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Real-time loop-mediated isothermal amplification assay for rapid detection of Rift Valley fever virus

by

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Submitted in partial fulfillment of the requirements for the degree

Magister Scientiae

(Microbiology)

Department of Microbiology and Plant Pathology

in the Faculty of Natural and Agricultural Sciences

University of Pretoria

Pretoria

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February 2010

I declare that the thesis, which I hereby submit for the degree MSc at the University of Pretoria, South Africa, is my own work and has not been submitted by me for degree purposes at any other university.

Chantel le Roux

ACKNOWLEDGEMENTS

1. The experimental work was performed in the Special Pathogens Unit, National Institute for Communicable Diseases, of the National Health Laboratory Service, Sandringham. The study was done in collaboration with the Nagasaki University. I would like to thank Prof. Kouichi Morita and Dr. Toru Kubo for technical assistance and design of all of the primers used in the RVF RT-LAMP assay.
2. The project was supervised by Prof. Louis Nel, Prof. Janusz Paweska and Dr. Jacqueline Weyer and I would like to express my sincere thanks and appreciation for their guidance, support and shared knowledge throughout the study.
3. A special thank you to Dr. Jacqueline Weyer for fruitful discussions, advice and continuous encouragement.
4. I would like to thank Antoinette Grobbelaar and Pat Leman for technical laboratory assistance as well as Petrus Jansen Van Vuren for providing serial bleed samples from sheep experimentally infected with Rift Valley fever virus.
5. I would like to thank Guy Hall from the National Health Laboratory Service for photographical assistance.
6. My thanks are extended to other colleagues and friends at the National Institute for Communicable Diseases – especially Dr Jenny Rossouw, Dr Nicky Page, Tersia Kruger, and Dr Lizette Koekemoer for advice and encouragement.
7. A special thank you to my family, loved ones and friends, especially my parents and Jan for constant encouragement, love and support.
8. I am grateful for the financial support of the study by the Polio Research Foundation (grant number 06/07).
9. I would like to thank the National Health Laboratory Service for providing a bursary during my study.

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SUMMARY

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For the degree MSc (Microbiology)

Rift Valley fever (RVF) belongs to the group of viral haemorrhagic fevers (VHFs), most of which are zoonotic diseases causing outbreaks in animals and humans all over Africa. In the absence of haemorrhagic or specific organ manifestations, these diseases are clinically difficult to diagnose. Rapid laboratory confirmation of cases is therefore essential for timely execution of supportive treatment, appropriate case management, infection control, and tracing of contacts. Rift Valley fever virus (RVFV), a mosquito-borne pathogen, is responsible for high mortality rates and abortion in domestic ruminants, resulting in significant socio-economic losses. Furthermore, the virus is potentially infectious by aerosol, can replicate in a wide range of mosquito species and poses a bioweapon threat. The recent spread of the virus outside of the African continent, demonstrates its ability to move northwards to RVF free regions, e.g. to Europe and Northern America. Such fears fuel the international demand for reliable and validated diagnostic tools for rapid diagnosis of RVF.

The aim of this study was to develop a rapid and accurate molecular tool for the detection of RVFV. A real-time loop-mediated isothermal amplification assay (LAMP) targeting the L segment of RVFV, was developed and evaluated. The assay proved to be highly specific and able to detect RVFV strains representing the genetic spectrum of the virus. Furthermore, the assay did not amplify the RNA of other genetically and antigenically related phleboviruses. The sensitivity of the assay was compared to that of a previously published TaqMan RTD-PCR protocol and found to be equal. Similarly, the assay demonstrated very high diagnostic sensitivity and specificity in various clinical human and animal specimens, collected during natural outbreaks of the disease in Africa. The detection of specific viral genome targets in positive clinical specimens was achieved in less than 30 minutes. As a highly accurate, rapid and very simple nucleic acid detection format, the RT-LAMP assay has the potential to be used in less well equipped laboratories in Africa. The assay format can be adapted to a portable device that can be utilized during RVF outbreaks in remote areas, and can be a valuable tool for differential diagnosis of VHFs.

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LIST OF ABBREVIATIONS

α	Alpha
β	Beta
AMV	Avian myeloblastosis virus
B3	Backward outer primer
BIP (B1c B2)	Backward inner primer
BHK	Baby hamster kidney cells
bp	Base pair
BSL-3	Biosafety level three
BSL-4	Biosafety level four
Bst	*Bacillus stearothermophilus*
CAR	Central African Republic
CCHFV	Crimean-Congo haemorrhagic fever virus
CDC	Centres for Disease Control and Prevention
CER	Chicken embryo reticulum
CF	Complement fixation
CO_2	Carbon dioxide
cRNA	Complementary ribonucleic acid
Da	Dalton
DDBJ	DNA Data Bank of Japan
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTPs	Deoxynucleotide triphosphates
dsDNA	Double stranded DNA
EBOV	Ebola virus
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assays

LIST OF ABBREVIATIONS (continued)

EMEM	Eagle's minimum essential medium
F3	Forward outer primer
FDR	Fluorescent detection reagent
FIP (F1c F2)	Forward inner primer
F-test	Fisher test
g	Gravitational force
G1	Glycoprotein 1
G2	Glycoprotein 2
HI	Haemagglutination-inhibition
IF	Immunofluorescence
IFNs	Interferons
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IU	International Unit
L	Large segment
LAMP	Loop-mediated isothermal amplification
M	Medium segment
MARV	Marburg virus
$MgCl_2$	Magnesium Chloride
mM	Millimolar
mRNA	Messenger ribonucleic acid
mol wt	Molecular weight
N	Nucleocapsid protein
n	Number
NASBA	Nucleic acid sequence-based amplification
nm	Nanometre

LIST OF ABBREVIATIONS (continued)

NSm	Non-structural proteins (M segment)
NSs	Non-structural proteins (S segment)
OIE	Office International des Epizooties
ORF	Open reading frame
P	Statistically significant
PFU	Plague forming units
pmol	Picomole
QT-NASBA	Real-time quantitative NASBA
R^2	Coefficient of determination
RNase H	Ribonuclease H
RNA	Ribonucleic acid
RNPs	Pseudohelicoidal ribonucleoproteins
RSA	Republic of South Africa
RT-PCR	Reverse transcriptase polymerase chain reaction
RTD-PCR	Real-time detection PCR
RT-tHDA	Reverse transcription-thermophilic helicase dependent amplification
RVF	Rift Valley fever
RVFV	Rift Valley fever virus
S	Small segment
SDA	Strand displacement amplification
SPU-NICD	Special Pathogens Unit of the National Institute for Communicable Diseases
ssDNA	Single stranded DNA
T7 DdRp	T7 DNA dependent RNA polymerase
TFIIH	Transcription factor II H
tHDA	Thermophilic helicase dependent amplification
Tp	Time of positivity

LIST OF ABBREVIATIONS (continued)

Tte-UvrD	Thermostable UvrD helicase
Tth	*Thermus thermophilus*
TCID	Tissue culture infectious dose
USA	United States of America
USDA	United States Department of Agriculture
UV	Ultraviolet
V4	Version four
Vero	African green monkey kidney cells
VHFs	Viral haemorrhagic fevers
VNT	Virus neutralization test
wt/vol	Weight/volume
µl	Microliter

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Brief overview of viral haemorrhagic fevers

1.1.1. Aetiology

The term viral haemorrhagic fever (VHF) describes a severe multi system syndrome that can be caused by several different negative-sense RNA viruses. The syndrome is characterized by damage to the integrity of the vascular system and multiple organ failure. VHF is accompanied by visible bleeding disorders, although haemorrhagic symptoms do not always occur. VHFs are zoonotic diseases, which cause outbreaks often associated with severe epidemics in humans and animals. Several characteristics of VHF agents are summarized in table 1 (Adapted from Peters, 2000).

TABLE 1: General properties of viruses causing haemorrhagic fever

1.) Small RNA viruses (genome mol wt, 1 x 10^6 -2 x 10^6)
2.) Lipid enveloped and acid sensitive
3.) Aerosolation possible, but not the main route of transmission
4.) Persists in nature, using different strategies: all VHFs are zoonotic and circulate in different reservoirs, including mosquitoes, ticks, rodents and bats.
5.) Negative, ambisense replication strategies
6.) Different morphologies and morphogenesis
7.) Interactions with cells differ: cytopathic effects, interferon sensitivity
8.) Cause similar disease syndrome, but of different pathogenic mechanisms
9.) Induce different immune responses in humans

VHF agents belong to four distinct families:

- The *Arenaviridae* including Lassa -; Lujo -; Junin -; Machupo -; Guanarito viruses. Certain arenaviruses are associated with rodent transmitted diseases in humans. Humans become infected through contact with urine, saliva, fecal material or body excretions from infected rodents (Gonzalez *et al*., 2007).
- The *Flaviviridae* including Yellow fever -; Dengue - and Kyasanur Forest disease viruses. Flaviviruses are spread by mosquito (yellow fever and dengue) and tick bites (Kyasanur Forest disease virus) (Miller, 2008).
- The *Bunyaviridae* including Hantaan-; Crimean-Congo haemorrhagic fever- (CCHFV) and Rift Valley fever viruses (RVFV). Bunyaviruses are normally carried and transmitted by arthropods or rodents (Miller, 2008).
- The *Filoviridae* including Marburg virus and Ebola viruses. Recent studies have shown that certain bat species might be the natural or intermediate hosts of Marburg and Ebola viruses (Towner *et al*., 2009). Marburg virus specific RNA and immunoglobulin G (IgG) have been detected in a fruit bat, *Rousettus aegyptiacus* (Swanepoel *et al.,* 2007; Towner *et al.,* 2007), as well as two insectivorous bat species, *Miniopterus inflatus* and *Rhinolophus eloquens* (Swanepoel *et al.,* 2007). Presence of Ebola virus specific antibodies were detected in serum and nucleotide sequences were found in spleen and livers of three fruit bat species: *Myonycteris torquata*, *Hypsignathus montrosus* and *Epomops franqueti* (Leroy *et al*., 2005; Pourrut *et al*., 2007).

Most of the VHF viruses are classified as high or maximum biohazard agents, primarily due to the severity of disease caused in humans in the absence of effective vaccination and treatment and the possibility of their aerosolization. Biosafety level 3 (BSL-3) and at BSL-4 facilities are high biocontainment laboratory facilities for testing and storage of these and other viral agents (viruses that are deemed exotic, viruses that have been eradicated and new or uncharacterized viruses with unknown biohazard). These facilities have specialized air handling requirements, waste management systems and

personal protective equipment requirements to prevent exposure of laboratory personnel and to prevent release of these agents to the environment (CDC and NIH, 2007).

1.1.2 Transmission

Haemorrhagic fever viruses are zoonotic and are maintained in animal reservoirs but may spillover to cause infection in humans. Nosocomial outbreaks of VHFs have also been reported. Mode of transmission to the humans varies for the different VHF agents. Hanta- and Arenaviruses are commonly spread through aerosolization of the virus particle that has been excreted in rodent urine or faeces and direct rodent to human transmission may occur (Lednicky, 2003; Monath, 1975; Murphy and Walker, 1978; Walker *et al*., 1975). Flaviviruses are most frequently transmitted via mosquito bites and human-to-human transmission is not common (Calisher and Gould, 2003; Gould and Solomon, 2008). CCHF is transmitted through tick bites (Hoogstraal, 1979), although exposures to infected animal blood and tissue may also lead to infection. Human-to-human transmission normally occurs through contact of infected blood (such as needle stick injuries or during operations) (Burney *et al*.,1980; Fisher-Hock *et al*., 1995) and aerosol transmission plays a relatively small role in causing of infections. The exact mode of transmission of filoviruses from its proposed reservoir to the human population is still under speculation and, most human filovirus outbreaks are associated with hospital settings, resulting from lack of adequate facilities and poor practices in barrier nursing (Baron *et al*., 1983; Francesconi *et al*., 2003; Gear *et al*., 1975; Johnson *et al*., 1996; Muyembe-Tamfum *et al*., 1999; Smith *et al*., 1982; World Health Organization, 1978).

1.1.3 Geographic distribution of viral haemorrhagic fevers in Africa

Marburg virus outbreaks occur in Sub-Saharan Africa and have been reported from Uganda, Zimbabwe, Kenya, Democratic Republic of Congo (previously Zaire) and Angola and Ebola virus outbreaks in Democratic Republic of Congo, Sudan, Gabon and Uganda. Lassa virus is carried by *Mastomys natalensis* in the Western parts of Africa

and CCHF is widely spread through different species of ticks in Africa, parts of Eastern Europe, Middle East, Asia as well as Afghanistan, Iran and Turkey (Fisher-Hock, 2005). (A world map displaying reported VHF cases for 2005 is shown in figure 1.)

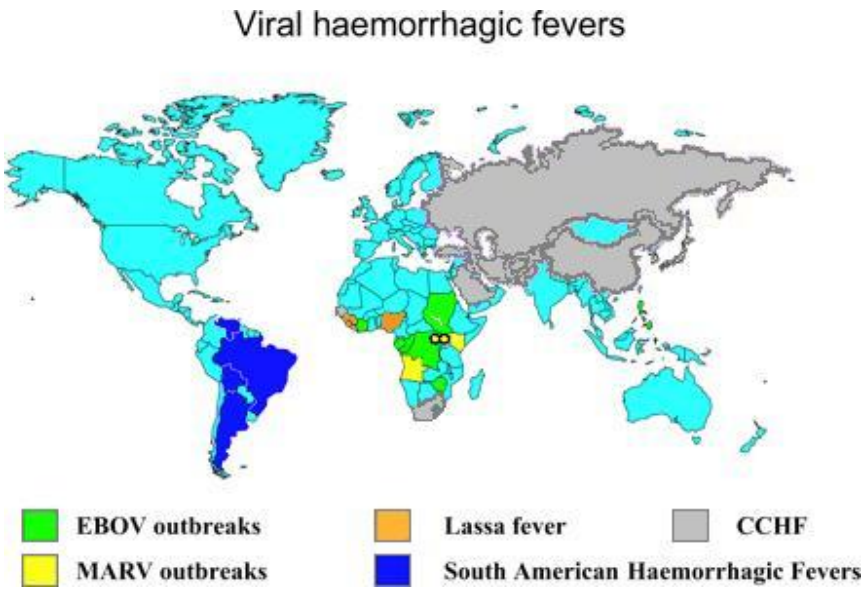


FIGURE 1: Map of the world showing countries reporting VHF in 2005. CCHF - Crimean-Congo haemorrhagic fever; EBOV - Ebola virus, MARV- Marburg virus. (Adapted from Fisher-Hock, 2005).

Rift Valley fever (RVF) is widespread throughout Africa (RVF map is shown in figure 3), but also occurs in Madagascar and Saudi Arabia. RVFV causes periodical outbreaks in ruminant species including cattle, sheep, goat and humans. It can also cause high mortality in new-born animals and abortion in pregnant animals (Swanepoel and Coetzer, 2004).

1.1.4 Signs and symptoms

Initial symptoms of VHFs are non-specific and may include: fever, sore throat, headache, nausea and vomiting, muscle aches, loss of strength and dizziness. The disease may progress to shock; haemorrhage which may manifest as a petechial rash or ecchymoses (bleeding under the skin); bleeding from internal organs, ears, eyes and mouth is less common. Patients often suffer from acute abdominal pain and may have coffee - ground diarrhoea. Other effects of VHF infection will vary based on the type of VHF. Lassa and Ebola virus infection can cause deafness; Lassa virus infection can cause pericarditis (chest pain); Marburg virus infection can cause uveitis (inflammation of the inner layer of the eye) whilst RVF infections have been associated with retinitis (inflammation of the retina), uveitis and encephalitis. Most VHFs can cause renal failure and severe hepatitis.

Outbreaks of filoviruses are associated with mortality rates of up to 90% of the cases (Fisher-Hock., 2005). Lassa virus is responsible for a high number of primary infections, with a 16% mortality rate in untreated hospitalized cases (Fisher-Hock, 2005; McCormick, 1987). The probability of humans becoming infected with CCHF and becoming clinically sick, is 20% (Ergonul, 2007) with mortality rates reported as 33% in Kosovo and 5% in Turkey (Ergonul and Whitehouse, 2007).

In the absence of haemorrhagic or specific organ manifestations, infections by VHF viruses are clinically difficult to recognize, with the implication that definitive diagnosis depends mainly on reliable laboratory tests (Drosten *et al.,* 2002). Although the range of possible VHF agents can be narrowed down by the patient's travel and exposure history, a suspected case and the causative virus must be rapidly identified to initiate specific or supportive treatment and to implement appropriate case management, infection control and tracing of contacts.

