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EVALUATION OF THE RIFT VALLEY FEVER VACCINATION PROGRAMME IN MOZAMBICAN CATTLE

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...all our science, measured against reality, is primitive and child-like and yet it is the most precious thing we have.

Albert Einstein

IN MEMORIAM
to Igor Roberto,

who was given a very short mission on Earth and was taken away by a mysterious illness. Rest in peace!

ABSTRACT

Rift Valley fever (RVF) is a viral disease that is spread by various arthropods (primarily mosquitoes) and affects ruminants and humans. RVF has led to tremendous losses of livestock in many African countries, Saudi Arabia, and Yemen, and its zoonotic impact on human deaths has been documented in most of the endemic countries where large outbreaks have occurred.

The RVF virus (RVFV) is composed of three single-stranded RNA gene segments (designated S, M, and L) with negative polarity, and it is transmitted mainly by mosquitoes of the genera *Aedes* and *Culex*, and various biting flies.

Outbreaks are associated with heavy rainfall and expansion of vegetation, both of which favour increases in mosquito population and thus lead to a high risk of infection in livestock and humans.

In Africa, control of RVF is based on immunization with the formalin-inactivated vaccine or the Smithburn attenuated vaccine, the former of which has been administered to cattle in Mozambique since 2002.

In the first part of the present research project, we evaluated the effect of transportation and storage conditions on the efficacy of the formalin-inactivated vaccine in cattle: in Maputo Province, three groups were immunized with vaccine stored at 4 °C (group A), at 25 °C (group B), and at temperatures alternating between 4 and 25 °C (group C), respectively; in Zambezia Province, animals were vaccinated as stipulated by the Directorate of the National Veterinary Services (group D).

Antibodies against RVFV were monitored by indirect enzyme-linked immunosorbent assay (ELISA), immunofluorescence assay (IFA), and the plaque reduction neutralization test (PRNT).

Pre-vaccination screening of cattle for neutralizing antibodies showed seropositivity in 17% and 7% in Maputo and Zambezia Provinces, respectively, and those animals were excluded from the study.

After initial inoculation with the RVFV vaccine, neutralizing antibodies were detected in more than 74% of the cattle in all groups, and levels of those antibodies were even higher after booster immunization. ELISA detected a response to anti-RVFV N protein antibody in about one third of the cattle in all groups after primary vaccination, and almost 80% of the animals were seropositive after booster immunization. Also, after both primary and booster

vaccinations, the anti-RVFV N protein antibody titres were higher in group D compared to groups A, B, and C.

These results demonstrate that the current storage and transportation conditions in Mozambique have no influence on the efficacy of the formalin-inactivated RVFV vaccine given to cattle.

The second stage of the research focused on a cross-sectional study aimed at evaluating the circulation of RVFV, by detection of neutralizing antibodies by PRNT in 404 cattle serum samples collected from different herds in six districts in Maputo Province, during 2010-2011.

The PRNT results revealed that 36.9% (95% CI 32.2%–41.6%; n=149), of cattle sera had RVFV neutralizing antibodies, which is high for an area where RVFV disease has not been reported for several decades. These findings suggest that RVFV is actively circulating among the cattle in the six districts.

LIST OF PAPERS

I. Lagerqvist N.[1], **Moiane B.[1],** Bucht G., Neves L., Fafetine J., Paweska J.T., Lundkvist Å., Falk K.I. (2012). Stability of a formalin-inactivated Rift Valley fever vaccine: Evaluation of a vaccination campaign for cattle in Mozambique. *Vaccine*, **30:** 6534-6540.*

[1]These authors contributed equally to this work.

