EVALUATION OF THE SAFETY AND IMMUNOGENICITY OF RIFT VALLEY FEVER MP-12 AND arMP-12ΔNSm21/384 VACCINE CANDIDATES IN GOATS (*CAPRA HIRCUS*), SHEEP (*OVIS ARIES*) AND CALVES (*BOS INDICUS*) FROM TANZANIA

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A THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGEREE OF MASTER OF PHILOSOPHY IN VIROLOGY OF THE SOKOINE UNIVERSITY OF AGRICULTURE. MOROGORO, TANZANIA.

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ABSTRACT

Rift Valley fever virus (RVFV) is an arbovirus that causes Rift Valley fever (RVF), a disease that causes morbidity and mortality in livestock and humans. Among the measures considered, vaccines are the most effective control strategy against this RVF disease. While we have available vaccines, effective vaccines and better routes of vaccination are needed to prevent RVF among livestock and humans. Therefore, the aim of this study was to evaluate the safety and immunogenicity of a live attenuated RVFV MP-12 and a derivative recombinant RVFV arMP-12ΔNSm21/384 vaccine using the intramuscular (IM) route of vaccination in Tanzanian calves, and goats. Also, a proof of concept study was conducted to evaluate the safety and immunogenicity of the RVFV arMP-12ΔNSm21/384 vaccine using the intranasal (IN) route of vaccination in the same species, as well as sheep. Overall, a total of 61 animals (goats, sheep and calves) aged 6 to 9 months old were used in this study. Twenty five animals, including 5 goats and 3 calves were vaccinated IM with a dose of 1×10^5 plaque forming units (PFU)/ml of RVF MP-12 and 8 goats and 5 calves were vaccinated with the RVF arMP-12ΔNSm21/384 vaccine, and 2 goats and 2 calves received a placebo to serve as controls. Afterward, rectal temperatures were recorded on day 1, 2, 3, 4, 5, 7 then weekly throughout the study. Blood samples were collected on day 14 before vaccination and on the days 0, 3, 5, 7, 14, 21, 28, 35, 70, 84 and 87 post vaccinations (PV). At day 87 PV, all IM vaccinated animals were revaccinated via the IM route with 1×10^4 PFU/ml of RVF MP-12 vaccine and blood samples were again collected on days 94, 101 and 108 PV. As a proof of concept study, 7 sheep, 10 goats and 10 calves were vaccinated intra-nasally (IN) with 50 µl of 1×10^5 PFU of arMP-12ΔNSm21/384 vaccine and 2 sheep were vaccinated with 100 ul of $1x\ 10^5$ PFU of the arMP-12ΔNSm21/384 vaccine, and 7 animals (2 goats, 3 sheep and 2 calves) received a placebo to serve as controls. Rectal temperatures were recorded and blood samples were collected

14 days before and on day, 0 immediately before vaccination and on days 3, 5, 7, 14, 21, 28 and 35 PV. Samples collected in both studies on the day -14 before vaccination, day 0 immediately before vaccination, 3 and 5 were tested for viremia by virus isolation attempted in Vero E6 cells and samples collected on days 7, 14, 21, 28, 35, 70, 84, 87, 94, 101 and 108 PV were tested for RVFV neutralizing antibody by the plaque reduction neutralization test (PRNT). None of the animals had detectable viremia and clinical manifestations throughout the study. All IM vaccinated animals and 70% of each species in the IN vaccinated animals had the first detectable antibody on either day 5 or 7 PV, respectively and antibody titers ranged from 1: 10 to 1:40. Afterwards, antibody titers increased and ranged from 1:10 to 1:640 for the IN and 1:40 to 1:640 for IM vaccinated animals. The antibody response was lower for the IN vaccinated animals, but goats that were vaccinated both by the IM and IN routes responded better than other species while calves had the lowest antibody titers. Therefore, these findings demonstrated that the IN route of vaccination is promising for use in place of the IM route to avoid the use of needles that can cause needle stick injuries and the IN route may prove to be a safer and more efficient route of vaccination especially in mass vaccination campaigns. However, based on the preliminary results of this study, the volume of the vaccine dose for IN vaccination may need to be increased from 50 µl to 100 ul per animal to improve the immunogenicity of the vaccine.

DECLARATION

I, SALAMA BURHAN NYUNDO, do hereby declare to the Senate of Sokoine University of Agriculture that this dissertation is my own original work done within the period of registration and that it has neither been submitted nor currently being submitted for a higher degree award in any other institution.

_____ _____

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The above declaration is confirmed by;

_____ _____

Prof. P. Wambura Date

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DEDICATION

This work is dedicated to my father (Burhan Nyundo) and my mother (Joharia Hassani Kidumu) for their unconditional love, help and support, which has made me to be what I am today.

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LIST OF ABBREVIATIONS, ACRONYM AND SYMBOLS

Δ	Deletion
ABSL-2	Animal Biosafety Level 2
APC	Antigen Presenting Cells
BSL-2	Biosafety Level 2
CDC	Centers for Disease Control
CL 13	Clone 13
CPE	Cytopathic Effect
CTL	Cytotoxic T lymphocyte
DC	Dendritic Cells
DIVA	Difference between natural Infected from Vaccinated Animals
DP	Days Post vaccination
EBME	Eagle’s Basal Media Earle’s salt
ELISA	Enzyme Linked Immunosorbent Assay
EMEM	Eagles Minimum Essential Media
FBS	Fetal Bovine Serum
Gc	Glycoprotein C
Gn	Glycoprotein N
HBSS	Hanks Balanced Salt Solution
IACUC	Institutional Animal Care and use Committee
IFN	Interferon
IM	Intramuscular
IN	Intranasal
Kb	Kilo Base

L	Large RNA segment
LP	Large protein
M	Medium RNA segment
MHC	Major Histocompatibility Complex
mRNA	messenger Ribonucleic acid
N	Nucleocapsid protein
nm	Nanometer
NP	Nucleoprotein
NSs	Nonstructural S protein
PBS	Phosphate Buffered Saline
PFU/ml	Plaque Forming Unit/millimeter
PRNT	Plaque Reduction Neutralization Test
PRNT80	Plaque Reduction Neutralization $_{80\%}$
PV	Post Vaccination
qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribo nucleic acid
RNP	Ribo nucleoprotein
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
RVF	Rift Valley Fever
RVFV	Rift Valley Fever virus
S	Small RNA segment
SC	Subcuteneous
SUA	Sokoine University of Agriculture
Th	T helper cells
TNF	Tumor Necrosis Factor
USAID	United States Agency for International Development

USD	United States Dollar
UTEP	University of Texas at El Paso
VNA	Virus Neutralization Assay

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Rift valley fever (RVF) is an acute viral zoonotic disease caused by the RVF virus (RVFV) that affects both ruminants and humans. The disease was first characterized during an outbreak of RVF among exotic wool sheep that had been imported into Kenya (Daubney *et a*l., 1931). RVFV virus belongs to a genus *Phlebovirus* of the family *Phenuiviridae* of the order *Bunyavirales* that includes a group of enveloped RNA-viruses (Linthicum *et a*l., 1985; Pepin *et a*l., 2010). The enveloped virion is 100 nm in diameter with glycoproteins protruding from the surface. It is spherical and composed of single-stranded RNA, including a small (S), medium (M) and large (L) segment that is surrounded by nucleocapsid proteins.

RVFV is transmitted primarily by mosquitoes of genus *Aedes*. These mosquitoes transmit RVFV from infected animals to non-infected animals including humans; however, an important risk of human infection and disease is also through direct contact with blood, body fluids, or tissues of RVFV-infected animals, mainly livestock (Taylor *et al*., 2001; Woolhouse and Gowtage-Sequeria, 2005; Mohamed *et a*l., 2010; Ross *et a*l., 2012). In addition, exposure to infectious aerosols has also been confirmed as a source of RVFV infection (Reed *et a*l., 2013).

Susceptibility to RVFV infection depends on the age and animal species. In young domestic ruminants, RVFV causes mortality of up to 100% and abortions in 80 - 100% in pregnant ewes (Dar *et a*l., 2013). Clinical signs in adult goats, sheep and cattle are not consistent but may include a rise in body temperature, nasal discharge, unsteady gait,

excessive salivation, loss of appetite, and bloody diarrhea (Kahlon *et a*l., 2010; OIE Terrestrial Manual, 2012). High abortion rates along with febrile illness can be a reliable indicator of RVF outbreak, mainly following periods of heavy rainfall in East Africa when the mosquito vectors appear in large numbers (Williams *et a*l., 2016; Sang *et a*l., 2018).

In human, RVF present as a mild febrile illness that may progress to a severe illness characterized by fever, dizziness, weight loss and myalgia. However, in some patients, disease may progress to severe hemorrhagic fever, encephalitis, and ocular disease that can be fatal in about 1 to 4% of the humans .

The development of effective control and outbreak intervention measures for RVF is a global priority because of the devastating impact of outbreaks of RVF on human and animal health in Africa and the Arabian Peninsula (Faburay *et a*l., 2017). Among the measures considered, vaccines are the most effective strategies for reducing the impact of RVF on the health of livestock (Faburay *et a*l., 2017). While several vaccines are available, there is an urgent need to develop safer and more efficacious vaccines to prevent this RVF among livestock. As an example among the veterinary vaccines, there are two live attenuated vaccines, RVFV Smith-burn and RVFV Clone 13, that have been used more commonly and have contributed to the prevention of RVF and/or reduction in the transmission of RVFV (Faburay *et a*l., 2017). However, the Smithburn vaccine causes abortions and malformations in pregnant ewes similar to the wild-type RVFV and experimental studies suggested that Clone 13 may cause teratogenic effect among pregnant sheep (Hunter *et al.,* 2002; Makoschey *et a*l., 2016). Also, neither of these vaccines elicits an immune response that can be used to distinguish infected from vaccinated animals (DIVA). DIVA can be essential for identifying RVFV vaccinated animals and therefore makes it possible to avoid trade restrictions and exportation of an

infected animal in Africa (McElroy *et a*l., 2009). Currently, there are no approved vaccines for human use (Faburay *et a*l., 2017).

Among the promising candidate veterinary vaccines being developed and evaluated, the live attenuated RVFV MP-12 was tested in humans, sheep, cattle and non-human primates in the U.S.A. through subcutaneous or intramuscular routes and found to be safe, immunogenic and efficacious (Morrill *et al*., 1987, 1991; Bir*d et al.,* 2009). However, the vaccine is not DIVA compatible. Therefore, as an approach for developing a DIVA compatible vaccine, the MP-12 vaccine virus was used to develop a recombinant vaccine using reverse genetics technology to delete, nucleotides 21-384 of the non-structural gene from M segment (NSm) of the MP-12 virus to serve as a potential DIVA biomarker (Ikegami *et al*., 2006; Won *et al.*, 2007; Bird *et a*l., 2011). Studies showed that the immunogenicity of the RVFV arMP-12ΔNSm21/384 vaccine candidate in sheep was comparable to that of the parent RVFV MP-12 vaccine candidate (Morrill *et al*., 2013a). Also, studies in the U.S.A. showed that the RVFV arMP-12ΔNSm21/384 vaccine candidate was safe and immunogenic in sheep and cattle (Morrill *et al*., 2013a, b). These observations and a study in Canada that showed the RVFV arMP-12ΔNSm21/384 to elicit protective antibody against challenge with virulent RVFV in sheep, and the DIVA potential indicated that the latter candidate offered an advantage over the RVFV MP-12 candidate as a potential RVFV vaccine (Weingartl *et al*., 2014a).

Although safety and efficacy had been demonstrated for the RVFV arMP-12ΔNSm21/384 vaccine candidate in North American livestock, further studies are required to document the safety and efficacy of these vaccines. One of the priorities is to conduct similar studies in African livestock to generate critical information required for assessing and eventual consideration of the vaccine for use in Africa and the surrounding region. Such studies are

warranted because the relevance of the observations based on studies regarding the safety and efficacy of the RVFV arMP-12ΔNSm21/384 vaccine in North America livestock to African species of livestock is unknown. However, the possibility that the immune response of African species could differ is because of possible differences in nutritional and health status, genetics and non-specific resistance and other factors.

Therefore, the studies being conducted at SUA, including this project involving African livestock species are being designed to obtain safety and immunogenicity data based on parental intramuscular (IM) and subcutaneous (SC) routes of vaccination. While both routes are well documented to produce uniform, systemic immunity and sustained absorption of the inoculum, the IM route was selected for the present study because of the logistic advantages and the ease of administering the vaccine in comparison to the subcutaneous vaccination that requires more technical skills. However, the IM route may results in pain, bruises and muscle inflammation and necrosis (Hemsworth, 2000; Lie *et al.*, 2006). Therefore, in addition to the IM route, a proof of concept pilot study was conducted to evaluate needle-free vaccine delivery using a device designed to administer the RVFV arMP-12ΔNSm21/384 vaccine by the intranasal (IN) route.