1.2 Rift Valley fever virus classification and characteristics

1.2.1 Taxonomy of the *Phleboviruses*

RVFV belongs to the genus *Phlebovirus*, family *Bunyaviridae* (Nichol *et al*., 2005). The *Bunyaviridae* family is divided into 5 genera: *Orthobunyavirus* (Akabane virus; Bunyamwera virus), *Hantavirus* (Hantaanvirus), *Nairovirus* (CCHFV), *Phlebovirus* (Punto Toro virus; RVFV; Sandfly fever Naples virus; Uukuniemi virus; as well as tentative species such as: Arumowot virus; Chagres virus; Gabek Forest virus; Gordil virus; Saint-Floris virus; Sandfly fever Sicilian virus) and *Tospovirus* (Nichol *et al*., 2005). Some of the Phleboviruses have been linked to human disease including Chagres (Peralta *et al*., 1965; Srihongse *et al*., 1974; Tesh *et al*., 1974), RVF (Daubney *et al*., 1931), Sandfly fever Sicilian and Sandfly fever Naples (Sabin, 1955; Tesh *et al*., 1975) and Punto Toro viruses (Peralta *et al.*, 1965; Tesh *et al*., 1974). Most of the viruses in the Bunyaviridae family share serological properties, but their genome and proteome sizes differ. Bunyaviridae are divided into two major serogroups: the Phlebotomus fever group which is transmitted by sandflies, mosquitoes or ceratopogonids of *Culicoides* and the Uukuniemi group of viruses carried by ticks (Nichol *et al*., 2005).

1.2.2 Physical characteristics and molecular biology

The phleboviruses are sensitive to pH values outside of the range 7-9 and are rapidly inactivated by high temperatures (above 56˚C), ultraviolet light and lipid solvents (Bishop *et al*., 1980).

Bunyavirus particles are pleomorphic/spherical (80-120 nm in diameter) and have a host-cell derived bilipid layer through which glycoprotein peplomers project (Murphy *et al*., 1973). RVFV has a tripartite single stranded RNA genome and the segments are designated small (S), medium (M), and large (L). The genome is contained in a separate nucleocapsid (N) within the virion with two glycoproteins (G1 and G2) and a viral transcriptase or L-protein (Rice *et al*., 1980). The virion structure of RVFV is displayed in figure 2.

The S segment (mol wt: 0.5-0.6X10^6) (Rice *et al*., 1980) is 1690 nucleotides in size and makes use of an ambisense coding strategy (Ihara *et al*., 1984). The gene coding for the N protein is negative sense in the 3' half of the viral RNA, and is transcribed into viral complementary mRNA, which is translated to form the N protein. During genome replication, viral-complementary RNA is produced. The gene coding for the NSs protein is negative-sense in the 3' half of the viral-complementary RNA. This is transcribed into viral-sense mRNA which then translated to form the NSs protein (Giorgi *et al.,* 1991).

The overlapping open reading frame (ORF) of the S segment encodes the N protein (28,000 Da) on the antigenomic (negative sense) strand and the non-structural protein (NSs, 31,000Da) on the genomic (positive sense) strand (Nichol, 2001). Billecocq *et al*., (2004) showed that the NSs protein is able to stop interferon (IFN) production, but does not hinder the activation of IFN-specific transcription factors. The NSs protein also interacts with the p44 subunit of transcription factor II H (TFIIH) (Le May *et al*., 2004). TFIIH is responsible for transcription of protein coding genes by RNA polymerase II.

The M segment (mol wt: 1.3-1.7X10^6) (Rice *et al*., 1980) has 3885 nucleotides and negative polarity, meaning the negative sense genome must be transcribed into positive sense mRNAs for protein production. The M segment encodes at least four viral proteins in a single ORF: two major envelope glycoproteins (G1, 65 000 Da; G2, 56 000 Da) (Gentsch and Bishop, 1979; Rice *et al*., 1980) which are exposed to the surface of the virus and play a role in viral transmission, infection, pathogenesis, immunity and serve as neutralizing and haemagglutinating inhibiting antibody targets (Battles and Dalrymple, 1988; Beaty *et al*., 1981; Keegan and Collett, 1986; Shope *et al*., 1981). A pre-glycoprotein coding region encodes the two non-structural proteins: NSm1 of 78kDa (Suzich *et al*., 1990) and NSm2 of 14 kDa (Fuller and Bishop, 1982) which were shown to be non-essential for virus growth in cell culture (Bird *et a*l., 2007; Gerrard *et al.,* 2007; Won *et al*., 2006), but act as virulence factors by inhibiting the host cell apoptotic pathway (Won *et al*., 2007).

The L segment (mol wt: 2.3-2.7X10^6) (Rice *et al*., 1980) has 6404 nucleotides and negative polarity. It codes for the L protein component of a viral transcriptase in a single

ORF: a 237 000 Da RNA-dependent RNA polymerase (enzyme that catalyzes the replication of RNA from an RNA template) in a viral complementary sense (Clerx-van Haaster *et al*., 1982; Elliot *et al*., 1992; Müller *et al*., 1994).

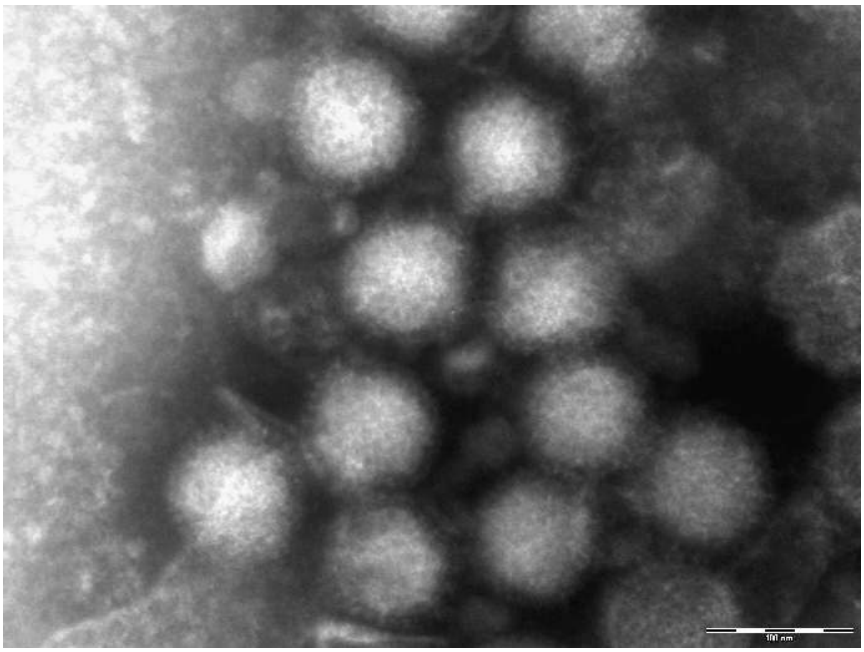


FIGURE 2: An electron microscope photograph displaying the RVFV virions.

1.2.3 Phylogeny

The advantage of a segmented RNA genome is that whole segments may be exchanged (or reassorted) in simultaneously infected cells, which may lead to the acquisition of competitive genetic characteristics. Reassortment in nature occurs when two different strains infect the same host at the same time (Sall *et al*., 1999). Evidence of genetic reassortment among members of the Bunyaviridae family has been shown *in vivo* and *in vitro* (Beaty *et al.,* 1985; Borucki *et al.,* 1999; Gerrard *et al.,* 2004) as well as in mosquitoes (Beaty *et al*., 1982). For RVFV, genome reassortment has been shown in tissue culture (Saluzzo and Smith, 1990) and in mosquitoes (Turell *et al*., 1990). Evidence of natural reassortment of RVFV has been shown in Egyptian strains isolated

between1977 and 1993 and between lineages of the virus from West African, Egyptian and Central East African origins (Sall *et al.*, 1999).

Evolution of RVFV in nature seems to be driven by point mutations and genome reassortments between the viruses in circulation and may result in new viral characteristics including enhanced virulence (Sall *et al*., 1999).

Due to the high error rate of RNA polymerase (which lead to point mutations), and segment reassortment that occurs in RNA viruses, studies have been conducted to determine the genomic variation between the Phlebotomus and Uukuniemi group of bunyaviruses. cDNA analysis done on the RNA of the S segment of RVFV, Sandfly fever Sicilian virus, Punto Toro virus, Tospovirus and Uukuniemi virus indicate that the N proteins of all of these viruses are comparable (Giorgi *et al*., 1991). In addition, the L protein sequences of RVFV and Uukuniemi virus showed 58% similarity (Elliot *et al*., 1992). More recently, Bird *et al*., (2007) compared the complete nucleotide and amino acid genome sequences as well as the individual S, M and L segments of 33 ecologically and biologically diverse RVFV strains. The overall genetic diversity of these strains was found to be low, with maximum differences of 5% and 2% for segments at nucleotide and amino acid level, respectively. Differences in the nucleotide and amino acid levels of the S, M and L segment are summarized in Table 2.

TABLE 2: Maximum pairwise sequence identity differences for the S, M and L segment of 33 ecologically and biologically different RVFV strains obtained from Bird *et al*., 2007.

Maximum pairwise sequence identity differences	Difference at % Nucleotide level	Difference at % Amino acid level
S segment	4	1
M segment	5	2
L segment	4	1

High variation of the M segment sequences is expected, due to the role of glycoproteins in inducing neutralizing antibodies and in cell attachment. However, Bird *et al*., (2007) showed high conservation between the nucleotide and amino acid levels for the M segment as well as for the S and L segment. In fact, the RNA polymerase activity proved to be completely conserved in all 33 RVFV strains (Bird *et al*., 2007; Müller *et al*., 1994).

Various detailed tests done by Bird *et al*., (2007) on 33 L, M and S full genome segments showed no statistically significant evidence for genetic recombination for the L and M segment and some evidence for the S segment, but not statistically enough to change the overall S segment phylogeny.

Thus, although RVFV has a segmented RNA genome, it is genetically very stable due to either overall low mutation tolerance or a relatively common ancestor. Bayesian analyses done on 33 complete RVF genomes estimated that a common recent ancestor was isolated 108-117 years prior to the year 2000 (Bird *et al*., 2007).

1.2.4 Replication cycle

The G1 and G2 glycoproteins of RVFV play an important role during viral infection and pathogenesis. G1 and G2 attach to the host cell receptors and play a role in fusion of the virus to the host cell. When the virus is used as an immunogen, the G2 protein plays a role in activating the formation of protective neutralizing antibodies which act against the virus (Keegan and Collett, 1986). The RVFV is endocytosed by the host cell and after uncoating, the L polymerase starts primary transcription, leading to the production of mRNAs. This initiates secondary transcription which is involved in replication and leads to production of complementary ribonucleic acid (cRNA), which in turn act as an intermediate for synthesis of viral RNA (Le May *et al*., 2005). Viral RNA and cRNA are associated with copies of the N protein and a few molecules of the L polymerase to form pseudohelicoidal ribonucleoproteins (RNPs) and serve as templates for transcription and replication (Le May *et al*., 2005). All of this occurs in the cytoplasma of the host cell. Virions mature primarily by budding through the endoplasmic reticulum in the Golgi region into cytoplasmic vesicles and fuses to the plasma membrane, releasing virus particles. The NSs protein of RVFV, synthesized during replication, enters the host cell nucleus and forms filamentous structures (Struthers and Swanepoel, 1982; Yadani *et al.,* 1999). The NSs protein seems to play two roles. The one role is to interact with the p44 subunit of TFIIH, a transcription factor involved in DNA repair and cell cycle regulation and has several enzymatic activities. When the NSs interact with the p44 unit of TFIIH, there is a drop in host RNA synthesis (Le May *et al.,* 2004). The other role of the NSs protein is to block IFN α/β production in virus infected cells at transcriptional level (Billecocq *et al*., 2004).

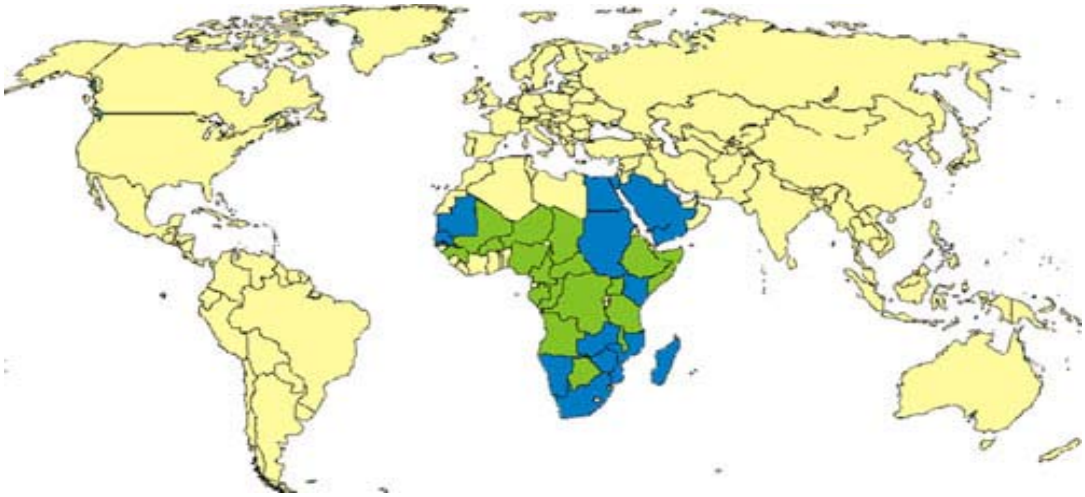
1.3 Epidemiology of Rift Valley fever

RVFV is known to cause large epidemics in domestic ruminants and humans throughout Africa and recently, in the Arabian Peninsula and the Archipelago of Comoros (RVF distribution is shown in figure 3). Outbreaks in ruminants are characterized by high levels of mortality and abortion in pregnant animals with mortality in new born animals approaching 100% (Coetzer, 1982; Easterday *et al*., 1962a; Rippy *et al*., 1992; Swanepoel and Coetzer, 2004).

In 1910, after heavy rains in the Great Rift Valley of Kenya, there were reports of illness in sheep and man, including fatalities in lambs. However the causative agent, RVFV, was only isolated in July 1930 after high mortality in newly born Merino lambs in the Rift Valley, northwards of Lake Naivasha. These outbreaks were also accompanied by heavy rainfall in this area, with a total mortality in lambs and ewes reaching about 3500 and 1200, respectively as well as abortions in pregnant animals (Daubney *et al*., 1931). RVFV was first recognized in South Africa in 1950-1951 after a large epidemic in cattle and sheep. The occurrence of serious ocular sequelae (loss of the central vision) was reported for the first time during this outbreak after a pathologist became infected with RVFV while working in a laboratory (Freed, 1951; Schrire, 1951). The second major outbreak of RVF in SA occurred in 1974-76 with major losses of ruminants. Deaths from encephalitis and haemorrhagic fever with necrotic hepatitis were reported for the first time during this epizootic (Swanepoel and Coetzer, 2004). A major epidemic occurred along the Nile Delta in Egypt in 1977-1978, causing deaths and abortions in sheep, cattle, goats, water buffalo and camels with an estimated 18 000 human infections and 598 deaths (El-Akkad., 1978; Meegan and Bailey, 1989). RVFV was isolated for the first time in Madagascar in 1979. Large outbreaks of RVFV occurred in 1987 in Mauritania and Senegal, affecting almost 89 000 individuals (Jouan *et al.,* 1989). In the Garissa district of Kenya and parts of Somalia, 170 human deaths were reported in 1997-1998 (Woods *et al.*, 2002). Between 2000 and 2001, an RVFV outbreak occurred for the first time outside of the African continent, in Jizon province in South West Saudia Arabia and Yemen, with an estimated 2000 human cases, 245 deaths and loss of sheep and goats (CDC, 2000a; CDC, 2000b; Jupp *et al*., 2002; Shoemaker *et al*., 2002). In 2006-2007,

after heavy rainfall in Somalia, Tanzania and Kenya, RVFV re-emerged, leading to 1062 reported human cases, 315 deaths and loss among livestock (CDC, 2007). During 2007 and 2008, RVF transmission was found in the Archipelago of Comoros and the French Mayotte (Sissoko *et al*., 2009).

Since RVFV is transmitted by a wide range of mosquito species and livestock are known to develop high levels of viremia, the virus has proven to cross geographic boundaries. To date, RVFV has caused three epidemics in areas not previously infected with RVFV. In 1977, RVFV was detected for the first time north of the Sahara desert in Egypt (Meegan *et al*., 1981), in 2000, the virus was isolated for the first time in Yemen and Saudi Arabia (CDC, 2000a; CDC, 2000b) and during 2007 and 2008, RVF was reported in the Archipelago of Comoros and the French Mayotte (Sissoko *et al*., 2009). Based on complete genome data sets, Bird *et al.,* (2007) showed that there is a firm phylogenetic linkage between RVFV strains from Saudi Arabia, Madagascar and strains circulating earlier within the African continent. In addition, RVFV strains from Central-African Republic, Zimbabwe or South Africa fall within multiple virus lineages, which implies that genotype movement occurs over large geographic distances.