II. Lagerqvist N, **Moiane B,** Mapaco L, Fafetine J, Vene S, Falk KI (2013). Antibodies against Rift Valley Fever virus in cattle, Mozambique. [letter]. *Emerg Infect Dis*., **19:**.1177-1179.*

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CONTENTS

LIST OF ABBREVIATIONS

CDC	Centers for Disease Control and Prevention (Atlanta, GA, USA)
CDW	Communicable Diseases Watch
ELISA	enzyme-linked immunosorbent assay
FAO	Food and Agricultural Organization of the United Nations
IFA	immunofluorescence assay
MLVV	modified live virus vaccine
MP-12	12th passage mutant vaccine
NDBR 103	National Drug Biological Research 103 vaccine
NSm	medium-sized non-structural protein
NSs	small non-structural protein
PRNT	plaque reduction neutralization test
RT-PCR	reverse transcriptase polymerase chain reaction
RVF	Rift Valley fever
RVFV	Rift Valley fever virus

1 INTRODUCTION

Rift Valley Fever (RVF) is a viral zoonotic disease that is spread by arthropods (primarily mosquitoes) and affects livestock (sheep, goats, cattle, buffaloes, and camels) and humans in Africa (Meegan, 1979; Ksiasek *et al*., 1989; Morvan *et al*., 1992) and the Arabian Peninsula (Meegan *et al*., 1979; Balhky & Memish, 2003; Davies & Martin, 2006).

RVF is responsible for significant economic losses in livestock production (Cagnotali *et al*., 2006), because it is associated with a high mortality rate, chiefly in young animals, and it also induces abortions among gestating females (Davies, 1990).

The incubation period for the RVF virus (RVFV) 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 RVFV is seen in young animals, and hence symptoms are severe in this group. Sudden onset of fever (up to 41 °C), 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. The severe form of RVF is characterized by fever, anorexia, and lymphadenopathy followed by weakness, and death usually occurs in about 15% of affected livestock.

1.1 RIFT VALLEY FEVER EPIDEMICS

1.1.1 Animals

RVFV was first identified by Daubney and Hudson in1931 based on observations made in the field and 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.

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 (Valadão, 1969).

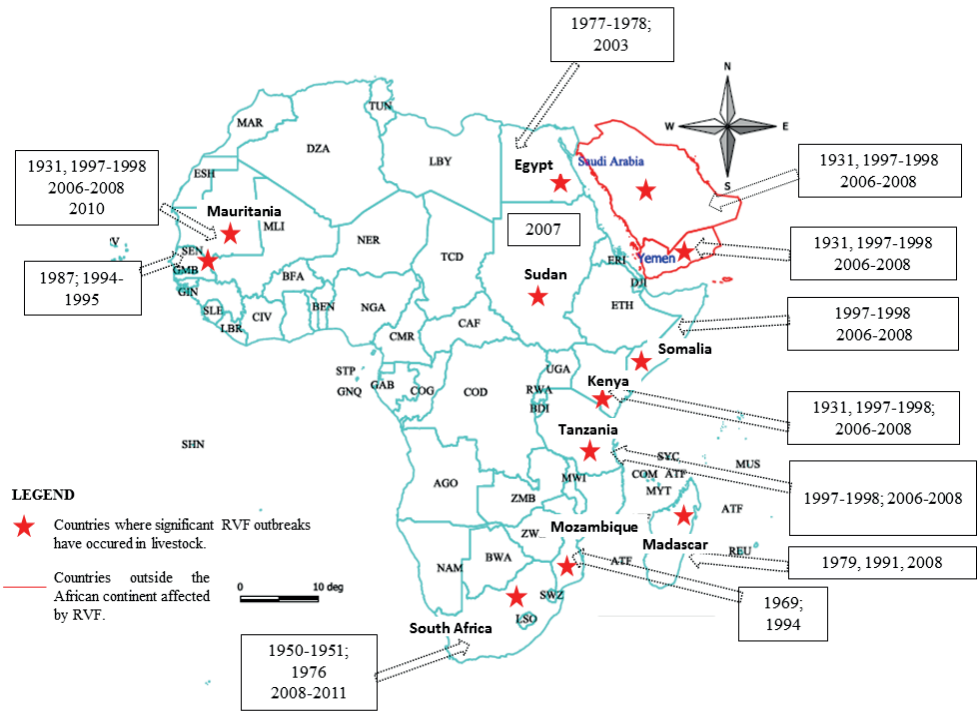


Figure 1: Map of Africa, Saudi Arabia, and Yemen showing where large outbreaks of RVF have been documented. The map was drawn using the Manifold program (Manifold System 8.0. http://www.manifold.net/personaledition:8.0.26.0.211) and the database was obtained from Geo Community (http://data.geocomm.com/catalog/SF/datalist.html.2011).