Although needle and syringe devices are inexpensive and easily adaptable to different settings, needle-free technology offers advantages as compared to the use of needles to deliver vaccines (Mitragotri, 2005; Giudice and Campbell, 2006). Also, the development and evaluation of needle-free delivery of RVFV vaccines is a priority goal of the World Organization for Animal Health. In this regard, needle-free vaccine delivery technology can be easily administered by lay-personnel such as farmers to enhance safety, and immunogenicity using a small amount of vaccine dose to induce an immune response delivers vaccine/drug without piercing the skin together with fewer injection site lesions.

The needle-free route has the potential to elicit mucosal and systemic immunity that may afford protection to livestock. Therefore, using a non-invasive vaccine delivery route, such as the IN route that doesnt require the use of needles could prove to represent an improved vaccination method for the prevention of RVF disease among African livestock. The potential value of the IN route is in that it induces a humoral immune response that ensures protection at the local site and at the distal parts of the body by inducing antibody production at these sites (Kozlowski *et a*l., 2002; Neutra and Kozlowski, 2006; Chen and Cerutti, 2010; Morrill and Peters, 2011).

These studies reported herein are confined to the evaluation of safety and immunogenicity of the RVFV MP-12 and RVFV arMP-12ΔNSm21/384 vaccine in goats and cattle using the IM route of vaccination. The RVFV MP-12 was included to also serve as an immune response positive control. because of the extensive amount of vaccination information available for this vaccine that showed it to be safe, immunogenic, and efficacious in human volunteers and North America livestock species and in sheep in Tanzania (Morrill *et al*., 1987, 1991; Bird and Ksiazek, 2009; Pittman *et a*l., 2016a, 2016b; Adamson *et a*l., 2018). In contrast, the RVFV arMP-12ΔNSm21/384 is the candidate vaccine being considered for use in Africa and has only limited evaluations.

The exclusion of sheep in this study involving intramuscularly vaccinated animals was because the RVFV arMP-12ΔNSm21/384 vaccine was already evaluated in Tanzanian sheep and the results were published for this species (Adamson *et al*., 2018). In addition to the evaluation of the IM route of vaccination, a pilot proof of concept study was conducted to evaluate the safety and immunogenicity of RVFV arMP-12ΔNSm21/384 using the IN route of vaccination of goats, sheep, and calves. However, the overall aims of this study were not intended to compare the IM route with the IN route of vaccination. Since this

route of RVFV vaccination has never been reported for domestic ruminants, it is important to emphasize that the evaluation of the IN route of vaccination was a proof of concept pilot study designed to generate preliminary data as to whether or not the RVFV arMP-12ΔNSm21/384 vaccine would elicit an immune response using this route of vaccination. If so, the intentions are to conduct further experiments beyond the scope of this study to gain a better understanding of the potential of IN route for possible use to vaccinate livestock with RVFV vaccines.

1.2 Objectives

1.2.1 Overall objective

To evaluate the safety and immunogenicity of a live attenuated RVFV MP-12 and arMP-12ΔNSm21/384 vaccine candidates in domestic Tanzanian ruminants using the IM and IN routes of vaccination.

1.2.2 Specific Objectives

i. To evaluate the safety and immunogenicity of RVFV MP-12 and arMP-12ΔNSm21/384 vaccines in Tanzanian goats and calves using the IM route of vaccination.

ii. To evaluate the safety and immunogenicity of RVFV arMP-12ΔNSm21/384 using the IN route of vaccination in Tanzanian goats' sheep and calves

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Rift Valley Fever

The Rift Valley Fever (RVF) is a zoonotic disease primarily affecting domestic ruminants and humans. In ruminants, the severe form of disease is characterized by the development of high viremia, severe prostration and death. Mortality is always high in young animals, where up to 80% succumb to the disease and is more severe in newborns (<1 week old) having an almost 100% mortality rate (Kamal, 2009; Kahlon *et a*l., 2010; Baudin *et a*l., 2016). This acute severe form of the disease is less common in older sheep, cattle and goats with a mortality rate of approximately 10-30%.Clinical signs in adult sheep, goats and cattle are not consistent but may include elevated body temperature, nasal discharge, unsteady gait, excessive salivation, loss of appetite, diarrhea and high abortion rate (80-100%) (Centers for Disease Control, 2000). RVF is caused by a virus known as Rift Valley Fever Virus (RVFV). The virus belongs to the genus *Phlebovirus*, family *Phenuiviridae* of the order *bunyavirales* (Pepin *et a*l., 2010). Epidemics and epizootics of RVF in east Africa are normally due to heavy rainfall (Davies *et a*l.,1985) that results in very high population density of mosquitoes, especially of the genus *Aedes* as well as other genera of mosquitoes that may also serve as vectors of the virus (Anyamba *et a*l., 2010). In West Africa, outbreaks have been associated with imported cattle and closing of the diama dam on the Senegal river and in Egypt, outbreaks have been associate with irrigation schemes after RVFV was apparently introduced from Sudan (Linthicum *et al.*, 2016).

2.2 RVF in Tanzania and Outside Countries

Currently, all of the necessary animal hosts such as sheep, cattle and goats and mosquito vectors required for the transmission and spread of RVFV are present in Tanzania (Sindato

*et a*l., 2011; Redding *et a*l., 2017). RVFV infection among Tanzanian domestic ruminants reportedfor the first time in 1930 and then during 1947, 1957, 1960, 1977, 1997 and the latest outbreak occurred in 2006/07 (Fyumagwa *et a*l., 2011; Sindato *et a*l., 2014). Therefore, since independence, Tanzania has experienced three RVFV outbreaks but the most noticeable and well-documented outbreak occurred during 2006/07 (Sindato *et a*l., 2011; Chengula *et a*l., 2013). In the 2006/07 outbreak, RVFV emerged following heavy rains in eastern Africa, including Somalia, Kenya and Tanzania and caused a total of 1,062 reported human cases and 315 deaths in Tanzania. The outbreak also resulted in economic losses among livestock in southern Somalia, Kenya, and northern Tanzania (Martin *et a*l., 2008). Despite the history of repeated outbreaks of RVF in Tanzania, the spatial and temporal ecological factors as well as the epizootiology, epidemiology and habitat suitability for the disease occurrence are not fully understood (Anyamba *et a*l., 2010; Sindato *et a*l., 2014).

In 1997, another epizootic/epidemic of RVF occurred in Kenya and Somalia following heavy rains (Fyumagwa *et a*l., 2011; Muga *et a*l., 2015; Taylor *et a*l., 2016) which led to the deaths of 300 people and a large number of animals (Munyua *et a*l., 2010). However, in Tanzania, the disease was confined to the northern regions with mild and little pathology in animals (Kozlowski *et a*l., 2002; Mohamed *et a*l., 2010; Fyumagwa *et a*l., 2011). The major risk factors identified to be associated with RVF animal cases in Tanzania, was due to an increase in the population density of arthropods and a high amount of precipitation with flooding in areas with high density of livestock that created a conducive environment for RVF outbreak to occur (Himeidan *et a*l., 2014). In human, the RVF cases were associated more-so with direct contact with sick animals and animal products, including blood and milk (Kahlon *et a*l., 2010; Mohamed *e*t *a*l., 2010).

In 1977, RVFV emerged for the first time to cause a devastating outbreak among humans and livestock in Egypt, which was associated with an increase in agricultural flooding that produced an unusually high population density of mosquitoes. In 2000, an outbreak of RVF occurred for the first time outside of Africa in Saudi Arabia and Yemen that raised serious concern regarding the possibility of the global spread of the virus (Morvan *et al*., 2004). Subsequent, RVF outbreaks in Africa were reported in 2003 in Egypt, 2007 in Sudan, 2018-19 in Madagascar, 2010 in South Africa, 2012 Mauritania, and 2016 in Niger. The introduction of RVFV in all of the countries was triggered by the movement of infected animals, trade and changes in weather conditions including land use (CDC, 2000).

2.3 RVFV Structure

The RVFV virion has an icosahedral structure with a T=12 triangulation number, ranging from 80–110 nm in diameter and can be observed under 40 000× electron microscopy magnification (Freiberg *et al*., 2008). The virion envelope is composed of a lipid bilayer and two glycoproteins, Gn and Gc protruding from the envelope surface forming 5-8 nm length sub-units. Among the *Phlebovirus* genus, only one related virus, Uukuniemi virus is known to have this structure (Freiberg *et a*l., 2008; Sherman *et a*l., 2009; de Boer *et a*l., 2012). Inside the envelope, the RNA genome is surrounded by nucleocapsid proteins and the genome is composed of three segments each attached to the RNA dependent RNA polymerase. The tripartite genome consists of a small (S), medium (M) and large (L) RNA segment having the size of 1.6 kb, 3.8 kb and 6.4 kb, respectively. The S segment encodes a nucleocapsid protein (NP) in the negative sense and a non-structural protein (NSs) in a positive sense. The NP is highly immunogenic and several antibody-based RVFV detection assays are based on this protein (Balamurugan *et a*l., 2010). The viral RNA M segment is 3885 nucleotides that encode four nested proteins in a single open reading frame, two structural glycoproteins Gn and Gc and 2 non-structural proteins, NSm and 78

kDa (Pepin *et a*l., 2010; Weingartl *et a*l., 2014b). The viral RNA L segment encodes for the viral RNA-dependent RNA polymerase. Both non-structural genes, NSs and NSm function as virus virulence factors (Billecocq *e*t *a*l., 2004; Bird *et a*l., 2007; Bird *et a*l., 2008; Ikegami and Makino, 2009). Viruses within the *Bunyaviridae* family doesn't possess matrix proteins; however, the linkage of matrix proteins to the envelope with the virus core is compensated by the interactions between ribonucleoprotein (RNP) and glycoproteins since a layer of RNP is situated proximal to the inner leaflet of the membrane which represents the cytosolic tail of the glycoproteins (Lo, 2010; Pepin *et a*l., 2010).

2.3.1 Functions of the RVFV genome segments

2.3.1.1 Large segment

The L RNA segment is 6404 nucleotide and encodes for an L protein. The protein mainly serves as an RNA-dependent RNA polymerase but also is responsible for enzymatic activities such as endonuclease and transcriptase (Gauliard *et a*l., 2006; Ikegami *et a*l., 2006; Bouloy and Flick, 2010). Also, the L protein plays a role in viral replication, transcription, and maturation and packaging of new virions (Liu *et al*., 2008).

2.3.1.2 M segment

The M RNA segment is 3880 nucleotide and encodes precursor of many proteins (Bouloy *et a*l., 2001) with different lengths depending on five start codons. These proteins can, in turn, be divided into four distinct proteins (Suzich *et a*l., 1990). The four proteins include two structural proteins, glycoproteins (Gn) and (Gc), and two non-structural proteins; NSm and large glycoprotein (LGp).

2.3.1.3 Structural proteins (Glycoproteins)

Structural proteins in the RVFV M RNA segment are normally translated as protein precursors. There are five in-frame AUG codons in the M segment, the first AUG generates 78 kD proteins, the second generates the NSm-Gc-Gn precursor proteins and Gc, and third, fourth, or fifth AUG can be cleaved to produce mature Gn and Gc by the host cells (Bird *et a*l., 2007; Liu *et a*l., 2008). The protein precursor from the second AUG is more efficient than from the third AUG codons for Gn expression (Won *et a*l., 2006; Bird *et a*l., 2007). Gn contains a Golgi maintenance motif which is responsible for localization of the Gn/Gc glycoprotein dimer to the Golgi apparatus during the viral replication cycle. These proteins are responsible for RVFV envelope formation, related to RVFV attachment and can induce neutralizing antibody which is critical for protection against virus infection. Therefore, the glycoproteins of RVFV are perfect targets for vaccine development as it has been reported that a Gn/Gc subunit vaccine can elicit strong antibody responses in sheep (Faburay *et a*l., 2014).

2.3.1.4 Non-structural protein (78 kDa and NSm)

The 78 kD precursor proteins generated from the first AUG cleavage in RVFV is essential for the transmission of RVFV by mosquitoes to the ruminant host that was demonstrated in C6/36 mosquito cell and virus in Vero E6 derived virus (Won *et al*., 2006; Liang *et al*., 2014; Weingartl *et al*., 2014b). The NSm protein in RVFV plays a major role in virus apoptosis suppression of infected cells.

2.3.1.5 Small segment

The S RNA segment is 1690 nucleotides long and encodes a nucleocapsid protein in the negative sense and a non-structural protein (NSs) in a positive sense (Bird *et a*l., 2007).

2.3.1.6 N Protein

The N protein is 27 kDa encoded by the S RNA segment which has ambiance polarity (Suzich *et a*l., 1990; Liu *e*t *a*l., 2008). The proteins play an important function in RVFV replication, transcription and replication by RNA directed RNA polymerase (RdRp) and packaging of the virions.