Countries with endemic disease and substantial outbreaks of RVF:	Gambia, Senegal, Mauritania, Namibia, South Africa, Mozambique, Zimbabwe, Zambia, Kenya, Sudan, Egypt, Madagascar, Saudi Arabia, Yemen.
Countries known to have some cases, periodic isolations of virus, or serologic evidence of RVF:	Botswana, Angola, Democratic Republic of the Congo, Congo, Gabon, Cameroon, Nigeria, Central African Republic Chad, Niger, Burkino Faso, Mali, Guinea, Tanzania, Malawi, Uganda, Ethiopia, Somalia.

FIGURE 3: Distribution of RVF after 2000. (Adapted from internet publication CDC, Atlanta, USA).

1.4 Rift Valley fever virus vectors, mammalian hosts and transmission cycles

RVFV has been isolated from at least 40 species of mosquitoes representing 8 genera (Fontenille *et al*., 1998; Meegan and Bailey, 1989). In South Africa, *Culex theileri* appears to be the main epidemic vector, but RVFV has been isolated from 12 species in the subcontinent, including *Aedes, Culex, Anopheles* and *Eretmapodites* (Sall *et al*.,

1999; Swanepoel and Coetzer, 2004). Ticks were suspected vectors of RVFV and it was shown that *Hyalomma truncatum* ticks are able to transmit the virus infection by bite after inoculation with RVFV. However, transmission studies with several other species of ticks indicated that ticks are not involved in outbreaks of RVF (Linthicum *et al*., 1989).

RVF outbreaks have been associated with heavy rainfalls in generally dry areas (Daubney *et al*., 1931; Davies *et al*., 1985; Woods *et al*., 2002). The floodwater *Aedes* species can overwinter as eggs and survive for long periods in mud. Flooding of generally dry areas allow for hatching of transovarially infected *Aedes* species which feed on nearby mammals (Linthicum *et al*., 1989). Flooded areas in Kenya are shown in figure 4. The transmission cycle of RVFV is shown in figure 5. The efficacy of transmission of the RVFV depends on the characteristics of each mosquito species and, is not influenced by the strain of RVFV. However, the dose of RVFV and warm weather results in higher transmission rates, leading to outbreaks of RVF (Faran *et al.,* 1988; Turell *et al*., 1984; Turell *et al*., 1985; Turell, 1993).

After ingestion of a RVFV infected bloodmeal, in most mosquitoes, the virus is confined to the midgut (mesenteronal barrier). RVFV replicates in the midgut/mesenteronal epithelial cells. The existence of a dose-dependent mesenteronal barrier of infection threshold level which influence mosquito susceptibility to infection with arboviruses was recognized by Chamberlain and Sudia., (1961). If the virus is able to pass the mesenteronal barrier – which seems to be viral dose dependent, independent of the infection rate, the virus migrates to the haemocoel, and via the haemolymph reaches secondary target organs such as the salivary glands and ovaries. If there are no salivary gland barriers or the virus is able to pass the salivary gland barrier if there is one, the virus is able to be transmitted from the salivary gland secretions while the mosquito is feeding on animals or humans and if there are salivary gland barriers and the virus does not succeed in passing the barrier, the mosquito is not able to transmit the virus to animals or humans.

Turell *et al*., (2008) conducted studies to determine the vector competence of different species of mosquitoes to transmit RVFV by testing eight species (*Aedes palpalis; Aedes mcintoshi, Aedes circumluteolus, Aedes calceatus, Aedes aegypti, Culex antennatus, Culex pipiens,* and *Culex quinquefasciatus*). Although all eight of the species were susceptible to infection with RVFV, all except *Aedes calceatus, Aedes Aegypti* and *Culex quinquefasciatus* transmitted RVFV by bite after oral exposure. Different "barriers" such as midgut escape and salivary glands seemed to be the determinant of vector competence for the different species (Faran *et al.,* 1986; Faran, 1988; Kramer *et al*., 1981; Romoser *et al*., 1992; Turell *et al*., 1984; Turell *et al*., 1985). From the study done by Turell *et al*., (2008) it was found that the principal determination of vector competence in the Culex specie is the midgut escape barrier (more severe midgut escape barrier) and the salivary gland barrier is the principal determination of vector competence in the Aedes specie (more severe salivary gland barrier and a moderate midgut escape barrier). Kramer *et al*., (1981) found that for instance*, Aedes Stegomyia* can become infected and develop a disseminated infection after oral exposure to RVFV, but due to their salivary gland barriers, these specie can not transmit the virus by bite, thus they are inefficient vectors. In contrast, *Culex pipiens* was implicated as the principal vector during the 1977-1978 outbreak in Egypt and seemed not to have salivary gland barriers, but midgut infection and midgut escape barriers determine vector competence. From the studies done by Turell *et al.*, (2008), it was found that when mosquitoes were exposed to higher RVFV doses, the infection rate of the mosquitoes were higher and the percentage of infected individuals that developed disseminated infection increased. Turell *et al*., (2008) also found that for each species, mosquitoes with a disseminated infection had more virus particles than those mosquitoes (from the same specie) without disseminated infection.

FIGURE 4: Flooded areas during the 2006-2007 RVF outbreak in Kenya

Different mammalian hosts such as sheep, cattle, goats, buffalo, various species of antelopes, camels and humans are susceptible to RVFV, with sheep being the most susceptible and lambs being slightly more susceptible than adult sheep (Allam *et al*., 1986; Easterday, 1965). RVFV is spread from viremic animals to uninfected susceptible animals by infected mosquitoes (Hoogstraal *et al*., 1979). When a lamb becomes infected with RVFV, there is a 2 to 4 day incubation period before the lamb becomes lethargic and, does not want to move or feed, followed by fever and bloody diarrhoea. The case fatality rate in lambs ranges from 90 to 100% while in adult sheep it drops to ranges between 20 and 30% (Woods *et al*., 2002). Abortions and high neonatal fatalities are characteristic of RVFV in pregnant sheep, cattle and goats.

Human RVFV infections normally occur due to bites of infected mosquitoes, exposure to body fluids, blood, amniotic fluid, tissues of infected animals and especially those having contact with the virus through abraded skin, mucous membranes or wounds during handling or slaughtering of infected animals (Brown *et al*., 1981 ; Easterday, 1965; Francis and Magill, 1935; Hoogstraal *et al*., 1979); during necropsies and during laboratory studies (Abu-Elyazeed *et al*., 1996; Gear *et al*., 1951; Joubert *et al*., 1951; Zaki *et al*., 1995). RVFV has been isolated from raw milk (Haig *et al*., 1953; Jouan *et al.,*

1989) and ingestion of raw milk has been suggested as a risk factor in studies done by Alexander., (1951), Jouan *et al*., (1989) as well as Woods *et al*., (2002), but historically there has been no direct evidence that the RVFV has been transmitted from the raw milk to cause human or animal infection during epidemic periods (Easterday., 1965). There have been no reports of human-to-human transmission via these modes (Francis and Magill, 1935; Joubert *et al*., 1951). There have been many reports of humans that became infected while investigating RVF in the field (Francis and Magill, 1935; Hoogstraal *et al*., 1979; Joubert *et al*., 1951; Kitchen *et al*., 1934; Schwentker and Rivers, 1934; Smithburn *et al*., 1949) as well as in house wives that handled raw meat on farms (McIntosh *et al*., 1980).

Figure 5: RVFV transmission cycle adapted from Geering *et al*., (2002).

RVF is characterized in humans as a mild to severe influenza-like illness of 2 to 5 days duration, but in 1 to 2% of infected cases, the disease can progress to hepatitis, encephalitis, retinitis, ocular sequelae or a haemorrhagic syndrome, with a hospitalization case fatality rate of 10 to 20% (Madani *et al*., 2003; McIntosh *et al*., 1980; Meegan *et al*., 1981; Swanepoel and Coetzer, 2004). Human illness, disability and suffering due to RVF outbreaks, can place a huge burden on the public health infrastructure to provide adequate medical care. In addition, RVF outbreaks lead to devastating economic losses among livestock with owners reporting losses of up to 70 %, with the greatest losses among sheep and goats (Bird *et al*., 2007; Meegan *et al.*, 1981; Woods *et al*., 2002) (RVFV infected patient is shown in figure 6).

FIGURE 6: Patient from the Garissa district of Kenya receiving treatment after being infected with RVFV.

1.5 Control, prevention and treatment of Rift Valley fever

RVFV is regarded as a potential bioterrorism and bioweapon agent and has been classified as a category B select agent by the CDC *(http:www.bt.cdc.gov/agent/agent list-category.asp)*, although the U.S. Department of Health and Human Services, Department of Agriculture (USDA) and National Institute for Allergy and Infectious Diseases (*http:www.niaid.nih.gov/dmid/biodefense/bandc_priority.htm*) classify RVF as a category A select agent. The Office International des Epizooties (OIE) of the World Organization for Animal Health listed RVFV as a high-consequence agent with the potential to spread internationally (Le May *et al*., 2004) and, therefore, effective vaccines are necessary to control and prevent RVF from spreading.

Control and prevention of RVFV infection in animals: RVF outbreaks are associated with abnormally heavy rainfall. The period and region where the outbreak might occur can be predicted, mosquito populations can be controlled (Woods *et al*., 2002) and an efficient, cost-effective, single-dose vaccine, is available (Bird *et al*., 2008).

An example of a live-attenuated vaccine is the Smithburn entropic strain. While live-attenuated vaccines do not need booster vaccinations, the molecular mechanisms of attenuation are not very clear, but might rely on single base substitutions for attenuation, thus, raising the possibility of reversion to the virulent phenotype due to RNA polymerase errors that often occur. It has been shown that live-attenuated vaccines cause abortions, teratogenic effects and neural pathology in animal models and livestock, limiting their use in the field (Hunter *et al*., 2002; Morrill *et al*., 1997a; Morrill *et al*., 1997b; Morrill and Peters, 2003). In addition, when live attenuated vaccines are applied, one can not differentiate between vaccinated and naturally infected animals, which make it difficult to keep track of “RVF disease free” countries and endemic RVF regions (Bird *et al*., 2008).

Prevention of RVFV infection in animals and humans: TSI-GSD-200 is an example of an inactivated RVF vaccine where inactivation of the live wild-type virus was done by using

formalin. While inactivated vaccines provide protective immune responses in humans and animals, they unfortunately need to be applied two to three times for initial inoculations and require regular booster vaccinations for effective protection (Pittman *et al*., 2000; Swanepoel and Coetzer, 2004), making the vaccination costly, and logistically difficult to administer. Another disadvantage of inactivated vaccines, are the possibility of improper inactivation, which might result in RVF disease outbreaks.

Treatment of RVFV infection in humans: Ribavirin is currently listed as the drug of choice for RVF prophylaxis and treatment in humans by the WHO, due to its ability to inhibit RVFV infection in mice, cell culture and Rhesus monkeys (Peters *et al*., 1986; Stephen *et al*., 1980). Unfortunately, Ribavirin has serious side effects such as anemia, neurotoxicity, gastrointestinal effects and bronchospasms and, therefore, has not been approved by the U.S. Food and Drug Administration (Inuoye *et al*., 2002; Sidwell and Smee, 2003). Interferon and hyperimmune serum have also been used, but are not cost effective and not readily available (Peters *et al*., 1986).

It is difficult to control and prevent RVF outbreaks due to all of these unresolved problems. This scenario emphasizes the need for rapid, accurate, reliable, cost-effective detection techniques that ensure early identification of RVF outbreaks so that appropriate control measures can be applied to prevent spread of the virus.

1.6 Diagnosis of Rift Valley fever

1.6.1 Pathological features of Rift Valley fever

RVF presents clinically as uncomplicated or complicated infection in humans:

- Uncomplicated RVF

It is a mild to severe influenza-like illness with a sudden onset of fever, malaise (depression), rigor, headaches, lower back muscle pains or gastroenteritis symptoms including abdominal pain, nausea, vomiting, diarrhea and anorexia. Clinical laboratory

tests normally show leukocytosis (raised white blood cells), followed by leucopenia (reduced white blood cells which can increase the risk of infections).

- Complicated RVF

RVF disease can cause serious complications such as a) meningo-encephalitis: acute onset of febrile disease, followed by disorientation, vertigo and hallucinations; b) haemorrhagic manifestations: sudden onset of febrile disease, jaundice, melena, petechial and purpuric skin lesions, bleeding of gums, liver necrosis and eventually death; c) ocular complications due to vasculitis (inflammation of blood vessels), retinal lesions leading to vision loss; d) hepatitis: renal failure, clinical laboratory tests showing elevated liver enzyme levels leading to liver failure, thrombocytopenia (low platelets in the blood), elevated lactate dehydrogenase and creatine kinase enzymes (Madani *et al.*, 2003; McIntosh *et al*., 1980; Meegan *et al*., 1981).

In livestock, pathological features may include leucopenia during the first three to four days of RVF infection (with viremia and fever) and may be followed by leukocytosis during early recovery (Daubney *et al*., 1931; Easterday., 1965., Swanepoel *et al*., 1986b). Raised levels of enzymes such as sorbitol dehydrogenase and glutamate dehydrogenase (indicative of hepatocyte necrosis) or aspartate aminotransferase (suggestive of hepatocyte degeneration) can be found in serum (Swanepoel *et al*., 1986b). Hepatic lesions of RVF are similar in animals and humans and differ according to the age of the infected animal (Coetzer., 1982; Daubney *et al.,* 1931; Easterday *et al*., 1962b; Van Velden *et al*., 1977). The most severe lesions are found in aborted sheep foetuses and new-born lambs in which the liver is enlarged; haemorrhages and oedema of the wall gall bladder; peripheral and visceral lymph nodes are enlarged, may have petechia; visceral haemorrhages; mild to moderate effusion of fluid; congestion of lungs; hepatic necrosis is the most striking lesion of RVF (Coetzer., 1982; Daubney *et al*., 1931., Van Velden *et al*., 1977).

RVFV is found in the blood of infected mammalian hosts for a short period after infection. IgM antibody is the first to appear after initial or recent exposure to protect the host against the infection and cannot be passed across the placenta. IgG antibody is

involved with secondary humoral immune response, protecting the host against infections and, can cross the placenta, protecting the foetus from infections and persisting indefinitely at high titers. After natural RVFV infection of domestic animals, the IgM antibody wanes within 4 months (Morvan *et al*., 1992) and, therefore, testing for IgM antibodies during the acute phase, or shortly thereafter is an important diagnostic tool to differentiate between recent and previous infections (Niklasson *et al*., 1984). It has been found that high RVFV viremia and antibodies levels develop in infected sheep (Easterday, 1965). Virus has been detected in the spleen of sheep 21 days after infection (Yedloutschnig *et al*., 1981), indicating that the virus can persist in the host for some time after the symptomatic phase. The danger of this includes the slaughtering of apparently healthy animals that may be infected with RVFV (Easterday *et al*., 1962a). RVF has a short incubation period of 12-36 hours and lambs less than two weeks are likely to die within 36-42 hours after onset of illness. In older sheep, cattle and goats, viremia is detected on day 1 or 2 after infection, with high titers of viremia found between day 2 and 5, and usually persisting until day 7 (Easterday., 1965; Easterday *et al*., 1962a; Easterday *et al*., 1962b; Swanepoel *et al*., 1986a). Antibodies can be detected from day 3, using a virus neutralization test (VNT) and from day 4 to 7, using haemagglutination-inhibition (HI) or enzyme-linked immunosorbent assays (ELISAs) (Jansen van Vuren *et al*., 2007; OIE, 2008; Paweska *et al*., 2003a; Paweska *et al*., 2003b; Paweska *et al*., 2005a; Paweska *et al*., 2005 b). In humans, RVFV antigen is detected from day 2 to day 5 after infection. IgM and IgG antibody can be detected from day 5, IgM antibody is absent in 50% of patients by day 45 and cannot be detected 4 months later, whereas IgG can persist indefinitely at a high titer (Madani *et al*., 2003; Van Velden, 1977). Graphical presentation of viremia, IgM and IgG kinetics in humans infected with RVF is shown in figure 7.

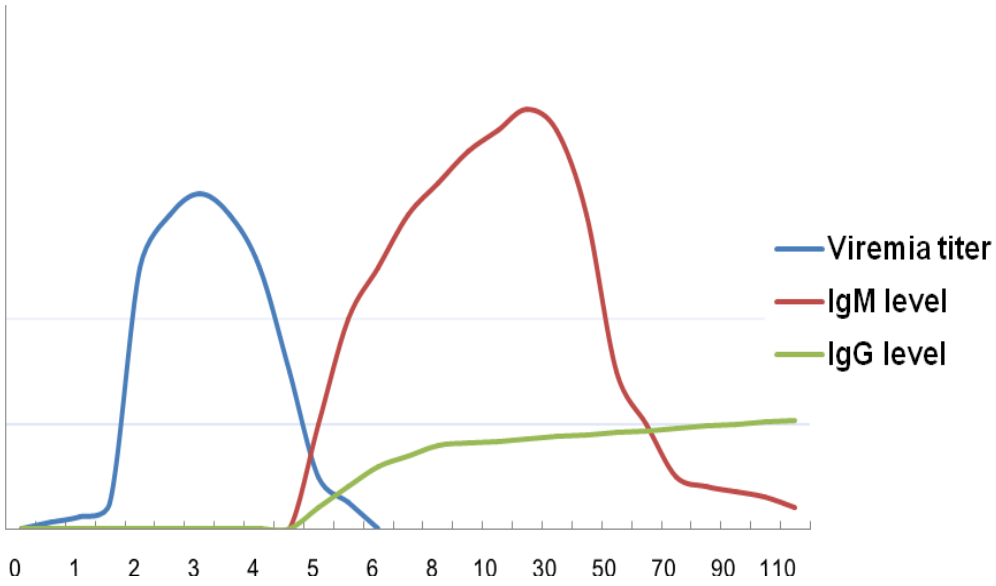


FIGURE 7: Kinetics of viremia and antibody levels in patient infected with RVFV drafted based on information from Madani et al., (2003) and Van Velden, (1977).

