Rift Valley fever in Mozambique - epidemiology, diagnostics and vaccine use

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Cover: Cattle grazing in a wetland in Chibuto District (Gaza Province), South Mozambique
(Photo: Belisário Moiane)

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Abstract

Background: Rift Valley fever (RVF) is an arthropod-borne disease that causes huge losses among livestock in Africa and Arabic Peninsula, due to high death and abortion rates. RVF phlebovirus (RVFPV) also infects humans, raising public health concerns worldwide. RVF is endemic in Mozambique however, outbreaks were just reported in the south. In this thesis, we have analyzed the serological status of domestic ruminants, and African buffaloes, identified RVFPV mosquito vectors in Gaza province, evaluated the stability of a formalin RVFPV vaccine stored at different temperatures in Mozambique, and evaluated RVFPV antigens for oral immunization of mice.

Materials and methods: Blood samples were collected from cattle (n = 2724), goats (n = 1283), sheep (n = 148), and African buffaloes (n = 69), between February 2010 and May 2011 (study I), in 2013 and 2014 in the three regions of Mozambique (study II), and 2014 to 2016 in Gaza (study III), furthermore, mosquito trapping was performed for morphological identification and RVFPV detection (study III). Serological status of the herds was achieved by ELISA and PRNT. In study IV we evaluated the stability of a formalin-inactivated RVFPV-vaccine treated in four different temperature conditions, by assessing the antibody response in animal groups A-D. Two RVFPV antigens (N and ΔGn proteins) were expressed in *Arabidopsis thaliana* plants and administered orally to mice in two experiments (n =4, each group and n = 8 in the control) (study V).

Results and discussion: RVFPV seropositivity rate ranged from 7% to 36.9%. The seroprevalences in livestock and African buffaloes were high suggesting that RVFPV is actively circulating in Mozambique. *Culex, Anopheles,* and *Mansonia* were abundant potential vectors of RVFPV; Members of *Culex* genus were found to carry RVFPV, however, identification down to species was not performed; the formalin-inactivated vaccine elicited strong and long lasting antibody response in cattle, irrespectively of the storage condition; *A. thaliana* plants successfully expressed RVF antigens, which in turn induced strong anti-body responses in mice. Further studies on the use of these antigens in ruminants are pending investigations.

Conclusions: (1) RVFPV is actively circulating among livestock and African buffaloes in Mozambique which requires a continuous and intensified surveillance; (2) a member of *Culex* genus is a candidate vector for RVFPV in South Mozambique; (3) formalin-inactivated RVFPV vaccine used for cattle in Mozambique is stable; (4) RVFPV antigens expressed in *A. thaliana* plants elicited strong antibody response in mice and are eligible for use as vaccine candidates.

Keywords: Rift Valley fever phlebovirus, Arabidopsis thaliana, RVF antigens, seroprevalence, mosquito vectors, cattle, goats, sheep, African buffaloes, Mozambique

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Rift Valley feber i Mozambique - epidemiologi, diagnostik och vaccinering

Abstrakt

Bakgrund: Rift Valley feber (RVF) är en artropod-vektorburen sjukdom i Afrika och på Arabiska halvön, RVF orsakar hög dödlighet och hög abortfrekvens och därmed stora förluster bland boskap. RVF phlebovirus (RVFPV) infekterar också människor, vilket globalt uppmärksammas som ett folkhälsoproblem. RVF är endemisk i Moçambique men utbrott har bara rapporterats i de södra delarna. I denna studie har vi analyserat serologisk status hos tama idisslare och hos Afrikansk buffel, identifierat myggvektorer för RVFPV i Gaza-provinsen, utvärderat stabiliteten hos ett formalininaktiverat RVFPV-vaccin lagrat vid olika temperaturer under fältförhållanden i Moçambique och utvärderat RVFPV-antigener för oral immunisering av möss.

Material och metoder: Blodprover insamlades från nötkreatur (n = 2724), getter (n = 1283), får (n = 148) och Afrikansk Buffel (n = 69), mellan februari 2010 och maj 2011 (studie I), under 2013 och 2014 i tre regionerna i Moçambique (studie II) och under 2014-2016 i Gaza (studie III). Vidare så fångades mygg för morfologisk identifiering och för påvisande av RVFPV (studie III). I studie IV utvärderade vi stabiliteten hos en formalininaktiverad RVFPV-vaccin som behandlats vid fyra olika temperaturer, genom att bedöma antikroppssvaret i djurgrupperna A-D. Serologisk undersökning av djuren gjordes med ELISA och PRNT. Två RVFPV-antigener (N och ΔGn-proteiner) som uttrycktes i *Arabidopsis thaliana*-växter administrerades oralt till möss i två experiment (n = 4 i varje grupp och n = 8 i kontrollen) (studie V)

Resultat och diskussion: Seroprevalensen för RVFPV varierade från 7% till 36,9% och den höga prevalensen hos boskap och Afrikansk buffel tyder på en aktiv cirkulation av RVFPV i Moçambique. *Culex, Anopheles* och *Mansonia* var rikligt förekommande potentiella vektorer för RVFPV. *Culex*-släktet påvisades bära RVFPV, men identifiering ned till underart utfördes inte; det formalininaktiverade vaccinet framkallade ett starkt och långvarigt antikroppssvar hos nötkreatur, oberoende av lagringsförhållandet. *A. thaliana*-växter uttryckte framgångsrikt RVF-antigener, vilket i sin tur gav upphov till ett starkt antikroppsvar hos möss. Ytterligare studier behövs om användningen av dessa antigener hos idisslare.

Slutsatser: (1) RVFPV cirkulerar aktivt bland boskap och Afrikansk buffel i Moçambique vilket kräver en intensiv övervakning; (2) en underart av *Culex* släktet är en kandidat som vektor av RVFPV i södra Moçambique; (3) formalininaktiverat RVFPV-vaccin som används till boskap i Moçambique är stabilt; (4) RVFPV-antigener uttryckta i *A. thaliana*-växter framkallar ett starkt antikroppssvar hos möss och är en tänkbar vaccinkandidat.

Nyckelord: Rift Valley feber phlebovirus, *Arabidopsis thaliana*, RVF antigener, seroprevalens, myggvektorer, nötkreatur, getter, får, Afrikansk buffel, Moçambique

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Dedication

To my family, Quitéria Moiane, Lenna Ndindasse and Sáskia Nwety, my parents Tomé Moiane and Sarita Moiane for their strong support in this journey. I extend my gratitude to the farmers from the study areas, particularly the small holders, for allowing our research team to work in their farms.

The meaning of life is to find your gift. The purpose of life is to give it away.
Author Unknown

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Lagerqvist N*, **Moiane B**, Mapaco L, Fafetine J, Vene S, Falk KI (2013). Antibodies against Rift Valley fever virus in Cattle, Mozambique [letter]. *Emerging Infectious Diseases*, 19 (7), 1177-1178. doi: eid1907.130332.

II **Moiane B*,** Mapaco L, Thompson P, Berg M, Albihn A, Fafetine J (2017). High seroprevalence of Rift Valley fever phlebovirus antibodies in domestic ruminants and African Buffaloes in Mozambique shows need for intensified surveillance. *Infection Ecology & Epidemiology* (accepted).

III **Moiane B*,** Mapaco L, Fafetine J, Macandza G, Choleti H, Dustone B, Berg M, Albihn A (2017). Identification of the Rift Valley fever phlebovirus vectors in South Mozambique (submitted).

IV Lagerqvist N*[1], **Moiane B[1]**, Bucht G, Fafetine J, Paweska JT, Lundkvist Å, Falk KI (2012). Stability of a formalin-inactivated Rift Valley fever vaccine: Evaluation of a vaccination campaign for cattle in Mozambique. *Vaccine*, 30, 6534-6540. doi: 10.1016/j.vaccine.2012.08.052.

[1]These authors contributed equally to this work.

V Kalbina I, Lagerqvist N, **Moiane B**, Ahlm C, Andersson S, Strid Å*, Falk KI** (2016). *Arabidopsis thaliana* plants expressing Rift Valley fever virus antigens: Mice exhibit systemic immune responses as the result of oral administration of the transgenic plants. *Protein Expression and Purification*, 127, 61-67. doi: 10.1016/j.pep.2016.07.003.

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The contribution of Belisário Moiane (BM) to the papers included in this thesis was as follows:

I- BM contributed to the design of the study, field work (experimental and sampling), laboratory work, data analysis (with support from the co-authors and statistician), drafted and finalised the manuscript.

II and III- BM contributed to the design of the studies, planned and performed field and laboratory work, compiled the data, performed the analysis with support from the co-authors and statistician, drafted and finalized the manuscripts, and was also handling the correspondence with the Journals' Editorial managers during the manuscripts submission process.

IV- BM contributed to the design, planning of field and laboratory work, participated in the data analysis with the support of a statistician, compilation of data and drafted the manuscript.

V- BM contributed to the study design, participated in the laboratory work and finalization of the manuscript.

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Abbreviations

BL	Basal labyrinth
CDC	Centres for Disease Control
c-DNA	Complimentary Deoxyribonucleic acid
CVL	Central Veterinary Laboratory
DINAP	Direcção Nacional de Pecuária [Natonal Directorate of Livestock]
DNSV	Direcção Nacional dos Serviços de Veterinária [Directorate of the National Veterinary Service]
dsRNA	Double stranded RNA
ECL	Enhanced chemiluminescent liquid
EEV	Equine encephalitis virus
eiF-2	Eukaryotic initiation factor two
ELISA	Enzyme linked immunosorbent assay
FAO	Food and Agricultural Organization
FBXW11	Ubiquitin E 3 ligase sub-unit
GIS	Geographic information system
IFA	Immunofluorescence Assay
IgG	Immunoglobulin G
IgM	Immunoglobulin M
Imd/jnk	Immunodeficiency/c-Jun N-terminal kinases, signalling pathways
INF	Interferon
JAK/STAT	Janus kinase/Signal Transducer and Activator of Transcription
JEV	Japanese Encephalitis Virus
LGP-2	Laboratory of Genetics and Physiology 2
MDA5	Melanoma Differentiation-Associated protein 5
NF-kβ	Necrosis factor kappa beta
Ni-NTA	Nitrilotriacetic acid
NSm	Non-structural protein of medium size

NSs	Non-structural of small size
PAMPS	Pathogen-associated molecular patterns
PCR	Polymerase chain reaction
PDVDF	Polyvinylidene difluoride membrane
piRNA	Piwi-interacting RNA
PKR	Protein Kinase R
PRNT	Plaque reduction neutralization test
PRRs	Pattern recognition receptors
RIG-I	Retinoic acid-inducible gene I
RNA	Ribonucleic Acid
RT-PCR	Reverse-transcription polymerase chain reaction
RVF	Rift Valley fever
RVFPV	Rift Valley fever phlebovirus
SAP-30	Sin3A-associated protein
SCF	Skp1/Cul1/F-box complex (ubiquitin ligase)
SFV	Semliki forest virus
SiRNA	Small interfering RNA
SLEV	St. Louis encephalitis virus
ssRNA	Single stranded RNA
TFIIH	Transcription factor II Human
VLPs	Virus-like particles
WEEV	Western equine encephalitis virus
WHO	World Health Organization
XPB	Xeroderma pigmentosum type B
YY1	Yin Yang 1 transcription repressor

1 Background

Rift Valley Fever (RVF) is a viral zoonotic disease that is spread by arthropods (primarily mosquitoes) and affects livestock (e.g. sheep, goats, cattle, buffaloes, and camels) and humans in Africa (Morvan *et al.*, 1992; Ksiazek *et al.*, 1989; Meegan, 1979) and the Arabian Peninsula (Davies & Martin, 2006; Balkhy & Memish, 2003). RVF may be a problem to handle in the close future also in other parts of the world than Africa and the Arabic peninsula. RVF antibodies were detected among camels *(Camelus dromedairus)*, gazelle (Gazella *subgutturosa subgutturosa*), and buffaloes (*Bubalus bubalis linneaus*) in Turkey (Gur *et al.*, 2017).

RVF is responsible for significant economic losses in livestock production (Chengula *et al.*, 2013), because it is associated with a high mortality rate, chiefly in young animals, and it also induces abortions among gestating females (Davies, 1990; Kondela *et al.*, 1985).

The incubation period for the RVF phlebovirus, RVFPV (ICTV, 2016), can vary from one to six days, essentially determined by the age and species of the infected animal. Among the different species that serve as hosts, domestic ruminants are the most susceptible to infection.

Also, the greatest sensitivity to RVFPV is seen in young animals, and hence symptoms are severe in this group. Sudden onset of fever (up to 41 ℃), disinclination to move, anorexia, and death within 36 hours are common symptoms in infected lambs and calves.

In adult animals, symptoms are usually not noticeable, and livestock show only mild or no signs of the disease. However, a severe form of RVF is characterized by fever, anorexia, and lymphadenopathy followed by weakness, and thereafter death usually occurs in about 15% of affected livestock.

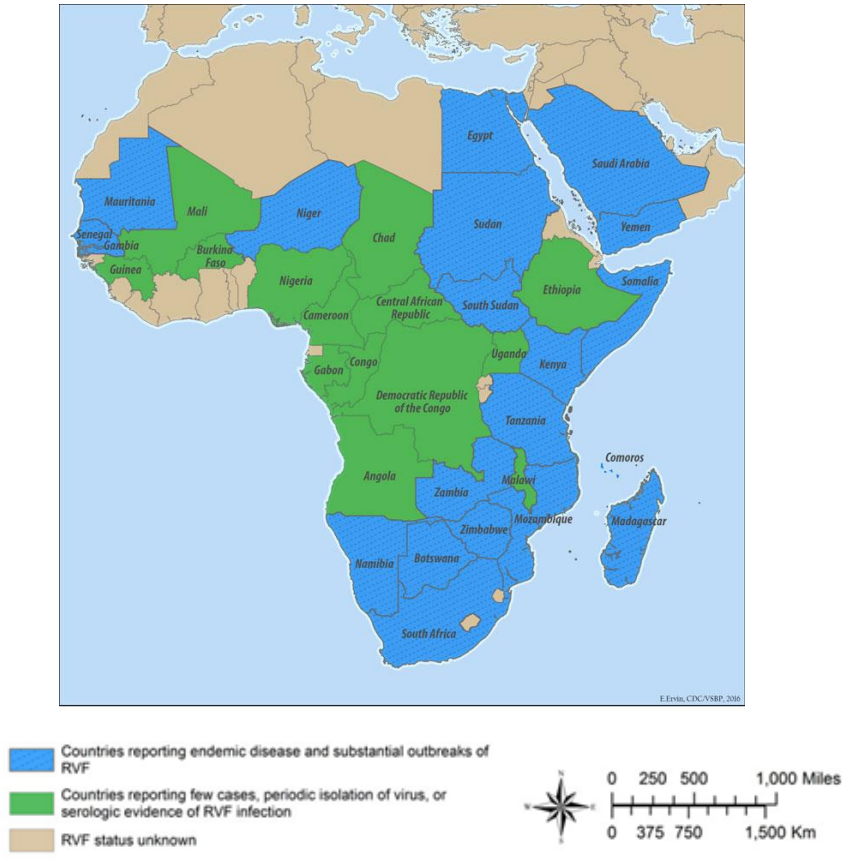
Rift Valley fever can also be a serious diseases for humans, and yet there are no licensed vaccines for wide use, except some experimental vaccines used under experimental conditions, and thus with strong restrictions (Ikegami & Makino, 2009).

1.1 Rift Valley fever epidemics

1.1.1 Animals

RVFPV was first identified in 1931 based on observations made in the field. Also in lambs, goats, and cows given experimental inoculations of extracts obtained from affected animals during an epidemic that occurred in sheep on a farm in the Rift Valley in Kenya at that time (Daubney & Hudson, 1932).

During subsequent decades, several RVF outbreaks were reported to cause huge numbers of deaths in livestock in sub-Saharan Africa, Egypt, and the Arabian Peninsula (Saudi Arabia and Yemen) (Figure 1). In Mozambique, outbreaks have been reported in cattle herds in the provinces of Gaza and Maputo in the southern part of the country (Fafetine *et al.*, 2016; Valadão, 1969).



https://www.cdc.gov/vhf/rvf/outbreaks/distribution-map.html, accessed on 07.06.2017

Figure 1: Rift Valley fever distribution Map

1.1.2 Humans

Humans can contract RVFPV through the bites of infected haematophagous (blood-feeding) mosquitoes or by direct or indirect contact with body fluids or organs from infected animals.

Thus during an outbreak of RVF, cattle herders, farmers, slaughterhouse workers, and veterinarians are at high risk of infection due to their close contact with infected animals.

Table 1 summarizes aspects of significant epidemics involving humans in Africa and the Arabian Peninsula. It is important to note the zoonotic relevance

of RVFPV highlighted by that all of these outbreaks are also leading to human deaths.