2.4 RVF Transmission

As an arbovirus, RVFV is transmitted horizontally by mosquitoes. The virus is also thought to be transmitted vertically by mosquitoes via transovarial transmission whereby the virus is transmitted from the infected mosquitoes to their offspring via eggs (Miller *e*t *a*l., 2015). After heavy rainfall in East Africa, the infected *Aedes* species mosquito eggs hatch and adults emerge to transmit the virus to animals and humans. Other species of mosquito's vectors such as *Culex, Mansonia*, and *Anopheles* can transmit RVFV from infected animals to healthy animals or to humans through blood feeding (Miller *et a*l., 2015). In addition, blood-sucking insects such as sandflies, midges, and ticks can also serve as vectors for RVFV transmission (Miller *et a*l., 2015). Moreover, field and laboratory studies have confirmed that RVFV can be transmitted via aerosol to humans (CDC, 2000) and direct contact with infected body fluid or tissues can also result in human and ruminant infections (Reed *et a*l., 2013).

2.5 The Immune Response to RVFV

2.5.1 Innate immune response

Innate responses contribute to the clearance of RVFV in infected animals (Nfon *et a*l., 2012). The innate immune response is composed of effectors such as TNF, IFN, chemoattractant and soluble chemical factors (Clem, 2011) that are important and

responsible for initiating the response to microbes that may prevent, control, or eliminate infection. Moreover, it is also required for stimulation of the adaptive immune response.

Innate immunity against RVFV is mostly based on results from experimental models (do Valle *et a*l., 2010; Nfon *et a*l., 2012) whereby innate immune cells, and in particular cell subsets such as dendritic cells (DCs), natural killer (NK) cells, and neutrophils play crucial roles in early antiviral defense. Although DCs are not involved in early pathogen clearance, they are potent antigen-presenting cells and are a source of viral protective type I interferon (IFNs) (Iwasaki and Medzhitov, 2004). Type I IFNs response is mediated through the IFN-α and -β (IFNAR1/2) heterodimer receptors, and the downstream induction of IFN-stimulated genes (ISGs) responsible for an effective antiviral defense (do Valle *et a*l., 2010; Nfon *et a*l., 2012). Non-human primates (NHP) that were challenged with RVFV, secreted IFN-α within 12 hours and did not develop the disease (Morrill *et a*l., 1990). Therefore, RVFV replication is inhibited by the type I IFNs. However, RVFV NSs protein inhibits IFN-α and IFN-β induction, thereby enabling early virus replication and viremia (Bouloy *et a*l., 2001).

In addition to the observations in NHP, IL-12 and IFN-y interleukins were detected in serum samples of RVFV infected goats and were shown to be responsible for the protection of the animals against RVFV infection (Nfon *et a*l., 2012). IL-12 and IFN-y peaked at days 2–4 post inoculation (DPI) suggesting an otherwise functional innate immune response to RVFV in goats. Also, it has been revealed that human IFN-y had minimal in vitro antiviral effect against RVFV infection (Habjan *et a*l., 2009). Therefore, it is possible that IFN-y and IL-12 may have played a role in the rapid clearance of viremia in infected goats. In addition, sheep infected with RVFV, the virus was cleared from the

blood before the detection of neutralizing antibodies indicating that innate immunity was likely to have been responsible for this early clearance and protection.

2.5.2 Adaptive immune response

In addition to the innate immune response, adaptive immunity is mediated by humoral and cell-mediated immune responses and is also responsible for the clearance of RVFV in infected animals (Fearon and Locksley, 1996; Medzhitov *et a*l., 1997; Sun *et a*l., 2009). Adaptive, also known as acquired immunity is the subsystem of the overall immune system. The system is capable of recognizing and providing a more rapid and stronger response to repeated exposures to the same microbe. It is composed of highly specialized, systemic cells and processes that prevent and eliminate pathogens such as RVFV.

Following RVFV infection, the adaptive immune system is normally triggered and initiated by antigen presenting cells (APC) such as DC and macrophages (Berke, 1995; Appay *et a*l., 2002). When the viral antigen is presented to APC cells, the cells engulf and digest virus into small peptides, and then transport the peptides to lymphatic nodes and present them to major histocompatibility complex (MHC)I or II and to naive T cells in the spleen and peripheral lymph nodes (Walter and Barr, 2011).

Upon antigen presentation, to MHC I, the cytotoxic T cells (CD8+T cells) are activated and differentiate into effector cytotoxic T lymphocytes and B memory cells (Springer, 1990; Appay *et a*l., 2002). After differentiation, the RVFV infected cells are lysed by releasing complexes of perforin and granzymes that enters the cytoplasm of infected cell and induces apoptosis (Dommelen *et a*l., 2006).

Moreover, following the presentation of the epitope to MHC class II molecules, CD4+ helper T cells are activated which then activates the CD4+ lymphocytes. Once the CD4+ cells are activated, interleukin IL-2 receptors are released and expressed on the surface of the CD4+ lymphocyte surface. IL-2 stimulates its own receptors as well as those of mononuclear phagocytes, increasing their microbicidal activity. Also, following the release of IL-2, B cells are also stimulated to synthesize antibodies. The B cells recognize antigens using their antigen receptors on their membrane. Once the receptor recognizes the antigen, it activates B cells to process the antigen to peptides and then displays them on the MHC class II molecules and then activates T helper cells. After activation of T helper cells, the cells secrete different signals to the B cell that proliferate and produce antibodies by shifting from IgM or IgD isotypes to IgA, IgE, or IgG (Bon *et a*l., 2001). At the same time, B cell differentiates into memory B cells that provide a faster immune response when the specific antigen reappears again in human's and/or animal's body. These antibodies are useful for RVFV neutralization and therefore for the prevention of RVFV infection (Medzhitov *et a*l., 1997; Kozlowski *et a*l., 2002).

Several studies have documented the role of humoral immunity in protecting animals and human against RVFV (Morrill 1987, 1991, 1997a, b). Protection is believed to be achieved by neutralizing antibodies, including serum IgM and IgG antibodies against RVFV. Apart from humoral immunity, the role of cell-mediated immunity in relation to RVFV protection was also studied in Canada (Weingartl, 2014b). Following vaccination of sheep with the arMP-12-NSm21/384 vaccine, the animals were shown to be protected based on the detection of antigen-specific IFN-response. Moreover, cell-mediated immunity was shown to be involved in long term protection of sheep, goats and mice from RVFV infection (Nfon, 2012). Therefore, both humoral and cell-mediated immunity contributes to the protection of livestock against RVFV. Additionally, N protein of RVFV was

identified as a potential CD8+ T cell immunogen, capable of eliciting a cell-mediated immunity against RVF in mice. The CD8+ activation was observed in mice immunized with modified vaccinia Ankara (MVA) expressing RVFV Gn/Gc glycoproteins, indicating that cell-mediated immunity directed against the glycoproteins may also contribute to protection against RVFV (Lopez, 2013).

2.5.3 Mucosal immunity

Mucosal immunity protects the mucosal surfaces against RVFV but also prevents the spread of infection (Salmon, 1999 & Neutra Kozlowski, 2006). Immunity is mediated by dimeric IgA (dIgA) antibody that has the Fc fragment joined with J-chain (Kozlowski *et a*l., 2002). Following entry of the RVFV into the animal/ human body via attachment to the oral or nasal mucosal membranes, the antigen is taken up by microfold (M) cells that are epithelial cells covering the APC cells and presented to APC, such as dendritic cells, B cells and macrophages. Once presented on the APC cells, the virus antigen is processed and presented to the CD4 T helper cells. T helper cells interact with B-cells to produce IgA. When IgA interact with B cells, they move to effector sites such as nasal passage where they differentiate into IgA producing plasma cells and secrete IgA in dimers (DIgA). DIgA transforms to sIgA and is transported to the effector sites which bind to the polymeric Ig receptor. The SIgA is important in protection against RVFV infection in the mucosal surfaces of the upper respiratory by neutralizing the virus and preventing colonization of the mucosal surfaces by the virus.

2.6 Disease Diagnosis

Currently, RVF laboratory diagnosis is based on, cell culture, serological and molecular methods (Sall *et a*l., 2002; Kamal, 2009; Vuren and Paweska, 2009). The gold standard for RVFV antibody detection is the neutralization assay (VNA) which can be used to quantify

the amount of antibody present in serum samples. The assay is very specific but it takes several days to weeks to obtain results. Although ELISA is not virus antibody specific, it is commonly used to screen for RVFV infection and early detection of antibodies. Cross-reaction between RVFV and other *Phleboviruses* may occur in ELISA assay (Vuren and Paweska, 2009; Seo *et a*l., 2013). Molecular methods are economical and provide rapid methods for detecting RVF viral RNA. Currently, many highly sensitive nucleic acid-based molecular tests have been developed and proven useful to diagnose RVF during outbreaks (Ibrahim *e*t *a*l., 1997; Walter and Barr, 2011). However, molecular techniques cannot be used in the field because molecular reagents and chemicals are not stable at ambient temperatures. Therefore further studies should focus on improving thermostability and simplification of the machines when developing new methods of RVFV diagnostic techniques for use under field conditions.

2.7 Prevention and Control of RVF

Vaccination is considered to be the most effective tool for RVF prevention and control in humans and ruminants (Faburay *et al*., 2017). Other preventive measures include public health education, mosquito vector control and restriction of animal movements (Pepin *et a*l., 2010; Glancey *et a*l., 2015). Protective gear such as gloves and outer garments should be worn and care should be taken when handling RVF sick animals, managing RVF patients and their tissues or any other associated biological materials to prevent infection (Anyamba *et al*., 2010). Vector control, either by using insecticides or pesticides is among the most commonly used practice for controlling RVF (Anyamba *et al*., 2010). Although insecticides kill mosquitoes, it is not an effective option because of the expense, manpower requirements, and the very limited areas that can be covered and sustained, considering the extensive and diverse terrain that support RVFV transmission. Additionally, there are no specific treatment measures for RVFV infection in humans and

animals, and therefore, management of clinical cases is only through supportive therapy (Pepin *et a*l., 2010).

2.8 Vaccination

The fundamental mechanism for vaccination is based on the adaptive acquired immune system (Daouam *et a*l., 2015). Once exposed to an antigen via vaccination, the system can recall the epitopes, if re-exposed to the specific antigen and respond rapidly and with an enhanced immune response (Medzhitov and Janeway, 1997). Vaccines are the most effective control strategy against RVF outbreaks. Currently, there are many vaccines available, including inactivated and live attenuated vaccines (Hunter *et a*l., 2002; Dungu *et a*l., 2010; Von Teichman *et a*l., 2011). The most Common used vaccines include the live attenuated RVFV Smithburn and Clone 13. The Smithburn vaccine was developed in South Africa by serial passages in mouse brain that resulted in the attenuation of field isolate of RVFV called Smithburn (Smithburn, 1949). The vaccine induces early and long-term immunity after a single injection (Oreshkova *et a*l., 2013) but is not recommended for use in the early stages of pregnant ewes because of the risk of abortions and stillbirths .

Clone 13 (CL13) is a natural live attenuated RVFV mutant that was isolated from a non-fatal human case of RVF (Billecocq *et a*l., 1996). The vaccine has a deletion in the non-structural protein coded by the viral RNA S segment (NSs) that has been identified as a major determinant of virulence (Bird *et a*l., 2008). In the evaluation of efficacy and safety of CL13 vaccine in ewes at different stages of pregnancy, results revealed that clinical manifestation of RVF such as abortions in pregnant ewes was not observed when animals were vaccinated (Dungu *et a*l., 2010). Also vaccinated animals were protected from clinical RVF following virulent challenge (Njenga *et al*., 2015). However, subsequent experimental studies showed that CL 13 was able to cross the ovine placental barrier to

cause fetal infections, malformations, and stillbirths (Makoschey *et a*l., 2016). Also, both Smithburn and CL 13 vaccines are not DIVA compatible and therefore, cannot be used to differentiate naturally infected animals from vaccinated animals and thus they cannot help with the problem of animal trade restriction in RVFV enzootic countries. Furthermore, the mode of delivering these vaccines is through the subcutaneous route using needle injections. The needle injections involve the piercing of the skin and require a large volume of vaccine dose in order to elicit antibody (Chen and Cerutti, 2010). The immunity offered by this route of vaccine delivery is only systemic immunity (Giudice and Campbell, 2006) whereas, vaccine delivery such as intranasal route can generate both mucosal and systemic immunities that may prove to be an effective alternative vaccine route for the protection of livestock against RVFV.

The live-attenuated RVFV MP-12 vaccine was developed from a wild type RVFV ZH548, which encodes the virulent S RNA segment and attenuated M and S segments (Ikegami *et a*l., 2015). In developing the vaccine, the wild-type ZH548 strain received 12 serial passages in human lung diploid cells (MRC-5) in the presence of a chemical mutagen, 5-fluorouracil (Caplen *et a*l., 1985; Vialat *et a*l., 1997). Serial passaging of ZH548 resulted in attenuating mutations in all three RVFV RNA segments based on virulent testing in mice (Saluzzo and Smith, 1990). The master seed and vaccine lots of the MP-12 vaccine candidate have received extensive safety and efficacy testing in ruminants and in non-human primates. Currently, the MP-12 vaccine is conditionally licensed for veterinary purposes in the United States and Canada and has also been tested and found to be safe and immunogenic for human use in phase I and II clinical trials (Ikegami *et a*l., 2015; Pittman *et a*l., 2016a, b). Although MP-12 is highly immunogenic in ruminants, the vaccine is not DIVA compatible. Thus, without a DIVA marker, studies were conducted that led to the use of the MP-12 vaccine to develop a recombinant veterinary vaccine

candidate with a deletion in the non-structural nucleotides of the viral RNA M segment to serve as a potential DIVA marker and was named RVFV arMP-12ΔNSm21/384.