1.6.2 Laboratory confirmation of Rift Valley fever

Clinical diagnosis of RVF may not be simple because of the co-circulation of other diseases with similar clinical manifestations (i.e. brucellosis). Abortion storms in domestic species, especially after high rain fall are strongly indicative of RVF. However, definitive diagnosis of the disease relies on specific laboratory testing. Reliable and rapid diagnostic techniques for early identification of the disease may be used to control spread and prevent large economical losses associated with death in animals and may be useful for patient management.

Different diagnostic techniques are available to identify the disease at different stages of infection.

1.6.2.1 Virological methods

During the febrile stage of the disease, the virus can be isolated from serum, blood, spleen, liver or brain of dead animals or from aborted Foetus tissues (OIE, 2008). If

BSL-3 or 4 facilities are available, primary virus isolation can be performed by inoculating the virus into adult or suckling mice, hamsters, embryonated chicken eggs or 2-day old lambs (OIE, 2008). Histopathological lesions in the liver are characteristic of RVF and can assist with diagnosis. Alternatively, the virus can be inoculated into baby hamster kidney cells (BHK), African green monkey kidney cells (Vero); Chicken embryo reticulum (CER), or primary kidney or testis cells of calves and lambs. After 12-24 hours post inoculation, rounding of the cells and destruction of the whole cell sheet can be observed; within 18-24 hrs after infection, the RVFV antigen can be detected by making use of immunoflourescent techniques and after 5 to 6 days, the cytopathic effect of RVFV can be observed under a microscope (Jansen van Vuren and Paweska, 2009; OIE, 2008).

1.6.2.2 Serological methods

The viremic stage for RVF occurs for a short period and, therefore, specimens are often tested retrospectively, using serological techniques to detect RVFV-specific antibodies. Classical serological methods include various forms of haemagluttinin inhibition (HI), complement fixation (CF), indirect immunofluorescence tests (IF) and, VNTs which are useful for measuring antibody levels after RVFV infection (Niklasson *et al*., 1984). HI is used in non endemic areas and, has high sensitivity and specificity, but individuals previously infected with other phleboviruses, might generate a false-positive result (Clarke and Casals, 1958). Humans and animals produce neutralizing antibodies that give lifelong immunity and VNTs are useful for verification of RVF infection or for detecting antibodies after vaccination (Tesh *et al.,* 1982) and are seen as the gold standard for IgG (not for IgM) comparative serology (Paweska *et al*., 2003a). In addition, VNTs, are highly sensitive and specific (Swanepoel *et al*., 1986a), but are expensive, time-consuming, needs cell culture or animal facilities, special containment facilities (Scherer *et al.*, 1980), include health risks to laboratory personnel (Smithburn *et al*., 1949), and are restricted for use outside RVF endemic areas (OIE, 2008).

Highly sensitive and specific ELISAs to detect antibodies for RVFV have been developed (Meegan et al., 1987; Niklasson *et al*., 1984; Paweska *et al*., 2003b), based

on β-propiolactone inactivated and/or gamma irradiated antigens derived from tissue culture or mouse brain by sucrose-acetone extraction. Disadvantages of utilizing the ELISAs include poor binding of antigens to ELISA plates. To overcome these problems, a sandwich ELISA for detection of IgG and a capture ELISA for detection of IgM antibody was developed by Paweska et al., (2003a; 2005a); as well as an inhibition ELISA (Paweska *et al.*, 2005b). Production of antigen requires expensive biocontainment facilities and involves culturing of the virus prior to inactivation, the possible exposure of laboratory staff to incomplete inactivation and high production costs (Kitchen, 1934; Smithburn *et al*., 1949; McIntosh *et al*., 1980). Moreover, restrictions on production outside endemic areas are prohibitive (OIE, 2008). Development of an IgG and IgM indirect ELISA based on recombinant N protein of RVFV might overcome most of these problems (Jansen van Vuren *et al*., 2007).

During the early stage of the disease, antigen detection techniques can be used and are useful in laboratories where tissue culture facilities are not available (OIE, 2008). Antigen detection techniques include immunofluorescent staining of impression smears of liver, spleen or brain; RVFV antigen identification after infection by immunoflourescent staining of cover-slip preparations; agar gel immunodiffusion detection of antigen from serum or tissues. Various ELISA formats for antigen detection have been reported (Niklasson *et al*., 1983; Meegan *et al*., 1989; Zaki *et al*., 2006), but were not validated according to current standards, reagents are costly and not easy to prepare and involved a high biohazard risk for laboratory personnel. A sandwich ELISA for antigen detection based on anti-nucleocapsid antibody capture system was designed to detect RVFV using thermo-chemically inactivated specimens (Jansen van Vuren and Paweska, 2009). The ELISA proved to be safe, highly accurate, rapid and robust and could be used in both endemic and non-endemic areas.

1.7 Nucleic acid detection methods

Different types of nucleic acid techniques have been described to detect RVFV genomic material. Although nucleic acid techniques do not detect viable virus (as with virus isolation), it has an advantage in situations where the storage temperature of the

specimen is compromised, resulting in loss of virus infectivity, but presence of its genomic material (Sall *et al*., 2001). Nucleic acid techniques are less time-consuming because results are obtained within a day and may provide information about the genome of the virus with further processing. Nucleic acid techniques are useful where antibodies are absent or antigen-antibody complexes interfere with detection of viral antigens in ELISAs. RT-PCR techniques were developed to detect RVFV RNA in mosquitoes (Ibrahim *et al*., 1997; Jupp *et al*., 2000) and RT-PCR and nested RT-PCR were developed to detect RVFV in human and animal sera (Sall *et al*., 2001; Sall *et al*., 2002). From serial dilutions, the detection limits of RT-PCR was found to be 50 plaque forming units (PFU) and nested RT-PCR was found to be 0.5 PFU (Ibrahim *et al*., 1997; Sall *et al.,* 2001). In the study done by Sall *et al*., (2001), the detection limit of the nested RT-PCR was found to be better than virus isolation and was used to detect RVFV during the Mauritanian outbreak in 1998. A nested PCR for detecting phlebovirus RNA of Sandfly fever Sicilian virus, Sandfly fever Naples virus, Toscanavirus, RVFV, Aguacate virus and Punta Toro virus proved to be useful for surveillance and control of phlebovirus infections (Sánchez-Seco *et al*., 2003). Advantages of the nested RT-PCR compared to HI, CF, VNT and ELISA includes a small volume of test serum, it is easy to perform, rapid and specific and did not show any cross-amplification with closely related phleboviruses.

A RTD-PCR, based on TaqMan® (or hydrolysis) probe technology, was designed by Garcia *et al*., (2001) to detect and quantify RVFV from serum or cells. Most of the previously described PCR detection assays are time-consuming since it requires an additional cDNA synthesis step prior to PCR and agarose gel analysis to confirm the band sizes of the PCR product. A nested PCR increase the risk of false-positive results due to carryover contamination. The RTD-PCR provided results within 3 to 4 hours due to fast temperature transition rates and online analyses of data, meaning product amplification is measured in real-time. The product is also detected in a closed system, reducing chances of contamination. Additional information such as an indication of the concentration of the virus can be detected (higher initial concentration increases more rapidly) and, if external quantitative standards (with known copy numbers) are used

simultaneously, the actual viral genome concentration can be determined, unlike agarose gel analysis results which only give the band size of the PCR product (Read *et al*., 2001). The sensitivity of RTD-PCR was found to be less than 100 RNA copies/run or less than 10 tissue culture infectious dose $(TCID)_{50}$/ ml (Garcia *et al*., 2001). A quantitative field RTD-PCR designed by Njenga *et al*., (2009) showed 100% agreement between RTD-PCR and virus isolation confirming results from Madani *et al*., (2003) in the serum of a RVF patient during the Saudi Arabia outbreak in 2000-2001. The clinical symptoms of different VHFs are very similar. Multiple RTD-PCRs with the same cycling conditions were designed by Drosten *et al.,* (2002) to detect Marburg, Ebola, Lassa, RVFV, Dengue and Yellow fever in one reaction, saving cost and time without compromising the accuracy of the test, but it was not validated. Optimization of multiplex PCRs can be difficult including determination of binding conditions for each primer pair to different target sequences which also must not have non-specific binding activity.

Although nucleic acid techniques such as RT-PCR, nested-PCR and RTD-PCR are less expensive and time consuming than virus isolation and serological methods, the use of thermocyclers and Real-time platforms as well as the high complexity of these molecular techniques still makes them costly for use in routine laboratories. There is an increased demand for rapid, cost-effective, simple methods to be used in the laboratory and field settings.

A thermophilic helicase dependent amplification (tHDA) assay makes use of a thermostable UvrD helicase (Tte-UvrD) enzyme. The energy generated by hydrolysis of nucleoside triphosphate breaks hydrogen bonds linking double-stranded DNA (dsDNA) to form single-stranded DNA (ssDNA) instead of making use of heat. Genome targets are selectively amplified at a constant temperature of 60˚C-65˚C by making use of DNA polymerase (An *et al*., 2005). A reverse-transcription tHDA (RT-tHDA) assay was developed by Goldmeyer *et al*., (2007), based on the tHDA platform, to detect and amplify RNA targets. These assays can be performed using cost-effective equipment

such as heating blocks or waterbaths because of isothermal temperature and results can be obtained within 30 minutes.

Other amplification methods which make use of a combination of RT and isothermal amplification include strand displacement amplification (SDA), nucleic acid sequence-based amplification (NASBA) and loop-mediated isothermal amplification assay (LAMP).

The SDA performed under isothermal conditions, as described by Walker *et al*., (1992), consists of three steps: 1. The target dsDNA within a sample is denatured by heat in the presence of primers and other reagents to form ssDNA; 2. Enzymes such as HincII and exo$^-$ Klenow have strand displacement activity and are added to generate target DNA copies with defined 5' and 3' ends; 3. The reaction is incubated at an isothermal temperature of 37˚C. The target copies of this process are amplified by SDA. The SDA technique can be used to amplify ssDNA or dsDNA.

The NASBA was originally introduced by Compton, (1991) and makes use of the simultaneous activity of three enzymes, each catalyzing a specific reaction: Avian myeloblastosis virus (AMV) reverse transcriptase; Ribonuclease H (RNase H); and T7 DNA dependent RNA polymerase (T7 DdRp). During the "non-cyclic" phase of NASBA, primer 1 anneals to the target RNA sequence, AMV reverse transcriptase uses deoxynucleotide triphosphates (dNTPs) to extend the 3' end of primer 1 to form a cDNA copy of the template. RNase H hydrolyses RNA from the RNA:DNA hybrid to form ssDNA (the original RNA is destroyed). Primer 2 anneals to the ssDNA and uses reverse transcriptase to synthesize the second strand of DNA, forming dsDNA and leading to a transcriptionally active promoter. The T7 DdRp transcribes RNA copies (10-100 copies of RNA from each template molecule) from the transcriptionally active promoter. During the "cyclic" phase of NASBA, each new RNA molecule acts as a template for the binding of primer 2 and, reverse transcriptase extends primer 2 to form a RNA:DNA hybrid. RNase H hydrolysis RNA from the RNA:DNA hybrid, leaving ssDNA. Primer 1 binds to ssDNA and reverse transcriptase synthesizes DNA to form

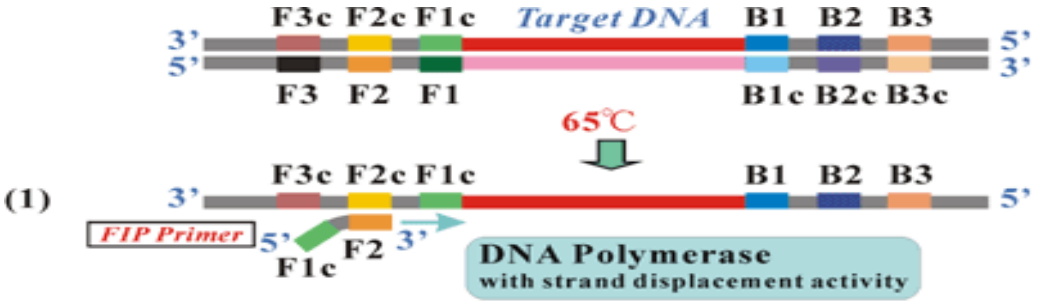
dsDNA, again leading to a transcriptionally active promoter. All of these enzymatic actions occur simultaneously under isothermal conditions at 41˚C (Compton, 1991). NASBA is a sensitive, transcription based amplification system, specifically designed to detect various kinds of RNA: genomic RNA, mRNA, rRNA, viriods as well as ssDNA (Gulliksen *et al*., 2004). Double-stranded DNA has been amplified in NASBA systems, but not very efficiently and only in absence of the RNA target (Deiman *et al*., 2002).

The amplification reaction of NASBA is more complicated than PCR, but uses less expensive equipment than PCR. In PCR assays, the number of amplified molecules doubles with each step of amplification, requiring ± 20 cycles to produce an amplification of one million-fold in 3-4 hours compared to NASBA that amplifies 10-100 copies of RNA from each transcription step and, requires only 4 to 5 cycles to achieve similar amplification results in ± 2 hours (Compton, 1991). The sensitivity of NASBA was found to be similar to nested-PCR for detecting enterovirus RNA (Heim and Schumann, 2002). Gulliksen *et al.,* (2004) added beacon probes to hybridize to the target RNA during amplification which made real-time monitoring of NASBA possible and applied NASBA to a microchip system, displaying results in a real-time graph (similar to RTD-PCR) using isothermal conditions at 41˚C, and producing more than 10^9 copies in 1½ hours. Schneider *et al*., (2005) adapted a quantitative NASBA assay (Tyagi and Kramer, 1996) to real-time quantitative NASBA (QT-NASBA) assay by applying molecular beacon technology and compared it to real-time QT-PCR to detect *P. falciparum* parasites. Both detection assays compared well and displayed similar detection limits of 20 parasites/ml of blood. The amount of extracted nucleic acid required in the QT-NASBA assay was less than in QT-PCR, which makes the sensitivity of QT-NASBA higher than QT-PCR. In the Schneider *et al*., (2005) study, 48 samples where quantified in 4 hours (including RNA extraction) compared to QT-PCR which took 16 hours. The QT-NASBA requires small volumes of samples; finger prick blood collection can be used compared to venous blood collection and filtering of blood samples for real-time QT-PCR. NASBA technology is specific for RNA detection and, therefore, there is no need for extraction of RNA to remove genomic DNA from samples (Schneider *et al*., 2005).

Although techniques such as tHDA, SDA and NASBA are more cost-effective than RT-PCR and RTD-PCR, they are less specific. SDA overcomes these deficiencies by using four primers and isothermal conditions, but increased background due to enzymatic digestion of non-specific DNA in the samples still compromise specificity of the assay (Notomi *et al*., 2000).

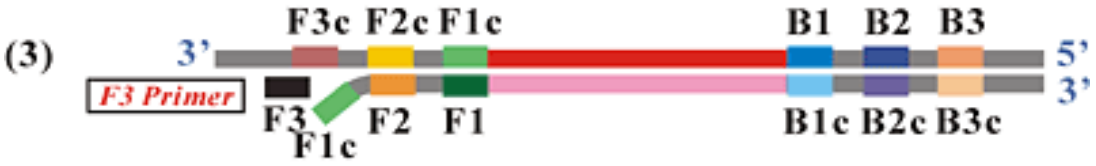
1.7.1 Loop-mediated isothermal amplification assay

LAMP has been shown to be a very rapid, specific and simple nucleic acid detection assay, and overcomes the tHDA, SDA and NASBA shortcomings. It has been successfully used to detect and amplify DNA viruses (Bista *et al*., 2007; Enomoto *et al*., 2005; Ihira *et al*., 2004; Iwata *et al*., 2006; Kaneko *et al*., 2005; Notomi *et al*., 2000; Okamoto *et al*., 2004) and RNA viruses (Mori *et al*., 2006; Okafuji *et al*., 2005; Parida *et al.,* 2006; Parida *et al*., 2007; Thai *et al*., 2004), differentiate viral serotypes and subtypes (Nakagawa and Ito, 2006; Parida *et al*., 2005), identify virus-carrying mosquitoes (Perera *et al.,* 2009), and for the rapid identification of bacterial infections (Iwamoto *et al*., 2003; Wei *et al*., 2008). LAMP amplifies target nucleic acid under isothermal conditions usually between 60˚C and 65˚C, thereby making use of simple, inexpensive equipment such as heating blocks or waterbaths (Notomi *et al*., 2000). RT-LAMP makes use of two enzymes in the reaction: AMV reverse transcriptase for converting target RNA to cDNA and *Bacillus stearothermophilus* (*Bst)* DNA polymerase for autocycling strand displacement DNA synthesis. LAMP relies on four primers (two inner and two outer primers) to recognize six nucleotide sequences on the target RNA or DNA (Notomi *et al*., 2000) and to increase the specificity of the LAMP assay, two loop primers can be added so that eight nucleotide sequences on the target are recognized (Nagamine *et al*., 2002). This makes, LAMP very specific and decreases the detection of background DNA. The amplification process of LAMP consists of 11 steps and is shown in figure 8.