Table 1: Summary of significant RVF epidemics, involving humans on the African continent and Saudi Arabia

Year(s)	Country	No. of cases	No. of deaths	Reference
2016	Niger	64	23	(WHO, 2016)
1977–1978	Egypt	18,000	598	(El-Akkad, 1978)
2010-2015	Mauritania	31-63	8-13	(Boushab *et al.*, 2016; Abdourahmane *et al.*, 2014; Faye *et al.*, 2014; WHO, 2012; El Mamy *et al.*, 2011)
1991	Madagascar	Unknown	1	(Morvan *et al.*, 1992)
1997–1998	Kenya, Tanzania, and Somalia	100,000	> 450	(CDC, 1998)
1998	Kenya	27,500	Unknown	(CDC, 1998)
2000–2001	Saudi Arabia and Yemen	882	124	(Balkhy & Memish, 2003)
2006–2007	Kenya, Somalia, and Tanzania	1,000	300	(WHO, 2007a)

2007–2008	Tanzania Kenya	684 264	155 109	(WHO, 2007b; WHO, 2007a)
2007	Sudan	747	230	(Hassan *et al.*, 2011)
2008	Madagascar	418	17	(WHO, 2008)
2009-2011	South Africa and Namibia	250	25	(Archer *et al.*, 2013; Monaco *et al.*, 2013; Métras *et al.*, 2012)

The main route of infection is through inoculation of the virus via wounds or abrasions caused by contaminated knives or other perforating tools used for slaughtering or necropsy of animal carcasses.

Another important mode of transmission entails inhalation of RVFPV-containing aerosols produced during the slaughter of infected animals, inappropriate handling of laboratory samples or when taking care of patients with RVF (Murphy *et al.*, 1999).

Ingestion of unpasteurized or uncooked milk from infected animals has also been described as a potential source of RVFPV (Balkhy & Memish, 2003).

Evidence of vertical transmission of RVFPV has been documented in Saudi Arabia, where IgM antibodies against this virus were detected in samples from a new-born and its mother soon after birth; the five-day-old infant presented with a skin rash and showed signs of liver and spleen swelling on palpation (Adam & Karsany, 2008), and in Sudan where 54% (15/28) women with acute infection aborted (Baudin *et al.*, 2016). This finding suggests that RVFPV has the ability to infect the foetus during pregnancy and cause severe lesions that can cause abortion or compromise survival after birth.

Notably, anti-RVFPV antibodies have been detected in 2% (28/1163) of pregnant women (Niklasson *et al.*, 2009) and 5% (10/200) of febrile patients (Gudo *et al.*, 2016) in Mozambique, although no outbreak in humans has ever been reported in that country.

1.2 The Rift Valley fever phlebovirus particle

RVFPV is a single-stranded RNA virus of the genus *Phlebovirus*, which is one of the four genera of the family *Phenuiviridae*. One should note that since 2016, there were changes in the taxonomic classification of some members of the order *Bunyavirales*. The former name Rift Valley fever virus, RVFV, was replaced by Rift Valley fever phlebovirus, and the family name *Bunyaviridae* was replaced by *Phenuiviridae*, according to International Committee on Taxonomy of Viruses (ICTV, 2016). Therefore, we have proposed, in this thesis, the abbreviation "RVFPV" based on the species name initials, although in the published studies the old terminology is still used.

In short, RVFPV is composed of three negative-stranded RNA segments, here simply designated small (S), medium (M), and large (L). The L segment encodes RNA polymerase; the M segment generates the envelope glycoproteins Gn/Gc and medium sized non-structural protein (NSm); and the S segment codes for nucleocapsid (N) protein and a small non-structural protein (NSs) (Murray *et al.*, 2009; Suzich & Collett, 1988).

Medium-sized non-structural protein (NSm) plays a role in suppression of apoptosis in the host cells (Won *et al.*, 2007), and NSs is responsible for disruption of the antiviral response of those cells (Bird *et al.*, 2008).

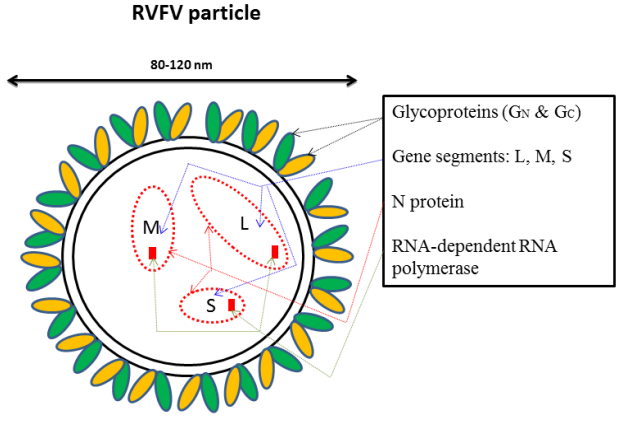


Figure 2. Schematic representation of the structure of the Rift Valley fever phlebovirus (RVFPV) showing the three genetic segments designated small (S), medium (M), and large (L). The yellow and green structures on the surface of the virus particle are the glycoproteins Gn and Gc. The red dots surrounding each gene segment represent N protein, and the red rectangles within the segments are the viral RNA-dependent RNA polymerase. *Adapted from*: Pepin *et al.* (2010).

Table 2: The components of the RVFPV and their roles

Protein	Molecular weight	Genome segment	Role	Reference(s)
Viral RNA polymerase	230	L	Viral replication and RNA transcription.	(Zamoto-Niikura *et al.*, 2009)
G_n/G_c	65/56	M	Participation in interactions of the virus with the host cell via membrane receptors, contribution to assembly of the virus particles, and interaction with N protein.	(Filone *et al.*, 2006; Garry & Garry, 2004)
NSm (NSm 1/NSm2)	78/14	M	Suppression of virus-induced apoptosis in the host cells.	(Won *et al.*, 2007)
N	27	S	Induction of humoral and T-cell immune responses.	(Jansen van Vuren *et al.*, 2011)
NSs	31	S	Function as an interferon antagonist by limiting IFN-mediated host antiviral responses, inhibiting cellular transcription, and degrading protein kinase PKR; interaction with specific DNA regions of the host genome to induce chromosome cohesion and segregation defects.	(Mansuroglu *et al.*, 2010; Bird *et al.*, 2008; Billecocq *et al.*, 2004)

1.3 The vectors and ecology

Mosquitoes of the genera *Aedes* and *Culex* are the main vectors of RVF (Fontenille *et al.*, 1998; Gad *et al.*, 1987b), however other biting flies (e.g., *Anopheles* spp.) and sandflies can also be involved in the amplification of RVF epidemics (Seufi & Galal, 2010b; McIntosh *et al.*, 1980).

The main breeding sites for the mentioned mosquitoes are ponds, temporary wetlands (dambos), and irrigation ditches that are filled with water during heavy rains and floods. The mosquitoes, especially *Aedes* spp., lay infected eggs at the edges of the short-lived water bodies; those eggs remain dormant over the dry season, and therefore the level of RVF in livestock is low during that period (the enzootic cycle). In the rainy season, the eggs hatch into adult RVFPV-infected mosquitoes that can transmit the virus to livestock (Davies *et al.*, 1992; Logan *et al.*, 1991).

Twenty days after the onset of flooding, the *Aedes* population decreases and is replaced by a secondary RVFPV vector (members of *Culicinae*, *Anophelinae*, and various haematophagous biting flies such as phlebotomines). The increase in the population of secondary vectors amplifies the RVFPV transmission cycle, and therefore large numbers of livestock and humans are infected thus causing an epidemic (Figure 3).

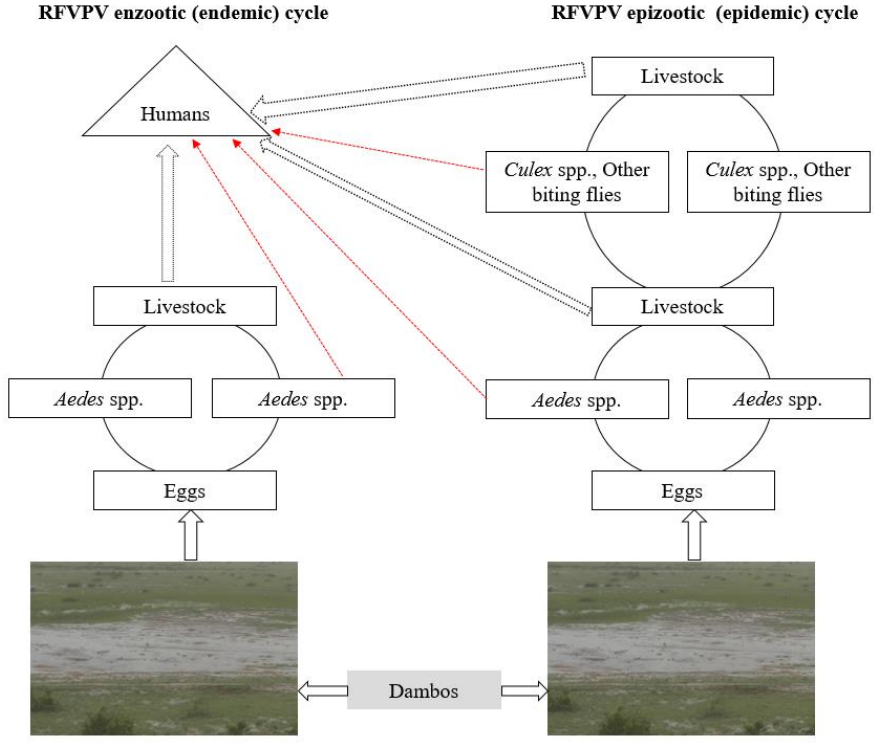


Figure 3: Schematic representation of the endemic and epizootic cycles of RVFPV showing the role of *Aedes* spp and other vectors on the occurrence of either of the two cycles. The thick black dot arrows show the interaction between livestock and humans in both cycles and the thin red dot arrows indicate how the biting flies can potentially transmit RVFPV to humans, either in enzootic or epizootic cycle.

There are several factors that have a substantial impact on the RVF vectors and consequently also affect the trade of live animals and the sales of meat and related products (from infected animals). Several of those factors are associated with the changing weather e.g. increased temperatures intensified by the *El Ninõ* phenomenon (Anyamba *et al.*, 2001), which has led to extreme weather events as desertification and floods. Furthermore, agricultural practices, including the construction of dams, have had significant influence on the spread of RVFPV vectors across the African continent and the Arabian Peninsula (WHO & FAO, 2009; Anyamba *et al.*, 2001; Linthicum *et al.*, 1999).

1.4 Clinical signs

1.4.1 Animals

The most evident signs of an RVF outbreak in farm animals are extensive occurrence of spontaneous abortions or stillbirths and particularly among young animals extremely high mortality rates (up to 100% in susceptible breeds).

The following signs and symptoms can be observed: acute fever, lymphadenitis, nasal and ocular discharge (mainly in adult livestock), haemorrhagic diarrhoea, vomiting, abdominal colic, jaundice, decreased milk production, and prostration occasionally followed by death due to severe liver impairment (Kapoor, 2008).

Symptoms may vary due to age, animal species, breeds and virus strain. Young animals are more susceptible to infection and develop a severe disease as very often is leading to death. In livestock, sheep are more susceptible than goats and cattle (Ikegami & Makino, 2011). The disease severity may also be influenced by the RVFPV strain involved (Wilson *et al.*, 2016). The local livestock breeds tend to be more resistant to RVF infection than the imported ones (Munyua *et al.*, 2010).

1.4.2 Humans

In humans, the RVFPV incubation period is two to six days, and the clinical features of infection vary from mild to severe manifestations of the disease, which occurs in ocular, meningoencephalitic, and haemorrhagic forms (Laughlin *et al.*, 1979).

Persons with the mild form experience a flu-like fever, muscle and joint pain, headache, and some also exhibit marked sensitivity to light (photophobia), anorexia, neck stiffness, and vomiting (Davies & Martin, 2006; Al-Hazmi *et al.*, 2003).

The severe (meningoencephalitic) form of RVF affects a small number of individuals, and it is characterized by either or both of two distinct syndromes: (i) eye disease involving ocular lesions that lead to decreased vision (Siam & Meegan, 1980); (ii) meningoencephalitis, which occurs one to four weeks after the onset of the first symptoms and is characterized by headache, amnesia, disorientation, convulsions, hallucination, and lethargy, in some cases followed by coma (Rakotoarivelo *et al.*, 2011; Alrajhi *et al.*, 2004).

The haemorrhagic form usually appears two to four days after the onset of disease, and the patients develop jaundice (indicating liver impairment, due to massive necrosis of hepatocytes), vomit blood, pass blood in faeces, and display ecchymosis of the skin and bleeding from the nose and gums. The case-to-fatality ratio in this form of RVF often rises to as high as 50%, and death frequently occur within three to six days of the onset of symptoms. Also, the virus is detectable in the blood for up to 10 days in patients with haemorrhagic jaundice (WHO, 2010).

There is still no cure, nor any licensed vaccine against RVF for general use in humans. In mild cases, the treatment is supportive and entails administration of fluids and pain relievers. In severe cases including encephalitis and bleeding, patients must receive ventilation and blood transfusions (Al-Hazmi *et al.*, 2003).

1.5 How does RVF phlebovirus interact and evade host defence mechanisms?

RVFPV is capable to overcome the host immune system and establishes itself in the target cells and organs through different mechanisms. In insect hosts RVFPV infection is more persistent, with elimination of virus over longer periods after infection, whereas in the vertebrate host the virus infection outcome is lytic, which means that it is shed for very short time following infection (Walter & Barr, 2011).

1.5.1 Mosquitoes

In mosquitoes RVFPV is able to replicate and spread both vertically, when the virus passes to the eggs in the female's ovaries, and horizontally during mating

of an infected male with a female mosquito. Primarily a competent mosquito vector takes a blood meal from a viremic vertebrate, thus the virus infects the midgut epithelial cells of the vector and replicates. Thereafter, RVFPV disseminates mainly to the salivary glands and/or ovaries. An infected mosquito will then transmit RVFPV to the next vertebrate host through a bite, or pass it vertically to the offspring (transovarial and transtadial transmissions). For RVFPV to be able to reach the target organs in the mosquito it has to overcome, among others, the gut escape barrier (extra-cellular basal lamina around the epithelial cells) and salivary gland barrier (basal lamina in the surrounding) (Romoser *et al.*, 2005).

After the RVFPV has overcome the mosquito midgut epithelia (primary anatomic barrier), through disruption of the basal labyrinth (BL), it disseminates into homocoel and replicates in the secondary tissues, such as salivary glands, and ovaries (Franz *et al.*, 2015). The BL is a non-cellular layer rich in proteins (collagen type IV, laminin, perlecan and entactin/nidogen), that separates the organism compartments or cell layers, and sustains the renewal of epithelium structure.

In the epithelial cells the viral RNA of arboviruses replicates in the endoplasmic reticulum, however the virions maturation site may vary, and apparently depends on the virus and mosquito species involved. Some virions accumulate and maturate in the BL and extracellular spaces between cell plasma membrane and BL (example, SLEV in *Culex pipiens*, EEV in *Aedes triseriatus*, WEEV in *Aedes dorsalis*), whereas some other arbovirus virions assembly occurs in the BL, and thus do not enter the cisterna of endoplasmic reticulum (example, WEEV in *Culex tarsalis* and Chikungunya virus in *Aedes aegypti*) (Franz *et al.*, 2015).

There is a range of virus-host cell molecules that prime the mosquito immune response to arboviruses, however the mechanisms involved in some of the pathways are not yet well understood. The JAK/STAT, imd/jnk, Toll, small RNA interference (siRNA) and autophagy pathways are the main that trigger the anti-viral responses in mosquito (Iranpour *et al.*, 2016; Moy *et al.*, 2014; Kingsolver *et al.*, 2013; Fragkoudis *et al.*, 2009).

Activation of the host-cell immune signalling pathways will lead to the transcription and expression of anti-viral effectors, however arboviruses are capable to counteract this process, thereby inhibiting either one or more signalling mechanisms. For instance, the Japanese Encephalitis virus (JEV)

blocks transcription of anti-viral genes through tyrosine phosphorylation of STAT in *Aedes albopictus* (Lin *et al*., 2006), Semiliki Forest virus (SFV) is responsible for the decrease of host cell gene transcription. The activation of Toll like receptors block the replication of Dengue virus, whereas JAK/STAT and imd/jnk block the replication of SFV when activated (Souza-Neto *et al.*, 2009).