1.1.2 Humans

Humans can contract RVFV 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 with the virus due to their close contact with infected animals.

Table 1 summarizes aspects of significant epidemics involving humans in Africa and the Arabian Peninsula, and it is important to mention that the majority of these outbreaks led to human deaths, which highlights the zoonotic relevance of RVFV.

Table 1: **RVF outbreaks in humans in African and Arabian Peninsula countries**

Year	Country	No. of cases	No. of deaths	Reference
1977–1978	Egypt	18,000	598	Meegan (1979)
1987	Mauritania	Unknown	> 200	Kane (2000)
1991	Madagascar	Unknown	1	Morvan *et al*. (1992)
1997–1998	Kenya, Tanzania, and Somalia	100,000	> 450	Woods *et al.* (2002)
1998	Kenya	27,500	Unknown	CDC (1998)
2000–2001	Saudi Arabia and Yemen	882	124	Balkhy & Memish (2003)
2003	Mauritania	25	9	Faye *et al*. (2007)
2006–2007	Kenya, Somalia, and Tanzania	1,000	300	CDC (2007), Breiman *et al.* (2008).
2007–2008	Tanzania Kenya	684 264	155 109	WHO (2007) WHO, 2008
2007	Sudan	747	230	Hassan *et al*. (2011)
2008	Madagascar	418	17	WHO (2008)
2010	South Africa	186	18	Fung & Ma (2010)
2010	Mauritania	63	13	El Mamy *et al.* (2011)

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 RVFV-containing aerosols produced during the slaughter of infected animals, inappropriate handling of laboratory samples, or care of patients with RVF (Murphy *et al*., 1999; Francis & Magill, 1935).

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

Vertical transmission of RVFV has been documented in Saudi Arabia, where IgM antibodies against this virus were detected in samples from a newborn 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). This finding suggests that RVFV has the ability to infect the foetus during pregnancy and cause severe lesions that can compromise survival after birth.
Notably, anti-RVFV antibodies have been detected in 2% (28/1163) of pregnant women in Mozambique (Niklasson *et al*, 1987), although no outbreak in humans has ever been reported in that country.

1.2 THE RVF VIRUS PARTICLE

RVFV is a single-stranded RNA virus of the genus *Phlebovirus*, which is one of the five genera of the family *Bunyaviridae* (Shope *et al*., 1980; Suzich & Collett, 1980).
In short, RVFV 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 (G1/G2); and the S segment codes for the nucleocapsid (N) protein and a small non-structural protein (NSs) (Murray *et al*., 2009; Suzich & Collet, 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).

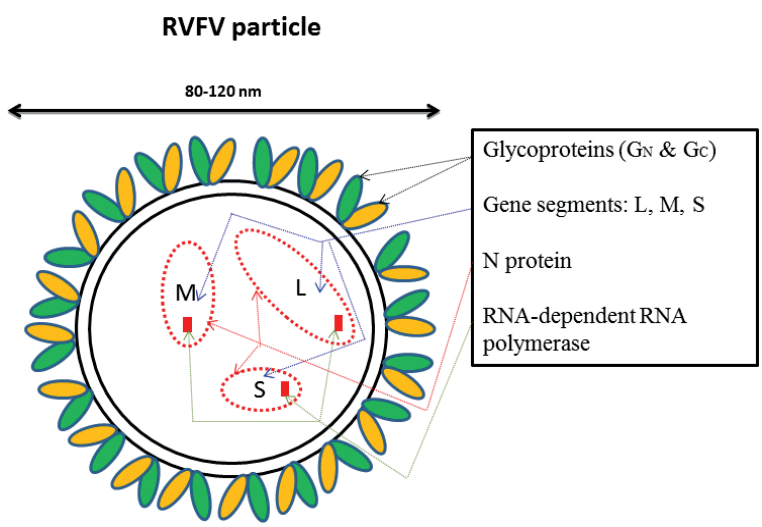


Figure 2. Schematic representation of the structure of the Rift Valley fever virus (RVFV) showing the three genetic segments designated small (S), medium (M), and large (L). The yellow and green structures on the surface of the virus particle are the glycoproteins G_N and G_c. The red dots surrounding 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 RVFV and their roles