The recombinant arMP-12ΔNSm21/384 vaccine candidate was generated from the MP 12 vaccine virus by reverse genetics (Won *et al.*, 2007) that produced a large deletion of the NSm nucleotide sequence at the pre-Gn site. Studies in the United States showed that this vaccine candidate was safe and induced protective immunity in ewes at 42 days of pregnancy and was safe and immunogenic in calves (Bird *et al*., 2011). In another study, a single vaccination of arMP-12ΔNSm21/384 vaccine afforded full protection to sheep when challenged four weeks post vaccination (Weingartl *et a*l., 2014a), therefore demonstrating that this vaccine was efficacious in protecting these animals from RVFV infection. Although the arMP-12ΔNSm21/384 vaccine candidate was not tested as extensively in sheep and calves and not at all in goats, the preliminary results in sheep revealed that this candidate was as safe and efficacious as the parent RVFV MP-12 vaccine candidate (Morrill *et al.*, 2013a). Therefore, since the arMP-12ΔNSm21/384 vaccine candidate has a potential DIVA marker, this vaccine was selected to replace the parent RVFV MP-12 vaccine for evaluation in the United States and Africa as a potential candidate for use to prevent RVF among livestock in Africa and surrounding RVFV enzootic countries.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

Since the arMP-12ΔNSm21/384 vaccine virus is classified as a BSL2 agent, all research activities required the use of an Animal Biosafety Level 2 (ABSL 2) holding facility and Biosafety Level 2 (BSL 2) virology laboratory (CDC BMBL, 2009). Therefore, all experiments involving goats, sheep and calves were conducted in Morogoro, Tanzania (6.8278° S, 37.6591° E) at Sokoine University of Agriculture (SUA) in an insect proof ABSL-2 facility and a BSL-2 virology laboratory. The animal facility is designed to prevent the entry of arthropods and to provide sanitation measures as well as ventilation and an incinerator for disposing of animal carcasses and animal waste. The laboratory is equipped with basic virology and cell culture instruments together with a water distiller and an autoclave for sterilizing equipment before use and autoclaving waste materials from the laboratory before disposal by incineration. All work in the laboratory and animal holding facility was performed by faculty and staff who had received biosafety training and demonstrated proficiency in performing all procedures involved with working with infectious disease agents in accordance to biosafety practices described in the SUA laboratory standard operating procedure manual.

3.2 Vero E6 Cells and Vaccine Viruses

3.2.1 Vero E6 Cells

The Vero E6 cells used in this study were provided by the University of Texas El Paso, Texas. The cells were provided for use to prepare working stocks of the RVFV MP-12 vaccine virus, and for testing the dose of the vaccine used to vaccinate animals, and for

performing tests for the detection of neutralizing antibody in serum samples obtained from experimental vaccinated animals.

3.2.2 Vaccine viruses

Importation of Vaccine viruses here in Tanzania was approved by the Directorate of veterinary Service of the Ministry of Livestock and Fisheries and Tanzania Food and Drugs Authorities. Aliquots of 1.0 ml of the freeze-dried form of the arMP-12ΔNSm21/384 vaccine (Lot No 15/3/2017) were approved by the relevant authorities here in Tanzania and provided by the Multi-chemical Industry (MCI) Santé Animale Biopharmaceutical Company in Mohammedia, Morocco. The identity of arMP-12ΔNSm21/384 virus was confirmed at MCI by qualitative real-time polymerase chain reaction assay (qPCR) that targeted the L and M viral RNA segments of the virus and then sequenced in Genewiz laboratories (GENEWIZ Global Headquarters; USA), using Next Generation Sequencing technology (NGS) Illumina method 1x50bp SR, HiSeq 2500, High Output, per lane (V4 chemistry). The infectivity titer of the arMP-12ΔNSm21/384 vaccine virus was $10^{5.5}$ TCID50/ml in Vero E6 cells. This vaccine candidate was used to vaccinate goats and calves.

The MP-12 virus was originally obtained by UTEP from the World Reference Centre for Emerging Viruses and Arboviruses, Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston Texas. At UTEP, the identity of the MP-12 vaccine virus was confirmed by plaque reduction neutralization test (PRNT) using RVF MP-12 specific monoclonal antibody (Mab). The Mab neutralized the infectivity titer of the MP-12 virus from 10^6 plaque forming units (PFU)/ml to 10^2 PFU/ml but did not neutralize the infectivity titer of Sindbis and/or West Nile viruses. The RVFV MP-12 obtained from UTEP was used at SUA to prepare a stock virus in Vero E6 cells with an

infectivity titer of 1.4 x 10^7 PFU/ml and stored in 0.5 ml aliquots at -80°C for use to vaccinate sheep, goat and calves and to perform the PRNT to detect RVFV neutralizing antibody in serum samples obtained from vaccinated animals.

3.3 Experimental Animals

Local livestock, Six to 9 months old goats (*Capra hircus*), sheep (*Ovis Aeris*), and calves (*Bos indicus*) were used in this study. A total of 61 animals, 25 animals for IM vaccination (15 goats and 10 calves) and 36 animals for IN vaccination (12 goats 12 sheep and 12 calves) were purchased from local livestock keepers in the Mvomero district of Morogoro region, Tanzania. A higher number of goats were used in the experiment to evaluate the response of the animals following IM vaccination because no studies had been conducted in goats using the RVFV MP-12 or the RVFV arMP-12ΔNSm21/384 vaccines. In contrast, both vaccines had been evaluated in calves and sheep in the USA (Morrill *et al*., 1997a, b; Morrill *et al*., 2013, b). Also, due to limited space in the ABSL2 facility, it was not possible to house as many calves as goats because of the larger size of calves. Sheep were not included in the IM study because this species' response to IM vaccination with RVFV MP-12 and the RVFV arMP-12ΔNSm21/384 vaccines was performed in a separate study (Adamson *et al*., 2018). Animals were ear tagged with individual numbers for identification and treated with a dose of 1: 1000 Chlorfenvinphos for killing ectoparasites and 4 ml 2.5% Albendazole orally for killing endoparasite. All animals were acclimatized in the ABSL-2 facility for 14 days and monitored daily for signs of RVF disease. All animals were screened for presence of RVFV and antibody and found to be seronegative by virus isolation and PRNT assay before vaccination. Animal experiments were performed according to an experimental protocol approved by UTEP and SUA's Institutional Animal Care and Use Committee (IACUC) (ref # 559105-08 and SUA/CMVBS/R.1, respectively).

3.4 Experimental Design

The study was designed to address the 2 specific aims, including the first to evaluate the safety and immunogenicity of the MP-12 and MP-12ΔNSm21/384 vaccine in calves and goats using the IM route of vaccination and the second aim was to conduct a proof of concept study to evaluate the IN route of vaccination of sheep, goats and calves using the RVFV arMP-12ΔNSm21/384 vaccine. All animals for IM and IN groups were housed in the ABSL-2 facility and were randomized into test and control groups. As mentioned above, the ABSL-2 insect proof facility was used because the RVFV MP-12 and arMP-12ΔNSm21/384 are classified as BSL2 agents. Also, the IM and the IN vaccine route animal studies were conducted at different times and the facility was cleaned thoroughly and disinfected after the completion of the first IM study and before the animals were placed in the facility to perform the second or the IN study.

3.5 Intramuscular Vaccination

Goats and calves were randomly divided into 2 groups. 5 goats and 3 calves were used for vaccination with MP-12, and 8 goats and 5 calves for vaccination with the arMP-12ΔNSm21/384 vaccine. 2 animals in each species were used as negative controls. Each freeze dried vial of arMP-12ΔNSm21/384 was reconstituted in 2 ml of Eagle's Minimum Essential Medium (EMEM) containing 4% fetal bovine serum (FBS). Each reconstituted vial contained 1×10^5 PFU/ml of the arMP-12ΔNSm21/384 virus. The MP-12 vaccine virus was diluted in EMEM to yield a concentration of 1×10^5 PFU/ml from the initial concentration of 1.4×10^7 PFU/ml. One ml of each virus was loaded into separate 5 ml syringes in a class IIA2 biosafety cabinet in the virology laboratory and transported in a cool box on ice to the ABSL 2 animal facility. An 18 gauge needle was attached to each of the 5 ml syringes and the animals were vaccinated intramuscularly (IM) in the neck area with one ml per animal. The 2 control animals were vaccinated likewise with one ml of

EMEM containing 4% FBS. At day 87 PV, all IM vaccinated animals including the negative controls were revaccinated with a dose of 1×10^4 PFU/ml MP-12 vaccine. The animals were revaccinated to determine if a booster dose would elicit a more robust immune response based on an increase in antibody titers that would mimic being exposed to virulent RVFV in the field following vaccination. An increase in antibody would serve as a valuable indicator of possible protection afforded by one dose of the vaccine. Information was recorded for each vaccinated animal, including the date of inoculation, vaccine dose and route, identification numbers, sex, and the animal pen number.

3.6 Mystic Intra-nasal Needle Free Delivery Device

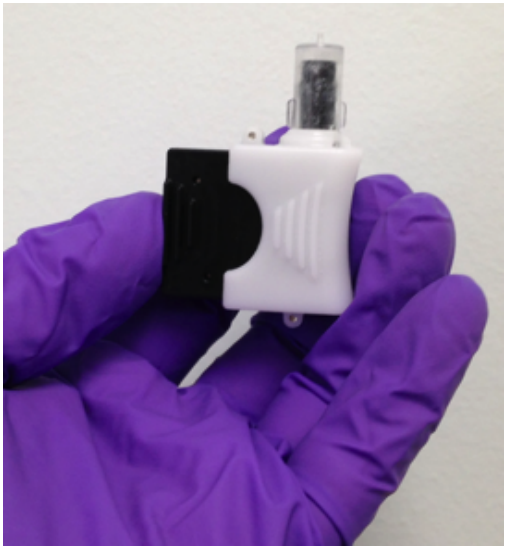


Figure 1: Mystic Intra-nasal Needle-Free Delivery device used for intranasal (IN) vaccination of goats, sheep and calves.

African breeds of sheep, goats and calves were vaccinated IN using a Mystic needle-free vaccine delivery device that was obtained from Mystic Pharmaceuticals, Inc., Austin, Texas (Fig. 1). As a mechanical dispensing device, the components consisted of a trigger, plunger, blister and a nozzle. The blisters were pre-loaded with the desired volume and concentration of the vaccine and then sealed leaving a small amount of compressible head

space inside each blister (the compressible head space is indicated by the white area above at the top of the internal black blister chamber). The vaccine was then dispensed by pressing the trigger to push the plunger to compress and crush the blister that expels the vaccine through the orifice of the internal nozzle cannula as a fine spray into the cavity of the animal's nasal cavity to penetrate and enters to the surrounding mucosal tissue. At the time of purchasing these Mystic devices, only 34 devices that could hold 50 ul each plus 2 devices that could hold 100 ul each were available.

3.7 Intra-nasal Vaccination

The animals used for IN vaccination were distributed into three groups. Group 1 had 10 goats, 7 sheep and 10 calves that were each vaccinated in the left nares using the Mystic device with 50ul each of 1 x 10^5 pfu/ml of the arMP-12ΔNSm21/384 vaccine.Group 2 had2 sheep which were vaccinated like-wise with 100ul of the arMP-12ΔNSm21/384 vaccine and group 3 included 2 goats, 3 sheep and 2 calves which each animal was vaccinated with 50 ul of phosphate buffered saline (PBS) to serve as negative controls. The vaccination procedure involved the manual restraint of each goat, sheep and calf by 2 or more technicians who secured each animal between their legs by gripping the horns or the base of the ear for the hornless animals while holding the head up at a 45-degree angle. The nozzle of the Mystic device loaded with a blister that contained 50ul or 100 ul of the 1 x10^5 PFUs of the arMP-12ΔNSm21/384 vaccine and 50ul of PBS was positioned into the left nare of each animal. The trigger of the device was activated by applying smooth firm pressure to dispense the dose of vaccine into the nare cavity. Each animal was then restrained in the same position for 5 seconds to allow the vaccine to penetrate the mucosal tissue. Information was recorded for each animal, including the date of vaccine inoculation to animals, vaccine type given to animals, dose of vaccine injected to animals and route of administration.

The evaluation of the RVFV arMP-12ΔNSm21/384 vaccine using the IN route of vaccination was a the first proof of concept study conducted in livestock using this route of vaccination. As a proof of concept study, the volume of the RVFV arMP-12ΔNSm21/384 vaccine dose was 50ul but contained the same concentration or 1 x 10^5 PFU/ml as the maximum dose used in other studies. The lower volume was used because 34 of the 36 the available intranasal Mystic devices were designed to take 50 µl of the volume and 2 devices were designed to hold 100 ul each as described above. Thus, the 50 ul dose of vaccine was consistent with that used in other studies, but the volume was lower and allowed for the determination if the lower volume would elicit an immune response, and if so, the lower volume would reduce the cost of manufacturing the vaccine and therefore, more affordable by the user. In case the lower volume did not elicit an immune response, 2 sheep were vaccinated with 100 ul or twice the 50 ul volume administered to the other animals to gain a preliminary understanding of the volume required eliciting an immune response.