Arbovirus infections also activate the RNA interference immune pathways in mosquitos, mosquito cell lines and other insects. RVFPV was efficiently inhibited through induction of exogenous siRNA and piRNA pathways in *Aedes* sp and *Culex quinquefasciatus* (Dietrich *et al.*, 2017; Sanchez-Vargas *et al.*, 2004). RVFPV specific siRNA are produced in mosquito cells in response to viral infection, and thus they block the key proteins of the exogenous siRNA and piRNA pathways. It was also shown that RVFPV was not capable of expressing viral effectors to circumvent siRNA produced in mosquito's cells in response to infection (Dietrich *et al.*, 2017). RVFPV and other RNA viruses evade mosquito innate immune response through mutations in regions targeted by RNAi, at every round of replication, which is caused by an error-prone RNA dependent RNA polymerase (Blair & Olson, 2015; Blair & Olson, 2014). It has also been shown elsewhere that in RVFPV infected *Aedes* mosquitoes cells, dicer-2- and piwi-RNAi pathways downregulate the synthesis of NSs during the acute phase of infection, and thus allowing persistent infection within the vectors (Léger *et al.*, 2013).

1.5.2 RVFPV and vertebrate host-interaction

In vertebrate hosts both innate and adaptive immunity are triggered after infection with RVFPV and other viruses. The early innate immunity involves the interferon (IFN) system, as a result of the interaction between the pattern recognition receptors (PRRs), encoded by the host cell, and pathogen-associated molecular patterns, PAMPs (Gack, 2014). RIG-I-like helicases (RIG-I, MDA5 and LGP-2) recognize viral dsRNA or uncapped 5´termini of ssRNA, and thus activate transcription factors in the nucleus, such as nuclear factor kB (NF-kB) and IFN-regulatory factor 3 (IFN-3). The activation of these transcription factors stimulates the expression IFN-β genes. Subsequently, specific anti-viral proteins are activated, which include Mx GTPases and protein kinase R, PKR (Gack, 2014; Errett *et al*., 2013; Walter & Barr, 2011).

Active Mx protein binds to N protein of many viruses and thereafter moves it from its intended location site, leading to overall disruption of viral replication, whereas, PKR induces the phosphorylation of the translation initiation factor eIF-2, and further downregulation of viral and host-cell translation (Walter & Barr, 2011).

RVFPV NSs protein is the major viral component that acts against the vertebrate host cells innate immune response. NSs inhibits the INF system, thereby acting directly in the nucleus where it is co-localized within large filamentous structures, together with p44 and XBP domains of the TFIIH transcription factor. The NSs protein interaction with these two domains blocks the formation of a complete TFIIH complex. Furthermore, RVFPV NSs binds to p66 domain of the TFIIH, by recruiting F-Box protein FBXO3, downregulating post-translation (Kainulainen *et al.*, 2014), which leads to a decreased abundance of p66 domain and consequent disruption of the host-cell transcription (Kalveram *et al.*, 2011).

RVFPV NSs binds to SAP-30-, YY1- and SIN3A- associated co-repressor factors within the filamentous compartments, blocking the host cell transcription. This will result in direct inhibition of IFN-β expression (Le May *et al.*, 2008); in addition to this, NSs protein is implicated in the downregulation of PKR, inducing the suppression of the eukaryotic initiation factor 2-alpha (eIF 2-α) phosphorylation (Ikegami *et al.*, 2009), thereby recruiting the SCF (Skp, Cul1, F-box protein) E-3 ubiquitin ligases containing FBXW11 and FXW1 (β-TRCP1) as substrate recognition units (Kainulainen *et al.*, 2016; Mudhasani *et al.*, 2016), however, it was also shown that NSs can inhibit host transcription without downregulating PKR (Kalveram *et al.*, 2013).

In vertebrate hosts, adaptive immune response to RVFPV is established within 4 to 8 days post-infection. Antibodies that primarily target RVFPV glycoproteins (Gn and Gc) are produced a few days after infection. IgM and IgG antibodies directed to both N and NSs proteins are produced, however N protein is highly immunogenic, so that RVFPV infected vertebrates produce high levels of anti-N proteins antibodies. Anti-RVFPV IgM levels are elevated in the blood a few days after infection and persist for a short time, indicating an acute phase, whereas IgG antibodies peak at the later stages post-infection and last longer (Pepin *et al.*, 2010).

1.6 RVF prevention and control

A one-health approach which may connect the veterinarians, physicians and other health professionals and stakeholders must be widely implemented, in order to develop and establish integrated methods to identify knowledge, attitudes, practices and challenges of the community towards a better understanding and improvement of the RVF control (Hassan *et al.*, 2017).

To date, the control of RVF in endemic countries, is based on the use of live-attenuated or inactivated vaccines, as described below.

1.6.1 Animals

Inoculation of livestock with the Smithburn live attenuated vaccine or a formalin-inactivated RVFPV vaccine prior to the rainy season has been implemented on the African continent, although not consistently in some countries. Furthermore, in Saudi Arabia and many countries in Africa, routine surveillance programmes are performed in which diagnostic tools based primarily on ELISA are used to detect anti-RVFPV IgG and anti-RVFPV IgM antibodies in farm animals.

Unfortunately, only a few African countries have BSL3 facilities equipped to handle biologically active virus, and therefore neutralization tests can only be conducted in a limited number of laboratories.

A study based on the use of geographic information system (GIS) software and analysis of satellite remote sensing data to predict outbreaks has been performed in Kenya (Linthicum *et al.*, 1999).

It is plausible that such methods can provide powerful support to achieve nearly real-time livestock vaccination prior to RVFPV outbreaks, but unfortunately those tools are currently only available in a small number of countries in Africa.

RVFPV vector control programmes have also been introduced in some African countries, although limited financial resources make it impossible to implement these measures in large areas with potential breeding habitats for the mosquito vectors.

Use of a combination of several such schemes (e.g., integration of programmes aimed at controlling RVFPV and malaria vectors) would significantly reduce the costs of strategies aimed at eradicating several vector-borne tropical diseases (e.g., RVF) in Africa.

It can also be mentioned that immunization with formalin-inactivated RVFPV vaccine is the main control strategy applied to control RVFPV infections in cattle in Mozambique.

1.6.2 Livestock vaccination in Africa

1.6.2.1 RVFPV live attenuated vaccines

The Smithburn vaccine is one of the live attenuated vaccines that is administered to livestock to control RVF (Ikegami *et al.*, 2009), and it has the side effects of inducing abortions and teratogenicity in pregnant animals (Kamal, 2009).

Another attenuated RVFPV immunization used in animals is based on the MP12 strain, but early tests using that vaccine indicated that it induces abortions in ewes (Ikegami *et al.*, 2009).

1.6.2.2 RVFPV inactivated vaccines

Inactivated vaccines have been developed in an attempt to address the safety issues, but they involve the major weaknesses of having low immunogenicity and offering only short-term protection, as well as requiring at least three inoculations at different time points during the same year, which is expensive and time consuming (Lubroth *et al.*, 2007).

Formalin-inactivated RVFPV vaccines derived from the Entebbe strain or from a pantropic strain passaged in baby hamster kidney cells have been used to immunize domestic ruminants (Ikegami *et al.*, 2009).

1.6.2.3 Other vaccine candidates

There are several RVF vaccine candidates that have been developed and tested under laboratory conditions in animal models. The list of such candidates includes virus-like particles, VLPs (Naslund *et al.*, 2009), recombinant viruses (Faburay *et al.*, 2016; Kortekaas *et al.*, 2012; Mandell *et al.*, 2010; Wallace *et al.*, 2006), attenuated RVFPV strains that show less severe side effects when compared to those caused by the Smithburn strain (Dungu *et al.*, 2010; Morrill *et al.*, 1997a; Morrill *et al.*, 1997b), and DNA vaccines (Bhardwaj *et al.*, 2010; Lorenzo *et al.*, 2010; Wallace *et al.*, 2006).

All these vaccines have been shown to induce a protective immune response in laboratory animals (Boshra *et al.*, 2011; Soi *et al.*, 2010; Ikegami *et al.*, 2009).

Three candidates, namely the NDV-GnGc vaccine (Newcastle disease virus-based vector that produces structural glycoproteins Gn and Gc), NSR vaccine (non-spreading RVFPV) and GNeS3 vaccine (purified ectodomain of the Gn structural glycoprotein) have been tested in lambs and induced a neutralizing antibody response within three weeks after a single inoculation. Furthermore the lambs were protected from viremia, pyrexia and death after they were challenged with a recombinant RVFPV nineteen days post-vaccination (Kortekaas *et al.*, 2012).

Of great concern is the fact that many of these vaccine candidates were developed on the premise that all strains of RVFPV possess identical neutralizing epitopes. Considering that phleboviruses epitope glycoproteins are strain specific and some of them have shown a high rate of genetic variability (Deyde *et al.*, 2006), these vaccines may only work against a limited number of RVFPV strains.

1.6.3 Vaccination in humans

As of yet, no vaccine has been licensed for broad use in immunization of humans against RVFPV. However, some forms developed for specific target groups are available, such as formalin-inactivated TSI-GSD 200 vaccine (Kark *et al.*, 1982) and the investigational NDBR 103 formalin-inactivated vaccine (derived from Entebbe strain, 184th passage), which have been used to immunize people enrolled in clinical trials (Rusnak *et al.*, 2011). These

vaccines are given only to veterinarians, laboratory workers, and people living in close contact with livestock, and they are not used in Mozambique.

1.7 RVF in Mozambique

1.7.1 RVF outbreaks and serological studies

In Mozambique, RVFPV was first reported forty years ago (Valadão, 1969), when high abortion and death rates occurred among cattle, goats and sheep in Gaza and Maputo provinces of south region of the country. Another outbreak of RVF among small ruminants, was reported in the same areas (Fafetine *et al.*, 2016), however only a few number of animals was affected.

During these two inter-epidemics, high seroprevalences of RVFPV among domestic ruminants were reported in Gaza (Engstrom, 2012) and Zambézia (Blomström *et al.*, 2016; Fafetine *et al.*, 2013; DINAP, 2002) provinces, in the south and central Mozambique, respectively.

1.7.2 RVF vaccination program

The former National Directorate of Livestock in Mozambique introduced a programme using a commercial formalin-inactivated RVFPV in Zambezia Province in 2002 in response to a survey of RVFPV antibodies conducted in 1996, which showed that 37% (152/412) of the cattle in the region were seropositive (DINAP, 2002).

It was also shown that 51% (71/140) of the water buffaloes and 52.6% (50/95) of the cattle were positive for anti-RVFPV IgG antibodies in 1999 and 2001 (DINAP, 2002). The programme initiated in Zambezia Province in 2002 was extended to Manica and Gaza Provinces during the same year, and this action was motivated by the heavy rains and floods that occurred in the central and southern parts of the country in 2000 (DINAP, 2003).

Subsequently, vaccination was performed only in Zambezia and Gaza Provinces in 2003 and 2005, and was restricted to Zambezia Province from 2006 to date.

The commercial formalin-inactivated RVFPV vaccine (Onderstepoort Biological Products) used in Mozambique is imported from South Africa and is stored at the Central Veterinary Laboratory in the city of Maputo according to the instructions of the manufacturer. It takes approximately one week to send the vaccine from Maputo and administer it to cattle in Zambezia Province, because the roads that access most of the districts in that region are still in poor condition. This delay in delivery of the vaccine makes it difficult to maintain the cold chain, since a supply of ice is lacking at many of the intermediate stops along the transport routes.

1.8 RVF diagnosis

1.8.1 Animals

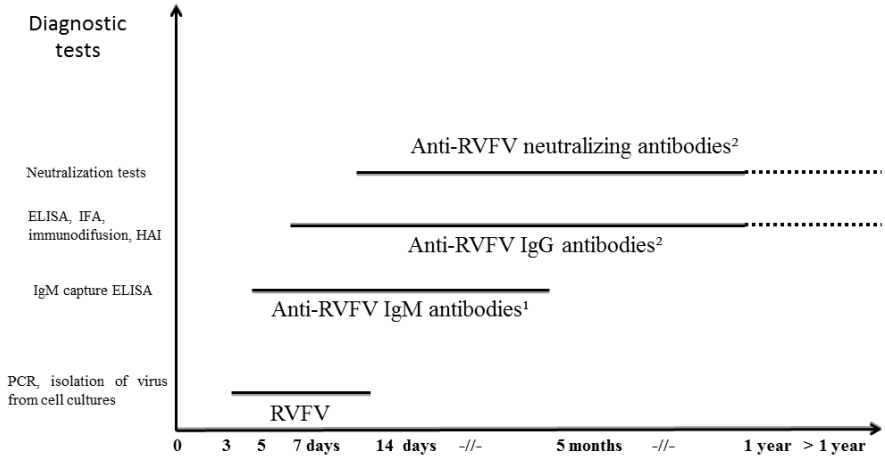
In the field, RVF cases can be suspected when heavy rainfalls are followed by a sudden rise in the mortality rate among new-born animals and an increased number of abortions (up to 100%) in ewes, cows, and goats, as well as other animal species such as camels. The preliminary diagnosis can be supported by necropsy results showing the presence of extensive liver lesions in aborted foetuses, neonatal animals, or adult livestock.

In the laboratory, RVFPV can be isolated from blood or serum collected during early stages of disease, or from liver, brain, and spleen collected *post-mortem* and the organs of aborted foetuses. Reverse transcriptase polymerase chain reaction (RT-PCR) is used for rapid detection of RVFPV in blood from day 1 to day 5 post-infection (Ibrahim *et al.*, 1997).

Isolation of the virus can be achieved by propagation in cell cultures (e.g., Vero cells, baby hamster kidney cells, chicken embryo reticulum, or primary cells of sheep or cattle origin) or in laboratory animals (e.g., hamsters, adult or suckling mice, or two-day-old lambs).

For serology, IgM capture ELISA (Williams *et al.*, 2011) is employed to diagnose RVF in the acute phase of illness (4–7 days post-infection), and the methods used during later stages include IgG ELISA, the plaque reduction neutralization test (PRNT), the haemagglutination inhibition test, and less often immunofluorescence assay (IFA), as well as complement fixation and immunodiffusion.

Figure 4 illustrates the diagnostic tests that are used in relation to the length of time elapsed after infection with RVFPV and the time to detection of RVFPV antigens and immunoglobulins M and G in infected animals.



[1](Morvan *et al*., 1992);[2](Swanepoel & Coetzer, 2004)

Figure 4: Diagnostic tests used in relation to the time elapsed after natural infection with RVFPV.

1.8.2 Humans

In humans, RVF causes an acute and febrile illness, although a few number of patients may have complications, such as haemorrhages, thrombosis, neurological disorders, retinopathy and blindness (Ikegami & Makino, 2011), as well as miscarriages in pregnant women (Baudin *et al.*, 2016).

RVFPV is detected by quantitative Real-Time PCR performed during the febrile phase of illness (up to 5 days after infection), and IgM capture ELISA is carried out to demonstrate an early antibody response a few days after infection. Diagnosis at a later stage of infection is accomplished by serological techniques, such as N protein based ELISA to detect RVF anti-N protein IgG antibodies and neutralization tests to reveal RVFPV neutralizing antibodies.

2 Aims of the thesis

The objectives of this thesis were as follows:

To estimate the seroprevalence of RVFPV in domestic ruminants and African buffaloes in Mozambique.

To identify potential mosquito vectors and understand their role in the transmission of RVFPV in Mozambique.

To investigate the effect of transport and storage conditions on the efficacy and stability of formalin-inactivated RVFPV vaccine given to cattle in Maputo and Zambezia Provinces in Mozambique.

To express RVFPV antigens in transgenic plants, so that these can be administered orally to mice and induce immune response.

3 Material and methods

The study number I aimed at determining the prevalence of RVF antibodies in unvaccinated cattle herds in Maputo Province in southern Mozambique; a later study (II) investigated the seroprevalence, not only in cattle, but also in goats, sheep and African buffaloes countrywide (sections 3.1 and 3.2, respectively).

In the study number III, identification of the RVFPV vectors and their possible role in the virus transmission among domestic ruminants in Gaza province, South Mozambique, was performed (section 3.3).

The study number IV was conducted to assess the immunity of cattle after vaccination with a formalin inactivated RVFPV vaccine in Mozambique and to evaluate the stability of the formalin-inactivated vaccine after storage under different conditions. Section 3.4, describes the study area, storage and transport of vaccine from South Africa to Mozambique (the city of Maputo) and from there to Namaacha District in Maputo Province, as well as to Zambezia Province and then to the district of Alto-Molocue.

We have also assessed the ability of transgenic plants to express RVFPV antigens and their immunogenicity after oral administration to mice, study V, (section 3.5).

Studies I, II, III and IV were performed under ethical permit number 555/DNSV/2008, issued by the Board of Ethics of the Directorate of the National Veterinary Service, Ministry of Agriculture and Food Security of Mozambique, and study V was performed under ethical permit number N105/12, approved by the Committee on the Ethics of Animal Experiments, at Karolinska Institute (Stockholm, Sweden).

3.1 Antibodies against Rift Valley fever phlebovirus in cattle in Mozambique (study I)

A total of 404 sera samples were collected from the same number of cattle of different ages and mixed breeds between February 2010 and May 2011 (Table 3). A number of 364 sera were obtained from biobanks at the Directorate of Animal Sciences of the Central Veterinary Laboratory in Maputo (Mozambique), and 40 were collected from cattle in the Moamba District in 2011.