Protein	Molecular weight	Genome segment	Role	Reference
Viral RNA polymerase	244	L	Viral replication and RNA transcription.	(Zammoto-Nikura *et al.*, 2009).
G_N/G_C	5,955	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.	(Garry & Garry, 2004; Filone *et al*., 2006).
NSm (NSm 1/NSm2)	7,814	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.	(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.	(Billecocq, 2004; Burton, 2004; Bird *et al*., 2008; Mansuroghu *et al.*, 2010).

1.3 THE VECTORS AND THEIR HABITATS

Mosquitoes of the genera *Aedes* and *Culex* are the main vectors of RVFV (Gad *et al*., 1987; Fontenille *et al*., 2008), however other biting flies (e.g., *Anopheles* spp.) and sandflies can also be involved in the amplification of RVF epidemics (Linthicum, 2009; McIntosh, 1980; Seufi & Galal, 2010).

The main breeding sites for the mentioned vectors 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 RVFV infection in livestock is low during that period (the enzootic cycle). In the rainy season, the eggs hatch into adult RVFV-infected mosquitoes that can transmit the virus to livestock (Logan *et al.,* 1991, Davies *et al.,* 1992).

Twenty days after the onset of flooding, the *Aedes* population decreases and is replaced by a secondary RVFV vector (members of Culicinae, *Anopheles* spp., and various haematophagous biting flies such as phlebotomines). The increase in the population of secondary vectors

amplifies the RVFV transmission cycle, and therefore large numbers of livestock and humans are infected thus causing an epidemic (Figure 3).

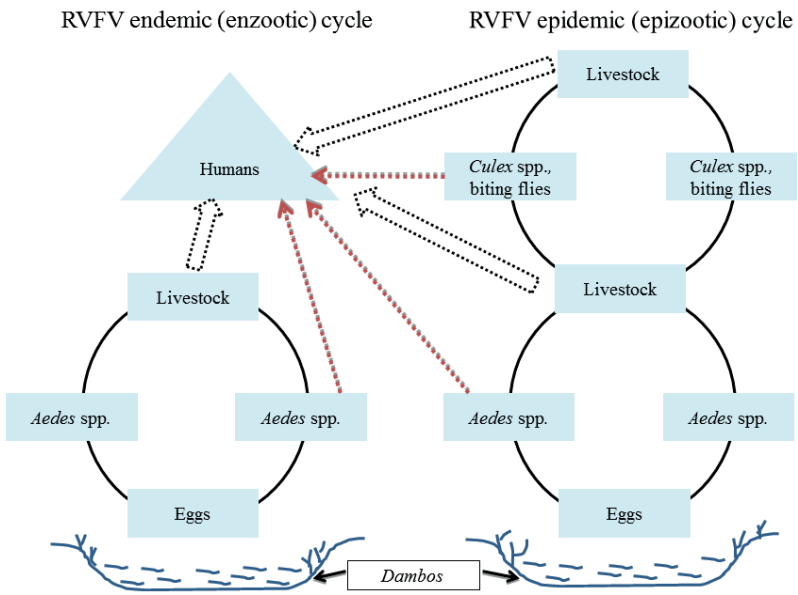


Figure 3: Schematic representation of the endemic and epizootic cycles of RVFV showing the role of *Aedes* spp. and other vectors, as well as the impact of climatic and environmental factors on the occurrence of either of the two cycles. The black dot arrows show the interaction between livestock and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

There are several factors that have a substantial impact on the RVFV 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 climate change caused by the *El Ninõ* phenomenon, which has led to desertification, global warming, and floods. Furthermore, agricultural practices, including the construction of dams, have had significant influence on the spread of RVFV vectors across the African continent and the Arabian Peninsula (Linthicum *et al*., 1999; Anyamba *et al*., 2001; FAO & WHO, 2009).

