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RIFT VALLEY FEVER VIRUS VACCINE STRATEGIES

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ABSTRACT

Rift Valley fever virus circulates throughout Africa and the Arabian Peninsula and is of great concern for animal and public health. Infections in humans are often manifested as mild self-limiting illness, although in some cases there are more severe symptoms such as neurological complications and hemorrhagic fever. Spontaneous abortions among livestock are a hallmark for Rift Valley fever virus outbreaks and disease in small ruminants often has a deadly outcome. At present, there is no vaccine available for use in humans and the ones used in livestock are either poorly immunogenic or cause severe adverse effects. The economic impact of this pathogen in the form of livestock losses and restrictions on the trade of animals and animal products as well as its significance in relation to public health underscores the importance of developing safe and effective vaccines. The main focus of this thesis was to evaluate existing vaccines and novel vaccine candidates, with special emphasis on vaccine platforms practical in resource-poor areas.

It is difficult to maintain a cold-chain during transit in Mozambique and the inactivated Rift Valley fever virus vaccine is transported more than 2000 km within the country before it is administered to livestock in Zambezia Province. For that reason, the vaccine was evaluated for its ability to induce antibodies in cattle after storage at ambient temperatures. Importantly, the storage and transport conditions used in Mozambique did not have an adverse effect on the antibody responses induced by the vaccine. When performing the aforementioned study, we found evidence of previous Rift Valley fever virus infections in livestock in Maputo Province, a region where there had been no recorded evidence of the virus since 1969. A cross-sectional seroprevalence study was undertaken to examine the need to implement a vaccination program in this particular province. Unexpectedly, seroconversion was observed in 37% of the investigated cattle, suggesting that this pathogen is widely distributed throughout Maputo Province.

Rift Valley fever virus is highly pathogenic and to circumvent the handling of replicating virus during the vaccine manufacturing process would be advantageous. Other highly desirable vaccine-characteristics are low production costs, high immunogenicity to reduce the number of doses, and a non-invasive delivery route to avoid the challenge of maintaining sterility of hypodermic equipment. To fulfill some of those requirements we developed and evaluated three different vaccine strategies *i)* DNA vaccines, *ii)* vaccine based on virus-like particles, and *iii)* plant-derived protein subunit vaccines. All candidates induced vaccine-specific antibody responses in mice and the DNA- and virus-like particle-based vaccines conferred protection against Rift Valley fever disease.

Here, we raise the question of extending the vaccination program in Mozambique to include Maputo Province. We show that the inactivated virus vaccine is well-suited for that purpose until more effective alternatives are available. In the search for such an alternative, we evaluated three vaccine candidates. One of those candidates, vaccine based on virus-like particles, was found to have good prospects as a future Rift Valley fever virus vaccine.

LIST OF PAPERS

I. **Lagerqvist N***, Moiane B*, Bucht G, Fafetine J, Paweska J.T., Lundkvist Å and Falk K.I. 2012. Stability of a formalin-inactivated Rift Valley fever vaccine: evaluation of a vaccination campaign for cattle in Mozambique. *Vaccine 30(46):6534-40.*

II. **Lagerqvist N**, Moiane B, Mapaco L, Fafetine J, Vene S and Falk K.I. High prevalence of antibodies neutralizing the Rift Valley fever virus in cattle in Maputo Province, Mozambique. *Accepted for publication in Emerging Infectious Diseases.*

III. **Lagerqvist N**, Näslund J, Lundkvist Å, Bouloy M, Ahlm C and Bucht G. 2009. Characterisation of immune responses and protective efficacy in mice after immunisation with Rift Valley fever virus cDNA constructs. *Virol. J. 17(6):6.*

IV. Näslund J*, **Lagerqvist N***, Habjan M, Lundkvist Å, Evander M, Ahlm C, Weber F and Bucht G. 2009. Vaccination with virus-like particles protects mice from lethal infection of Rift Valley fever virus. *Virology 385(2):409-15.*

V. **Lagerqvist N**, Kalbina I, Moiane B, Ahlm C, Strid Å, Andersson S and Falk K.I. Mice fed transgenic *Arabidopsis thaliana* expressing Rift Valley fever virus antigens exhibit systemic immune responses. *Manuscript.*