The samples were gathered in the Districts of Matutuíne, Boane, Marracuene, Manhiça, Moamba, and Magude in Maputo Province (Figure 5) as part of a routine surveillance programme for several diseases. In May 2011, additional blood sampling within this project was performed on 40 cattle in the Moamba District.

Table 3: Number of serum sample collected from cattle in different districts in Maputo Province

District	Number of samples	Year	Source
Boane	28		
Magude	34		
Manhiça	65	2010	CVL* Maputo
Marracuene	82		
Matutuíne	131		
Moamba	24	2010	CVL* Maputo
	40	2011	Serology Laboratory Veterinary Faculty
Total	**404**		**-**

*CVL = Central Veterinary Laboratory in Maputo, Mozambique.

In this study we determined the overall prevalence in the province, which gave us an indication of the distribution of RVFPV at a district level. Maputo Province is subdivided into 7 districts, and samples from 6 of these districts were available for analysis: Boane (n = 28), Magude (n = 34), Manhiça (n = 65), Marracuene (n = 82), Matutuine (n = 131), and Moamba (n = 64). The livestock populations in Magude, Manhiça, Matutuine, and Moamba Districts range in size from 20,000 to 70,000 animals, and Boane and Marracuene Districts have smaller populations of 6,000 and 9,000, respectively.

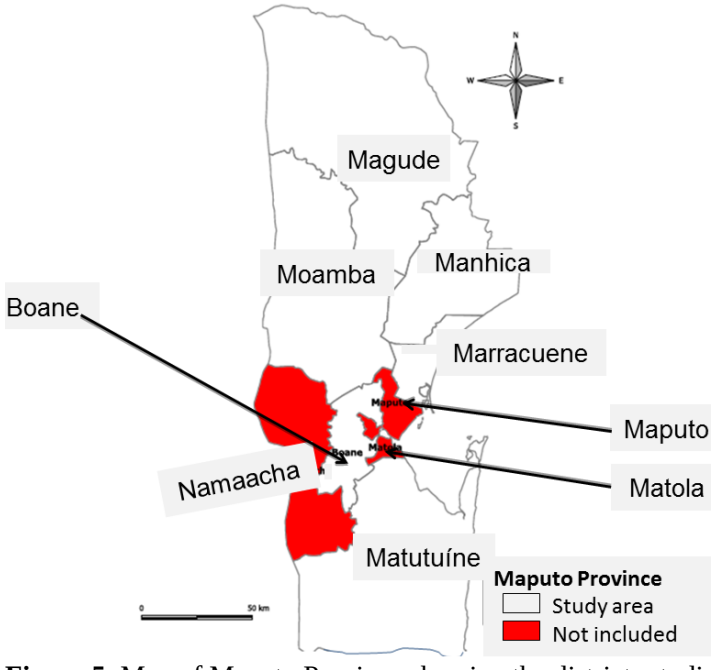


Figure 5: Map of Maputo Province showing the districts studied.

Blood samples from the caudal vein of each animal were collected in 5-ml vacuum tubes without any additive, and stored on ice. Upon arrival at the laboratory (after transportation for approximately 1 hour from the most distant sampling area), the samples were centrifuged at 1500 x g, and each of the extracted sera was divided into two aliquots, which were placed in 1.5-ml Eppendorf tubes in a water bath and heat inactivated at 56 °C for 30 min, and then stored at –20 °C pending analysis.

All the samples were transported frozen (on dry ice) to the Swedish Institute for Communicable Disease Control (Solna, Sweden), where they were heat inactivated in a Biosafety Level 3 (BSL3) containment facility before being analysed.

The samples were tested by PRNT as described in paper I. RVFPV seropositivity was defined as 80% reduction of virus infectivity at a serum dilution of 1:40.

3.2 High seroprevalence of Rift Valley fever phlebovirus antibodies in domestic ruminants and African buffaloes in Mozambique shows need for intensified surveillance (study II)

In this study we have performed a cross-sectional study on farms located in 7 of 10 Provinces of Mozambique, grouped in three geographic regions, respectively, north (Cabo Delgado and Niassa), central (Sofala and Tete) and south (Inhambane, Gaza and Maputo) to investigate the seroprevalence of RVFPV among cattle, goats, sheep and African Buffaloes (*Syncerus cafer*) (Figure 1, study II).

The age estimate for young cattle was <2 years and the cut-off for young sheep and goats was <12 months. African buffaloes, both young (< 2.5 years) and adults (≥ 2.5 years) were chemically immobilized by aerial darting, for subsequent sampling.

To achieve this goal, we have collected a total of 1581 blood samples from cattle, 1117 from goats, 85 from sheep and 69 from African buffaloes, in the years 2013 and 2014, as described in section 3.1. Sera were tested for anti-RVFPV antibodies using a commercial RVF competition multi-species ELISA (IDvet, France), at the Central Veterinary Laboratory in Mozambique.

3.3 Identification of the Rift Valley fever phlebovirus vectors in South Mozambique (study III)

This study was performed in southern Mozambique, and included nine localities within Xai-Xai, Chibuto, Chóckwè and Bilene-Macia districts in Gaza Province, and one locality in Moamba district within Maputo Province (figure 1, study III). All the districts of Gaza Province are located in the South whereas Moamba district lies in the central-western region of Maputo Province (figure 1, study III).

A total of 897 blood samples were collected from cattle (n=668), goats (n=166) and sheep (n=63) from 2014 to 2016. Sera were prepared and analyzed for RVFPV antibodies using competition multi-species and IgM capture ELISAs (IDvet, France).

CDC mini light traps (BioQuip Products, Inc, USA) were set near the pens and stables where animals (either sheep, goat or cattle) were kept overnight, with maximal protection against the winds. The traps were set 1.5 meters above the ground, between 6 pm and 6 am each trapping day. Three collections events were performed in October 2014, four in November 2015, and nine in March-April, 2016. In the first two years, collections were limited to Xai-Xai district (Patrice Lumumba and Bassopa localities). In 2016 collections were also performed in Chibuto (Macalawane) and Chóckwè (Bombofu and Chalucuane) districts (figure 1, study III).

After transportation to laboratory (approximately 20-40 minutes), the mosquitoes were knocked down at 4 ^{o}C for 30 minutes, and sorted by trap number, place, sex, genus, and species as far as possible. Classification was achieved by morphology (Jupp, 1996; Gillet, 1972).

Specimens were pooled (5-20 mosquitos) in 2 ml cryogenic vials, RNA later solution was added, following transportation on dry ice to the Veterinary Faculty Laboratory in Maputo, and storage at -80 ^{o}C for further analysis.

Sera samples from domestic livestock, were collected and analysed as previously described for investigation of the RVFPV seroprevalence in the trapping areas.

3.4 Stability of a formalin-inactivated Rift Valley fever vaccine: evaluation of a vaccination campaign for cattle in Mozambique (study IV)

In this study, a formalin-inactivated RVFPV vaccine was divided into portions (separate vials) that were stored at three different temperatures over a period of one week: 4 ^{o}C (vial A), in accordance with the manufacturer's instructions; 25 ^{o}C (vial B); alternating between 4 and 25 ^{o}C (vial C). Thereafter, in the district of Namaacha (Maputo Province), the vaccine was administered twice at an interval of 21 days to 25 cattle divided into three groups (A, n = 9; B, n = 8 and C, n = 8), which corresponded to the storage temperatures for the vaccine. ELISA and IFA were performed to monitor the antibody response of the immunized cattle, and the PRNT was used to determine the titres of neutralizing antibodies in the test sera.

To assess the efficacy and duration of protective immunity induced by the vaccine in cattle, the same batch was used under field conditions to inoculate 38 animals in the Alto-Molocue District, Zambezia Province (Figure 6). The immunized animals were grazing in community pastures that were included in a free-range livestock production system in the highlands of this district.

Figure 6: Map of Mozambique showing the study area in Maputo and Zambezia Provinces.

The vaccine was imported from South Africa to the city of Maputo in Mozambique by plane and then transported by car to the Directorate of the National Veterinary Services, where it was stored at 4 °C as stipulated by the manufacturer.

The transport was subsequently continued to the district of Namaacha and the city of Quelimane (the latter in Zambezia Province), which are, respectively, located about 75 and 1,565 km from Maputo.

Thereafter, the vaccine was delivered by car from Quelimane to Alto-Molocue (Figure 6), where it was stored at 4 °C or on ice when possible. At this point, the vaccine was again refrigerated until the next day, when it was transported on ice by car approximately 60 km to the area of Milevane and about 30 km to the area of Nivava, and then administered to the cattle.

Blood samples were collected as described previously. PRNT and RVFPV manipulation for IFA test were performed in a BSL3 laboratory, at the Swedish Institute for Communicable Diseases, Solna, Sweden.

3.5 Mice fed with Rift Valley fever phlebovirus antigens expressed in transgenic *Arabidopsis thaliana* show high level of immune response (study V)

We amplified the DNA encoding full length N protein and a deletion mutant of Gn protein (ΔGn) from S-segment (GenBank ID DQ 380151) and M-segment (GenBank ID DQ 380206) of RVFPV cDNA, using specific primers (see table 1, paper V). Platinum Taq DNA polymerase (Invitrogen) was used for DNA amplification.

Xbal or BamHI restriction enzymes (New England Biolabs) were used for digestion of the amplified PCR products. Ligation was performed in the 35S-CaMV cassette of the pGreen 0229/N plant vector. Sequencing was performed to verify the correctness of the binary vectors pGreen 0229/N and pGreen 0229/ ΔGn (GATC Biotech).
Agrobacterium tumefaciens (strain EHA105) was transfected with pGreen 0229/N and pGreen 0229/ΔGn plasmids by electroporation. Kanamycin was used to select transformed bacteria, and PCR was performed to verify the transgenes using Sseg, Mseg1 and 35S primers. *Arabidopsis thaliana* (*A. thaliana*) wild type (WT) plants of the Columbia-O Ecotype (The European Arabidopsis Stock Centre) were transformed using floral dip method of *Agrobacterium*-mediated gene transfer. Genomic DNA was extracted from leaf tissue, to check the presence of the N and ΔGn inserts in the transformed plants, using RED Extract-N-Amp Plant PCR kit (Sigma-Aldrich), followed by amplification using the 35S primers. The reaction mixtures were cycled at 94 ºC for 10 min, followed by 40 cycles of 94 ºC 60 s, 58 ºC 60 s and 72 ºC, and a final extension at 72ºC for 10 min.

To analyze the PCR products, agarose electrophoresis was performed, and thereafter sequencing of the purified products (GATC Biotech). RVFPV antigens were checked using western blotting.

Plant tissues from N-transformant lines were ground in liquid nitrogen, mixed with Tris buffer, supplemented with Complete™ protease inhibitor cocktail (Roche Diagnostics). Extracts were loaded on to 12% SDS PAGE electrophoresis gels (Life Technologies), and thereafter transferred to PDVDF membrane (Millipore), followed by blocking with 10% fat-free milk in TBS with 0,1% tween-20. After 1h incubation at 22 ºC, peroxidase conjugated anti-mouse IgG antibody (Jackson Immuno Research), diluted in 5% milk in TBS-T, was added and incubation was perfomed as decribed above. To detect bound antibody, ECL reagent (GE Health Care) was used.

For RNA detection in transgenic plant, fresh tissues (0,1-0,15 g) of each ΔGn transformant line were homogenized in Tissue Lyzer (Qiagen) by using Trizol LS reagent (Life Technologies), according to manufacturer´s instructions. Ambion TURBO DNase (Life Technologies) was used to remove DNA contamination. Superscript III Platinum One Step Quantitative RT-PCR system (Invitrogen) was used to analyze the presence of RNA and DNA, using Mseg2 fwd and Mseg2 rev primer pairs (see table 1, study V). The PCR products were analyzed by electrophoresis.

C57/BI female mice, aged between 6-8 weeks old, were immunized according to the scheme shown in table 2 of paper V. The animals were kept four and four in cages with access to food and water *ad-libitum* during the whole immunization routine, except the days of immunization, when normal food was replaced by fresh plants for 24 h. Mice were provided 21 g fresh weight intact plant per four animals, in the first experiment; blood samples were collected from tail vein two weeks after immunization.

In the second experiment, mice were given 26 g of fresh leaves during each of the three immunization rounds, and thereafter sampled at weeks 0, 2, 4, 5 and 6 (see table 2, paper V). Sera were stored at -20 ºC until further analysis.

To prepare viral antigens for ELISA, RVFPV strain ZH548 grown in Vero cells (ATCC no. CCL-81) was centrifuged at 2500 xg at 4 ºC for 10 min. Viral particles were purified by centrifugation through a 20% sucrose cushion (w/w)

in Tris-EDTA (pH7.5) using Beckman ultra-clear ultracentrifuge tubes in a SW41 rotor at 36,000 rpm at 4 ºC for 2 h in a Beckman L-80 ultracentrifuge. The pellet was re-suspended in Tris-EDTA and thereafter stored at -70 ºC for further use. Experiment involving RVFPV was performed in biosafety level 3 containment laboratories. Recombinant N protein with a 6-His tag was expressed from the prokaryotic expression vector pET14b (Novagen). Protein expression from transformed BL21 Escherichia coli (Invitrogen) was induced for 4 h using 0.5 mM isopropyl-b-D-thiogalactopyranoside, IPTG (Calbiochem) and purification was performed under native conditions by gravity-flow chromatography using Ni-NTA Agarose (Qiagen) according to the manufacturer's instructions. The extracted recombinant protein was dialysed twice against sterile phosphate-buffered saline (PBS) using SLIDE-a-Lyzer Dialysis Cassette with 10 K molecular-weight cut-off (Pierce), following determination of the concentration using ND-1000 NanoDrop spectrophotometer (NanoDrop Technologies).

For detection of immunization-induced antibody responses, ELISA was performed using 96 well-plates (Nunc MaxiSorp) coated with 50mL/well of either 3 mg/mL recN diluted in 0.05 M Na_2CO_3 (pH 9.6) or sucrose-extracted RVFPV diluted 1:200 in PBS. ELISA based on recombinant N protein and purified RVFPV was performed as described elsewhere (Näslund *et al.*, 2008; Vene *et al.*, 1994). Serum was two-fold serially diluted (1:50 and 1:51200) and reactive antibodies were detected by HRP conjugated goat anti-mouse IgG (Jacksson ImmunoResearch), goat anti-mouse IgG1 (Dako) or goat anti-mouse IgG2c (Thermo Scientific) antibodies diluted 1:5000, 1:4000 or 1:3000, respectively. Antigen-antibody complexes were visualized by adding TMB substrate (Sigma-Aldrich) and the reaction was stopped with H_2SO_4; the absorbance was measured at 450 nm. Negative and positive control samples were present in duplicate on each plate. The cut-off was calculated as the mean absorbance value plus three times the standard deviation obtained from dilutions of pre-immunization sera. The ELISA titer was determined as the reciprocal of the highest serum dilution that resulted in a reading above the cut-off in duplicate samples.

3.6 Statistical methods

Fisher's exact test was used to compare seroprevalences in different species and regions (studies I, II and III), and multivariable analysis to assess the

association between potential risk factors (location, species, sex and age) and RVF seropositivity among the animals was performed using logistic regression (studies II and III).

In study III, the mosquito genera were compared for different localities using Fisher exact test. Frequencies were calculated using IBM SPSS statistics software Version 20 (IBM, USA).

In study IV, statistical correlations were analysed using Spearman´s ranking test, furthermore the initial comparisons of the antibody responses in the four vaccination groups was performed by using Kruskal Wallis test, and the Mann-Whitney U test was carried out if systematic variations were found, to compare the vaccine induced titers in animals assigned to each group. The statistical analysis was performed using *Statistica*™ 10 software (StatSoft, Inc).

4 Results

4.1 Antibodies against Rift Valley fever phlebovirus in cattle in Mozambique (study I)

A total of 404 cattle serum samples were evaluated by PRNT, and 36.9% (149/404) were found to be positive for RVFPV-neutralizing antibodies. Seropositive animals were observed in all of the herds in the six districts under consideration.

The districts with highest seroprevalence were Manhiça, 61.5% and Marracuene, 62.2%, suggesting that RVFPV activity is high in these areas.

The seropositivity rate in the Southern District of Matutuine was 19.8%, the Northern and Western Districts of Magude and Moamba had seroprevalences of 26.5% and 29.7%, respectively. Surprisingly no RVF outbreak has ever been reported in both cattle and humans on the districts of Matutuíne, Namaacha, Magude and Moamba. Furthermore, livestock raised in those districts were not affected during the 1969´s RVF outbreaks. Boane District which has the smallest cattle population in Maputo had high seroprevalence, 14.3%.