1.4 CLINICAL SIGNS

1.4.1 Animals

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

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

1.4.2 Humans

In humans, the RVFV 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, anorexia, neck stiffness, and vomiting (Al-Hazmi *et al*., 2003; Davies & Martin, 2006).

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 (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), 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 RVFV 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 THE DISEASE: PREVENTION AND CONTROL STRATEGIES

1.5.1 Animals

Inoculation of livestock with the Smithburn live attenuated vaccine or a formalin-inactivated RVFV vaccine prior to the rainy season has been implemented on the African continent. 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-RVFV IgG and anti-RVFV 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 RVFV outbreaks, but unfortunately those tools are currently only available in a small number of countries in Africa.

RVFV 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 RVFV 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 RVFV vaccine is the main control strategy applied to control RVFV infections in cattle in Mozambique.

1.5.2 Livestock vaccination in Africa

1.5.2.1 RVF virus live attenuated vaccines

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

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

1.5.2.2 RVF virus 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 RVFV 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 & Makino, 2009).

1.5.2.3 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 (Kortekaas *et al*., 2010; Mandell *et al.,* 2010; Wallace *et al*., 2006), attenuated RVFV strains that show less severe side effects when compared to those caused by the Smithburn strain (Dungu *et al*., 2010; Morrill *et al*, 1997a; Morril *et al*., 1997b), and DNA vaccines (Wallace *et al*., 2006; Bhardwaj *et al.*, 2010; Lorenzo *et al.*, 2010).
All these vaccines have been shown to induce a protective immune response in laboratory animals (Boshra *et al*., 2011; Soi *et al.,* 2010; Ikegami & Makino, 2009; Takehara *et al*., 1989).
Three candidates, namely the NDV-GnGc vaccine (Newcastle disease virus-based vector that produces structural glycoproteins Gn and Gc), NSR vaccine (non-spreading RVFV) 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 RVFV 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 RVFV possess identical neutralizing epitopes. Considering that Bunyaviruses 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 RVFV strains.

1.5.3 Vaccination in humans

As of yet, no vaccine has been licensed for broad use in immunization of humans against RVFV. 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.

1.6 THE RVF VACCINATION PROGRAM IN MOZAMBIQUE

The former National Directorate of Livestock in Mozambique introduced a programme using a commercial formalin-inactivated RVFV in Zambezia Province in 2002 in response to a survey of RVFV antibodies conducted in 1996, which showed that 37% (152/412) of the cattle in the region were seropositive (Direcção Nacional de Pecuária, 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-RVFV IgG antibodies in 1999 and 2001 (Direcção Nacional de Pecuária, 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 (Direcção Nacional de Pecuária, 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 RVFV 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.

1.7 RVF DIAGNOSIS

1.7.1 Animals

In the field, RVF can be suspected when heavy rainfalls are followed by a sudden rise in the mortality rate among newborn 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.

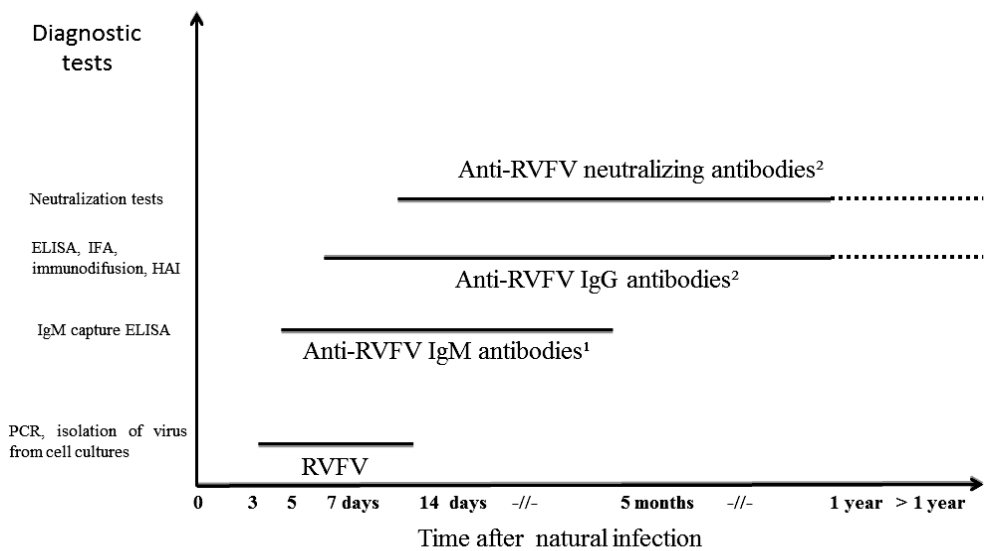
In the laboratory, RVFV can be isolated from blood or serum collected during early stages of disease, or from liver, brain, and spleen collected *post-mortem* and the organs of aborted foetuses. Reverse transcriptase polymerase chain reaction (RT-PCR) is used for rapid detection of RVFV in blood from day 1 to day 5 post-infection (Ibraim *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 RVFV and the time to detection of RVFV antigens and immunoglobulins M and G in infected animals.