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CONTENTS

List of abbreviations

APC	Antigen presenting cell
BSL	Biosafety level
cRNA	Complementary RNA
CTLs	Cytotoxic T lymphocytes
DCs	Dendritic cells
DIVA	Differentiating infected from vaccinated animals
ELISAs	Enzyme-linked immunosorbent assays
Gc	C-terminal glycoprotein
Gn	N-terminal glycoprotein
Ig	Immunoglobulin
INF	Interferon
iVLP	Infectious virus-like particles
L	Large
LSDV	Lumpy skin disease virus
M	Medium
MHC	Major histocompatibility complex
mRNA	Messenger RNA
N	Nucleocapsid
NCRs	Non-coding regions
NDV	Newcastle disease virus
NSm	Nonstructural protein, M segment
NSm1	Nonstructural protein 1, M segment
NSm2	Nonstructural protein 2, M segment
NSs	Nonstructural protein, S segment
PAMPs	Pathogen associated molecular patterns
PRNT	Plaque reduction neutralization test
PRRs	Pattern recognition receptor
RNP	Ribonucleoprotein
RT-PCR	Reverse transcriptase polymerase-chain reaction
RVF	Rift Valley fever
RVFV	Rift Valley fever virus
S	Small
SPV	Sheep pox virus
Th	T helper
VLPs	Virus-like particles
VNT	Virus neutralization test
VRPs	Viral replicon particles
vRNA	Viral RNA

1 AIMS

The aims of this thesis were to evaluate existing and novel Rift Valley fever virus vaccines with the specific objectives being:

I. To evaluate if different storage and transport conditions affect the ability of formalin-inactivated vaccine to induce immune responses in cattle.

II. To perform a seroprevalence study in livestock with the aim of investigating the need for a vaccination campaign in Maputo Province in Mozambique.

III. To construct DNA vaccines based on the viral nucleocapsid protein and the glycoproteins and to evaluate the efficacy of those vaccine candidates in mice.

IV. To evaluate if vaccination with virus-like particles confers protection in mice.

V. To produce protein subunit vaccines in *Arabidopsis thaliana* and evaluate if these proteins are immunogenic when given orally to mice.

2 INTRODUCTION

The *Bunyaviridae* family of viruses currently comprises about 100 species [1]. They are assigned, based on their serological relationships supported by biochemical analyses, to one of the five genera in the family [2]. The *Tospovirus* genus solely includes viruses that infect plants, while members of the *Hantavirus*, *Nairovirus*, *Orthobunyavirus*, and *Phlebovirus* genera infect animals and some of them also cause severe and sometimes fatal disease in man *e.g.* Crimean-Congo hemorrhagic fever virus, Rift Valley fever virus (RVFV), and Sin Nombre virus [3-5]. With the exception of hantaviruses, which are transmitted by persistently infected rodents or insectivores [6], members of the *Bunyaviridae* family are arboviruses that rely on arthropod vectors such as mosquitoes, ticks, and sandflies for their transmission [7]. The *Bunyaviridae* family is an ecologically diverse group of viruses and as of today, members of this family have been found on all inhabited continents [8].

2.1 EPIDEMIOLOGY

RVFV was first isolated in 1930 on a farm in Rift Valley in Kenya, where a disease caused high mortality in lambs and abortions in pregnant ewes [9]. In the late 1950s, an acute and fatal disease in cattle and sheep was observed in southern Africa. In South Africa, RVFV caused and the death of estimated 100,000 sheep and 500,000 abortions in an area predominated by sheep farming [10]. The disease was not previously known by veterinarians working in the affected area and the lack of pre-existing immunity among farmers suggested a recent introduction [11]. Short thereafter, RVFV emerged to the neighboring countries Mozambique and Namibia [12, 13] and Zambia and Zimbabwe [14, 15].

In 1979, RVFV was again isolated from a new area. Previous outbreaks had been restricted to the African mainland, however, Madagascar now reported its first isolate from mosquitoes, but in the absences of severe disease in animals and humans [16, 17]. Years later, characteristic RVFV outbreaks with abortions in cattle and disease in man were recorded in Madagascar [18-21]. RVFV was confined to countries in Sub-Saharan Africa until the late 1970s, when the virus emerged into northern Africa. The outbreak in Egypt in 1977-1978, with an estimated 200,000 human cases (18,000 clinical cases were officially reported [22]), is to date one of the largest RVFV epidemics ever recorded.