4.2 High seroprevalence of Rift Valley fever phlebovirus antibodies in domestic ruminants and African buffaloes in Mozambique shows need for intensified surveillance (study II)

The overall seroprevalence of RVFPV was high 25.6%. Within the regions, animals in the central Mozambique had the highest overall prevalence of antibodies against RVFPV, 51.2% (395/771). With regards to species, cattle

with 37.3% (590/1581) and African buffaloes with 30.4% (21/69), had higher seroprevalence than other species. The highest was observed in the central province of Sofala, 64.5% (350/543) for cattle and 38.3% (18/47) for African buffaloes (table 1, study II).

Seroprevalence of RVFPV in goats, 20.2% (45/223), was higher in south province of Gaza than those observed in the province of Tete, and the northern provinces of Cabo Delgado and Niassa (table 1, study II); meanwhile, no differences were observed between Maputo and Tete, as well as between Tete and the north provinces for this species.

Sheep were sampled in Gaza and Maputo (south), and no difference was observed in the seroprevalence of RVFPV for this species between the two provinces.

The seroprevalence of RVFPV, 38.3% (18/47) (table 1, study II), was higher in African buffaloes from Sofala province (figure 1, study II), than that observed in Tete province (table 1 and figure 1, study II). African buffaloes were only available for sampling in the central Mozambique, in Marroumeu National Reserve and Gorongosa National Park (Sofala) and Tchuma-tchato special conservation area (Tete).

Cattle were more likely to be seropositive in Sofala and Maputo, respectively, two and four times higher than in Gaza province, whereas the odds of seropositivity were lower in Inhambane and Tete (table 2, study II).

Adult animals, for all species, were nine times more likely to have been exposed to RVFPV than the younger ones (OR = 8.92, $p < 0.0001$) (table 2, study II).

4.3 Identification of the Rift Valley fever phlebovirus vectors in South Mozambique (study III)

The overall observed RVFPV seroprevalence was high in Gaza province, 30.4% (256/825; p =0,000) and Maputo (Moamba district), 23.6% (17/72; p = 000), similar to findings in studies I and II, for Gaza and Moamba, respectively.

Comparisons between different species, in Gaza province, showed a higher seroprevalence in cattle, 39.4% (252/416) than sheep (12.7%, 8/63) and goats (7.8%, 13/166).

Across all district localities for each district, Bocovela (47.5%, 19/40) had higher seroprevalence than Chalucuane (42.3%, 30/71), Bombofu (34.7%, 60/113) and Mapapa (29.7%, 22/55) in Chóckwè district; in Chibuto district the locality of Gogoti (26.1%, 12/34) had higher seroprevalence of RVFPV than Macalawane (22%, 40/182); in Xai-Xai district, Patrice Lumumba had a seroprevalence (37.5%, 33/55) as was higher than Bassopa (27.3%, 27/72) ($p \leq$ 0,011). In Bilene-Macia district, one locality (Dzimbene) was assigned for sampling. Furthermore, cattle were the only animals available for sampling from this locality and the seroprevalence was high 25% (13/52).

All the positive samples to ELISA test that detects simultaneously IgM and IgG, were tested for IgM, in order to assess the acute cases. The prevalence of IgM antibodies to RVFPV was 1.3% (3/229). All IgM positive sera were from cattle, of which two were collected in Chóckwè and one in Bilene-Macia district. No differences were observed between species and localities, with regards to RVF IgM seropositivity.

The animal species (likelihood ratio $\chi^2 = 22.9$, df = 2, p = 0.000) and sex (likelihood ratio $\chi^2 = 9.4$, df= 2, p = 0.005) were the main risk factors that increased the odds of RVFPV seropositivity. Cattle were more likely to be seropositive than sheep and goats; female animals were more likely to be seropositive than males.

Regarding vector identification, a total of sixteen trapping events were performed in three districts of Gaza Province, respectively, ten in Xai-Xai (Bassopa and Patrice Lumumba), three in Chóckwè (Bombofu and Chalucuane) and three in Chibuto (Macalawane) (figure 1, study III). Overall, 1888 mosquitoes were collected (682 during three trapping events in October 2014, 168 in November 2015, during four trapping events, and 1038 mosquitoes during the nine trapping nights of March-April 2016). We observed the highest recovery rate in the last nine trapping nights.

In the first two years, the mosquito trapping was only performed in Xai-Xai district. In 2016 the trapping events included one locality in Chibuto (Macalauane) and two in Chóckwè (Bombofu and Chalucuane).

Xai-Xai had a total of 1328 trapped mosquitoes, the highest overall abundance during the study. However, for the trapping events performed during 2016, Chóckwè had the highest number of collected mosquitoes (534/1038). There was very low mosquito recovery for the trapping events performed in Chibuto district (table 1, study III).

Culex was the most abundant genus overall, with 42.3% (798/1880), followed by *Anopheles* and *Mansonia* genera, with proportions of individuals of 24.5% (462/1880) and 19.3% (364/1880), respectively.

During the year 2016, *Anopheles* spp was the most abundant genus in Chóckwè district (303 individuals), whereas *Culex* and *Mansonia* genera predominated in Xai-Xai district, with 592 and 350 individuals, respectively (figure 3, study III).

RNA extraction was performed in 45 pools of female mosquitos (each of which had 5 individuals) belonging to the genera *Anopheles* (n = 17), *Culex* (n = 13), *Mansonia* (n = 2), *Aedes* (n = 2), and other genera (n = 11). RVFPV NSs gene segment (accession number: MF 405141, www.ncbi.nlm.nih.gov/Genbank) was detected in *Culex* genus (2.2%, n = 45). The positive pool was that from mosquitoes collected in Chibuto district. We observed that the strain detected in this study clustered together with East African RVFPV strains (figure 4, study III).

4.4 Stability of a formalin-inactivated Rift Valley fever vaccine: evaluation of a vaccination campaign for cattle in Mozambique (study IV)

In this study, antibodies against RVFPV were detected in cattle sera by ELISA, IFA, and the PRNT. Seven per cent (n = 38, group D animals) of the samples from Zambezia Province and 17% (n = 25, groups A, B, C) from Maputo Province were seropositive prior to vaccination, and therefore these animals were excluded from the study.

Primary doses of the formalin-inactivated RVFPV vaccine induced a neutralizing antibody response in more than 74% of the immunized cattle in all groups. Also, similar results were obtained regarding anti-RVFPV N protein IgG antibodies.

Increases in anti-RVFPV N protein and neutralizing IgG antibodies were observed in serum samples collected soon (within three weeks) after booster vaccination, although for some animals the peak were seen later (i.e., from day 30 to day 45).

In the cattle in group D, a peak in anti-RVFPV neutralizing IgG antibodies occurred later (on day 45), and titres remained high longer (until day 147) compared to what was observed in the animals in the other groups (Figure 3 in study IV). On days 267 and 630 after primary vaccination, respectively, 60% and 73% of the animals in groups A, B, and C still had detectable neutralizing antibodies. The anti-RVFPV N protein antibody response lasted for only a short time (less than four months) in approximately 74% of the animals in all groups (Figure 2B in study IV), whereas neutralizing antibodies persisted throughout the entire study period.

In this investigation, no correlation could be found between the duration of anti-N protein and neutralizing antibody responses towards formalin-inactivated RVFPV vaccine in cattle.

4.5 Mice fed with Rift Valley fever phlebovirus antigens expressed in transgenic *Arabidopsis thaliana* show high level of immune response (study V)

The genes encoding RVFPV antigens sub-cloned into the 35S cassette of the binary vector pGreen 0229, resulted in pGreen0229/N and pGreen 0229/ΔGn. The first construct contained the gene that encoded the full length RVFPV N protein (25KDa, 760 bp) and the ΔGn insert (905 bp), encoding amino acids 91-382 of the mature Gn protein.

Agrobacterium tumefaciens mediated the transfer, insertion and integration of DNA encoding both N and ΔGn proteins at random sites in *A. thaliana* nuclear chromosomal DNA. The expression in the transgenic plants was mediated by cauliflower mosaic virus 35S promoter.

The extracted genomic DNA was used for PCR analysis using primers that targeted the 35S cassette in the pGreen vector (figure 1, study V). The 1038 bp for N (figure 1A, study V) and 1059 bp for ΔGn (figure 1B, study V) inserts

were analysed by PCR. Transformation was efficient in approximately 70-90% of the plants.

The N protein was detected in leaf tissue of all four PCR positive N transformant lines, using anti-N antibody (figure 2A, study V). Western blotting analysis showed the highest concentration of N protein in leaf tissues, 3.8 µg N/g fresh weight, as compared to that of roots, 3.3 µg N/g, and stem, 1.9 µg N/g fresh weight, respectively (figure 2B, study V). On the contrary, we could not detect ΔGn transformant by western blot, therefore we performed RT-PCR to analyse specific mRNA expression. The ΔGn2 line contained specific transgenic cDNA of 189 nuleotides (figure 2C, study V).

To check for contamination, reverse transcriptase was omitted and replaced by DNA polymerase. In the absence of the transcriptase, no DNA was detected in the mRNA samples of ΔGn2 positive lines (figure 2C, study V). N2 and ΔGn2 plant lines were chosen for immunization trials in mice.

Two weeks after immunization of mice with fresh plant material, two of four animals in the N(a) vaccine group had anti-N antibodies (figure 3A, study V) or anti-RVFPV antibodies (titers ranging from 1600-6400), when analysed by ELISA (table 2 and figure 3, study V).

After detailed analysis of IgG1 and IgG2C subclasses of antibodies, the animal that responded with the highest IgG antibody titer (figure 3A) had lower level of IgG1 (titer 50), however, it did not seroconvert for IgG2C antibody sub-class. The remaining animals in N(a) vaccine group did not respond.

In the ΔGn (a) vaccine group, two animals seroconverted after single exposure, titers 1600-3200 (figure 3B), and of those animals had an IgG1 antibody titer of 200. IgG2C antibodies were not detected in any of the animals. The mice that were fed with WT plants (control group), did not develop any antibody responses (figures 3A and 3B, study V).

When the doses was increased for the animals in the second experiment from 21g (given to mice assigned to the first experiment) to 26 g of plant material (table 2, study V) and vaccination was extended over four weeks, which included one primary and two booster doses (table 2, study V), 2 of 4 animals fed with plants expressing N protein had detectable IgG antibodies, while 3 of

4 mice in the group immunized with ΔGn plants seroconverted (figure 4, study V).

Both the positive animals assigned to the N(b) vaccination group, had IgG1 antibodies, ranging from 200-1600, whereas two of the three positive mice in group fed ΔGn(b) plants seroconverted to IgG1, titers between 200-400. Furthermore, IgG2C antibodies (titers, 200-1600) were produced by 2 animals in this group. The animals in the control group did not seroconvert.

5 Discussion

5.1 Seroprevalence of RVFPV in domestic ruminants and African Buffaloes

In study IV, seropositive animals were found prior to vaccination suggesting an active circulation of RVFPV in those areas. The investigation was performed in locations where cattle herds had not previously been surveyed to detect RVFPV antibodies or vaccinated. In subsequent surveys (studies I, II and III) we found high seroprevalences of RVFPV among domestic ruminants and African buffaloes throughout the countries, between 2010 and 2014.

The first report of RVF among cattle and goats in Mozambique was released five decades ago (Valadão, 1969) and concerned southern provinces of Gaza and Maputo, and later a small outbreak occurred in 2014 (Fafetine *et al.*, 2016). Despite the longer inter-epidemic period, studies I, II and III indicate that RVFPV is actively circulating, and possibly unnoticed outbreaks may have occurred overtime.

RVF has also been reported among humans in Mozambique. The first report, revealed 2% (28/1163) of pregnant women in 8 of 10 provinces as positive to RVFPV antibodies (Niklasson *et al.*, 1987) and a seroprevalence of 5% (10/200) RVFPV IgG antibodies, was found among febrile patients in south, during the heavy rains in 2013 (Gudo *et al.*, 2016). These reports, in combination with the results in studies of animals in I, II and III, as well as other studies performed in Mozambique, suggest that surveillance must be intensified and vaccination of livestock must be considered countrywide.

5.2 Identification of RVFPV vectors and their potential role in the vectorial transmission

Culex, Mansonia, and *Anopheles* were found in Chibuto, Chóckwè and Xai-Xai districts, except for *Aedes* that were only caught in Xai-Xai, all in Gaza Province, South Mozambique (table 1, study III). All these mosquitos' genera are known to be able for transmission of RVFPV to livestock and wild animals (Ndiaye el *et al.*, 2016; Seufi & Galal, 2010a; Turell *et al.*, 1996; Gad *et al.*, 1987a; Meegan *et al.*, 1980).

To the best of our knowledge, the study III is the first study that focus on trapping of RVFPV potential vectors in Mozambique, targeting animal pens where livestock are kept overnight for resting. *Culex, Mansonia,* and *Anopheles* female mosquitoes are commonly night feeders that can feed either on human or animal blood (Sande *et al.*, 2016; Fall *et al.*, 2011; Silver, 2008; Reisen *et al.*, 1997)

In study III, the three genera were the most predominant during the night trapping for the entire study period (figure 2, study III), as well as during 2016 (figure 3 Study III). On the other hand, *Aedes* was the less abundant genus, which may be related to its feeding behaviour. *Aedes* is mostly a day feeding mosquito (Paupy *et al.*, 2009), and thus performing night trapping reduces the chance to recover this genus.

In earlier studies *Anopheles* was collected in studies of insecticide resistance to *Plasmodium* vectors in Mozambique (Abílio *et al.*, 2015; Casimiro *et al.*, 2006a; Casimiro *et al.*, 2006b); *Culex* and *Mansonia* were identified in Zambezia Province, in the central Mozambique (Cholleti *et al.*, 2016); some *Aedes* were identified in Maputo Province and northern Mozambique (Higa *et al.*, 2015; McIntosh, 1975; McIntosh, 1971). But, we could not find any reports of *Aedes, Culex* and *Mansonia* from any district of Gaza province.

RVFPV RNA was detected from a pool of *Culex* mosquitoes, which suggests that this genus may play an important role on the viral transmission to livestock. This positive mosquito sample was collected in Chibuto district, in a region where RVFPV virus outbreaks were reported (Fafetine *et al.*, 2016; Valadão, 1969), and recently RNA from RVFPV strain belonging to lineage C was detected from goat sera and tissues (Fafetine *et al.*, 2016).

The RVFPV strain detected in study III (figure 4), seems to have spread from East Africa into Mozambique through livestock trade.

5.3 Evaluation of the stability of a formalin-inactivated RVFPV vaccine used for a vaccination campaign in Mozambique

Analysis of individual and between-group variations did not reveal any significant overall differences regarding the response to the vaccine stored under different temperature conditions, although the animals in group D (immunized with vaccine transported as stipulated by the Mozambican Directorate of Livestock) tended to show a better response compared to those in the other groups.

This suggests that the amplitude of temperature variations in Mozambique does not have a significant impact on the stability of the formalin-inactivated RVFPV vaccine, if the vaccine is distributed and administered to cattle within one week of dispatch from refrigerated storage. This is of large importance in some areas where, due to practical reasons, it is difficult to keep the vaccine refrigerated.

The use of formalin-inactivated vaccine to protect cattle in Mozambique against RVFPV should be accompanied by longitudinal studies that monitor the immune response in the animals to ascertain the need for annual vaccination.

In study IV, neutralizing antibodies against formalin-inactivated RVFPV vaccine remained detectable for more than one year. If this observation can be confirmed in areas where regular immunization with this vaccine is performed (in Zambezia Province), it would be possible to reduce the costs related to purchase of the vaccine, which in turn would decrease the number of doses needed per year or allow expansion of the vaccination coverage to new areas with the current number of doses.

5.4 Expression of RVFPV antigens in *Arabidopsis thaliana* and potential use as vaccine candidates

In study V, RVFPV antigens were produced in *A. thaliana* transgenic plants for oral delivery to mice. Oral delivery of plant-expressed recombinant viral subunit vaccines for human and animal use has been demonstrated elsewhere (Ma *et al.*, 2003). We have chosen the highly immunogenic RVFPV N protein and a deletion mutant of the Gn protein, a viral surface protein responsible for the attachment to the target cell (Rusu *et al.*, 2012) and known to induce and

interact with virus-neutralizing antibodies (Flick & Bouloy, 2005). Viral capsid proteins have previously been shown to be easily expressed in plants (Lindh *et al.*, 2009; Kehm *et al.*, 2001; Mason *et al.*, 1996) and in study V it was also shown for the RVFPV N protein, which was expressed at high concentrations.

The expression of RVFPV antigens in plants was demonstrated for the first time in study V. We could not be able to detect ΔGn protein expression in the transgenic lines, neither as a soluble protein nor as inclusion bodies in the insoluble fraction of the plant extracts, although we have removed the transmembrane elements that could negatively affect the expression, which indicates that the truncation did not lead to insolubility of the protein.