3.8 Specimen Collection and Preparation

The immune response as a measure of the immunogenicity of the RVFV vaccines was determined for animals vaccinated IM and IN routes by testing the serum component of blood samples for antibody obtained following vaccination. However, since the sheep, goats and calves used in the vaccine trials were free-ranging animals obtained from local sources in Tanzania, it was possible that they may have been infected with RVFV, and therefore were either viremic or positive for an antibody. Therefore, to ensure that the animals were not viremic and antibody negative at the time of vaccination, 4 ml blood samples were obtained from the jugular vein of each animal on day 14 before vaccination and on day 0 immediately before vaccination using an 18 gauge needle attached to a 6 ml vacutainer. Two to 3 ml of serum was obtained from each blood sample after leaving the

samples overnight at 4°C followed by centrifugation at 1200×G for 10 minutes. Aliquots of 0.5 to 1.0 ml of each serum sample were transferred to sterile pre-labeled vials and stored at -80°C freezer until tested for RVFV and/or RVFV neutralizing antibody to ensure that animals selected for the vaccine trials had not been infected with RVFV in the field.

After selecting goats and calves that were negative for RVFV and for RVFV antibody, the animals were vaccinated via the IM route with the RVFV MP-12 and the RVFV arMP-12ΔNSm21/384 vaccines as described above to assess the safety and immunogenicity of the two RVFV vaccines. Blood samples were then obtained from each animal on days 3, 4, 5, 7, 14, 21, 28, 35, 70, 84 and 87 PV including the 4 controls, 2 goats and 2 calves that were vaccinated with EMEM. Also, after the goats and calves, including the control animals received a booster with the RVFV MP-12 vaccine on day 87 PV, and blood samples were obtained from each animal on days 94, 101 and 108 PV. As described above, 2 to 3 ml of serum was obtained from each blood sample and stored in aliquots of 0.5 to 1.0 ml at -80°C freezer until tested for RVFV and/or RVFV neutralizing antibody.

Serum samples that were obtained from all animals used in both the IM and IN route of vaccination study on days 3, 4 and 5 PV were tested for RVFV in Vero cell cultures as possible evidence that the vaccines caused a viremia. Samples obtained from the IM vaccinated animals on days 7, 14, 21, 28, 35, 70, 84 and 87, and on days 94, 101 and 108 PV following the booster vaccination on day 87 PV were tested by the $PRNT_{80}$ to determine the neutralizing antibody response. Samples obtained from the IN vaccinated animals on days 5, 7, 14, 21, 28, and 35 PV were tested by the $PRNT_{80}$ to determine the neutralizing antibody response. Any serum sample obtained from animals vaccinated with either of the vaccines that were negative for antibody by the $PRNT_{80}$ was retested for RVFV IgG antibody by the ELISA.

3.9 RVF Reverse Transcription Polymerase Chain Reaction

Prior to performing the RVF reverse transcriptase polymerase chain reaction (RT-PCR) assay, RNA was extracted from serum samples collected from goats, sheep and calves on days 14 before vaccination and on day 0 immediately before vaccination, and on days 3, 4, and 5 PV following the manufacturer's instructions using the QIAamp® Viral RNA Mini kit (Qiagen, Hilden, Germany). The RNA from serum samples were pooled (P) in groups of 2 and the RNA extracted from the RVFV MP-12 virus-positive control (P19) and negative control sample (P22) were stored at -80°C until tested for RVFV RNA by the RT-PCR assay.

The QIAGEN One-Step RT-PCR assay kit was used to test RNA samples for RVFV RNA. Primers targeting the M segment (551bp): RVF forward 5'TGT GAA CAA TAG GCA TTG G'3 and RVF reverse 3'GAC TAC CAG TCA GCT CAT TAC 5' (Ibrahim *et a*l., 1997) were used with a concentration of 0.1 µM. Thermocycler conditions were as follows: 50°C for 30 minutes initial PCR activation at 95°C for 30 minutes, followed by 40 cycles at 95°C for 30 seconds, 58°C for 1 minute, and 72°C for 2 minutes, followed by a final extension for 10 minutes. The MP-12 RNA as positive (P19, 20 and 21) and master mix (Buffer) as negative controls (P18) were included in the RT-PCR assay. The PCR amplicons including positive and negative controls together with Hi-Lo ™DNA Marker (Bio nexus, Inc.) were run in 10 ul of gel red stain and 1.5% agarose at 120 voltages for 45 minutes and visualized under UV-transilluminator.

3.10 Viremia

Serum samples collected from goats, sheep and calves were diluted in 1:2 in EMEM supplemented with 4% FBS. The Confluent monolayer of Vero E6 cells was propagated in 24-well plates and cultures in each well were inoculated in duplicate using 50 ul of each serum sample per culture. The culture and inoculum were incubated for one hour at 37°C and agitated after every 15 minutes for virus adsorption. After adsorption, 0.5 ml of EMEM supplemented with 4% FBS was added to each culture and incubated at 37° C with 5% CO_2. Cultures were observed once daily for 10 days under a microscope for cytopathic effect (CPE). After 10 days, all CPE negative cultures were frozen and thawed to make a blind passage in Vero E6 cells using the same procedure and observed for 10 days for CPE. Any cultures that developed CPE were harvested and stored in aliquots of 1.0 ml for further study using RT-PCR to determine if the CPE was caused by RVFV. If there was evidence of RVFV, all aliquots and any remaining cultures were destroyed by heat in an autoclave at 112°F because of biosafety requirements that RVFV as a select agent must not be kept in a BSL 2 laboratory. The specific animals were isolated and quarantined in a holding facility separate from the ABSL 2 facility and not used in this study.

3.11 Immunological Method for Antibody Detections

3.11.1 Plaque Reduction Neutralization TEST-80 (PRNT-80)

The PRNT was used to evaluate the immunogenicity of the RVFV MP-12 and the RVFVarMP-12ΔNSm21/384 vaccine based on the detection and quantification of neutralizing antibody in sera samples collected from the vaccinated animals. As an assessment of immunogenicity, the PRNT results provided the number of animals that developed antibody and the antibody titer elicited against the particular vaccine that was used to vaccinate the animals. Each test sera was diluted 1:5 followed by 4-fold dilutions

(1:5, 1:20, 1:80, 1:320, 1:1280 and 1:5120) in Hanks Balanced Salt Solution (HBSS) supplemented with one % HEPES, penicillin and streptomycin and heat-inactivated FBS in a 96-well plate. An equal volume of 75 ul of each diluted test sera was mixed with an equal volume of 75 ul the virus dose (60-80 PFUs) such that the final serum dilution were 1:10, 1:40, 1:160, 1:640, 1:2560 and 1:10240, and virus dose ranged from 30 to 40 PFU. The controls consisted of a mixture of an equal volume of 60-80 PFU with a 1:10 dilution of RVFV antibody positive and a negative animal serum from either goats, sheep or calves. The virus dose serum dilution mixtures were incubated at 37°C in the absence of CO_2 for one hour.

Next, Vero E6 cells were seeded in 24-well tissue culture plates and incubated for 4-5 days at 37°C and 5% $CO_2$to provide 90% confluence monolayers. The growth media was then discarded from the Vero E6 cell monolayers and 50 ul of each virus dose – serum dilution mixture was inoculated onto each of 2 cell monolayers per sample. The mixture of the virus dose and the antibody positive control serum mixture were inoculated onto each of 20 cultures and the virus dose - antibody negative control serum mixture was inoculated onto 4 cultures. The cultures and virus – sera mixtures were incubated for one hour at 37°C and 5% CO_2 while agitating after every 15 minutes. One percent Seakem agarose with an equal volume of 2X Eagle's Basal Medium with Earle's salt (EBME), with HEPES, sodium bicarbonate, 8% FBS, and 1% penicillin, streptomycin and L-glutamine was prepared and 0.5 ml was overlaid onto each cell culture. The agarose overlay was allowed to solidify and plates with cells and inoculum were incubated for 2 days at 37°C with 5% CO_2. Each culture of cells and inoculum was then overlaid with 0.5 ml of 1% agarose mixed with an equal volume of 2x EBME supplemented with 5% neutral red and incubated overnight at 37°C. The PFUs were counted and recorded for both the controls and animal sera test samples, and the antibody positive and negative controls. The dilution

of serum for each sample that reduced the RVF MP-12 virus dose by 80% based on the number of PFU observed for the virus dose and antibody negative serum sample was considered as the neutralizing antibody titer.

3.11.2 Enzyme-linked immunosorbent assay

The purpose of testing selected sera samples by the ELISA was to detect and measure antibody in sera samples that tested negative for antibody by PRNT test. The ELISA can be a more sensitive test than the $PRNT_{80}$ for detecting antibody as observed for the results of testing sera obtained from sheep and goats during a study conducted in South Africa (Njenga *et a*l., 2015). Sera from some animals were positive by virus neutralization and negative by ELISA assay and vice versa. Therefore, the decision to test selected sera sample by the ELISA was based on data supported by the present study and data reported by others, and therefore the reason for using both the ELISA and the PRNT to test selected sera samples for antibodies (Paweska *et a*l., 2005). Therefore, all serum samples collected from the vaccinated animals that were negative by $PRNT_{80}$ for RVFV neutralizing antibody in this study were further tested for RVFV IgG antibody using an indirect competitive ELISA kit (ID screen® Rift Valley Fever Competition Multi-species- ID vet). The testing procedures were in accordance with the manufacturer's instructions for performing the ID Screen Rift Valley Fever Competitive Multi-species kit protocol (Appendix 2). The contents of the microplate (virus test sample and viral antigen and an enzyme substrate and an enzyme) were read and recorded at the O.D of 450 nm whereby, for each sample, the competitive percentage was calculated by taking the O.D of sample divide by OD of the negative control and presented in percentage. All samples that had less than or equal to 40% were considered as positive, samples greater than 40% and less than or equal to 50% were considered as doubtful and all samples that had 50% were considered as negative.

3.12 Clinical Assessment of Animals

Rectal body temperature for each vaccinated and control animal was taken at the time of blood collection up to day 35 PV. In addition, general health status including body temperature, animal movement, mortality and abortions were assessed by veterinary personnel and recorded once a day. Animals that developed any sign of illness during the study were given a clinical examination by a veterinarian and samples were collected for analysis and diagnosis.

3.13 Data Analysis

Data analyses were done after normalizing the different number of animals used in the experiments. All the data were analyzed using R statistical analysis software version 3.4.1. Analysis of mean $PRNT_{80}$ titers of goats and calves and antibody response between MP-12 and arMP-12ΔNSm21/384 vaccines following the first IM vaccination and after the IM MP-12 booster vaccination were done using Welch two-sample t-test and one-way ANOVA test. Comparison of antibody response between animal species vaccinated IN was also done using Welch two-sample t-test with a significance level of $p = \leq 0.05$.

CHAPTER FOUR

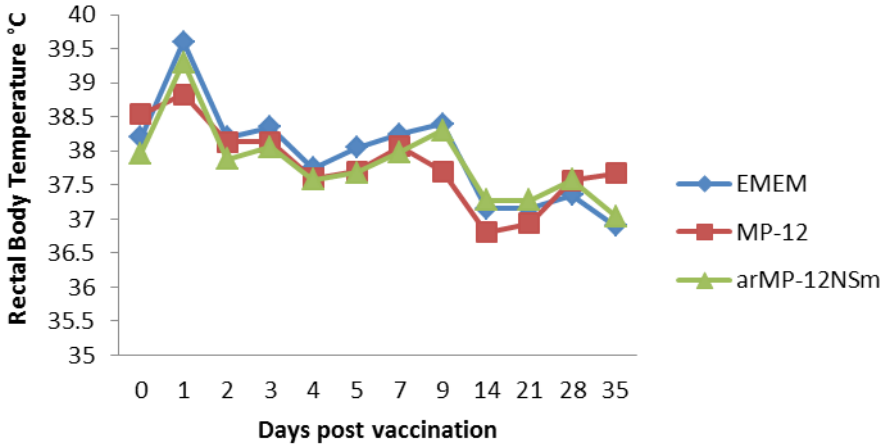
4.0 RESULTS

4.1 Intramuscular Vaccination

4.1.1 Clinical assessment of the experimental animals

All goats and calves that were vaccinated by IM route with RVFV MP-12 and arMP-12ΔNSm21/384 vaccines remained healthy and no significant adverse effects such as diarrhea and nasal discharges were observed during this study. The rectal temperatures ranged from 38 to 38.5°C before the animals were vaccinated and on day one PV, the temperature of all vaccinated animals had increased to 39°C and the control animals had a temperature of up to 40°C **(**Fig. 2). However, on day 2 PV and thereafter throughout the study, the temperature of the animals ranged from 37°C to 39.5°C, including the control animals (Fig. 2).