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



¹Morvan *et al*. (1992); ²S´wanepoel & Coetzer (2004)

1.7.2 Humans

In humans, RVFV 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 RVFV neutralizing antibodies.

2 THE PRESENT PROJECT

The first study (Paper I) was conducted to assess the immunity of cattle after vaccination with a formalin inactivated RVFV vaccine in Mozambique and to evaluate the stability the formalin-inactivated vaccine after storage under different conditions. Section 2.2 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.

The second investigation (Paper II) aimed at determining the prevalence of RVF antibodies in unvaccinated cattle herds in Maputo Province in southern Mozambique.

The study area (Figure 6) and the sampling for serological survey of RVF antibodies in cattle herds in Maputo Province are discussed in section 2.3.

2.1 AIMS

The objectives of the current research were as follows:

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

To estimate the prevalence of RVF antibodies in cattle herds in Maputo Province.

2.2 PAPER I: STABILITY OF A FORMALIN-INACTIVATED RIFT VALLEY FEVER VACCINE: EVALUATION OF A VACCINATION CAMPAIGN FOR CATTLE IN MOZAMBIQUE

In this study, a formalin-inactivated RVFV 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 5). 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 5: 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 5), 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.

2.3 PAPER II: ANTIBODIES AGAINST RIFT VALLEY FEVER VIRUS IN CATTLE, MOZAMBIQUE

Study area, animals and sampling

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). Three hundred and sixty four 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 6) 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	2010	CVL* Maputo
Magude	34		
Manhiça	65		
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 RVFV 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.

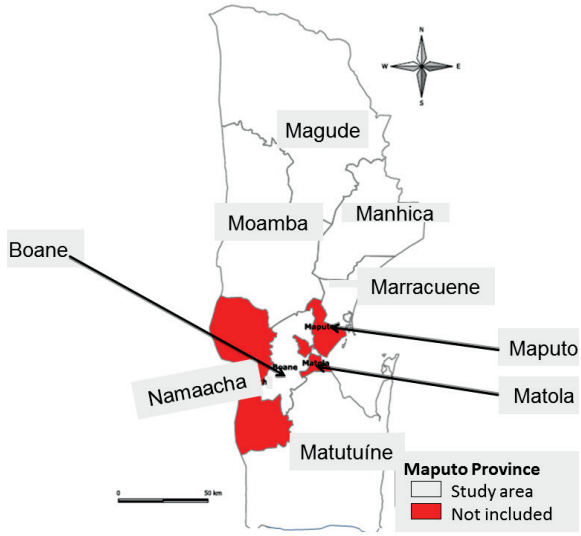


Figure 6: Map of Maputo Province showing the districts studied.

Blood samples from the caudal vein of each animal were collected in 5-ml vacuum tubes without any additive. Upon arrival at the laboratory, 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 subjected to heat inactivation in a Biosafety Level 3 (BSL3) containment facility before being analysed.

The samples were tested by PRNT as described previously by Lagerqvist *et al*. (2012). RVFV seropositivity was defined as 80% reduction of virus infectivity at a serum dilution of 1:40.

3 RESULTS AND DISCUSSION

This section provides a brief summary of the results reported in Papers I and II, and a discussion of the key findings of both studies.