Step 1: In the case of RNA detection and amplification, the RNA is converted to cDNA by the activity of AMV reverse transcriptase in the LAMP reaction. At temperatures between 60˚C and 65˚C, the forward inner primer (FIP: F1cF2) anneals to the complimentary sequence of dsDNA target (F2c region) and initiates DNA synthesis by using *Bst* DNA polymerase with strand displacement activity. Displacement occurs, releasing a single stranded DNA.

Step 2: From the 3' end of the FIP primer, through the activity of *Bst* DNA polymerase, a complementary DNA strand to the template is synthesized, forming a dsDNA.

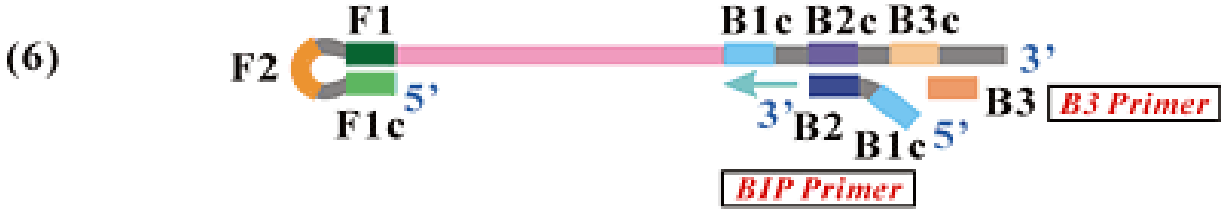


Step3: The forward outer primer (F3) anneals to the F3c region (on the original target DNA), outside of the FIP and initiates strand displacement DNA synthesis, releasing the FIP-linked complementary strand.

FIGURE 8 (Step 1-3): The amplification process of RT-LAMP (adapted from the Eiken Chemical website: *http//loopamp.eiken.co.jp/e/lamp/principle.html).*

Step 4: From the F3 primer (annealed to the F3c region of DNA target), a DNA strand is synthesized to form a dsDNA strand.

Step 5: From the FIP-linked complementary strand that was released in step 3, the F1c and F1 regions share complementary sequences and form a stem-loop structure.



Step 6: The ssDNA containing the stem-loop structure (produced in step 5) serves as a template for the backward inner primer (BIP: B1cB2) initiated DNA synthesis and subsequent backward outer primer (B3)-primed strand displacement DNA synthesis. The BIP primer anneals to the B2C region of DNA (produced in step 5), starting from the 3'end of BIP, and synthesis of complementary DNA takes place, reverting the loop structure into linear DNA. The B3 primer anneals to the outside region of BIP, through the activity of *Bst* DNA polymerase and the DNA synthesized from the BIP is displayed and released as ssDNA strand before DNA synthesis begin from the B3 primer.

FIGURE 8 (continued, step 4-6): The amplification process of RT-LAMP (adapted from the Eiken Chemical website: *http//loopamp.eiken.co.jp/e/lamp/principle.html).*

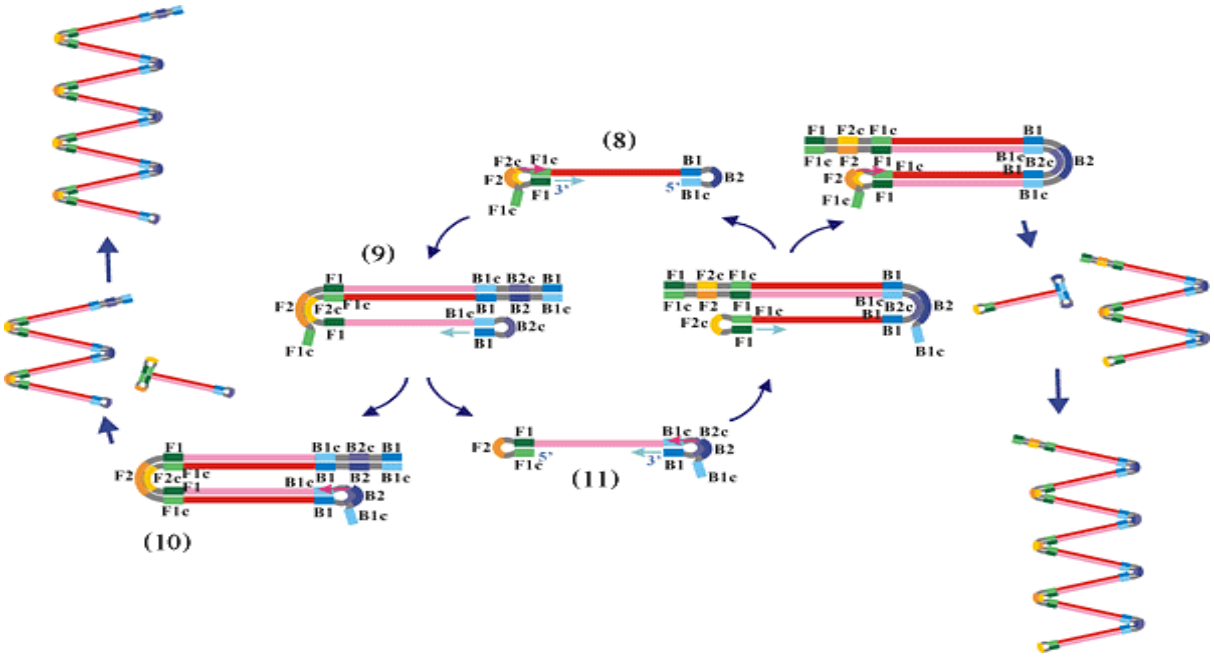
(7)

Step 7: dsDNA is formed.

(8)

Step 8: The BIP-linked complementary strand displayed in step 6, has complementary sequences, B1 and B1c, and forms a "dumbbell-like" structure which serves as the starting structure for the amplification cycle of the LAMP assay.

FIGURE 8 (continued, Step 7-8): The amplification process of RT-LAMP (adapted from the Eiken Chemical website: *http//loopamp.eiken.co.jp/e/lamp/principle.html).*



Step 8 – 11: The “dumbbell-like” structure is converted into stem-loop DNA by self primed DNA synthesis. FIP anneals to the single strand region in the stem-loop DNA and primes strand displacement DNA synthesis, releasing the previously synthesized strand. Because of complementary B1c and B1 regions, a stem-loop structure at the 3’ end of the released strand is formed. Starting from the 3’ end of the B1 region, DNA synthesis starts using self-structure as a template and releases FIP-linked complementary strand (step 9). The released FIP-linked complementary strand has complementary F1-F1c and B1c-B1 regions and forms a “dumbbell-like” structure (step 11) (This structure is the “turn-over” structure formed in step 8). Similar to steps 8 to 11, the structure in step 11 leads to self-primed DNA synthesis starting from the 3’ end of the B1 region. BIP anneals to the B2c region and primes strand displacement DNA synthesis, releasing the B1-primed DNA strand (forming similar structures as in step 9, 10 and 8). From the structure produced in step 10, the BIP anneals to the single strand B2c region, DNA synthesis continues by displacing a dsDNA sequence.

FIGURE 8 (continued, Step 8-11): The amplification process of RT-LAMP (adapted from the Eiken Chemical website: *http//loopamp.eiken.co.jp/e/lamp/principle.html).*

The final products of the LAMP reaction are DNA molecules with a cauliflower-like structure of multiple loops consisting of repeats of the target sequence.

LAMP products can be analyzed using agarose gel analysis, visual inspection under natural or ultraviolet (UV) light or real-time monitoring using a real-time turbidimeter.

Agarose gel analysis displays a typical ladder-like pattern, but due to high copy numbers amplification, the chances of contamination in the laboratory are increased when reaction tubes are opened to load DNA products on to agarose gels.

During DNA polymerization by DNA polymerase in the LAMP reaction, large amounts of pyrophosphate ions are released from dNTPs as by-products. The pyrophosphate ions react with magnesium ions from the reaction buffer to form magnesium pyrophosphate which can be visualized as a white precipitate at the bottom of the reaction tubes for positive LAMP products. The addition of a fluorescent detection reagent (FDR) such as SYBR Green I, which contains a fluorescent metal indicator (calcein) to the pre-LAMP reaction, allows the visualization of positive reactions. The calcein is complexed with manganese ions and remains quenched. Pyrophosphate formed during the LAMP reaction deprives calcein off the manganese ions and “free” calcein leads to emission of fluorescence. An even stronger emission of fluorescence occurs when magnesium ions bind to the “free” calcein (Yoda *et al*., 2007). Thus, visual detection of positive LAMP reactions under natural light result in change from orange to yellow and under UV light, from orange to bright fluorescent green. A negative reaction remains orange (Iwamoto *et al*., 2003; Mori *et al*., 2001; Mori *et al*., 2004).

For quantification of the LAMP assay, real-time monitoring of the LAMP can be performed using a real-time turbidity meter. Amplification results of a few to 10^9 DNA copies in less than an hour have been reported (Notomi *et al*., 2000).

1.8 Justification of the study

RVFV is seen as a highly feared veterinary pathogen and is considered an important zoonotic agent that poses a potential bioterrorism threat (Lim *et al*., 2005; Peters, 2000). RVFV has been shown to be highly infectious by aerosol (Brown *et al*., 1981; Easterday and Murphy., 1962; Miller *et al*., 1962) and have caused numerous laboratory infections (Hoogstraal., *et al*., 1979; McIntosh *et al*., 1980; Smithburn *et al*., 1949). RVFV causes outbreaks all over Africa and has the potential to spread outside of its traditional geographic boundaries. RVFV is easy to grow and maintain in various cell cultures or animal species and can replicate in a range of mosquito vectors (Turrel *et al*., 2008).

There is an increased international demand for validated molecular tools for the rapid laboratory diagnosis of RVF. Since the LAMP technique makes use of simple, inexpensive equipment such as heating blocks or waterbaths at isothermal amplification temperatures, and is relatively simple to use in the laboratory and field setting during outbreak situations. This study focused on the development and validation of the RT-LAMP assay to detect RVFV RNA in a range of clinical specimens.

1.9 Objectives arising from the study

The aim of the study was to develop a rapid and sensitive molecular tool for routine detection of RVFV in clinical specimens, that is cost-effective, simple to perform and could be used both in medical and veterinary laboratories, including its application in the field.

The specific objectives of the study included:

1.9.1 The design and optimization of a RT-LAMP assay for RVFV.

1.9.2 Determine the analytical sensitivity and specificity of the RT-LAMP assay.

1.9.3 Determine the diagnostic accuracy of the assay using a range of different clinical specimens from humans and animals.

CHAPTER 2

MATERIALS AND METHODS

2.1 The design and optimization of a Real-time loop-mediated isothermal amplification assay for Rift Valley fever virus

2.1.1 The design of Real-time loop-mediated isothermal amplification primers to detect Rift Valley fever virus RNA

The oligonucleotide primers for RT-LAMP assay amplification of RVFV were designed based on the complete sequences of segments S, M and L of RVFV strain-548. The nucleotide sequences of this prototype strain of RVFV were retrieved from GenBank (accession numbers DQ380151.1; DQ380206.1 and DQ375403.1), and were aligned with the available sequences of other isolates to identify conserved regions on the RVFV genome using CLUSTALW software version 1.83 (DNA Data Bank of Japan [DDBJ], Japan; http://clustalw.ddbj.nig.ac.jp/top-e.html). A potential target region for each segment was selected from the aligned sequences, and six primers comprising two outer (F3 and B3), two inner (FIP and BIP), and two loop primers (LF and LB) that recognize eight distinct regions on the target sequence (Table 3), were designed for the S segment (primer set S2 and N) and L segment (primer set L3). An additional five primers comprising two outer (F3 and B3), two inner (FIP and BIP), and one loop primer (LB) were designed for the M segment (primer set M2), the S segment (primer set NS) and L segment (primer set L) using LAMP primer software, PrimerExplorer V4 (Eiken Chemical Co., Japan; http://primerexplorer.jp/elamp4.0.0/index.html), as described previously by Notomi *et al.,* (2000).

TABLE 3. Primers designed for the RT-LAMP assay for the S segment (N and NSs genes), M segment (G1, G2 and NSm genes) and L segment (RNA polymerase gene)

Segment	Primer name	Genome position	Length (bp)	Sequences (5'-3')
S (S2)	S2-F3	1351-1369	19	TCATCCCGGGAAGGATTCC
	S2-B3	1568-1586	19	CAGTGGGTCCGAGAGTTTG
	S2-LF	1416-1440	25	GATGATGAAAATGTCGAAAGAAGGC
	S2-LB	1513-1536	24	CCACCATACTGCTTTAAGAGTTCG
	S2-F1c	1443-1462	40	GTGGCAACAAGCCCAGGAGG
	S2-F2	1389-1408		GTTGATGAGAGCCTCCACAG
	S2-B1c	1492-1512	40	GCATCCTTCTCCCAGTCAGCC
	S2-B2	1542-1560		AGGGTTTGATGCCCGTAGA
S (N)	N-F3	1120-1239	20	TCATATGCCTCGGGTATGCA
	N-B3	1433-1452	20	GCCCAGGAGGATGATGATGA
	N-LF	1272-1291	20	TCTTGAGTGAGTGGCTTCCT
	N-LB	1377-1401	25	TAGCTTATACTTGTTGATGAGAGCC
	N-F1c	1305-1324	40	CCTTGGCTGGCTGGACATGC
	N-F2	1251-1271		TCCATGGTAGTCCCAGTGAC
	N-B1c	1352-1373	42	CATCCCGGGAAGGATTCCCTTC
	N-B2	1403-1422		AGAAGGCAAAGCAACTGTGG

TABLE 3 (continued). Primers designed for the RT-LAMP assay of the S segment (N and NSs genes), M segment (G1, G2 and NSm genes) and L segment (RNA polymerase gene)

Segment	Primer name	Genome position	Length (bp)	Sequences (5'-3')
S (NS)	NS-F3	557-574	18	ATTGACCTGTGCCTGTTG
	NS-B3	735-754	20	TGGAATCCAGTTGTTTCTCC
	NS-LB	699-715	17	GCCTGATGCTGCGCTCA
	NS-F1c	622-642	43	TCTTTTGCTGCTTGCAGAAGC
	NS-F2	582-603		TTGATCTCATGTATGAGGTTGC
	NS-B1c	652-681	42	TAATGCTGTAGTTCCAAACTCAGCCA
	NS-B2	716-734		CATGCTGGGAAGTGA
M (M2)	M2-F3	2201-2220	20	GGCAGAGGCTTGTTTGATGT
	M2-B3	2379-2397	19	CCCTCCAAGACAGACACCT
	M2-LB	2328-2351	24	AAATGTTTGAGCTCAAGGAGATGC
	M2-F1c	2281-2302	41	CTCTGGCCCTCCCTGCATGATA
	M2-F2	2221-2239		TGAAGGGGGTCAAGGAAGA
	M2-B1c	2306-2327	40	TTGGACTGGGTCCTTTAGCCCT
	M2-B2	2352-2369		GCATTCCCCGACAAGGTG

TABLE 3 (continued). Primers designed for the RT-LAMP assay of the S segment (N and NSs genes), M segment (G1, G2 and NSm genes) and L segment (RNA polymerase gene)

Segment	Primer name	Genome position	Length (bp)	Sequences (5'-3')
L(L)	L-F3	2330-2347	18	CGTCCCTTTCTGGGATGT
	L-B3	2496-2517	22	CTCCATGAAATTCTGTCCATAG
	L-LB	2449-2470	22	TACTTGAAGGAGGCTTGCAAT
	L-F1c	2392-2414	46	GGATCTTTGTAACCCAAGAAGGC
	L-F2	2352-2374		GAAAATCATAGAACTTGAGCACC
	L-B1c	2426-2448	41	GAATGCATGAGTTCAGTGTTTCC
	L-B2	2478-2495		AGGCTCCTCAAGACTAGC
L(L3)	L3-F3	4081-4100	20	TGGGGATCTAGGAAGAAGTT
	L3-B3	4269-4289	21	GAGGCCATGACTTTACAAACT
	L3-LF	4123-4147	25	GTTCAATCCAGTTCTCTGGTAGTT
	L3-LB	4212-4234	23	CATTGCAGAGAAAGTCCATAGCC
	L3-F1c	4149-4172	44	AGCACCTCTGGATTCTCATTTATT
	L3-F2	4100-4121		CAGAAATTGAGAGACCGTTT
	L3-B1c	4186-4207	41	AGAACAGGCCCAGAAATATTGT
	L3-B2	4238-4256		GACAATGATGACACAACAC

2.1.2 Optimization of Real-time loop-mediated isothermal amplification primers and assay conditions

To determine the optimal incubation temperature for each primer set (S2, N, NS, M2, L and L3) used in the RVF RT-LAMP assay, RNA was extracted from (SPU 22/07 0121) a Kenya strain of RVFV and 10 pg of RNA was used per reaction for assay optimisation. Negative human serum and tissue culture supernatant were used as controls. The assays were run using the Loopamp® RNA Amplification kit (Eiken Chemical Co., Ltd. Tokyo, Japan) as described by the manufacturer, at temperatures ranging from 60ºC - 65ºC. To determine the optimal primer set for the assay, ten-fold serial dilutions of RNA prepared from infectious tissue culture supernatant containing $10^{6.8}$ $TCID_{50}$ /ml of the AR20368 RSA 81 isolate of RVFV were tested in duplicate.