2a.



2b.

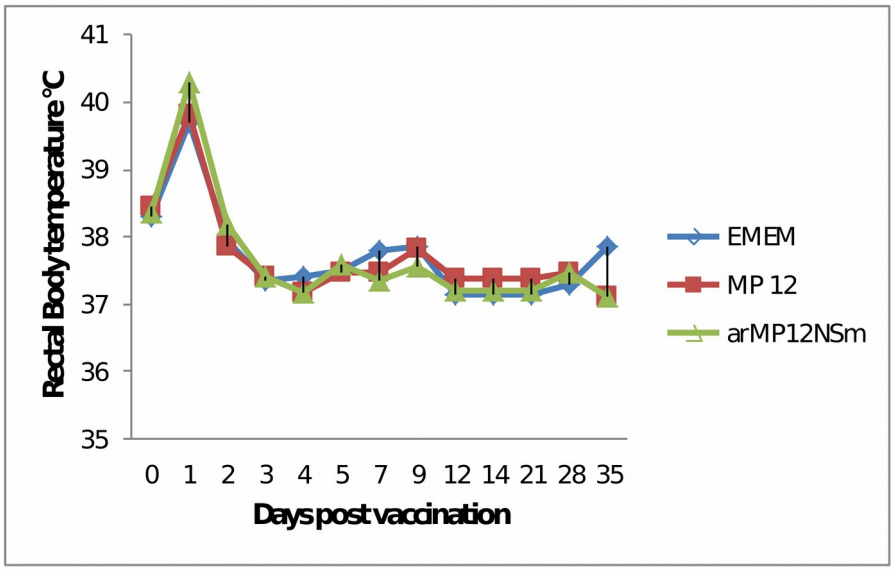


Figure 2: Mean rectal temperatures of 2a) calves and 2b) goats following (intramuscular) IM vaccination with 1×10^5 PFU/ml of RVFV MP-12 and arMP-12ΔNSm21/ 384 and Eagle's Minimum Essential Medium (EMEM).

4.1.2 Viremia

Serum samples obtained from all goats and calves 14 days before IM vaccination and on day 0 immediately prior to vaccination with the RVFV MP-12 and arMP-12ΔNSm21/384 were negative for RVFV RNA by RT-PCR (Fig. 3) and for virus isolation attempts in Vero E6 cells. Also, RVFV was not detected in any of the sera sample obtained on days 0, 3, 4, and 5 PV, nor from blind passages in Vero E6 cells. Therefore, there was no detectable viremia in the goats and calves as a result of IM vaccination with MP-12 and MP-12-Nsm-del vaccines. In addition, sera samples obtained from the goat and calves on day 14 before vaccination and on day 0 immediately prior to vaccination of the animals were negative for RVFV neutralizing antibody. The latter observation indicated that the animals had not been infected in the field with virulent RVFV.

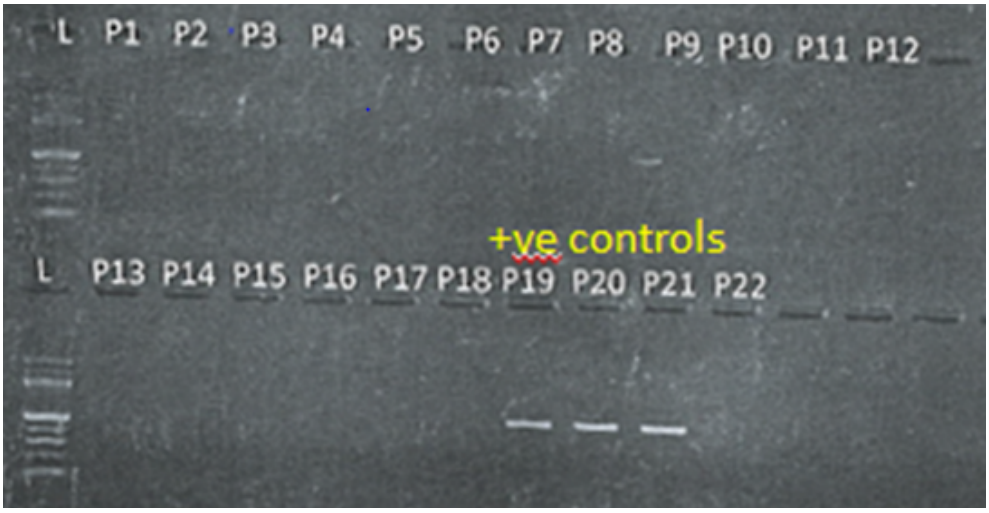


Figure 3: A gel red stained agarose showing DNA amplicons of sera samples collected on a day -14 and day 0 before vaccination of the MP-12 and arMP-12NSm21/384 vaccine. L and P numbers stand for ladder and pools of samples respectively. For the positive controls (P19, P20, and P21), RVF MP-12 RNA was used and their results are indicated by visible bands with an expected size of around 550 base pair (bp). The negative controls, (P22 andP18) and all pooled samples did not produce any band and are thus indicated by clear areas.

4.1.3 Immunogenicity of the vaccines

All goats and calves vaccinated by the IM route with MP-12 and arMP-12ΔNSm21/384 developed neutralizing antibody and the 2 control goats and 2 calves that received EMEM supplemented with 4% FBS did not produce neutralizing antibody. Seroconversion started on day 5 PV, whereby 5 goats vaccinated with MP-12 had neutralizing antibody titers of 1:10. On day 14 PV, the antibody titers of all animals increased to 1:40 or 1:160, which was either sustained or decreased through day 87 PV. After the RVFV MP-12 booster on day 87 PV, the immune response in these revaccinated animals was characterized by a rapid increase to peak titers of 1:640 on day 7 (94 PV), and on days 14 (101 PV) and 21 (108 PV), titers ranged from 640 to 10 240 (Table 1).

Among the arMP-12ΔNSm21/384 vaccinated goats, 5 of 8 animals had neutralizing antibody with titers of 1:10 on day 5 PV. By day 7 PV, all animals had antibody titers that ranged from 1:10 to 1:160. The antibody titers remained about the same until day 87 PV before the MP-12 booster. After revaccination with MP-12 vaccine virus on day 87, titers increased and ranged from 1:160 to 1:640 on days 94 and 101 PV, and from 1:160 to 1:2,560 on day 108 PV. The antibody titers for the 2 EMEM control animals vaccinated with MP-12 during booster was 1:10 and 1:40 on day 7 (day 94 PV), and then increased to 1:160 for both animals on day 21 (108 PV) (Table 1), thus resembling the titers observed for the initial vaccinated animals with MP-12 and arMP-12ΔNSm21/384 vaccines. Comparison of antibody response between goats vaccinated with MP-12 and arMP-12ΔNSm21/384 vaccine were not significant ($p \geq 0.10$). However, the antibody titers for the goats that were revaccinated with MP-12 were significantly higher for animals that initially received the MP-12 than those vaccinated with arMP-12ΔNSm21/384 ($p \leq 0.03$).

One RVFV MP-12 and arMP-12ΔNSm21/384 IM vaccinated calves had the first detectable neutralizing antibody starting on day 7 PV with a titer of 1:10 and by day 14 PV all animals had antibody titers that ranged from 1:10 to 1:40. These antibody titers were maintained in all animals through day 87 before MP-12 booster. After revaccination of all IM animals on day 87 PV with 1×10^4 PFU/ml of MP-12 vaccine, antibody titers on day 94 to 108 PV increased and ranged from 1:160 to 1:640 in all revaccinated animals. The 2 control goats and 2 calves that were vaccinated with the MP-12 vaccine developed antibody with a 1:40 titer on day 7 (94 PV) and day 14 (101 PV) and the titer for animal #39 had an increased to 1:160 on day 21 (108 PV) (Table 1).

Although the number of IM vaccinated animals varied between species and overall the number of animals used was low, statistical analysis indicated that the antibody titers between MP-12 vaccinated goats and calves following the first vaccination showed that, the mean antibody titers were significantly higher for goats ($p=0.01$) than for calves. However, the antibody titers were not significantly different after MP-12 booster ($p=\geq0.13$). Mean antibody titers between arMP-12ΔNSm21/384 vaccinated calves and goats did not differ significantly however; the mean titers of goats were higher than those observed in calves. Moreover, the antibody titers differed significantly between EMEM animals vaccinated with MP-12 as compared to the animals vaccinated with arMP-12ΔNSm21/384 ($p\leq0.02$).

Table 1: RVF neutralizing antibody titers for calves and goats that were vaccinated IM with 1×10^5 PFU/ml of MP-12 and arMP-12ΔNSm21/384 vaccine and EMEM

			Days post-vaccination													
Species	**Vaccine**	**Animal #**	**-14**	**0**	**5**	**7**	**14**	**21**	**28**	**35**	**70**	**84**	**87**	**94**	**101**	**108**
Calf	EMEM	39	0	0	0	0	0	0	0	0	0	0	0	10	40	160
Calf	EMEM	106	0	0	0	0	0	0	0	0	0	0	0	10	40	40
Goat	EMEM	58	0	0	0	0	0	0	0	0	0	0	0	10	40	160
Goat	EMEM	112	0	0	0	0	0	0	0	0	0	0	0	40	160	10240
Calf	MP12	38	0	0	0	0	10	10	40	40	40	40	40	640	640	640
Calf	MP12	102	0	0	0	0	10	10	10	10	10	10	10	160	160	160
Calf	MP12	100	0	0	0	10	40	40	40	40	40	40	40	160	160	160
Goat	MP12	56	0	0	10	10	160	160	640	640	640	160	160	640	640	2560
Goat	MP12	59	0	0	10	10	40	160	160	160	160	160	160	640	2560	2560
Goat	MP12	60	0	0	10	40	40	160	160	640	160	160	160	640	10240	10240
Goat	MP12	70	0	0	10	40	40	40	160	160	40	40	40	640	2560	2560
Goat	MP12	73	0	0	10	40	160	160	640	160	160	160	160	640	2560	2560
Calf	MP12NSm	41	0	0	0	10	40	10	40	40	40	40	40	160	640	640
Calf	MP12NSm	42	0	0	0	0	10	40	40	160	40	40	40	160	640	640
Calf	MP12NSm	43	0	0	0	0	40	160	640	640	640	160	160	640	640	640
Calf	MP12NSm	44	0	0	0	0	10	10	40	640	10	10	10	40	160	160
Calf	MP12NSm	46	0	0	0	0	40	40	10	10	40	10	10	40	160	160
Goat	MP12NSm	57	0	0	10	160	160	160	160	160	160	160	40	640	640	640
Goat	MP12NSm	66	0	0	0	10	10	40	40	40	40	40	40	160	160	160
Goat	MP12NSm	67	0	0	10	40	40	40	160	160	160	40	160	160	160	160
Goat	MP12NSm	68	0	0	10	40	160	160	160	640	40	40	40	640	640	2560
Goat	MP12NSm	71	0	0	0	10	40	10	40	40	160	40	40	640	640	640
Goat	MP12NSm	108	0	0	10	40	40	40	40	40	10	40	10	160	640	640
Goat	MP12NSm	110	0	0	10	40	40	40	40	160	160	160	160	640	160	160
Goat	MP12NSm	111	0	0	0	40	160	160	640	640	160	160	160	640	640	2560

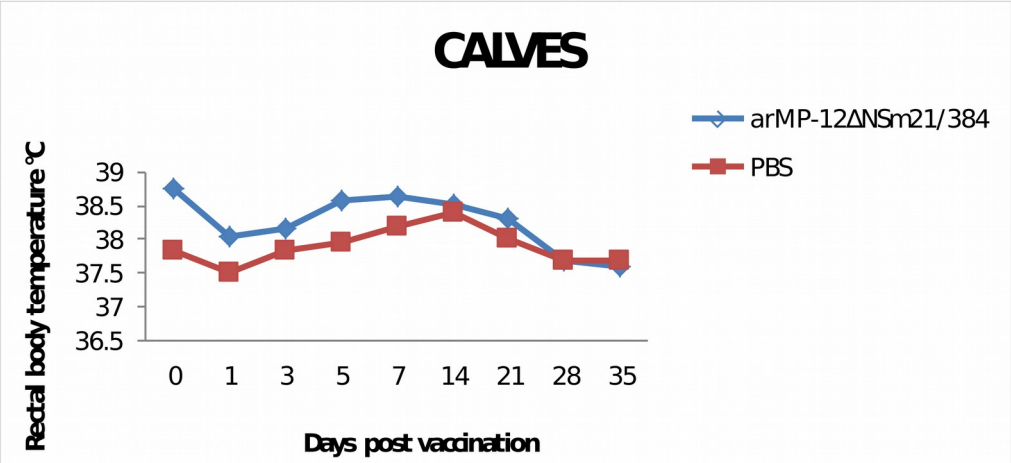
Data are expressed as the reciprocal of $PRNT_{80}$ titers.