There may be a number of other reasons for the lack of detectable ΔGn protein. One is that the truncation itself affected the presentation of the protein to the antibodies of the sera from RVFPV-infected animals that was used for its detection allowing it to escape identification. The lack of a signal (Faburay *et al.*, 2013) in the ΔGn polypeptide directing it into the plant cell endoplasmic reticulum may also be one of the reasons. Therefore, the protein may have been formed in a non-glycosylated form in the cytoplasm and to a large degree been degraded. However, independently of why the protein escaped immunoblot detection in our hands, the amounts of ΔGn protein formed in the plants were large enough for oral induction of a systemic immune response in mice.

Instead, to follow ΔGn expression, we assayed the occurrence of Gn specific mRNA in the ΔGn transgenic Arabidopsis lines. Although mRNA is not a proof of the existence of the corresponding protein, we decided to continue working with the mRNA positive ΔGn transgenic line. To explore the possibility of using these transgenic plants as vectors for vaccine antigen administration, we conducted two limited mice experiments. In the first experiment, half of the animals in vaccine groups N(a) and ΔGn(a) sero-converted after a single dose of fresh transgenic plants oral consumption. Due to the lack of detection of the ΔGn protein, it is of particular interest that animals consuming those plants did indeed sero-convert.

To determine if it was possible to increase the response rate, we conducted yet another experiment, increasing the amount of plant material shared by the four animals. Two booster doses were also applied for the same reason. Again two of four animals responded in the N(b) vaccine group. However, three of four seroconverted in the ΔGn(b) vaccine group, out of which one mouse did so only after two immunizations. Hence, the response rate was similar after one

immunization in the two different experiments. The increased dose and the repeated feedings used in the second experiment did not influence the observed IgG titers. The feeding procedure may have influenced in proportion of the animals that seroconverted in each vaccination group. As the animals were grouped 4 in each cage, it was not possible to ensure equal consumption of plant material on an individual basis. It is therefore likely that the non-responders had lower intake, or none at all, compared with the responders. Other studies, have previously shown that plant extracts delivered by gastric intubation (Mason *et al.*, 1996) or pelleted plant material (Ghiasi *et al.*, 2011) are feasible alternatives to free feeding, and thus ensure equal intake of plant materials.

We analysed IgG subclasses IgG1 and IgG2c which are indicators for a Th2 or Th1 cellular response, respectively (Mosmann & Coffman, 1989). A large proportion of IgG1 positive mice were overrepresented, compared to mice with IgG2c antibodies. This indicates that Th2 responses are elicited after oral immunization using these antigens.

6 Conclusions

RVFPV is endemic and a high seroprevalence was observed among cattle, sheep, goats and African buffaloes' herds in Mozambique.

The high seroprevalence observed among livestock and African buffaloes are an indication that RVF surveillance need to be intensified countrywide, and vaccination of animals should be considered.

Culex sp, *Anopheles* sp, *Mansonia* sp were the most abundant potential RVFPV mosquitoe vectors identified in the catches from the trapping events performed between 2014 and 2016, in Gaza province, south Mozambique.

Culex genus was the only potential vector found to carry RVFPV.

Transport and variation in storage temperature had no significant impact on the efficacy and stability of the formalin-inactivated RVFPV vaccine administered to cattle in Maputo and Zambézia.

Accordingly, it seems that this vaccine is eligible for use in areas of Mozambique where it is difficult to maintain the cold chain. The present results also showed that some of the cattle developed long-lasting neutralizing antibody responses, which suggests that the revaccination period can be extended to more than one year.

RVFPV antigens expressed in *Arabidopsis thaliana* elicited strong antibody response in mice after they were fed orally with the plant extracts. Therefore, potential use of genetically transformed plants as a vaccine for livestock should be further investigated.

7 Future perspectives

To further understand the distribution of RVFPV vectors in other areas at high risk in Mozambique, such as the Central Mozambique, the vectors study should be continued. Such a study should focus on the mosquito morphological and molecular identification to species level, and detection of RVFPV from the vectors. In addition to this, the investigation of the role of different vector species in the transmission of RVFPV must be continued.

Experiments aimed at evaluating the immunization of domestic livestock with transgenic plant materials that express RVFPV antigens should be performed in Mozambique, as a mean to find alternative to the formalin-inactivated RVFPV vaccine and expand the options for livestock vaccination.

To increase the awareness of RVF, signs and symptoms in livestock must be recognized by the farmers. Then, farmers can warn the local veterinary authorities. To facilitate this, an efficient information system must be put in place. Furthermore, health care officers should work in collaboration with the veterinarians in a One Health approach, to improve the data management of RVF from both animal and human side, which may boost efficiency of risk communication with regards to RVF in Mozambique.

8 Popular science summary

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Rift Valley fever (RVF) is a viral disease transmitted primarily by mosquitoes to domestic livestock (sheep, goats and cattle), wildlife (buffaloes and camels) and other animals. RVF also affects humans in contact with fluids from infected animals during slaughtering procedures, through bites from infected mosquitoes or when taking care of other infected individuals at a viremic stage of the disease, and thus it is a concern not only for the livestock production and trade but also for public health at local, regional and global level.

RVF is widespread in Africa, and Arabic Peninsula (Saudi Arabia and Yemen), where it often causes death in large numbers of livestock and also in a significant numbers of humans. Due to the increasingly global warming, the expansion of RVF vectors have been documented in parts of Europe. Also, for example in Turkey antibodies have been detected in wildlife and in China RVF infection was detected in a patient who had been to Africa. So RVFPV may be of concern also for the public health systems in regions outside Africa and Arabic Peninsula.

Animals affected by RVF may experience fever up to 41 ^{o}C, high rate of abortion, which may reach 100% in a short time –abortion storm, neonatal mortality and liver damage. Other signs may comprise hemorrhages, decreased milk yield, lacrimation, salivation, listlessness, disinclination to move or feed, enlargement of lymph nodes and abdominal pain. Susceptible, older and non-pregnant animals may have an unapparent infection.

Humans affected with RVF may show a moderate to severe illness with high fever, and a small proportion of individuals may develop retinal lesions, encephalitis, or signs of severe liver damage with hemorrhagic manifestations.

Rift Valley fever is endemic in Mozambique, where two outbreaks have been reported in goats, sheep, cattle and water buffaloes. RVF antibodies where also

detected from humans within the country. Vaccination is the main control strategy for RVF. Formalin-inactivated RVF phlebovirus vaccine, designed for sheep, goats and cattle, is used only to immunize cattle. However, vaccination is not done consistently since there has been no systematic serologic data to support immunizations of livestock.

In the studies numbers I, II, III and IV we have evaluated the RVF seroprevalence in sheep, goats, cattle and African buffaloes (*Syncerus* caffer) in Mozambique, between 2010 and 2016, and we found it to be high (7% - 36.9%).

Furthermore, in the study number IV, we evaluated the stability of the formalin-inactivated RVF vaccine that is used for cattle, stored under different temperatures. Therefore, four experimental groups, assigned to vaccine that had been stored for 1 week at 4 ^{o}C (n = 9, group A), at 25 ^{o}C (n = 8, group B), or alternating between 4 and 25 ^{o}C (n = 8, group C) in Maputo Province, or under the temperature conditions ordinarily occurring during transportation within Mozambique (n = 38, group D). Irrespectively of the temperature conditions the vaccine was found stable and induced a long-lasting immunity (about 21 months) in all vaccination group.

In study number III we have also performed the identification of the potential RVF vectors in southern Mozambique, between 2014 and 2016. *Culex, Anopheles,* and *Mansonia* genera were found to be the most abundant vectors.

RVF antigens were expressed in *Arabidopsis thaliana* plants, and the plant extracts were used to feed 8 mice (4 in each of the two experimental groups). The mice produced a strong antibody response after oral intake of the plant extracts (study number V).

From study numbers I to IV we can conclude that RVF is occurring countrywide in Mozambique, therefore the control measures, such as testing and vaccination, must be intensified. Furthermore, the effect of formalin-inactivated RVFPV vaccine is not adversely affected by different temperature conditions, therefore it is appropriate to use for livestock vaccination in Mozambique (study IV).

Of the four genera of mosquitoes trapped from the year 2014 up to 2016, in Gaza province in south Mozambique, *Culex* was found the only one to carry RVFPV (study III).

RVF antigens produced in *Arabidopsis thaliana* plants showed a potential for use as an alternative for oral vaccination of livestock against RVFPV. Therefore, field experiments using these antigens for livestock should be undertaken (study V).

8 Populärvetenskaplig sammanfattning

Rift Valley fever (RVF) är en virussjukdom som främst överförs av mygg till husdjur (får, getter och nötkreatur), vilda djur (bufflar och kameler) och andra djur. RVF kan smitta människor t.ex. vid slakt av infekterade djur, genom bett från smittade myggor eller vid omvårdnad av infekterade individer som är i ett viremiskt stadium av sjukdomen. RVF är av relevans inte bara föranimalieproduktion och handel utan även för folkhälsan på lokal, regional och global nivå.

RVF är utbredd i Afrika och på Arabiska halvön (Saudiarabien och Jemen), där det vid sjukdomsutbrott orsakat många dödsfall både för boskap och människor. På grund av den globala uppvärmningen har en expansion av RVF-vektorer dokumenterats i delar av Europa. Vidare har till exempel i Turkiet påvisats RVF-antikroppar hos vilda djur och i Kina har RVF-infektion diagnosticerats hos en patient som varit i Afrika. Därav är RVF av betydelse även för folkhälsoorganisationer utanför Afrika och Arabiska halvön. Djur som drabbas av RVF kan få högfeber (upp till 41 ^{o}C), aborter som kan uppgå till 100%, neonatal dödlighet och leverskada. Andra symtom kan vara blödningar, minskad mjölkproduktion salivation, nedsatt allmäntillstånd, ovilja att röra sig och att äta, förstorade lymfknutor och buksmärtor. Mindre känsliga djur få en symtomlös infektion.

Människor som drabbas av RVF kan uppvisa en måttlig till svår sjukdom med hög feber, och en mindre andel individer kan utveckla skador på ögon, hjärna och lever samt även orsaka blödningar.

Rift Valley feber är endemisk i Moçambique, där två utbrott har rapporterats på getter, får, nötkreatur och vattenbufflar. RVF-antikroppar har också diagnosticerats hosmänniskor i landet. Vaccination av boskap är den huvudsakliga strategin för kontroll av RVF. Ett formalininaktiverat RVF-vaccin, utformat för får, getter och nötkreatur, används. Vaccination görs inte konsekvent eftersom det inte funnits några systematiska serologiska data för att stödja immuniseringar av boskap.

I studie nummer I, II, III och IV har vi utvärderat RVF-seroprevalensen hos får, getter, nötkreatur och afrikanska bufflar (*Syncerus caffer*) i Moçambique mellan 2010 och 2016, och vi fann att seroprevalensen var hög (7% - 36,9%).

Vidare utvärderade vi stabiliteten hos det formalininaktiverade RVF-vaccinet som används för boskap, under lagring vid olika temperaturer. Fyra experimentella grupper vaccinerades med ett vaccin som hade lagrats i 1 vecka vid 4 ^{o}C (n = 9, grupp A) vid 25 ^{o}C (n = 8, grupp B) eller alternerande mellan 4 och 25 ^{o}C (n = 8, grupp C) i Maputo-provinsen eller under de temperaturförhållanden som vanligtvis uppkom under transport inom Moçambique (n = 38, grupp D) i Zambézia-provinsen, fick två immuniseringar (den andra administrerades tre veckor efter den förstavaccinationen). Oavsett temperaturförhållanden var vaccinet stabilt och framkallade en långvarig immunitet (cirka 21 månader) i alla vaccinationsgrupper (studie nummer IV).

I studie III har vi också utfört identifieringen av de potentiella RVF-vektorerna i södra Moçambique, mellan 2014 och 2016. Culex, Anopheles och Mansonia-genera befanns vara de vanligast förekommandvektorerna.

RVF-antigener uttrycktes i *Arabidopsis thaliana*-växter, och växtextrakten användes för att mata 8 möss (4 i vardera av de två experimentella grupperna). Mössen producerade ett starkt antikroppssvar efter oralt intag av växtextrakten (studie nummer V).

Från studie I till IV kan vi dra slutsatsen att RVF förekommer landsomfattande i Moçambique, därför måste kontrollåtgärderna, såsom testning och vaccination, intensifieras. Effekten av formalininaktiverat RVFPV-vaccin påverkas inte av olika temperaturförhållanden, därför är det lämpligt att använda för vaccinering av boskap i Moçambique (studie IV).

Av de fyra släkten av mygg som fångades från år 2014 fram till 2016, i Gaza-provinsen i södra Moçambique, var Culex den enda som skulle bära RVFPV (studie III).

RVF-antigener framställda i Arabidopsis thaliana-växter visade en potential för användning som ett alternativ för oral vaccination av boskap mot RVFPV. Därför bör fältförsök som använder dessa antigener för boskap genomföras (studie V).

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References

Abdourahmane, S., Ousmane, F., Yamar, B., Hampathé, B., Diawo, D., Oumar, F., Cheikh, L., Mohamed, B., Yahya, B., Mawlouth, D. & Amadou Alpha, S. (2014). Rift Valley Fever Outbreak, Southern Mauritania, 2012. *EmergInfect Dis,* 20(2), p. 296.

Abílio, A., Marrune, P., de Deus, N., Mbofana, F., Muianga, P. & Kampango, A. (2015). Bio-efficacy of new long-lasting insecticide-treated bed nets *Anopheles funestus* and *Anopheles gambiae* from central and nothern Mozambique *Malaria Journal,* 14, p. 352.

Adam, I. & Karsany, M.S. (2008). Case report: Rift Valley Fever with vertical transmission in a pregnant Sudanese woman. *J Med Virol,* 80(5), pp. 929-929.

Al-Hazmi, M., Ayoola, E.A., Abdurahman, M., Banzal, S., Ashraf, J., El-Bushra, A., Hazmi, A., Abdullah, M., Abbo, H., Elamin, A., Al-Sammani, E.-T., Gadour, M., Menon, C., Hamza, M., Rahim, I., Hafez, M., Jambavalikar, M., Arishi, H. & Aqeel, A. (2003). Epidemic Rift Valley Fever in Saudi Arabia: A Clinical Study of Severe Illness in Humans. *Clin Infect Dis,* 36(3), pp. 245-252.

Alrajhi, A.A., Al-Semari, A. & Al-Watban, J. (2004). Rift Valley Fever Encephalitis. *Emerg Infect Dis,* 10(3), pp. 554-555.

Anyamba, A., Linthicum, K.J. & Tucker, C.J. (2001). Climate-disease connections: Rift Valley Fever in Kenya. *Cad Saude Publica,* 17 Suppl, pp. 133-40.

Archer, B.N., Thomas, J., Weyer, J., Cengimbo, A., Landoh, D.E., Jacobs, C., Ntuli, S., Modise, M., Mathonsi, M., Mashishi, M.S., Leman, P.A., le Roux, C., Jansen van Vuren, P., Kemp, A., Paweska, J.T. & Blumberg, L. (2013). Epidemiologic Investigations into Outbreaks of Rift Valley Fever in Humans, South Africa, 2008–2011. *Emerg Infect Dis,* 19(12), pp. 1918-1925.

Balkhy, H.H. & Memish, Z.A. (2003). Rift Valley fever: an uninvited zoonosis in the Arabian peninsula. *Int J Antimicrob Agents,* 21(2), pp. 153-7.

Baudin, M., Jumaa, A.M., Jomma, H.J.E., Karsany, M.S., Bucht, G., Naslund, J., Ahlm, C., Evander, M. & Mohamed, N. (2016). Association of Rift Valley fever virus infection with miscarriage in Sudanese women: a cross-sectional study. *Lancet Glob Health,* 4(11), pp. e864-e871.

Bhardwaj, N., Heise, M.T. & Ross, T.M. (2010). Vaccination with DNA Plasmids Expressing Gn Coupled to C3d or Alphavirus Replicons Expressing Gn Protects Mice against Rift Valley Fever Virus. *PLoS Negl Trop Dis,* 4(6), p. e725.

Billecocq, A., Spiegel, M., Vialat, P., Kohl, A., Weber, F., Bouloy, M. & Haller, O. (2004). NSs protein of Rift Valley fever virus blocks interferon production by inhibiting host gene transcription. *J Virol,* 78(18), pp. 9798-806.

Bird, B.H., Albarino, C.G., Hartman, A.L., Erickson, B.R., Ksiazek, T.G. & Nichol, S.T. (2008). Rift valley fever virus lacking the NSs and NSm genes is highly attenuated, confers protective immunity from virulent virus challenge, and allows for differential identification of infected and vaccinated animals. *J Virol,* 82(6), pp. 2681-91.

Blair, C.D. & Olson, K.E. (2014). Mosquito immune responses to arbovirus infections. *Curr Opin Insect Sci,* 3, pp. 22-29.