3.1 STABILITY OF A FORMALIN-INACTIVATED RIFT VALLEY FEVER VACCINE: EVALUATION OF A VACCINATION CAMPAIGN FOR CATTLE IN MOZAMBIQUE (PAPER I)

In this study, antibodies against RVFV 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, suggesting that RVFV is circulating among cattle in those areas. The investigation was performed in locations where cattle herds had not previously been surveyed to detect RVFV antibodies or vaccinated.

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

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

In the cattle in group D, a peak in anti-RVFV 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 Paper I). After day 267 post-vaccination (i.e., 630 days after primary vaccination), 60% of the animals in groups A, B, and C still had detectable neutralizing antibodies. The anti-RVFV 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 Paper I), 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 RVFV vaccine in cattle.

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) did 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 RVFV vaccine, if the vaccine is distributed and administered to cattle within one week of dispatch from refrigerated storage.

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

In the present investigation, neutralizing antibodies against formalin-inactivated RVFV 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 doses.

3.2 ANTIBODIES AGAINST RIFT VALLEY FEVER VIRUS IN CATTLE, MOZAMBIQUE (PAPER II)

Four hundred and four cattle serum samples were evaluated by PRNT, and 36,9% (149/404) were found to be positive for RVFV-neutralizing antibodies. The seropositivity was observed in all of the herds in the six districts under consideration.

The districts with highest seroprevalence were Manhiça, 61,5% (95% CI 47,7% - 73,4%) and Marracuene, 62,2% (95% CI 51,7% - 72,7%), suggesting that RVF virus activity is high in these areas. The existence of areas with ponds, flood plains, and wetlands in combination with agricultural practices (e.g., building irrigation ditches for sugar cane companies) is associated with the spread of RVFV in the North-Eastern districts of Maputo (including Manhiça and Marracuene). The reason for this is that such factors enhance the breeding of the vectors of the virus (i.e., mosquitoes) in areas where livestock are raised, which is believed to increase the risk of infection.

The seropositivity rate in the Southern District of Matutuine was 19,8% (95% CI 13,0% - 26,7%), the Northern and Western Districts of Magude and Moamba had seroprevalences of 26,5% (95% CI 11,6% - 41,3%) and 29,7% (95% CI 18,5% - 40,9%), respectively. These disctricts share borders with South Africa (Kwazulu Natal, Mpumalanga and Limpopo, Provinces) which had been consecutively recording RVF outbreaks between 2008 and 2011 (OIE, 2009; OIE, 2010; OIE 2011a; OIE, 2011b; Métras *et al*., 2012).

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 (Valadão, 1969), which

seem to have expanded from Gaza province on the north into east-central districts of Maputo province, namely Manhiça, Marracuene.

Boane District which has the smallest cattle population in Maputo had high seroprevalence, 14.3% (95% CI 1,3% - 27,2%). RVFV seems to have spread into this district either from Namaacha or Matutuine, and it may be related to the trade of cattle into Boane, but it could also be linked to the movement of vectors among those districts which are very close to one another.

In Maputo Province, immunization against RVFV in cattle was performed only in the Namaacha District, and the purpose of this action was to evaluate the stability of the formalin-inactivated RVFV vaccine for the previous study (Lagerqvist *et al*., 2012). It is plausible that the National Veterinary Services extend the immunization programme to all districts in Maputo Province, in order to reduce the probability of an outbreak.

Moreover, surveillance programmes should (whenever possible) include the use of GIS tools to map the risk areas and the vectors, and to monitor the livestock raised within those areas (Soumare *et al*., 2007).

4 CONCLUDING REMARKS AND PERSPECTIVES

Paper I

RVFV is endemic in Zambezia and Maputo Provinces, which are located in the central and the southern region of Mozambique, respectively.

Variation in temperature had no impact on the formalin-inactivated RVFV vaccine administered to cattle in these two provinces

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.

Paper II

Vaccination against RVFV in cattle in Maputo Province should be considered.

It is not yet known what factors determine exposure of cattle to infection with RVFV in this province, and hence it is essential to initiate studies aimed at identifying and mapping the vector species responsible for transmission of this virus as soon as possible.

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