Prior to the outbreak in Egypt, RVFV was mainly considered a veterinary concern [23]. The outbreak in Egypt and later outbreaks in Tanzania, Somalia and Kenya in 1997-1998 [24], when the number of human infections was estimated to 27,500 in the Garissa district in Kenya alone [25], established RVFV as a severe human pathogen. The geographic range of RVFV has expanded substantially during the past century and the first appearance outside Africa was evident in the turn of the twenty-first century. Hundreds of human cases were recorded and thousands of animals were affected in southwest Saudi Arabia and in the adjacent regions of Yemen [26, 27]. It has been speculated that the virus was introduced by imported livestock or infected mosquitoes [28]. This is supported by sequence similarities between isolates from the Arabian Peninsula and virus isolated during the outbreak in eastern Africa in 1997-1998 [29, 30]. The geographic distribution of RVFV is outlined in Figure 1.

Figure 1. *Countries affected by Rift Valley fever virus. Modified from* [31].

2.2 IMPACT ON ECONOMY

Despite attempts to minimize the impact of RVFV through livestock vaccinations, surveillance, and vector control measurements, recent outbreaks in Sudan and Kenya caused an estimated 75,000 and 180,000 infected individuals, respectively [32, 33]. These figures clearly illustrate the direct impact on the public health. RVFV also affects the socio-economical situation because of labor layoffs, livestock mortality, and trade restrictions in regions already highly affected by poverty [34]. To illustrate the economical consequences that arise in this context it can be mentioned that the 16 months trade ban on livestock imposed by Saudi Arabia during the outbreak in Somalia in 1997-1998 resulted in losses estimated to US$100 million [35] and in Sudan, where livestock and livestock products are the second most important export after oil, the outbreak in 2007 was estimated to have huge economical impact especially in the rural areas where the majority of the animals are raised [32]. Other examples include the outbreak in Kenya in 2007 that resulted in total economical losses of US$32 million, whereof US$9.3 million was ascribed animal mortality [36]. During the same year, the economical losses in Tanzania were estimated to US$6 million [37].

2.3 TRANSMISSION

The emergence of RVFV is believed to be caused by a broad range of mosquito vectors combined with large numbers of susceptible animal hosts with a level of viremia sufficiently high to infect mosquitoes [23]. Epizootics are associated with periods of extensive rainfall [38]. The flooding of ground pools creates optimal breeding conditions for the mosquito vector. Nowadays, it is widely recognized that RVFV is maintained in arthropod vectors during inter-epizootic periods [39]. However, it is still unclear which, if any, animal reservoirs that are involved in the inter-epizootic maintenances. The role of birds was early excluded [40, 41], but other animal

reservoirs such as bats and rodents have been implicated [42, 43]. Davies *et al.* examined wild primates and found it unlikely that they are involved in the maintenance cycles [44]. A plausible setting is inter-epizootic cycling with low-level transmission in livestock and/or wild ruminants [45-47].

2.3.1 Vectors

RVFV has been isolated from a remarkable collection of vectors including mosquitoes, ticks, and a variety of flies [48-50]. Most studies have focused on the role of mosquitoes and it has been shown that mosquitoes of several genera including *Aedes, Culex, Mansonia,* and *Anopheles* can transmit the virus [42, 51-53]. Basically, the vectors of RVFV can be classified into two categories: maintenance and amplifying vectors [10, 54]. The maintenance vectors such as certain *Aedes* species [10] reside in temporary flooded ground pools [55]. RVFV has been shown to survive in eggs of *Aedes* mosquitoes during inter-epizootic periods [10]. The eggs require a period of dehydration and after heavy rainfall when their habitats are filled with water, they hatch and develop into virus-infected mosquitoes [54]. Once RVFV has been transmitted to, and replicated in, the host animal the amplifying vectors *e.g. Culex* mosquitoes become involved in the transmission [10]. When the rainfall stops the *Aedes* population decreases [29]. Since *Culex* mosquitoes breed in more permanent fresh-water bodies, their number remains high and they continue to spread the virus [29, 54, 56]. This scenario is based on observations from an outbreak in Saudi Arabia. In other endemic countries, outbreaks may occur independently of rainfall and transmission may involve other vectors [23].