Blair, C.D. & Olson, K.E. (2015). The role of RNA interference (RNAi) in arbovirus-vector interactions. *Viruses,* 7(2), pp. 820-43.

Blomström, A.-L., Scharin, I., Stenberg, H., Figueiredo, J., Nhambirre, O., Abilio, A., Berg, M. & Fafetine, J. (2016). Seroprevalence of Rift Valley fever virus in sheep and goats in Zambézia, Mozambique. *Infect Ecol Epidemiol,* 6, p. 10.3402/iee.v6.31343.

Boshra, H., Lorenzo, G., Rodriguez, F. & Brun, A. (2011). A DNA vaccine encoding ubiquitinated Rift Valley fever virus nucleoprotein provides consistent immunity and protects IFNAR(-/-) mice upon lethal virus challenge. *Vaccine,* 29(27), pp. 4469-75.

Boushab, B.M., Fall-Malick, F.Z., Ould Baba, S.E.W., Ould Salem, M.L., Belizaire, M.R.D., Ledib, H., Ould Baba Ahmed, M.M., Basco, L.K. & Ba, H. (2016). Severe Human Illness Caused by Rift Valley Fever Virus in Mauritania, 2015. *Open Forum Infect Dis,* 3(4), pp. ofw200-ofw200.

Casimiro, S., Coleman, M., Hemingway, J. & Sharp, B. (2006a). Insecticide resistance in *Anopheles arabiensis* and *Anopheles gambiae* from Mozambique. *J Med Entomol,* 43(2), pp. 276-282.

Casimiro, S., Coleman, M., Hemingway, J. & Sharp, B. (2006b). Insecticide resistance in *Anopheles funestus (*Diptera: Culicidae) from Mozambique. *J Med Entomol,* 43(2), pp. 267-275.

CDC (1998). Rift Valley Fever--East Africa, 1997-1998. *MMWR Morb Mortal Wkly Rep,* 47(13), pp. 261-4.

Chengula, A.A., Mdegela, R.H. & Kasanga, C.J. (2013). Socio-economic impact of Rift Valley fever to pastoralists and agro pastoralists in

Arusha, Manyara and Morogoro regions in Tanzania. *SpringerPlus,* 2, p. 549.

Cholleti, H., Hayer, J., Abilio, A., Mulandane, F., Verner-Carlsson, J., Falk, K., Fafetine, J., Berg, M. & Blomstöm, A.-L. (2016). Discovery of novel viruses in mosquitoes from Zambezi Valley of Mozambique *PLoS ONE,* 11(9), p. e0162751.

Daubney, R. & Hudson, J.R. (1932). Rift Valley Fever. The Lancet, p. 2.

Davies, F.G. (1990). Rift Valley fever in the Sudan. *Trans R Soc Trop Med Hyg,* 84(1), p. 141.

Davies, F.G., Kilelu, E., Linthicum, K.J. & Pegram, R.G. (1992). Patterns of Rift Valley fever activity in Zambia. *Epidemiology and Infection,* 108(1), pp. 185-191.

Davies, F.G. & Martin, V. (2006). Recognizing Rift Valley Fever. *Vet Ital,* 42(1), pp. 31-53.

Deyde, V.M., Khristova, M.L., Rollin, P.E., Ksiazek, T.G. & Nichol, S.T. (2006). Crimean-Congo hemorrhagic fever virus genomics and global diversity. *J Virol,* 80(17), pp. 8834-42.

Dietrich, I., Jansen, S., Fall, G., Lorenzen, S., Rudolf, M., Huber, K., Heitmann, A., Schicht, S., Ndiaye, E.H., Watson, M., Castelli, I., Brennan, B., Elliott, R.M., Diallo, M., Sall, A.A., Failloux, A.-B., Schnettler, E., Kohl, A. & Becker, S.C. (2017). RNA Interference Restricts Rift Valley Fever Virus in Multiple Insect Systems. *mSphere,* 2(3).

DINAP (2002). *Relatório anual 2001 [Annual report 2001]*. Maputo: Direcção Nacional de Pecuária [National Directorate of Livestock].

DINAP (2003). *Relatório anual 2002 [Annual report 2002]*. Maputo: Direcção Nacional de Pecuária [National Directorate of Livestock].

Dungu, B., Louw, I., Lubisi, A., Hunter, P., von Teichman, B.F. & Bouloy, M. (2010). Evaluation of the efficacy and safety of the Rift Valley Fever Clone 13 vaccine in sheep. *Vaccine,* 28(29), pp. 4581-7.

El-Akkad, A.M. (1978). Rift Valley fever outbreak in Egypt. October--December 1977. *J Egypt Public Health Assoc,* 53(3-4), pp. 123-8.

El Mamy, A.B.O., Baba, M.O., Barry, Y., Isselmou, K., Dia, M.L., Hampate, B., Diallo, M.Y., El Kory, M.O.B., Diop, M., Lo, M.M., Thiongane, Y., Bengoumi, M., Puech, L., Plee, L., Claes, F., de La Rocque, S. & Doumbia, B. (2011). Unexpected Rift Valley Fever Outbreak, Northern Mauritania. *Emerg Infect Dis,* 17(10), pp. 1894-1896.

Engstrom, J. (2012). *Seroprevalence of Rift Valley Fever in Domentstic Sheep and Goat of Gaza Province, Mozambique*. Uppsala: Swedish University of Agriculture Science.

Errett, J.S., Suthar, M.S., McMillan, A., Diamond, M.S. & Gale, M., Jr. (2013). The essential, nonredundant roles of RIG-I and MDA5 in detecting and controlling West Nile virus infection. *J Virol,* 87(21), pp. 11416-25.

Faburay, B., Wilson, W., McVey, D.S., Drolet, B.S., Weingartl, H., Madden, D., Young, A., Ma, W. & Richt, J.A. (2013). Rift Valley fever virus structural and nonstructural proteins: recombinant protein expression and immunoreactivity against antisera from sheep. *Vector-Borne Zoonotic Dis*, 13(9), pp. 619-29.

Faburay, B., Wilson, W.C., Gaudreault, N.N., Davis, A.S., Shivanna, V., Bawa, B., Sunwoo, S.Y., Ma, W., Drolet, B.S., Morozov, I., McVey, D.S. & Richt, J.A. (2016). A Recombinant Rift Valley Fever Virus Glycoprotein Subunit Vaccine Confers Full Protection against Rift Valley Fever Challenge in Sheep. *Scientific Reports*, 6, p. 27719.

Fafetine, J., Coetzee, P., Mubemba, B., Nhambirre, O., Neves, L., Coetzer, J. & Venter, E. (2016). Rift Valley fever outbreak in livestock, Mozambique, 2014. *Emerg Infect Dis*, 22(12), pp. 2165-2167.

Fafetine, J., Neves, L., Thompson, P.N., Paweska, J.T. & Rutten, V.P. (2013). Serological evidence of Rift Valley fever virus circulation in sheep and goats in Zambezia Province, Mozambique. *PLoS Negl Trop Dis*, 7.

Fall, A.G., Diaite, A., Lancelot, R., Tran, A., Soti, V., Etter, E., Konate, L., Faye, O. & Bouyer, J. (2011). Feeding behaviour of potential vectors of West Nile virus in Senegal. *Parasites & Vectors*, 4, p. 7.

Faye, O., Ba, H., Ba, Y., Freire, C.C.M., Faye, O., Ndiaye, O., Elgady, I.O., Zanotto, P.M.A., Diallo, M. & Sall, A.A. (2014). Reemergence of Rift Valley Fever, Mauritania, 2010. *Emerg Infect Dis*, 20(2), pp. 300-303.

Filone, C.M., Heise, M., Doms, R.W. & Bertolotti-Ciarlet, A. (2006). Development and characterization of a Rift Valley fever virus cell-cell fusion assay using alphavirus replicon vectors. *Virology*, 356(1-2), pp. 155-64.

Flick, R. & Bouloy, M. (2005). Rift Valley fever virus. *Curr Mol Med*, 5(8), pp. 827-34.

Fontenille, D., Traore-Lamizana, M., Diallo, M., Thonnon, J., Digoutte, J.P. & Zeller, H.G. (1998). New vectors of Rift Valley fever in West Africa. *Emerg Infect Dis*, 4(2), pp. 289-93.

Fragkoudis, R., Attarzadeh-Yazdi, G., Nash, A.A., Fazakerley, J.K. & Kohl, A. (2009). Advances in dissecting mosquito innate immune responses to arbovirus infection. *J Gen Virol*, 90(Pt 9), pp. 2061-72.

Franz, A.W., Kantor, A.M., Passarelli, A.L. & Clem, R.J. (2015). Tissue Barriers to Arbovirus Infection in Mosquitoes. *Viruses*, 7(7), pp. 3741-67.

Gack, M.U. (2014). Mechanisms of RIG-I-Like Receptor Activation and Manipulation by Viral Pathogens. *J Virol*, 88(10), pp. 5213-5216.

Gad, A.M., Hassan, M.M., el Said, S., Moussa, M.I. & Wood, O.L. (1987a). Rift Valley fever virus transmission by different Egyptian mosquito species. *Trans R Soc Trop Med Hyg*, 81(4), pp. 694-8.

Gad, A.M., Hassan, M.M., El Said, S., Moussa, M.I. & Wood, O.L. (1987b). Rift Valley fever virus transmission by different Egyptian mosquito species. *Trans R Soc Trop Med Hyg,* 81(4), pp. 694-698.

Garry, C.E. & Garry, R.F. (2004). Proteomics computational analyses suggest that the carboxyl terminal glycoproteins of Bunyaviruses are class II viral fusion protein (beta-penetrenes). *Theoretical Biol Med Modelling,* 1(1), p. 10.

Ghiasi, S.M., Salmanian, A.H., Chinikar, S. & Zakeri, S. (2011). Mice Orally Immunized with a Transgenic Plant Expressing the Glycoprotein of Crimean-Congo Hemorrhagic Fever Virus. *Clin Vaccine Immunol,* 18(12), pp. 2031-2037.

Gillet, J. (1972). *Common African mosquitoes and their medical importance*. London William Heinemann Medical Books, LTD.

Gudo, E.S., Pinto, G., Weyer, J., le Roux, C., Mandlaze, A., José, A.F., Muianga, A. & Paweska, J.T. (2016). Serological evidence of rift valley fever virus among acute febrile patients in Southern Mozambique during and after the 2013 heavy rainfall and flooding: implication for the management of febrile illness. *Virol J,* 13(1), p. 96.

Gur, S., Kale, M., Erol, N., Yapici, O., Mamak, N. & Yavru, S. (2017). The first serological evidence for Rift Valley fever infection in the camel, goitered gazelle and Anatolian water buffaloes in Turkey. *Trop Anim Health Prod*.

Hassan, O.A., Affognon, H., Rocklöv, J., Mburu, P., Sang, R., Ahlm, C. & Evander, M. (2017). The One Health approach to identify knowledge, attitudes and practices that affect community involvement in the control of Rift Valley fever outbreaks. *PLoS Negl Trop Dis,* 11(2), p. e0005383.

Hassan, O.A., Ahlm, C., Sang, R. & Evander, M. (2011). The 2007 Rift Valley Fever Outbreak in Sudan. *PLoS Negl Trop Dis,* 5(9), p. e1229.

Higa, Y., Abilio, A., Futami, K., Lázaro, A., Minakawa, N. & Gudo, E. (2015). Abundant *Aedes* (*Stegomya*) aegypti aegypti mosquitoes in the 2014 dengue outbreak area of Mozambique *Trop Med Hyg,* 43(2), pp. 107-109.

Ibrahim, M.S., Turell, M.J., Knauert, F.K. & Lofts, R.S. (1997). Detection of Rift Valley fever virus in mosquitoes by RT-PCR. *Molecular and Cellular Probes,* 11(1), pp. 49-53.

ICVT (2016). Virus Taxonomy: 2016 Release. [online], htttps://talk.ictvonline.org/taxonomy/, [Accessed 20-11-2017].

Ikegami, T. & Makino, S. (2009). Rift Valley fever vaccines. *Vaccine,* 27S4, pp. D69-D72.

Ikegami, T. & Makino, S. (2011). The Pathogenesis of Rift Valley Fever. *Viruses,* 3(5), pp. 493-519.

Ikegami, T., Narayanan, K., Won, S., Kamitani, W., Peters, C.J. & Makino, S. (2009). Rift Valley fever virus NSs protein promotes post-

transcriptional downregulation of protein kinase PKR and inhibits eIF2alpha phosphorylation. *PLoS Pathog,* 5.

Iranpour, M., Moghadam, A.R., Yazdi, M., Ande, S.R., Alizadeh, J., Wiechec, E., Lindsay, R., Drebot, M., Coombs, K.M. & Ghavami, S. (2016). Apoptosis, autophagy and unfolded protein response pathways in Arbovirus replication and pathogenesis. *Expert Rev Mol Med,* 18, p. e1.

Jansen van Vuren, P., Tiemessen, C.T. & Paweska, J.T. (2011). Anti-Nucleocapsid Protein Immune Responses Counteract Pathogenic Effects of Rift Valley Fever Virus Infection in Mice. *PLOS ONE,* 6(9), p. e25027.

Jupp, P. (1996). *Mosquitoes of Southern Africa: Culicinae and Toxorhynchitinae*. Republic of South Africa Ekogilde Publishers.

Kainulainen, M., Habjan, M., Hubel, P., Busch, L., Lau, S., Colinge, J., Superti-Furga, G., Pichlmair, A. & Weber, F. (2014). Virulence factor NSs of rift valley fever virus recruits the F-box protein FBXO3 to degrade subunit p62 of general transcription factor TFIIH. *J Virol,* 88(6), pp. 3464-73.

Kainulainen, M., Lau, S., Samuel, C.E., Hornung, V. & Weber, F. (2016). NSs Virulence Factor of Rift Valley Fever Virus Engages the F-Box Proteins FBXW11 and beta-TRCP1 To Degrade the Antiviral Protein Kinase PKR. *J Virol,* 90(13), pp. 6140-7.

Kalveram, B., Lihoradova, O. & Ikegami, T. (2011). NSs protein of rift valley fever virus promotes posttranslational downregulation of the TFIIH subunit p62. *J Virol,* 85(13), pp. 6234-43.

Kalveram, B., Lihoradova, O., Indran, S.V., Lokugamage, N., Head, J.A. & Ikegami, T. (2013). Rift Valley fever virus NSs inhibits host transcription independently of the degradation of dsRNA-dependent protein kinase PKR. *Virology,* 435.

Kamal, S.A. (2009). Pathological studies on postvaccinal reactions of Rift Valley fever in goats. *Virol J,* 6(1), p. 94.

Kapoor, S. (2008). Resurgence of Rift Valley Fever. *Infectious Diseases in Clinical Practice,* 16(1), pp. 9-12.

Kark, J.D., Aynor, Y. & Peters, C.J. (1982). A Rift Valley fever vaccine trial. Side effects and serological response over a six-month follow-up. *Ameri J Epidemiol,* 116(5), pp. 808-820.

Kehm, R., Jakob, N.J., Welzel, T.M., Tobiasch, E., Viczian, O., Jock, S., Geider, K., Süle, S. & Darai, G. (2001). Expression of Immunogenic Puumala Virus Nucleocapsid Protein in Transgenic Tobacco and Potato Plants. *Virus Genes,* 22(1), pp. 73-83.

Kingsolver, M.B., Huang, Z. & Hardy, R.W. (2013). Insect antiviral innate immunity: pathways, effectors, and connections. *J Mol Biol,* 425(24), pp. 4921-36.

Kondela, A., Loretu, K. & Mella, P.N. (1985). Isolation of Rift Valley fever virus from cattle abortions in Tanzania. *Trop Anim Health Prod*, 17(3), pp. 185-6.

Kortekaas, J., Antonis, A.F.G., Kant, J., Vloet, R.P.M., Vogel, A., Oreshkova, N., de Boer, S.M., Bosch, B.J. & Moormann, R.J.M. (2012). Efficacy of three candidate Rift Valley fever vaccines in sheep. *Vaccine*, 30(23), pp. 3423-3429.

Ksiazek, T.G., Jouan, A., Meegan, J.M., Le Guenno, B., Wilson, M.L., Peters, C.J., Digoutte, J.P., Guillaud, M., Merzoug, N.O. & Touray, E.M. (1989). Rift Valley fever among domestic animals in the recent West African outbreak. *Res Virol*, 140(1), pp. 67-77.

Laughlin, L.W., Meegan, J.M., Strausbaugh, L.J., Morens, D.M. & Watten, R.H. (1979). Epidemic Rift Valley fever in Egypt: observations of the spectrum of human illness. *Trans R Soc Trop Med Hyg*, 73(6), pp. 630-633.