2.2. Analytical sensitivity and specificity

2.2.1 Cell culture and viruses

The analytical sensitivity of RT-LAMP was evaluated by testing 17 RVFV isolates, representing a wide geographic and phylogenetic spectrum of isolates recovered in the field over a period of the last 50 years (1944-2007) (Table 4). Five selected isolates representing the phylogenetic spectrum of RVFV, namely West African, Egyptian and Central, Eastern and Southern Africa (Table 4), were tested in the range 0.0065 – 65 000 $TCID_{50}$ per reaction volume. The analytical specificity of RT-LAMP was evaluated by testing 6 additional African phleboviruses antigenically and genetically related to RVFV: Akabane ($10^{6.8}$ $TCID_{50}$/ml); Bunyamwera ($10^{7.8}$ $TCID_{50}$/ml); Gabek Forest ($10^{7.0}$ $TCID_{50}$/ml), Saint Floris ($10^{5.8}$ $TCID_{50}$/ml), Arumowot ($10^{4.8}$ $TCID_{50}$/ml), Gordil ($10^{5.8}$ $TCID_{50}$/ml), and six other unrelated arboviruses: yellow fever ($10^{6.0}$ $TCID_{50}$/ml), dengue I ($10^{5.5}$ $TCID_{50}$/ml), West Nile virus lineage I ($10^{7.8}$ $TCID_{50}$/ml), West Nile virus lineage II ($10^{6.0}$ $TCID_{50}$/ml), chikungunya ($10^{7.5}$ $TCID_{50}$/ml) and CCHF ($10^{4.3}$ $TCID_{50}$/ml). All of the virus isolates were obtained from the Special Pathogens Unit of the National Institute for Communicable Diseases of the National Health Laboratory Service, South Africa (SPU-NICD/NHLS). The viruses were amplified either in Vero cells (CCL-81) or in suckling mice using standard procedures (OIE, 2008).

TABLE 4: Identification, year of isolation, source, and origin of RVFV isolates

Identification	Year isolation	Source	Origin	Genetic Lineage	Log_{10} $TCID_{50}$ Titer/ml
SNS UGA44	1944	Smithburn strain	Uganda	C[a]	$10^{5.2}$
Lunyo UGA55	1955	Mosquito	Uganda	C	$10^{6.3}$
ArB1976CAR69	1969	Mosquito	*CAR	C	$10^{6.8}$
VRL2373ZI[e]	1974	Bovine	Zimbabwe	C	$10^{6.3}$
H1825RSA75	1975	Human	South Africa	C	$10^{7.0}$
B1143KEN77	1977	Human	Kenya	C	$10^{6.8}$
ZH501EGY77[d]	1977	Human	Egypt	E[b]	$10^{5.8}$
ZH548EGY77	1977	Human	Egypt	E	$10^{6.3}$
VRL1187ZIM78	1978	Bovine	Zimbabwe	C	$10^{7.0}$
Ar811MAD79	1979	Mosquito	Madagascar	E	$10^{6.3}$
Ar20368RSA81[d]	1981	Mosquito	South Africa	C	$10^{6.8}$
ArD38388BF83	1983	Mosquito	Burkino Faso	W[c]	$10^{5.8}$
ArD38661SEN83[d]	1983	Mosquito	Senegal	W	$10^{7.3}$
R1662CAR85[d]	1985	Human	CAR	C	$10^{6.5}$
SPU384001KEN97	1997	Human	Kenya	C	$10^{7.3}$
Ar2199SA00	2000	Mosquito	Saudi Arabia	C	$10^{7.0}$
SPU22.118KEN07[d]	2007	Human	Kenya	C	$10^{6.8}$

[a] Southern, eastern and central African lineage; [b] Egyptian lineage; [c] West African lineage; [d] Strains selected for determination of analytical detection limit; [e] Strain selected for restriction endonuclease digestion with *Bst XI*; * CAR-Central African Republic.

2.3 Diagnostic accuracy of Rift Valley fever Real-time loop-mediated isothermal amplification assay

Serial bleed samples (n = 20) taken from two sheep from day 0 to day 12 after experimental infection with the Kenyan 2007 isolate (SPU22/07) of RVFV were tested, including serum derived from clotted blood, and plasma (n = 6) collected in ethylene diamine tetraacetic acid (EDTA) as well as in heparin blood collection tubes.

In addition, routine diagnostic submissions received by the SPU-NICD in 2006-2008 from suspected RVF human (n = 65) and animal (n =3) cases in East- and Southern Africa, were used: including serum, liver and kidney tissue specimens.

2.4 Virus titration and isolation

Virus titration of infected tissue culture supernatants and sera from experimentally infected sheep were performed as described previously (Swanepoel *et al.,* 1986a) with minor modifications. Briefly sera were diluted 10-fold in Eagle's minimum essential medium (EMEM, BioWhittaker, USA) containing 100 IU penicillin, 100 µg streptomycin and 0.25 µg amphotericin B (BioWhittaker, USA). Four replicates of each 100 µl dilution (from 10^{-1} to 10^{-7}) were transferred into flat bottom 96-well cell culture microplates (Nunc, Denmark), and equal volumes of Vero cell suspension in EMEM containing 2 x 10^5 cells/ml, 8% fetal bovine serum/ml (Gibco, Invitrogen, USA), and antibiotics were added. Inoculated microplates were incubated at 37°C in a CO_2 incubator and observed microscopically for cytopathic effects for 10 days post-inoculation. Virus titers, calculated by the Kärber method (Kärber, 1931), were expressed as $TCID_{50}$/ per ml of sample. The limit of detection was 0.75 $\log_{10}$ $TCID_{50}$ per volume of sample tested. Isolations of RVFV from clinical specimens were done either by inoculation of 48 hour old monolayers of Vero cells or intracranial inoculation in suckling mice using standard laboratory procedures (OIE, 2008).

2.5 RNA extraction

Animal tissues were homogenized to prepare 10% (wt/vol) suspensions in EMEM, supplemented with antibiotics as described in section 2.4 After centrifugation at 3 000 x *g*, supernatants free of cells were harvested and stored at -70°C until used. Genomic

viral RNA was extracted from 140 µl of infected tissue culture supernatants, liver and kidney tissue supernatants or sera using the QIAamp® Viral RNA Mini kit (QIAGEN, Germany) in accordance with the manufacturer's protocol. The extracted RNA was eluted in a total volume of 60 µl of AVE Buffer and stored at -70 ˚C until use.

2.6 TaqMan Real-time detection PCR

The TaqMan-RTD PCR assay was performed using the LightCycler® RNA Amplification kit HybProbe (Roche Diagnostics, Germany) and the LightCycler V1.5 platform (Roche Diagnostics, Germany). Amplifications were carried out in 20µl reaction mixtures containing 5 µl of the target virus RNA or the *in vitro* transcribed RNA standard, 1 µM each of the sense and antisense primers and 5mM $MgCl_2$. Cycling profiles, primers (RVS and RVA) and a TaqMan probe (RVP) targeting a region of the G2 glycoprotein as described by Drosten *et al*. (2002) were used.

2.7 Real-time loop-mediated isothermal amplification assay

The RT-LAMP assay was carried out in a final reaction volume of 25 µl using the Loopamp® RNA Amplification kit (Eiken Chemical Co., Ltd. Tokyo, Japan) with 5 pmol each of the primers F3 and B3, 20 pmol each of the primers LF and LB (except for reactions using primer sets NS, M2 and L which only used the LB primer), 40 pmol each of the primers FIP and BIP. Five µl of the extracted RNA was used as template per reaction. For real-time monitoring, the RT-LAMP reactions were incubated as follows: S(S2) primer set at 62˚C, S (N) primer set at 65˚C, S (NS) primer set at 65˚C, M (M2) primer set at 62.5˚C, L (L) primer set 65˚C and L (L3) primer set at 61˚C. All the reactions were incubated for 60 minutes on a LA-200 Loopamp® Real-time Turbidimeter (Teramecs, Japan) and inactivated at 80˚C for 5 minutes. Positive (addition of known positive specimen) and negative (addition of nuclease free water only) controls were included in each run of the assay.

2.8 Analysis of Real-time loop-mediated isothermal amplification assay

2.8.1 Real-time monitoring

A real-time turbidimeter (Eiken, Japan) was used to monitor the accumulation of magnesium pyrophospate every six seconds spectrophotometrically at 400 nm. The cut-off value for positive samples was determined when the turbidity increased above the threshold value fixed at 0.1 over time. Results were analyzed using the LA-200E software package (Teramecs, Japan).

2.8.2 Agarose gel analysis

Five µl of the RT-LAMP products were electrophoresed on a 2% molecular grade agarose gel prepared in 0.5 x Tris-borate EDTA buffer stained with 0.5 µg/ml ethidium bromide. A 100 Bp marker (Promega, Madison WI, U.S.A) was used.
The amplification products were visualized using a transilluminator (Spectroline TC-302) with ultraviolet light at 302 nm.

2.8.3 Visualization by naked eye

Reaction tubes were pulse centrifuged to deposit the magnesium pyrophosphate (white precipitate) at the bottom of the tube, thereby detecting amplification by naked eye.
Alternatively, 1 µl of FDR (Fluorescent detection reagent, Eiken Chemical Co., Ltd. Tokyo, Japan) was added to the LAMP reaction. For a positive reaction, the orange solution changed to yellow under natural light while under UV irradiation fluorescence or green fluorescence was noted. Negative reaction remained the orange colour of the unbound dye. The colour change could be observed by naked eye or with the aid of ultraviolet light at 302 nm and the results captured photographically.

2.8.4 Restriction endonuclease digestion

Amplification products were digested with *Bst XI* (Roche, Germany) as suggested by the manufacturer and analyzed on a 2% agarose gel as described in 2.8.2.

2.9 *In vitro* transcription and quantification

To obtain a quantitative RNA standard, the diagnostic target region was amplified utilizing a standard RT-PCR and RVFV RNA prepared from infected tissue culture supernatant and transcribed *in vitro*. The primers for the RVF RTD-PCR (RVS, RVA) were designed based on the G2 region of the M-segment and the RVF RT-LAMP outer primers (L3 primer set) were designed based on the L segment, and were used respectively to generate an amplicon representative of the RTD-PCR or LAMP targets. The target region PCR product was cloned into T7/SP6 polymerase expression vector, pCRII-TOPO (Invitrogen, USA), according to the manufacturer's instructions. In order to enhance transcription from the template, the insert was amplified with vector specific, universal M13 primers using standard PCR conditions. The PCR products were purified using the Wizard® SV Gel and PCR Clean Up System (Promega, USA), transcribed a *in vitro* and digested with DNase using the MegaScript SP6 kit (Ambion, USA) according to the manufacturer's protocol. The RNA was purified according to the instructions of the RNeasy Protect kit (QIAGEN, Germany), and quantified spectrophotometrically. Target RNA copy number was calculated and serial dilutions ranging from 10^0-10^6 RNA copies were used to determine the range of quantification.

2.10 Comparison of the analytical sensitivity of Real-time loop-mediated isothermal amplification assay and TaqMan Real-time detection PCR

To compare the analytical sensitivity of RT-LAMP and TaqMan RTD-PCR in detection of decreasing number of RNA copies, ten-fold dilution series of RNA standard, ranging from 10^0 to 10^6 per reaction, were tested in six separate runs of each assay. The coefficient of determination (R^2) and the Fisher test (F-test) (Fisher, 1925) were used to determine if the difference in the results were statistically significant.

CHAPTER 3

RESULTS

3.1 Optimization of the primers and Rift Valley fever Real-time loop-mediated isothermal amplification assay conditions

The optimal incubation temperature for the RVF RT-LAMP assay was determined for each primer set. This was achieved by testing detection of 10pg of RNA (extracted from SPU 22/07) with different primer sets at a range of incubation temperatures. (Table 5).

TABLE 5: Optimal incubation temperatures determined for the RVF RT-LAMP assay

Primer set	Optimal incubation temperature (°C)	Incubation time (min)	Inactivation temperature (°C)	Inactivation time (min)
S2	62	60	80	5
N	65	60	80	5
NS	65	60	80	5
M2	62.5	60	80	5
L	65	60	80	5
L3	61	60	80	5

Once the optimal incubation temperature for each of the different primer sets was determined for the RVF RT-LAMP assay, the sensitivity of each assay was evaluated in order to determine the optimal primer set for the RVF RT-LAMP assay. Ten-fold serial dilutions of RNA prepared from infective tissue culture supernatant containing $10^{6.8}$ $TCID_{50}$ /ml of AR20368 RSA 81 isolate of RVFV was used in each assay. Detection limits for each assay are given in Table 6.

TABLE 6: The sensitivity of the RVF RT-LAMP assay using different primer sets determined by comparing ten-fold serial dilutions of the AR20368, RSA 81 strain of RVFV

Primer set	Detection limit ($TCID_{50}$ per reaction volume)
S2	0.65
N	0.65
NS	6.5
M2	65
L	0.65
L3	0.065

The L3 primer set was the most sensitive with a detection limit of 0.065 $TCID_{50}$ per reaction volume with no non-specific amplification. A Tp value (time of positivity in minutes at which the turbidity increases above the threshold value fixed at 0.1) of ≤ 45 minutes and turbidity above the threshold value of ≥0.1 was considered a positive result (Notomi *et al*., 2000). Real-time monitoring of turbidity allowed the detection of amplification products as early as 16 minutes after reaction initiation, with the majority of positive specimens detected in less than 30 minutes.

3.2 Analytical sensitivity of Real-time loop-mediated isothermal amplification assay

The sensitivity of the RT-LAMP assay was compared with a TaqMan RTD-PCR by testing ten-fold serial dilutions of RNA prepared from infective tissue culture supernatant containing $10^{6.8}$ $TCID_{50}$/ml of AR20368 RSA 81 isolate of RVFV. Both assays were equally sensitive with a detection limit of 0.065 $TCID_{50}$ units per reaction volume (Figure 9 A & B).