4.2 Intra-nasal Vaccination

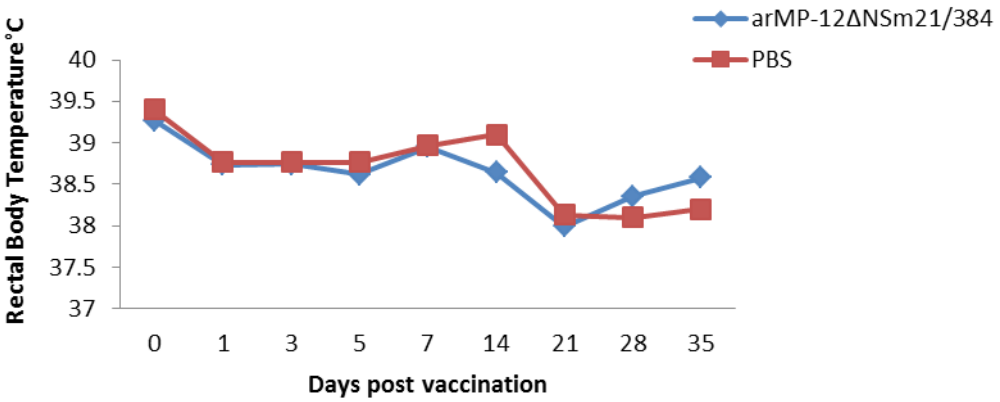
4.2.1 Clinical observation of the animals

Average body temperatures for goats, sheep and calves vaccinated IN with arMP-12ΔNSm21/384 as well as of negative control animals vaccinated with PBS are presented in (Fig. 4). The temperature ranged from 37.2 to 39.8°C in all arMP-12ΔNSm21/384 vaccinated animals and 37.5°C to 39.5°C in control animals. None of the animals developed clinical signs of illness such as fever throughout the study.

4a.



4b



4c

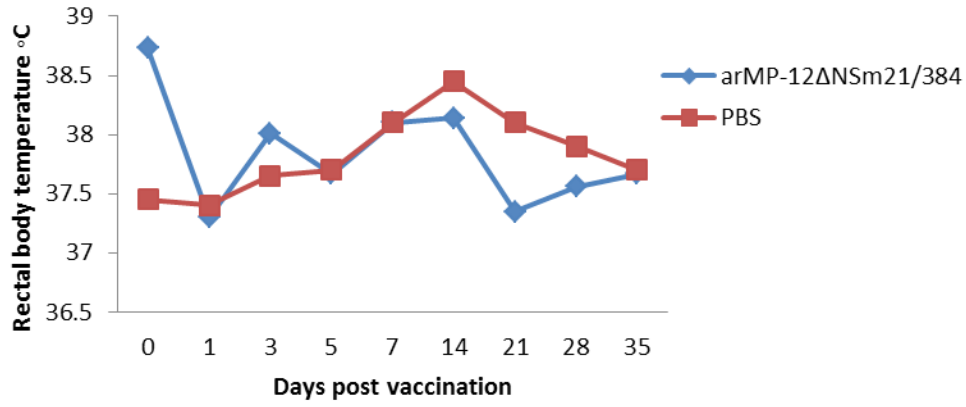


Figure 4: Mean rectal temperatures for calves (4a) sheep (4b) and goats (4c) vaccinated via the intranasal (IN) with 50 µl of 1×10^5 PFUs of arMP-12ΔNSm21/ 384 vaccines and Phosphate Buffered Saline (PBS).

4.2.2 Viremia

Viremia was not detected in any of the animals vaccinated IN with arMP-12ΔNSm21/384 or PBS, nor from cultures following blind passaged of samples in Vero cells. This observation was supported by results that showed the sera samples obtained from all goats, sheep and calves on 14 days before vaccination and on day 0 immediately prior to vaccination and days 0, 3, 4 and 5 PV were negative for RVFV based on virus isolation attempts in Vero cells.

4.2.3 Plaque Reduction Neutralization Test (PRNT-80)

A total of 70% of the goats, sheep and calves vaccinated with 50 µl of arMP-12ΔNSm21/384 vaccine via the IN route developed detectable neutralizing antibody (Table 2). Neutralizing antibody was observed beginning on day 5 PV in 3 of 7 calves and 1 of 9 goats (#979). None of the sheep had detectable neutralizing antibody on day 5 PV but antibody was detected in all sheep on day 7 and 14 PV together with the remaining goats and calves that were negative on day 5 (Table 2). Moreover, antibody titers in all 50 ul vaccinated calves and sheep ranged from 1:10 to 1:40, and 1:10 to 640 in goats respectively, (Table 2). A total of 9 vaccinated animals, 3 calves (#911, #918 and #902), 3 sheep (#938, #942 and #949) and 3 goats (#958, #965 and #969) did not develop detectable antibody through day 35 PV (Table 2). In addition, the mean antibody titers among goats, sheep and calves vaccinated with arMP-12ΔNSm21/384 did not differ significantly ($p= 0.30$). However, goats had the highest mean antibody titers as compared to sheep and calves.

For the 2 sheep that were vaccinated IN with 100 ul each of the arMP-12ΔNSm21/384 vaccine, the antibody titers were 1:160 for days 5 through 35 PV (Table 3).

Table 2: RVF neutralizing antibody titers of calves, sheep and goats vaccinated IN with 50 µl of 1×10^5 PFU of arMP-12ΔNSm21/384 vaccine and PBS

				Days post-vaccination							
Species	**Vaccine**	**Volume**	**Animal #**	**-14**	**0**	**5**	**7**	**14**	**21**	**28**	**35**
Calf	PBS	0.5	913	0	0	0	0	0	0	0	0
Calf	PBS	0.5	923	0	0	0	0	0	0	0	0
Sheep	PBS	0.5	943	0	0	0	0	0	0	0	0
Sheep	PBS	0.5	951	0	0	0	0	0	0	0	0
Sheep	PBS	0.5	954	0	0	0	0	0	0	0	0
Goat	PBS	0.5	974	0	0	0	0	0	0	0	0
Goat	PBS	0.5	980	0	0	0	0	0	0	0	0
Calf	arMP12ΔNSm	0.5	911	0	0	0	0	0	0	0	0
Calf	arMP12ΔNSm	0.5	921	0	0	10	10	10	10	10	10
Calf	arMP12ΔNSm	0.5	928	0	0	0	10	40	40	40	40
Calf	arMP12ΔNSm	0.5	918	0	0	0	0	0	0	0	10
Calf	arMP12ΔNSm	0.5	902	0	0	0	0	0	0	0	0
Calf	arMP12ΔNSm	0.5	908	0	0	0	0	10	10	10	10
Calf	arMP12ΔNSm	0.5	926	0	0	0	0	0	10	10	10
Calf	arMP12ΔNSm	0.5	915	0	0	10	10	40	40	40	40
Calf	arMP12ΔNSm	0.5	907	0	0	10	10	10	10	10	10
Calf	arMP12ΔNSm	0.5	924	0	0	0	0	10	40	40	40
Sheep	arMP12ΔNSm	0.5	933	0	0	10	10	10	10	10	10
Sheep	arMP12ΔNSm	0.5	938	0	0	0	0	0	0	0	0
Sheep	arMP12ΔNSm	0.5	939	0	0	0	0	40	40	40	40
Sheep	arMP12ΔNSm	0.5	942	0	0	0	0	0	0	0	0
Sheep	arMP12ΔNSm	0.5	949	0	0	0	0	0	0	0	0
Sheep	arMP12ΔNSm	0.5	955	0	0	0	0	40	40	40	40
Sheep	arMP12ΔNSm	0.5	957	0	0	0	0	0	10	10	10
Goat	arMP12ΔNSm	0.5	968	0	0	0	10	10	10	10	10
Goat	arMP12ΔNSm	0.5	958	0	0	0	0	0	0	0	0
Goat	arMP12ΔNSm	0.5	982	0	0	0	0	40	10	10	10
Goat	arMP12ΔNSm	0.5	977	0	0	0	0	40	40	40	40
Goat	arMP12ΔNSm	0.5	961	0	0	0	0	40	160	160	160
Goat	arMP12ΔNSm	0.5	965	0	0	0	0	0	0	0	0
Goat	arMP12ΔNSm	0.5	979	0	0	10	10	10	640	160	160
Goat	arMP12ΔNSm	0.5	976	0	0	0	0	10	10	40	40
Goat	arMP12ΔNSm	0.5	984	0	0	0	0	160	160	40	40
Goat	arMP12ΔNSm	0.5	969	0	0	0	0	0	0	0	0

Table 3: RVFV neutralizing antibody titers for 2 sheep vaccinated IN with 100 µl each of 1×10^5 PFU of arMP-12ΔNSm21/384 vaccine

				Days post-vaccination							
Species	**Vaccine**	**Volume**	**Animal #**	**-14**	**0**	**5**	**7**	**14**	**21**	**28**	**35**
Sheep	arMP12ΔNSm	100 µl	930	0	0	160	160	160	160	160	160
Sheep	arMP12ΔNSm	100 µl	935	0	0	160	160	160	160	160	160

A total of 68 serum samples from goats, calves, and sheep were negative for neutralizing antibody and 9 serum samples from goats and calves had low antibody titers to the RVFV arMP-12ΔNSm21/384 vaccine. These samples were retested by ELISA and of the 77 negative or with low antibody titers following vaccination with arMP-12ΔNSm21/384 vaccine, 4 were positive by both assays and 4 were positive by ELISA, but negative by $PRNT_{80}$ and 4 were positive by $PRNT_{80}$ but negative by the ELISA, thus demonstrating that the ELISA was slightly more sensitive assay for detecting antibody in sheep, goats and calves following vaccination with arMP-12ΔNSm21/384 vaccine.

CHAPTER FIVE

5.0 DISCUSSION

The results of this study indicated that the RVF MP-12 and arMP-12ΔNSm21/384 vaccine candidates were immunogenic as indicated by the development of neutralizing antibody in goats and calves following vaccination using the IM route. Regarding safety, although the animals had a slightly elevated temperature of 39°C to 40°C on day 1 PV all animals thereafter maintained normal body parameters such as appetite, well-being and normal rectal temperatures ranging between 37°C and 38°C. The transient, slightly elevated temperatures on Day 1 PV in all animals, including the negative control animals, suggested that this observation was not related to the vaccines. The most likely reason was stress caused by manual handling of the animals during vaccination. Other virulent RVFV infection-related symptoms such as hemorrhage, diarrhea, nasal and ocular discharge were not observed during the entire PV period. There was no evidence of virus shedding as the control animals remained negative while being confined in the same pens with the vaccinated animals. However, further studies are needed to exclude the possibility of shedding and/or spread of the vaccine virus, including experiments designed to evaluate viral shedding in excreta, such as nasal and ocular swabs, or testing for the potential spread to highly susceptible species, such as younger or immune-compromised animals.

The RVF Smithburn and Clone 13 vaccines, which are the more commonly, used vaccines in Africa, especially the Smithburn vaccine, warrant concern because of a link to fetal malformations, stillbirths and abortions during the first trimester of gestation. Moreover, experimental studies showed that Clone 13 had a potential teratogenic effect among pregnant sheep (Makoschey *et al*., 2016). Although this study did not assess the safety of the vaccines in pregnant goats and cattle, our preliminary results showed that both the MP-

12 and arMP-12ΔNSm21/384 vaccines were safe and the antibody titers induced were likely to be high enough to protect Tanzanian goats and calves against RVFV infection. The potential protective efficacy based on antibody titers is supported by the results of a study that showed antibody titers in sheep of approximately 1:100 following vaccination with arMP-12ΔNSm21/384 vaccine were protective against challenge with a virulent strain of RVFV (Weingartl *et al*., 2014a). Moreover, studies involving the parent MP-12 vaccine revealed that antibody titers ranging from 1:10 to 1:20 in hamsters and 1:20 in Rhesus Macaques afforded protection against challenge with a virulent strain of RVFV (Niklasson *et al*., 1984; Morrill and Peters, 2011).

All goats and calves vaccinated with MP-12 and arMP-12ΔNSm21/384 developed detectable neutralizing antibodies by day 5 or 7 PV, demonstrating that the vaccines elicited a rapid humoral immune response comparable to results reported for sheep and calves inoculated in the USA with a similar dose of arMP-12ΔNSm21/384 vaccine (Morrill *et al*., 2013a, b). Moreover, the results were similar to those observed for pregnant sheep vaccinated with RVF MP-12 vaccine that developed detectable neutralizing antibody from days 5 to 7 PV (Morrill *et al*., 1991).

Goats and calves vaccinated with the MP-12 vaccine developed neutralizing antibodies with peak titer between 1:160 and 1:640 for goats and 1:40 and 640 for calves by day 35 PV, which was either sustained or decreased through day 87 PV prior to being revaccination with the same vaccine. The rapid antibody immune response and overall in increasing pattern of antibody titer suggested that the vaccines may possibly protect animals, even if administered after the onset of an RVF outbreak, as reported previously by Bird *et al*. (2008). In our study, the rapid robust antibody response was observed in all goats and calves starting from day 94 PV after revaccination with the MP-12 vaccine. The

antibody titer for goats increased from 1:640 to 1:10,240 and from 1:40 to 1:640 in calves by day 108 PV, thus suggesting that the vaccine may afford protection to animals exposed to virulent RVFV in the field.