Le May, N., Mansuroglu, Z., Léger, P., Josse, T., Blot, G., Billecocq, A., Flick, R., Jacob, Y., Bonnefoy, E. & Bouloy, M. (2008). A SAP30 Complex Inhibits IFN-β Expression in Rift Valley Fever Virus Infected Cells. *Plos Pathogens*, 4(1), p. e13.

Léger, P., Lara, E., Jagla, B., Sismeiro, O., Mansuroglu, Z., Coppée, J.Y., Bonnefoy, E. & Bouloy, M. (2013). Dicer-2- and Piwi-Mediated RNA Interference in Rift Valley Fever Virus-Infected Mosquito Cells. *J Virol*, 87(3), pp. 1631-1648.

Lin, R.J., Chang, B.L., Yu, H.P., Liao, C.L. & Lin, Y.L. (2006). Blocking of interferon-induced Jak-Stat signaling by Japanese encephalitis virus NS5 through a protein tyrosine phosphatase-mediated mechanism. *J Virol*, 80(12), pp. 5908-18.

Lindh, I., Wallin, A., Kalbina, I., Savenstrand, H., Engstrom, P., Andersson, S. & Strid, A. (2009). Production of the p24 capsid protein from HIV-1 subtype C in Arabidopsis thaliana and Daucus carota using an endoplasmic reticulum-directing SEKDEL sequence in protein expression constructs. *Protein Expr Purif*, 66(1), pp. 46-51.

Linthicum, K.J., Anyamba, A., Tucker, C.J., Kelley, P.W., Myers, M.F. & Peters, C.J. (1999). Climate and satellite indicators to forecast Rift Valley fever epidemics in Kenya. *Science*, 285(5426), pp. 397-400.

Logan, T.M., Linthicum, K.J., Thande, P.C., Wagateh, J.N. & Roberts, C.R. (1991). Mosquito species collected from a marsh in western Kenya during the long rains. *J Am Mosquito Control Assoc*, 7(3), pp. 395-399.

Lorenzo, G., Folgar, R., Hevia, E., Boshra, H. & Brun, A. (2010). Protection against lethal Rift Valley fever virus (RVFV) infection in transgenic IFNAR-/- mice induced by different DNA vaccination regimens. *Vaccine*, 28(17), pp. 2937-44.

Lubroth, J., Rweyemamu, M.M., Viljoen, G., Diallo, A., Dungu, B. & Amanfu, W. (2007). Veterinary vaccines and their use in developing countries. *Rev Sci Tech*, 26(1), pp. 179-201.

Ma, J.K., Drake, P.M. & Christou, P. (2003). The production of recombinant pharmaceutical proteins in plants. *Nat Rev Genet*, 4(10), pp. 794-805.

Mandell, R.B., Koukuntla, R., Mogler, L.J., Carzoli, A.K., Freiberg, A.N., Holbrook, M.R., Martin, B.K., Staplin, W.R., Vahanian, N.N., Link, C.J. & Flick, R. (2010). A replication-incompetent Rift Valley fever vaccine: chimeric virus-like particles protect mice and rats against lethal challenge. *Virology*, 397(1), pp. 187-98.

Mansuroglu, Z., Josse, T., Gilleron, J., Billecocq, A., Leger, P., Bouloy, M. & Bonnefoy, E. (2010). Nonstructural NSs Protein of Rift Valley Fever Virus Interacts with Pericentromeric DNA Sequences of the Host Cell, Inducing Chromosome Cohesion and Segregation Defects. *J Virol*, 84(2), pp. 928-939.

Mason, H.S., Ball, J.M., Shi, J.J., Jiang, X., Estes, M.K. & Arntzen, C.J. (1996). Expression of Norwalk virus capsid protein in transgenic tobacco and potato and its oral immunogenicity in mice. *Proc Natl Acad Sci U S A*, 93(11), pp. 5335-5340.

McIntosh, B.M. (1971). The aedine subgenus Neomelaniconion Newstead (Culicidae, Diptera) in southern Africa with descritpions of two new species *J Ent Soc S Afr*, 34(2), pp. 319-333.

McIntosh, B.M. (1975). A taxonomic revision of certain *Aedes* species (Diptera: Culicidae) of subgenus *Aedimoprhus* in southern Africa. *J. Ent Soc S Afr*, 38(2), pp. 251-287.

McIntosh, B.M., Jupp, P.G., Dos Santos, I. & Barnard, B.J. (1980). Vector studies on rift valley fever virus in South Africa. *S Afr Med J*, 58(3), pp. 127-132.

Meegan, J., Khalil, G., Hoogstraal, H. & Adham, F. (1980). Experimental Transmission and Field Isolation Studies Implicating Culex Pipiens as a Vector of Rift Valley Fever Virus in Egypt. *Am J Trop Med Hyg*, 29(6), pp. 1405 - 1410.

Meegan, J.M. (1979). The Rift Valley fever epizootic in Egypt 1977-78. 1. Description of the epizzotic and virological studies. *Trans R Soc Trop Med Hyg*, 73(6), pp. 618-23.

Métras, R., Porphyre, T., Pfeiffer, D.U., Kemp, A., Thompson, P.N., Collins, L.M. & White, R.G. (2012). Exploratory Space-Time Analyses of Rift Valley Fever in South Africa in 2008–2011. *PLoS Negl Trop Dis*, 6(8), p. e1808.

Monaco, F., Pinoni, C., Cosseddu, G.M., Khaiseb, S., Calistri, P., Molini, U., Bishi, A., Conte, A., Scacchia, M. & Lelli, R. (2013). Rift Valley Fever in Namibia, 2010. *Emerg Infect Dis*, 19(12), pp. 2025-2027.

Morrill, J.C., Mebus, C.A. & Peters, C.J. (1997a). Safety and efficacy of a mutagen-attenuated Rift Valley fever virus vaccine in cattle. *Am J Vet Res*, 58(10), pp. 1104-9.

Morrill, J.C., Mebus, C.A. & Peters, C.J. (1997b). Safety of a mutagen-attenuated Rift Valley fever virus vaccine in fetal and neonatal bovids. *Am J Vet Res,* 58(10), pp. 1110-4.

Morvan, J., Rollin, P.E., Laventure, S., Rakotoarivony, I. & Roux, J. (1992). Rift Valley fever epizootic in the central highlands of Madagascar. *Res Virol,* 143(6), pp. 407-15.

Mosmann, T.R. & Coffman, R.L. (1989). TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol,* 7, pp. 145-73.

Moy, R.H., Gold, B., Molleston, J.M., Schad, V., Yanger, K., Salzano, M.V., Yagi, Y., Fitzgerald, K.A., Stanger, B.Z., Soldan, S.S. & Cherry, S. (2014). Antiviral autophagy restrictsRift Valley fever virus infection and is conserved from flies to mammals. *Immunity,* 40(1), pp. 51-65.

Mudhasani, R., Tran, J.P., Retterer, C., Kota, K.P., Whitehouse, C.A. & Bavari, S. (2016). Protein Kinase R Degradation Is Essential for Rift Valley Fever Virus Infection and Is Regulated by SKP1-CUL1-F-box (SCF)FBXW11-NSs E3 Ligase. *PLoS Pathog,* 12.

Munyua, P., Murithi, R.M., Wainwright, S., Githinji, J., Hightower, A., Mutonga, D., Macharia, J., Ithondeka, P.M., Musaa, J., Breiman, R.F., Bloland, P. & Njenga, M.K. (2010). Rift Valley Fever Outbreak in Livestock in Kenya, 2006–2007. *Am J Trop Med Hyg,* 83(2 Suppl), pp. 58-64.

Murphy, F., Gibbs, E., Horzinek, M. & Studert, M. (1999). *Veterinary Virology*. 3rd Edition. ed. USA: Academic Press (An imprint of ELSEVIER).

Murray, P., Rosenthal, K. & Pfaller, M. (2009). *Medical Microbiology*. 6th Edition. ed. Philadephia: Mosby (ELSEVIER).

Naslund, J., Lagerqvist, N., Habjan, M., Lundkvist, A., Evander, M., Ahlm, C., Weber, F. & Bucht, G. (2009). Vaccination with virus-like particles protects mice from lethal infection of Rift Valley Fever Virus. *Virology,* 385(2), pp. 409-15.

Näslund, J., Lagerqvist, N., Lundkvist, A., Evander, M., Ahlm, C. & Bucht, G. (2008). Kinetics of Rift Valley fever virus in experimentally infected mice using quantitative real-time RT-PCR. *J Virol Methods,* 151(2), pp. 277-282.

Ndiaye el, H., Fall, G., Gaye, A., Bob, N.S., Talla, C., Diagne, C.T., Diallo, D., B, A.Y., Dia, I., Kohl, A., Sall, A.A. & Diallo, M. (2016). Vector competence of Aedes vexans (Meigen), Culex poicilipes (Theobald) and Cx. quinquefasciatus Say from Senegal for West and East African lineages of Rift Valley fever virus. *Parasit Vectors,* 9, p. 94.

Niklasson, B., Liljestrand, J., Bergstrom, S. & Peters, C.J. (1987). Rift Valley fever: a sero-epidemiological survey among pregnant women in Mozambique. *Epidemiol Infect,* 99.

Niklasson, B., Liljestrand, J., Bergström, S. & Peters, C.J. (2009). Rift Valley fever: a sero-epidemiological survey among pregnant women in Mozambique. *Epidemiol Infect*, 99(2), pp. 517-522.

Paupy, C., Delatte, H., Bagny, L., Corbel, V. & Fontenille, D. (2009). Aedes albopictus, an arbovirus vector: From the darkness to the light. *Microbes and Infection*, 11(14–15), pp. 1177-1185.

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

Rakotoarivelo, R.A., Andrianasolo, R., Razafimahefa, S.H., Randremandranto Razafimbelo, N.S. & Randria, M.J. (2011). [Severe presentations of Rift Valley Fever in Madagascar]. *Med Mal Infect*, 41(6), pp. 318-21.

Reisen, W.K., Lothrop, H.D. & Meyer, R.P. (1997). Time of host-seeking by Culex tarsalis (Diptera: Culicidae) in California. *J Med Entomol*, 34(4), pp. 430-437.

Romoser, W.S., Turell, M.J., Lerdthusnee, K., Neira, M., Dohm, D., Ludwig, G. & Wasieloski, L. (2005). Pathogenesis of Rift Valley fever virus in mosquitoes--tracheal conduits & the basal lamina as an extra-cellular barrier. *Arch Virol Suppl*(19), pp. 89-100.

Rusnak, J.M., Gibbs, P., Boudreau, E., Clizbe, D.P. & Pittman, P. (2011). Immunogenicity and safety of an inactivated Rift Valley fever vaccine in a 19-year study. *Vaccine*, 29(17), pp. 3222-9.

Rusu, M., Bonneau, R., Holbrook, M.R., Watowich, S.J., Birmanns, S., Wriggers, W. & Freiberg, A.N. (2012). An Assembly Model of Rift Valley Fever Virus. *Frontiers in Microbiology*, 3, p. 254.

Sanchez-Vargas, I., Travanty, E.A., Keene, K.M., Franz, A.W., Beaty, B.J., Blair, C.D. & Olson, K.E. (2004). RNA interference, arthropod-borne viruses, and mosquitoes. *Virus Res*, 102(1), pp. 65-74.

Sande, S., Zimba, M., Chinwada, P., Masendu, H.T. & Makuwaza, A. (2016). Biting behaviour of Anopheles funestus populations in Mutare and Mutasa districts, Manicaland province, Zimbabwe: Implications for the malaria control programme. *J Vector Borne Dis*, 53(2), pp. 118-126.

Seufi, A. & Galal, F. (2010a). Role of Culex and Anopheles mosquito species as potential vectors of rift valley fever virus in Sudan outbreak, 2007. *BMC Infect Dis*, 10(65), pp. 1-8.

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

Shope, R.E., Peters, C.J. & Walker, J.S. (1980). Serological relation between Rift Valley fever virus and viruses of phlebotomus fever serogroup. *Lancet*, 1(8173), pp. 886-7.

Siam, A.L. & Meegan, J.M. (1980). Ocular disease resulting from infection with Rift Valley fever virus. *Trans R Soc Trop Med Hyg,* 74(4), pp. 539-541.

Silver, J.B. (2008). Mosquito Ecology, Field Sambpling Methods. In: Springer (ed. *Mosquito Ecology, Field Sambpling Methods*. Third Edition. ed. Netherlands Springer, pp. 845-946.

Soi, R.K., Rurangirwa, F.R., McGuire, T.C., Rwambo, P.M., DeMartini, J.C. & Crawford, T.B. (2010). Protection of sheep against Rift Valley fever virus and sheep poxvirus with a recombinant capripoxvirus vaccine. *Clin Vaccine Immunol,* 17(12), pp. 1842-9.

Souza-Neto, J.A., Sim, S. & Dimopoulos, G. (2009). An evolutionary conserved function of the JAK-STAT pathway in anti-dengue defense. *Procs Natl Acad Sci U S A,* 106(42), pp. 17841-17846.

Suzich, J.A. & Collett, M.S. (1988). Rift Valley fever virus M segment: cell-free transcription and translation of virus-complementary RNA. *Virology,* 164(2), pp. 478-86.

Swanepoel, R. & Coetzer, J. (2004). Rift Valley fever, . In: Coetzer, J.A.W. & Tustin, R.C. (eds). (*Infectious diseases of livestock*2). Cape Town: Oxford University Press, pp. 1037-1070.

Turell, M.J., Presley, S.M., Gad, A.M., Cope, S.E., Dohm, D.J., Morrill, J.C. & Arthur, R.R. (1996). Vector competence of Egyptian mosquitoes for Rift Valley fever virus. *Am J Trop Med Hyg,* 54(2), pp. 136-9.

Valadão, F.G. (1969). Previous note on the occurrence of a new virus disease in Moçambique- The Rift Valley fever. *Vetrin Moçamb Lourenço Marques,* 2(1), pp. 13-20.

Vene, S., Franzen, C. & Niklasson, B. (1994). Development of specific antibody patterns and clinical symptoms following Ockelbo virus infection. *Arch Virol,* 134(1-2), pp. 61-71.

Wallace, D.B., Ellis, C.E., Espach, A., Smith, S.J., Greyling, R.R. & Viljoen, G.J. (2006). Protective immune responses induced by different recombinant vaccine regimes to Rift Valley fever. *Vaccine,* 24(49-50), pp. 7181-9.

Walter, C.T. & Barr, J.N. (2011). Recent advances in the molecular and cellular biology of bunyaviruses. *J Gen Virol,* 92(Pt 11), pp. 2467-84.

WHO (2007a). Rift Valley fever in Kenya, Somalia and the United Republic of Tanzania. *Global Alert Response.* [online]. Available from: www.who.int/csr/don/2007_05_09/en/ [Accessed 2017.09.11].

WHO (2007b). Rift Valley fever, United Republic of Tanzania. *Wkly Epidemiol Rec,* 82, pp. 117-118.

WHO (2008). Rift Valley fever in Madagascar. *Wkly Epidemiol Rec,* 82(18), p. 157.

WHO (2010). Rift Valley fever. World Health Organization. [online]. Available from: http://www.who.int/mediacentre/factsheets/fs207/en/, [Accessed 2017.09.11].

WHO (2012). Rift Valley fever, Mauritania. *Wkly Epidemiol Rec*, 87 (45), p. 438.

WHO (2016). Rift Valley fever in Niger, 2016. [online]. Available from: http://www.who.int/csr/don/24-november-2016-rift-valley-fever-niger/en/, [Accessed 2017.09.04].

WHO & FAO (2009). Joint FAO-WHO experts' consultation on Rift Valley fever outbreak forecasting models, 2008. *Global Alert Response*. Rome, Italy: World Health Organization, pp. 1-22.

Williams, R., Ellis, C.E., Smith, S.J., Potgieter, C.A., Wallace, D., Mareledwane, V.E. & Majiwa, P.A. (2011). Validation of an IgM antibody capture ELISA based on a recombinant nucleoprotein for identification of domestic ruminants infected with Rift Valley fever virus. *J Virol Methods*, 177(2), pp. 140-6.

Wilson, W.C., Davis, A.S., Gaudreault, N.N., Faburay, B., Trujillo, J.D., Shivanna, V., Sunwoo, S.Y., Balogh, A., Endalew, A., Ma, W., Drolet, B.S., Ruder, M.G., Morozov, I., McVey, D.S. & Richt, J.A. (2016). Experimental Infection of Calves by Two Genetically-Distinct Strains of Rift Valley Fever Virus. *Viruses*, 8(5).

Won, S., Ikegami, T., Peters, C.J. & Makino, S. (2007). NSm protein of Rift Valley fever virus suppresses virus-induced apoptosis. *J Virol*, 81(24), pp. 13335-45.

Zamoto-Niikura, A., Terasaki, K., Ikegami, T., Peters, C.J. & Makino, S. (2009). Rift valley fever virus L protein forms a biologically active oligomer. *J Virol*, 83(24), pp. 12779-89.