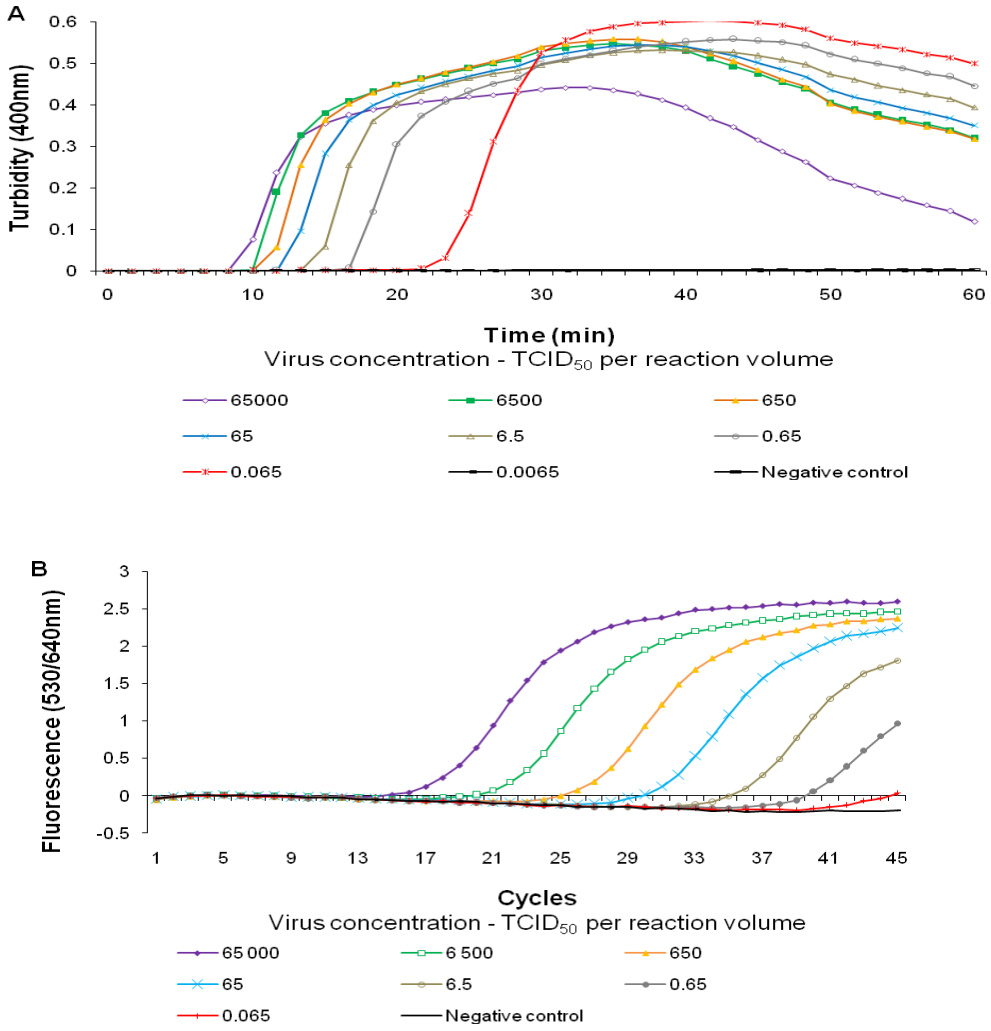
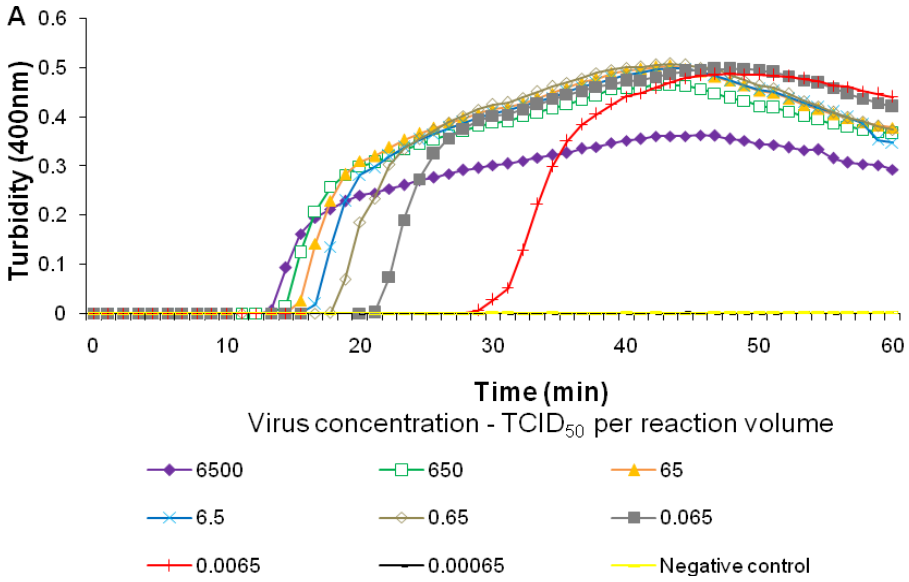


FIGURE 9: Real-time kinetics of RT-LAMP (A), and TaqMan RTD-PCR (B), in ten-fold serial dilutions of RNA prepared from infective tissue culture supernatant containing $10^{6.8}$ $TCID_{50}$/ml of the AR20368 RSA 81 isolate of RVFV.

The RT-LAMP assay detection limit of 0.065 $TCID_{50}$ units per reaction volume was further confirmed by testing ten-fold serial dilutions of four additional strains of RVFV (Table 4) representing the phylogenetic spectrum of the virus (Figure 10 A, B, C & D).



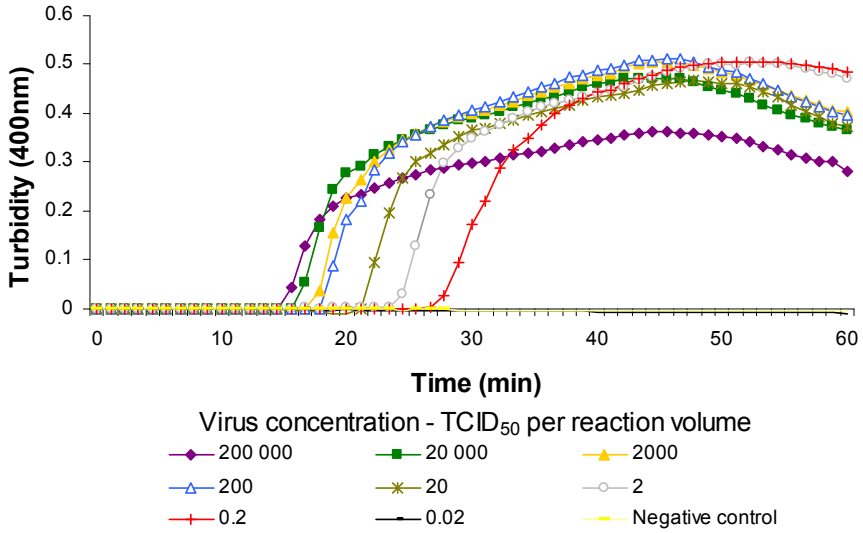


FIGURE 10 (A and B): Real-time kinetics of RT-LAMP in ten-fold serial dilutions of RNA prepared from infective tissue culture supernatant containing A: $10^{5.8}$ $TCID_{50}$/ml of ZH501EGY77 and B: $10^{7.3}$ $TCID_{50}$/ml of ArD38661SEN83.

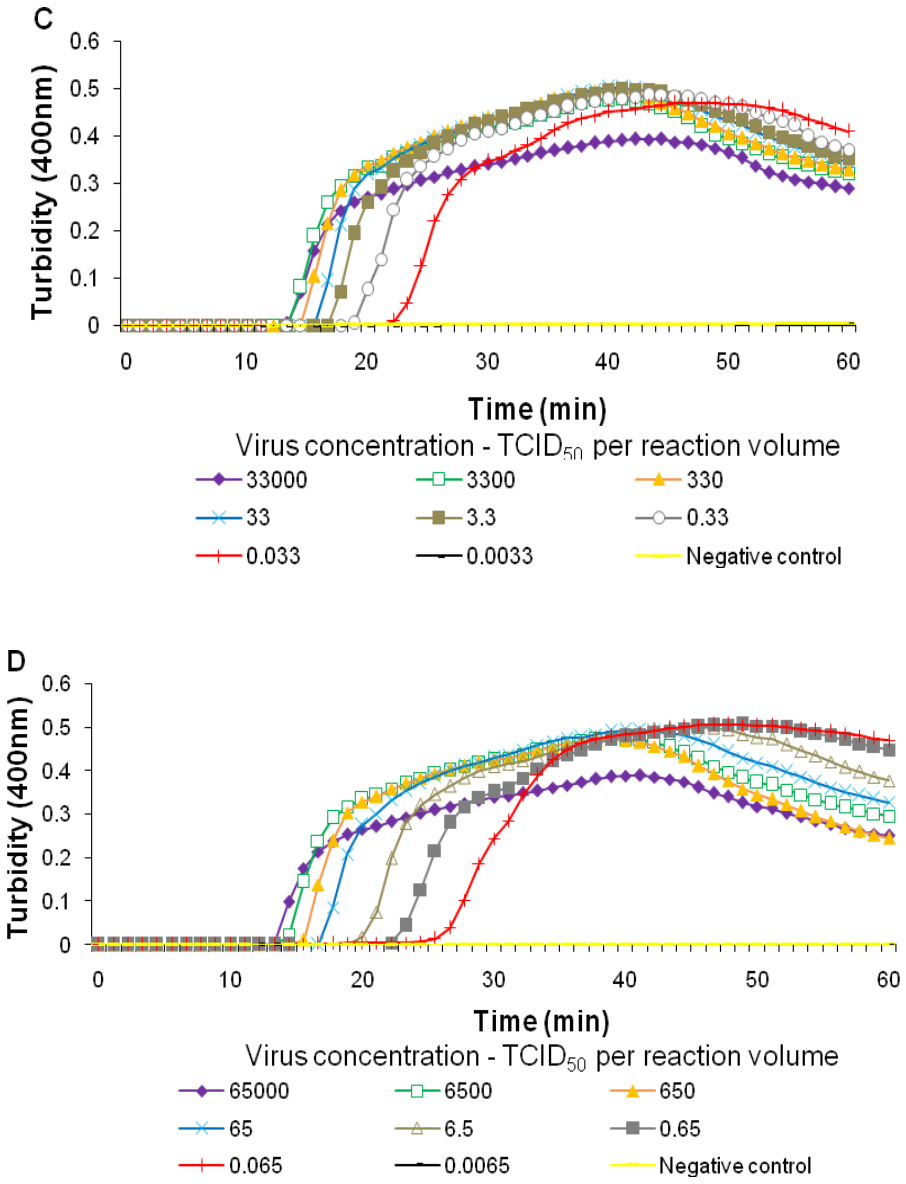


FIGURE 10 (C and D): Real-time kinetics of RT-LAMP in ten-fold serial dilutions of RNA prepared from infective tissue culture supernatant containing C: $10^{6.5}$ $TCID_{50}$/ml of R1662CAR85 and D: $10^{6.8}$ $TCID_{50}$/ml of SPU22.118KEN07.

The RVF RT-LAMP assay gave the following detection limits when tested with the different strains: ZH501EGY77: 0.0065 $TCID_{50}$/reaction volume; ArD38661SEN83: 0.2 $TCID_{50}$/reaction volume; R1662CAR85: 0.033 $TCID_{50}$/reaction volume and SPU22.118KEN07: 0.065 $TCID_{50}$/reaction volume. High analytical sensitivity of TaqMan

RTD-PCR and RT-LAMP was also demonstrated by measuring decreasing number of RNA copies. RNA standard dose-response curves for both assays were very similar (R^2 of 0.8386 and 0.8042, respectively) and had the expected characteristic slope (Figure 11). The borderline analytical detection limit, as measured by number of RNA copies per reaction, was 10 copies for both assays. During six runs, TaqMan RTD-PCR detected 10 RNA copies/reaction on four occasions (66.7 %), and the RT-LAMP on three occasions (50%); however, this difference was statistically not significant (P = 1.0000). Both assays had 100% sensitivity in detecting ≥100 RNA copies/per reaction (Figure 11). Despite distinct geographic and historic origins, all the RVFV isolates tested in this study were easily detectable by the RT-LAMP assay (Table 4), further confirming its high analytical sensitivity.

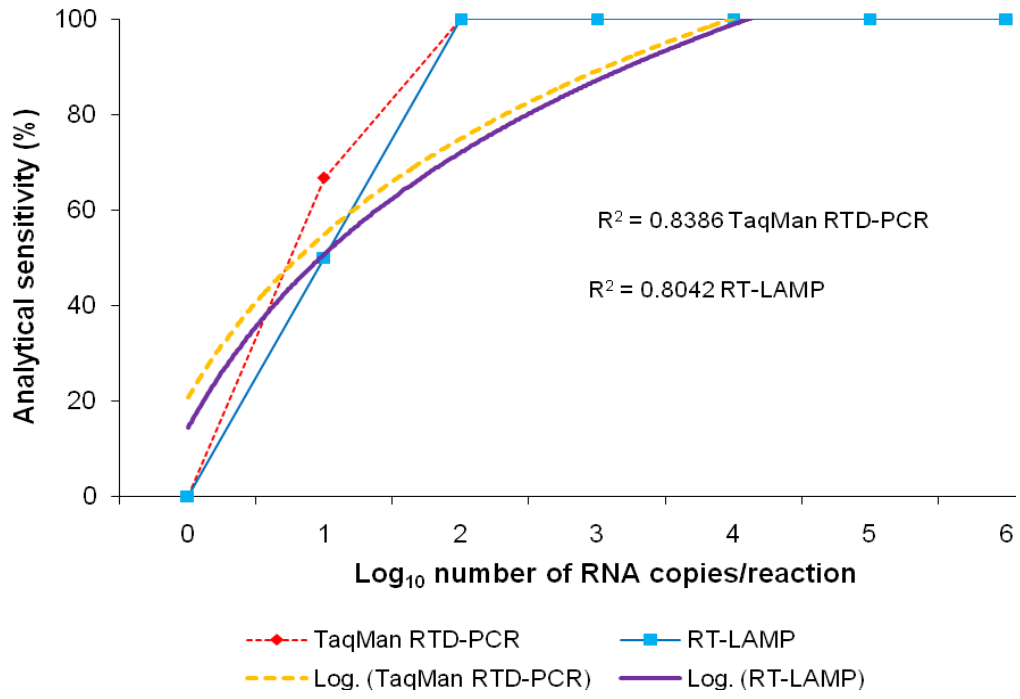


FIGURE 11: Analytical sensitivity of RT-LAMP and TaqMan RTD-PCR as measured by the detection of number of RNA copies of the target. Data were derived from testing ten-fold dilution series of RNA standard during six runs of both assays. Logarithmic trend lines are added and the coefficient of determination (R^2) calculated to determine the strength of linear data sets.

3.3 Analytical specificity of Real-time loop-mediated isothermal amplification assay

High analytical specificity of the assay was further confirmed by the absence of amplification products when using the RVFV specific L3 primer set with RNA extracted from highly concentrated stocks of six African phleboviruses related to RVFV, and six other unrelated arboviruses (results not shown). In addition, the specificity of the RT-LAMP amplification product was confirmed by restriction endonuclease digestion of VRL2373ZI (Table 4) with *Bst XI*, resulting in a product with the expected size of 209 bp as shown in figure 12.

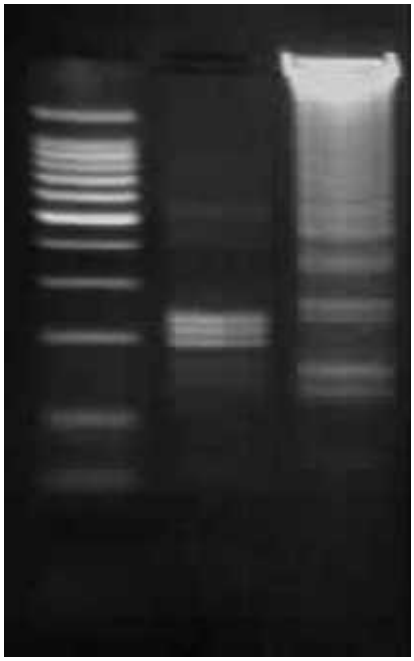


FIGURE 12: Restriction endonuclease digestion of the RT-LAMP product of VRL2373ZI with *Bst XI*. Lane 1: 100 bp marker; Lane 2: VRL2373ZI LAMP product – cut, resulting in a 209 bp product; Lane 3: VRL2373ZI LAMP product – uncut.

The red arrow is pointing at the middle band (209Bp) of the three bands. My hypotheses is that the other bands might be where the enzyme site was “masked” due to fold-over

structures/cauliflower-like structures that were produced during the RT-LAMP amplification assay as end-products, thus the enzyme was not able to cut these inverted repeats. We did expect a 209Bp band size when VRL2373ZI was cut with *BstXI,* thus our results confirm that it was VRL2373ZI that was amplified during the RT-LAMP assay to produce a corresponding LAMP product band size.

Alternative detection methods included agarose gel electrophoresis of the RT-LAMP products which displayed the typical ladder-like pattern (figure 13) as well as FDR detection of amplification (figure 14). The fluorescent dye became yellow in positive samples in natural light (figure 14A), and with UV irradiation, showed deep green fluorescence (figure 14B).

FIGURE 13: Agarose gel electrophoresis profile of RT-LAMP products in serially diluted RNA of RVFV. Lane M, 100 bp molecular weight marker; Lanes 1 to 8, RT-LAMP products yielded from ten-fold serial dilutions of RNA prepared from infective tissue culture supernatant containing $10^{6.8}$ $TCID_{50}$/ml of AR20368 RSA 81 isolate of RVFV. Lanes 1 to 7: from left to right: 65 000 $TCID_{50}$ to 0.065 $TCID_{50}$ /reaction volumes; Lane 8, 0.0065 $TCID_{50}$ /reaction volume – not detected. Lane 9, negative control.

Form these results, one would expect to see a decrease in brightness of the LAMP product (serial dilution made from 65 000 $TCID_{50}$ to 0.065 $TCID_{50}$/reaction volume), but instead we see an increase in brightness. My hypotheses is that these results are an example of an inverted-dilution effect: the reaction starts off with a high concentration of RNA, 65 000 $TCID_{50}$/reaction volume (figure 13, lane 1), the RT-LAMP reagents are depleted early during the amplification assay due to the high concentration of RNA and the exponential curve reaches its plateau very rapidly (see also figure 10 A, B, C and D), amplifying less LAMP product than when a lower concentration of RNA is used. When lower concentrations of RNA is used, for instance 0.065 $TCID_{50}$/reaction volume (figure 13, lane 7), the RT-LAMP reagents are not depleted so quickly, the amplification reaction takes longer in time and the exponential curve reaches a higher plateau (Figure 10 A, B, C and D), thus there are more amplification products made and this are displayed as brighter ladder-like pattern (figure 13, lane 7) than in for instance figure 13, lane 1. In figure 13, lane 8, there was no RVFV RNA detected and thus the sudden cut-off. Thus is expected, because LAMP is very specific, the amplification product is either displayed (if the RNA can be detected by the LAMP primers), or if the primers cannot detect the RNA, no amplification will take place and no LAMP product will be displayed.

