6	NM_009424	1.53 p = 0.06	1.72* p = 0.03	2.34 p = 0.06
Vav1	Vav 1 oncogene	NM_011691	2.20 p = 0.13	**7.50*** **p = 0.03**	**8.00*** **p = 0.02**
Was	Wiskott-Aldrich syndrome homolog (human)	NM_009515	1.53 p = 0.50	4.33 p = 0.07	**4.64*** **p = 0.004**
Wwp1	WW domain containing E3 ubiquitin protein ligase 1	NM_177327	-1.07 p = 0.38	**-9.16*** **p = 0.0001**	**-11.99*** **p = 0.0001**

WITSETD

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