A steady increase in neutralizing antibody titer was observed in goats and calves following vaccination with arMP-12ΔNSm21/384, with peak titer measured on day 35 PV ranging from 1:160 to 1:640 in goats and 1:40 to 1:640 in calves. These results demonstrated that the deletion of the non-structural region of the medium viral RNA segment (NSm) did not affect immunogenicity and that the vaccine activated B-cells and dendritic cells for initiation of antibody development. Following revaccination with the MP-12 vaccine, all goats and calves elicited a rapid humoral immune response, and antibody titers were significantly higher than when the animals were first vaccinated, thus further demonstrating the potential of the vaccine to elicit strong immune responses in the field, if the vaccinated animals were exposed to virulent RVFV.

The antibody responses of goats and calves following single IM vaccination with MP-12 or arMP-12ΔNSm21/384 did not differ, and therefore the arMP-12ΔNSm21/384, with its potential for use as a DIVA vaccine, could have an added advantage over the MP-12 vaccine. The results were comparable to those reported for studies conducted in sheep and calves in the USA following vaccination with MP-12 and arMP-12ΔNSm21/348 (Morrill *et al.*, 1987, 1991, 1997b, 2013a, 2013b), in which animals developed detectable neutralizing antibody by day 7 PV with a titer of 1:20. In this study, neutralizing antibody was detected in most vaccinated animals on either day 5 PV or 7 with titer ranging from 1:10 to 1:160, slightly higher than titers reported for sheep in the USA study. The observation that sheep vaccinated with arMP-12ΔNSm21/384 and challenged with virulent RVFV developed protective antibody titers that were comparable to those observed for

goats and calves in this study and therefore, suggested that these latter animals would also be protected following challenge with virulent RVFV (Weingartl *et al*., 2014a).

Overall, the antibody titres for goats and calves in this study, following IM vaccinations with MP-12 or the arMP-12ΔNSm21/384 vaccine candidate, were slightly lower than titers observed for sheep during a study in Canada and sheep and cattle inoculated with these vaccines in the USA (Morrill *et al*., 1987, 1991, 1997b, 2013a, b; Weingartl *et al.*, 2014a, b). However, the titers were comparable to those reported for goats, sheep and cattle vaccinated with RVF Clone 13, despite the difference in laboratory testing procedures (Dungu *et al*., 2010; Daouam *et al*., 2015). Comparison of antibody titers among different animal species and involving different laboratories must consider possible differences in genetics, age, nutritional and health status, environment and vaccination, as well as laboratory testing procedures. Susceptibility differences may also contribute to variations among animal species in their ability to elicit immune responses to RVFV infection. For example, goats were reported to be more resistant to developing RVF disease than sheep, attributed in part to a lower and shorter viremia (Nfon *et al*., 2012). Therefore, the reduced amount of antibody produced in goats following vaccination, as opposed to sheep, may have resulted in a lesser amount of the vaccine virus being available to stimulate B cell secretion of antibody and may, therefore, have elicited a lower immune response in goats.

While differences were observed in antibody titer elicited in goats vaccinated IM with either of the vaccines, the calves' antibody titer in this study were generally observed to be lower than those of goats. These results are in agreement with those observed in a previous study done on Clone 13 (Njenga *et a*l., 2015), whereby the antibody response was higher in goats followed by sheep and calves increasing orderly. Also, another study showed that cattle mounted lower antibody responses to RVFV vaccines (Barnard, 1979). While

differences were observed in antibody titers elicited in goats and calves, more critical criteria and promising feature regarding the assessment of the potential value of the MP-12 and arMP-12ΔNSm21/384 vaccines was the fact that the antibody responses were consistent with moderate and predictive protective titer. The importance of this observation is that numerous studies in the USA and Africa have demonstrated that antibodies are crucial for the protection of animals against infection with RVFV (Niklasson *et al*., 1984; Morrill and Peters, 2011; Dungu *et al*., 2010; Pepin *et al*., 2010; Njenga *et al.,* 2015).

As a proof of concept study, the aim was to determine if the IN route of vaccination of goats, sheep and calves with the RVFV arMP-12ΔNSm21/384 vaccine using a needle-free device would elicit neutralizing antibody. Neutralizing antibody titers ranging from 1:10 to 1:160 were detected in some but not all goats, sheep and calves on days 5 or 14 after vaccination with 50 µl or 100 µl of a 1×10^5 PFU arMP-12ΔNSm21/384 vaccine. The reasons for the failure of some animals do not develop detectable neutralizing antibody is unknown, but may have reflected the 50 µl volume of the dose of the vaccine. This is supported by the fact that both animals vaccinated with the larger volume of 100 µl of the vaccine responded with antibody titers of 1:160 by day 5 PV. Although the number of animals was only 2, the findings are very promising that a volume of 100 µl of 1×10^5 PFU/ml would be an appropriate dose for use to vaccinate domestic ruminants with the arMP-12ΔNSm21/384 vaccine using the needle-free delivery IN route of vaccination.

Studies conducted by others suggested that the 1:40 antibody titers and observed in this study are likely to be protective against virulent RVFV, including one study that showed antibody titers of less than 1:100 protected sheep vaccinated with the arMP-12ΔNSm21/384 vaccine and challenged with the virulent RVFV (Weingartl *et al*., 2014a). Also, antibody titers ranging from 1:10 to 1:20 elicited in Rhesus macaques and hamsters

by the RVFV MP-12 parent vaccine were protective against a lethal challenge with virulent RVFV (Niklasson *et al*., 1984; Morrill and Peters, 2011). Currently, the minimal standard $PRNT_{80}$ protective response for at-risk personnel working with RVFV is ≥1:40 (Pittman *et al*., 2016a). While neutralizing antibody is considered to be the main protective arm of the immune response that protect human and animals against RVFV infection (Mansfield *et a*l., 2015), the most appropriate methods for ascertaining the possible protective efficacy of the antibody titers observed in goats, sheep and calves in this study would be to perform challenge studies with virulent RVFV. Such studies were not conducted because of biosafety requirements, including Animal Biosafety Level 3 plus facilities that were not available for working with the virulent strain of the RVFV.

The evaluation of the RVFV arMP-12ΔNSm21/384 vaccine using the IN route of vaccination was the first study conducted in livestock using this route of vaccination. As a proof of concept study, the volume of the RVFV arMP-12ΔNSm21/384 vaccine dose was 50ul, but contained the same concentration or 1 x 10^5 PFU/ml as the maximum dose used in other studies (Morrill *et al*., 1997a; Wilson *et al*., 2014). Thus, the dose was not lower, but the volume was lower to determine if the lower volume would elicit an immune response and if so, the lower volume would indicate that the cost of manufacturing the vaccine would be substantially lower and therefore more affordable by the user. Moreover, IN route offers mucosal immunity that has the potential of affording protection to both local and distal sites of the body that could afford more robust protection of livestock against RVF.

From the previous scenario observed in sheep and goats during a study in South Africa, animals were positive by virus neutralization and negative by ELISA assay and vice versa (Njenga *et a*l., 2015). The same observation was also reported previously by Paweska *et a*l.

(2005) and findings at SUA showed the disagreement between ELISA and plaque reduction neutralization tests was about 10%. In this study, some PV days, samples including days 21 and 35 PV for animals #968, #982 and #918 were positive for RVFV antibody by either ELISA or PRNT80 while 8 animal's samples were either positive or negative by both assays indicating the sensitivity of the two tests in antibody detection. Although this observation is consistent with the previous observation, 10% disagreement between two assays is very minimal and therefore may not be accurate and reliable and may have occurred by chance. Also, sometimes ELISA detects antibody in the samples and thus the results always need to be confirmed by other tests including PRNT test, can always detect antibody that is specific against the virus used in the test and therefore termed to be a specific test and thus used as a gold standard for antibody detection.

Overall, the findings of this study revealed that the RVFV MP-12 and arMP-12ΔNSm21/384 vaccine candidates were safe and immunogenic in African breeds of goats and calves using the IM route of vaccination. The immune response was similar for the 2 vaccines and therefore indicated that the arMP-12ΔNSm21/384 with a DIVA marker would be the most appropriate and effective vaccine for use to vaccinate African livestock. As a proof of concept study, the IN route of vaccinating sheep, goats and calves with the arMP-12ΔNSm21/384 vaccine using a needle-free device elicited neutralizing antibody. However, preliminary results suggested that the volume of vaccine needs to be 100 ul or more to induce a consistent and robust immune response. This non-invasive routes using a needle-free vaccine delivery device has potential advantages over percutaneous vaccination using needles in mass vaccination scenarios because the needle-free route of vaccination offers a simple, efficient and rapid method to vaccinate livestock.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

Observations generated by this study revealed that the IM route of vaccination of goats and calves with the RVFV arMP-12ΔNSm21/384 vaccine consistently elicited neutralizing antibody in these species. Overall, the findings support previously reported data that the vaccine-induced antibody titers in calves that are likely to afford protection against virulent RVFV. These are the first reported findings for the RVFV arMP-12ΔNSm21/384 vaccine evaluation in goats and provide neutralizing antibody titers that are comparable or higher to those of sheep and calves. Overall, these are the first observations that strongly showed that the RVFV arMP-12ΔNSm21/384 vaccine is a very promising candidate for use to prevent RVF in African breeds of goats and calves.

The evaluation of the RVFV arMP-12ΔNSm21/384 vaccine using the IN route of vaccination was a proof of concept study and the first study conducted in livestock using this route of vaccination. Overall, the study achieved the aim and provided data needed to conduct a more in-depth designed study to further evaluate the RVFV arMP-12ΔNSm21/384 vaccine using the intra-nasal route of vaccination. More noteworthy observations were that this route of vaccination elicited neutralizing antibody ranging from 1:10 to mostly 1:160 in 70% of the animals tested using a volume of the only 50ul. An increase in volume to 100ul per animal enhanced the immune response with antibody titers of 1:100 being attained even though only 2 sheep were used in the trial. Although the data are preliminary, the intra-nasal route of vaccination of sheep, goat and calves with the RVFV arMP-12ΔNSm21/384 vaccine is very promising for eliciting an immune response and possible afford protection to these animals against virulent RVFV.

6.2 Recommendations

Based on the results of this study;

i. More studied should be done using a larger number of animals and escalating doses to test the safety and immunogenicity of the RVFV arMP-12ΔNSm21/384 vaccine via the IM and IN routes.

ii. The findings of this study may beused to design and conduct further studies needed to optimize the volume of inoculum and the vaccine dose for the IN route of vaccination.

iii. More Studies should be done to determine the sensitivity between the two assays, PRNT and ELISA in terms of early detection of RVFV antibody.

iv. Studies need to be expanded to evaluate the safety of the RVFV arMP-12ΔNSm21/384 vaccine in pregnant livestock, especially in pregnant sheep using different routes of vaccination.

v. Studies should be performed to evaluate the protective efficacy of the RVFV arMP-12ΔNSm21/384 vaccine at both laboratory using appropriape biosafety containment facility and field based environment.

REFERENCES

Adamson, E. K., Nyundo, S., Rowland, J., Palermo, P. M., Matiko, M. K., Bettinger, G. E., Wambura, P., and Watts, D. M. W. (2018). Safety and Immunogenicity of Rift Valley Fever MP-12 and a Novel arMP-12ΔNSm21/384 Recombinant Vaccine Candidate in Native Breed of Black Head Sheep (*Ovis Aries*) from Tanzania. *Journal of Vaccines Vaccine* 9: 394-399.

APPENDICES

Appendix 1: Innovative Diagnostic Screen Rift Valley Fever Competition Multi-species ELISA

Testing procedures

1. Add
   i. 50 ul of the Dilution buffer 19 to each well.
   ii. 50 ul of the Positive Control to well A1 and B1
   iii. 50 ul of the Negative Control to well C1 and D1
   iv. 50 ul of each sample to be tested to the remaining wells
2. Incubate 1 hour ± 4min at 37 °C
3. Empty the well. Wash each well 3 times with approximately 300 ul of the wash solution. Avoid drying of the wells between washings
4. Prepare the Anti-RVF-NP Conjugate 1× by diluting the Anti-RVF-NP-Po Conjugate 10× to 1/10 in Dilution Buffer 19
5. Add 100 ul of the Conjugate 1× to each well
6. Incubate 30 min ± 3 min at 21°C
7. Empty the wells. Wash each well 3 times with approximately 300 ul of the wash solution. Avoid drying of the wells between washings

8. Add 100 ul of the Substrate Solution to each well
9. Incubate 15 min ± 2 min at 21°C in the dark
10. Add 100 ul of the Stop solution to each well in order to stop the reaction
11. Read and record the O.D at 450nm.

Validation

The test is validated if:

12. The mean value of the Negative Control O.D is greater than 0.7
13. The mean value of the Positive Control is less than 30% of the O.D of Negative Control.

Appendix 2: Onderstepoort Journal of Veterinary Research

Appendix 3: Journal of Vaccines and Vaccination