FIGURE 14: Visual detection of RT-LAMP amplification products in ten-fold dilutions of RVFV RNA. Tube 1 to 8 from left to right, RT-LAMP products yielded from ten-fold serial dilutions of RNA prepared from infective tissue culture supernatant containing $10^{6.8}$ $TCID_{50}$/ml of AR20368 RSA 81 isolate of RVFV; Tube 9, negative control. Detection of the white precipitate (magnesium pyrophosphate) at the bottom of the tubes indicates a positive reaction, while the absence of precipitate indicates a negative reaction. After addition of FDR to the reaction mixture, fluorescence is detectable by the naked eye as a yellow color, while an orange color indicates a negative reaction (A); under UV irradiation, a positive reaction is indicated by a fluorescent bright green color (B).

3.4 Diagnostic accuracy of Real-time loop-mediated isothermal amplification assay

Results of viremia monitoring by RT-LAMP, TaqMan RTD-PCR and virus titration in two sheep experimentally infected with wild type isolate of RVFV are shown in Table 7. All three assays yielded negative results in sera collected before challenge. Of the sera collected on a daily basis post infection, 10 tested positive and 10 tested negative in all assays (Table 7). Results of viremia monitoring in experimentally infected sheep are shown in figure 15 A and figure 15 B. Both molecular assays yielded the same results in experimental sheep plasma collected either in EDTA or heparin, but there was a slight delay in the detection of RNA extracted from heparin treated blood by the RT-LAMP assay.

TABLE 7: Monitoring of viremia in sheep experimentally infected with wild-type RVFV by RT-LAMP, TaqMan RTD-PCR and virus titration

Experimental animal[a]	Day(s) after challenge	Result of: RT-LAMP[b]	Result of: TaqMan RTD-PCR[b]	Virus titer[c]
Sheep 1	0	-	-	
	1	+	+	4.75
	2	+	+	7.75
	3	+	+	7.0
	4	+	+	5.5
	5	+	+	4.5
	6	+	+	2.5
	9, 12	-	-	
Sheep 2	0	-	-	
	1	+	+	3.65
	2	+	+	6.25
	3	+	+	4.75
	4	+	+	1.0
	5-10	-	-	

[a] Sheep experimentally infected with the SPU22/07 Kenyan 2007 isolate of RVFV.
[b] -, Negative test result; +, Positive test result.
[c] Log_{10} $TCID_{50}$/ml of serum.

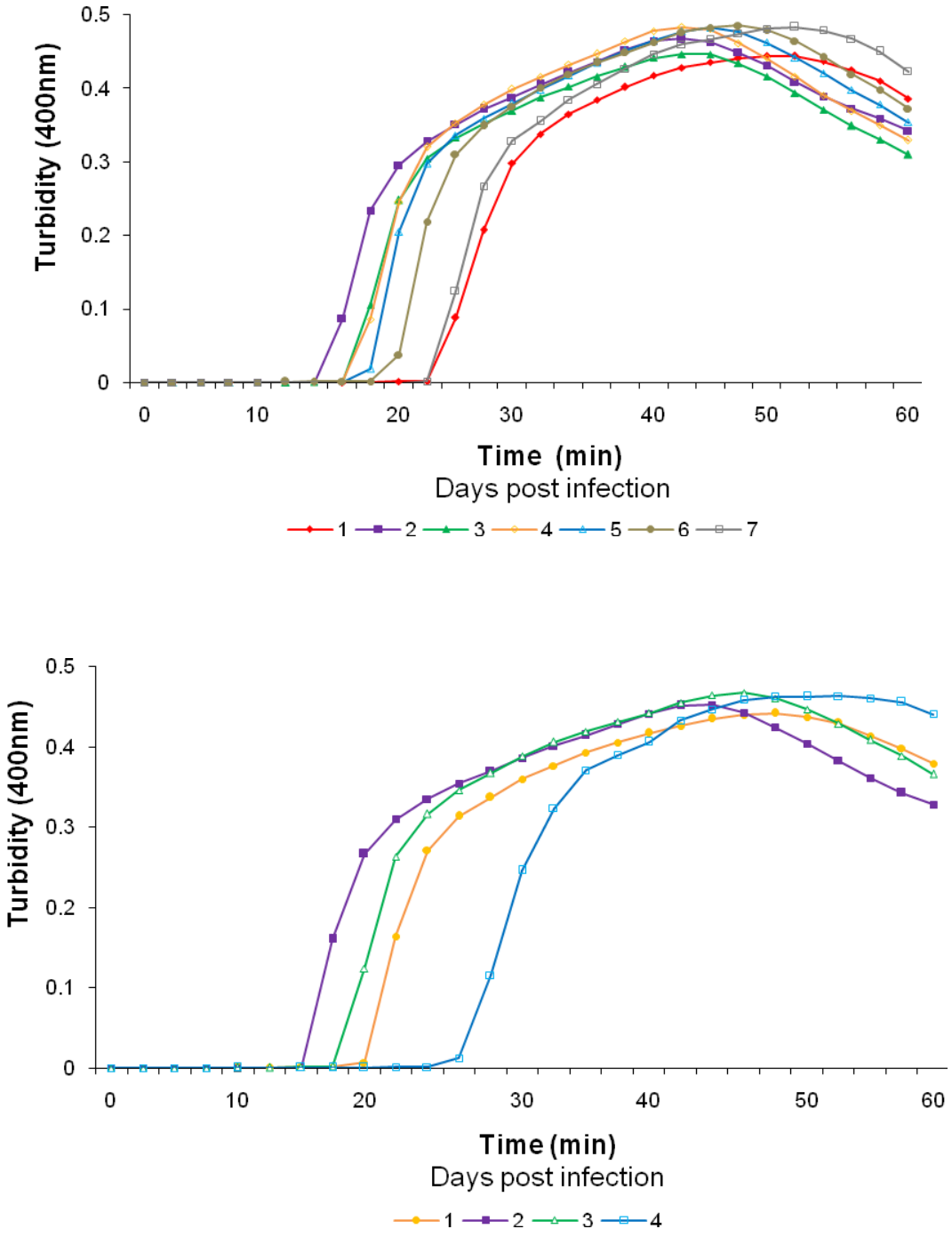


FIGURE 15: Monitoring of viremia in two sheep experimentally infected with wild-type RVFV by the RT-LAMP assay. A: Sheep 1; B: Sheep 2.

In a total of 32 human serum specimens which tested positive by virus isolation, all but one was positive by the RT-LAMP assay and TaqMan RTD-PCR. In addition, one human serum that tested negative by virus isolation yielded positive results in the two molecular assays (Table 8). There was 100% agreement between the RT-LAMP, TaqMan RTD-PCR and virus isolation results in detecting RVFV in liver and kidney tissues collected from 3 aborted buffalo foetuses (Table 8).

TABLE 8: Comparison of RT-LAMP, TaqMan RTD-PCR and virus isolation results for the detection of RVFV in various clinical specimens

			No. of specimens with indicated RT-LAMP, RTD-PCR, and virus isolation Results			
Type of specimen	Source tested	No. of samples	+,+, +[a]	-,-, -[b]	+,+, -[c]	-,-, +[d]
Serum[e]	Sheep	20	10	10	0	0
Plasma[e]	Sheep	6	6	0	0	0
Serum[f]	Human	65	31	32	1	1
Liver[f]	Buffalo[g]	3	3	0	0	0
Kidney[f]	Buffalo[g]	3	3	0	0	0
Total		97	53	42	1	1

[a] No. of specimens that tested positive (+) in all tests.

[b] No. of specimens that tested negative (-) in all tests.

[c] No. of specimens that tested positive by RT-LAMP and RTD-PCR, but negative for virus isolation.

[d] No. of specimens that tested negative by RT-LAMP and RTD-PCR, but positive for virus isolation.

[e] Specimens taken from sheep experimentally infected with wild-type RVFV.

[f] Specimens collected from suspected human and animal RVF cases during the disease outbreaks in Africa in 2006 to 2008.

[g] Aborted foetuses.

CHAPTER 4

DISCUSSION AND CONCLUDING REMARKS

4.1 Discussion

RVFV belongs to the group of RNA VHF agents, including Ebola-, Marburg-, Lassa- and other arenaviruses; CCHF, Yellow fever-, dengue-, and hantaviruses. VHFs are zoonotic disease which outbreaks occur all over Africa with certain VHFs also causing outbreaks in other parts of the world. Outbreaks in humans are most often associated with hospital settings and lead to high mortality, due to the severity of the disease and the absence of effective vaccines and treatment options. In the absence of haemorrhagic or specific organ manifestations, infections by VHF viruses are clinically difficult to recognize, with the implication that definitive diagnosis depends mainly on reliable laboratory tests (Drosten *et al*., 2003). Although the range of possible VHF agents can be narrowed down by the travel and exposure history, a suspected case and the causative virus must be rapidly identified to initiate specific or supportive treatment, implement appropriate case management, infection control, and tracing of contacts.

RVF is an important zoonotic disease. It has the ability to replicate in a wide range of mosquitoes and can be easily introduced by infected mosquitoes into domestic ruminants and humans leading to large outbreaks with high mortality rates, and abortion in pregnant animals. The effects of global climate changes, which facilitates the spread of arthropod-borne viruses (Purse *et al*., 2005), increase the risk of RVF crossing geographic boundaries. This possibility has been further emphasized when confirmed RVF outbreaks occurred recently outside of Africa, on the Arabian Peninsula and the Archipelago of Comoros and the French Mayotte. In addition to high socio-economic losses resulting from RVF outbreaks, RVFV is listed as a potential bioterrorism weapon. Also, the lack of efficient prophylactic and therapeutic measures increases international medical and veterinary demand for validated molecular tools for rapid diagnosis of RVF.

An array of molecular techniques for rapid detection and identification of RVFV have been published, but data on their routine diagnostic performance are not available (Drosten *et al.,* 2002; Garcia *et al*., 2001). RT-PCR, nested-PCR and RTD-PCR assays to detect RVF are available, but require expensive equipment and are highly complex making them costly and difficult for use in clinical diagnostic laboratories.

New rapid, cost effective, robust and simplified molecular methods have been developed which include tHDA, SDA, NASBA and LAMP. All of these methods amplify their target at an isothermal temperature. The disadvantage of methods such as tHDA, SDA and NASBA, however, is lower specificity.

The aim of this study was to develop a rapid and accurate molecular tool for the detection of RVFV RNA in clinical samples. The LAMP method proved to be a very rapid, sensitive, specific, simple and robust technique, which overcame the disadvantages of the previous molecular techniques. Including the time required for the extraction of nucleic acid, detection of RNA in different clinical specimens of human and animal origin could be achieved within 2 hours of arrival of the samples in the laboratory. Apart from high analytical and diagnostic accuracy and speed of detection, another important practical advantage of the LAMP assay is that it utilizes simple and relatively inexpensive equipment which renders it promising for use in resource poor settings. In addition, only basic molecular and technical skills are required for execution of the assay procedure, and interpretation of the results may be as simple as visual evaluation of colour change in the reaction mix. However, primer design for LAMP is more complex than for the conventional PCR-based assays and specialized training and software are required for their design. Moreover, the development of LAMP requires the use of a set of multiple primers spanning a highly conserved 300 bp genomic region. For RVFV this is easily achievable because there is no evidence of serological subgroups or major antigenic variation between isolates of disparate chronologic or geographic origins (Swanepoel and Coetzer., 2004), and isolates of RVFV show limited genetic diversity, irrespective of the genome segments analysed (Bird *et al*., 2007).

The first objective of the study was to design effective RT-LAMP primers and to optimize these primers for detection of RVF RNA. Six different primers sets targeting the S segment, M segment and L segment were designed and the RT-LAMP assay was optimized for each primer set by running the assay at temperatures ranging between 60˚C and 65˚C. The detection limits of the optimized assays were compared by using ten-fold serial dilutions of a known number of virus infectious doses. The L3 primer set was found to be the most sensitive (detecting 0.065 $TCID_{50}$/reaction volume), therefore, used in the RVF RT-LAMP assay to attain the study objectives.

The second objective of the study was achieved when the analytical sensitivity of the RT-LAMP assay compared favourable with the TaqMan RTD-PCR assay. Known number of virus infectious doses was detected for both assays at 0.065 $TCID_{50}$/reaction volume. The detection limit of 0.065 $TCID_{50}$/reaction volume was further confirmed for the RVF RT-LAMP assay when four additional strains of RVF (with known virus infectious doses), representing a phylogenetic spectrum of the virus, gave similar detection results. The standard dose-response curves for RT-LAMP and TaqMan RTD-PCR gave similar results when the *in vitro*-transcribed RNA standards were tested. The analytical detection limit, measured by number of RNA copies/reaction, was 10 copies for both assays. Similar analytical sensitivity for the TaqMan RTD-PCR assay based on the Superscript reverse transcriptase-platinum *Taq* polymerase enzyme mixture was reported by Drosten *et al*., (2002). All the RVFV isolates tested were detected by the RVF RT-LAMP assay. The RVF RT-LAMP assay was found to be very specific for detecting RVFV with no cross-reactivity of genetically related and unrelated arboviruses. Specificity of the RVF RT-LAMP assay was further verified by digesting one of the RT-LAMP products with a restriction enzyme and obtaining a product of the expected size.

The third objective of the study was achieved when RT-LAMP, TaqMan RTD-PCR and virus isolation results in clinical specimens from animals and humans, were compared. The diagnostic performance of the TaqMan RTD-PCR assay for the detection of RVFV is also reported in this study for the first time. Specific nucleic acid targets in all positive specimens could be detected in this study in less than 45 minutes with the majority

detected in less than 30 minutes. The study revealed that the RT-LAMP assay allows for rapid confirmation of clinical cases and early recognition of outbreaks. Visualization of amplification products by naked eye, fluorescence, or agarose gel electrophoresis may be appropriate for most laboratory settings, whilst real-time monitoring of the accumulation of magnesium pyrophosphate in the reaction mix potentiates quantification of the assay.

A similar study was done by Peyrefitte *et al.*, (2008) and revealed similar results than our study just before our results were published in March 2009. Their assay also proofed to be rapid, specific and sensitive in detecting RVFV with a detection limit of 10^1 RVFV RNA copies/assay. Their primers were also designed based on the L segment (at different sites than our primer design). Instead of using the Loopamp® RNA Amplification kit, they used individual reagents at 63°C and were also able to detect specific nucleic acid targets in positive specimens in less than 30 minutes. They compared the RT-LAMP assay with a TaqMan assay (Garcia *et al*., 2001) and the two assays compared very well. The study done by Peyrefitte et al., (2008) lacked human and animal RVFV positive sera and they had to use reconstituted sera (foetal calf serum mixed with RVFV) to test their RT-LAMP assay, which gave our study an advantage in evaluating the RT-LAMP assay on clinical specimens received during outbreaks and routine diagnostics from 2006 to 2008.

One has to emphasize, however, that definitive diagnosis or exclusion of RVF, as for any other suspected case of VHF, should not rely on a single molecular assay result. The LAMP assay should be run in parallel with additional tests such as virus isolation and detection of type-specific IgM and IgG antibodies to RVFV. In this context, it is important to emphasize that viremia in RVFV infected individuals is usually of very short duration (Swanepoel and Coetzer, 2004) and most infected patients and adult ruminants undergo subclinical or mild infections. IgM and IgG antibodies are easily demonstrable shortly after exposure to the virus and up to months after recovery from infection (Paweska *et al*., 2005a; Paweska *et al*., 2003a).

4.2 Concluding remarks

This study has shown that the RVF RT-LAMP assay compared very well with the TaqMan RTD-PCR. The RVF RT-LAMP assay is a rapid, simple, cost-effective molecular assay and is highly sensitive and specific for the detection of RVF in clinical samples. In addition, the RVF RT-LAMP assay is robust, meaning that the assay can be used in the clinical- as well as field laboratory setting, which makes it a valuable diagnostic tool for differential diagnosis of VHFs in less well equipped laboratories and during outbreak situations in remote areas in Africa and elsewhere.

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PUBLICATIONS

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

OTHER COMMUNICATIONS

Le Roux, C.A., Kubo, T., Grobbelaar, A.A., Jansen van Vuren, P., Weyer, J., Nel, L.H., Swanepoel, R., Morita, K., Paweska, J.T. A real-time reverse transcription-loop-mediated isothermal amplification assay for rapid detection of Rift Valley fever virus. Poster presented at the NICD Academic day, 11th of November 2008.