2.3.2 Animal hosts

RVFV has a wide range of hosts, and animals usually become infected by bites from infected mosquitoes [57]. The contact with infected bodily fluids also is an important route of transmission during outbreaks [54]. Aborted fetal material and placenta contain high viral load [54] and nasal, ocular, and rectal viral shedding have been reported in infected sheep [58]. Domesticated ruminants such as sheep, cattle, and goat seem to be the predominant hosts and it was early established that the newborn lamb was particularly susceptible [9]. Pigs have shown to be resistant to experimental infection with a low virus dose [9], but higher doses caused viremia [59] and one pig reported to have aborted during an outbreak [11]. Although an experimentally infected horse failed to react [9], the virus has been isolated from horse, donkey, and camels during an outbreak [60]. Indirect evidence of past RVFV infections has been presented for several wildlife species including rhinoceros, gazelle, warthog, impala, and waterbuck [61, 62], but of several carnivores tested, lion was found to be the only species with RVFV-neutralizing antibodies [63].

Although the natural circulation of RVFV seems to include mainly ungulates, the virus can cause viremia in other animal species. Experimental infections of monkeys, rats, and mice have repeatedly shown that those species are highly susceptible to RVFV infection [64, 65] and today they are the most commonly used animal models [66]. Cats, dogs, and grey squirrel are moderately susceptible [40], whereas infection is subclinical in rabbit and guinea-pig [40]. As of today, the only animal species that have been shown to be resistant to experimental and natural RVFV infection are birds, reptiles, and amphibians [10, 40, 67].

2.3.3 Human

Humans become infected from contact with infected animal tissues or bites from infected mosquitoes [68-70]. Farmers, abattoir workers, and veterinarians are more prone to contract the disease [68, 71] and close proximity to ruminants and the area density of livestock are important factors for seroconversion in human [72]. A number of accidental laboratory infections have been described in the literature, most of which occurred during animal experiments, postmortem examinations, or virus preparations [40, 42, 68, 73-78]. In those situations, RVFV infection presumably was a result of contact of virus with wounds or abraded skin. However, some laboratory acquired infections cannot be explained by direct contact with virus-infected blood or tissue [42, 73, 77]. Circumstantial evidence combined with experimental aerosol and intranasal infections that successfully have been performed in animals suggest that RVFV can be transmitted by aerosol [74, 79-81]. Although RVFV has been shown to be present in the upper respiratory tract of infected individuals [74], no human-to-human transmission has been documented to date. After that adequate infection control precautions have been put in place, there have been no reports on accidental infections among health-care workers or laboratory personnel [82].

RVFV has been suggested to be transmitted via intake of unpasteurized or raw milk from infected animals [82]. A few epidemiological studies have shown that drinking raw milk increases the risk for human infection [83, 84], whereas other groups have reported inconclusive results [25, 85]. Analysis of milk products from infected animals provides conflicting data as well. While viral shedding in milk from infected cattle has been documented [86], attempts to infect offspring via suckling and animals through ingestion of virus have failed [9, 87].

Vertical transmission is frequently observed in large mammalians and has lead to the suspicion of mother-to-child transmission in humans. An attempt to relate the occurrence of abortions to RVFV infections during an outbreak failed [88] and retrospective studies did not reveal an increase in the number of stillbirths among seropositive women in Mozambique [89]. However, two cases of suspected vertical transmission have been reported. A fatal case of a 6 day old infant with high levels of RVFV immunoglobulin (Ig) M antibodies and a mother with clinical signs consistent with RVFV infection shortly before childbirth [90] and a neonate who was found to be positive for RVFV IgM [91].

3 RIFT VALLEY FEVER

The course of events that follow inoculation of RVFV into the host are believed to resemble those observed for other arthropod-borne viruses [28]. After the virus has gained access into the host's body it spreads from the initial site of replication by lymphatic drainage to regional lymph nodes. Spillover of the virus to the circulation results in viremia, which leads to systemic infection and infection of critical organs [10]. RVFV replicates in a variety of cells, but the hepatocytes of the liver seem to be the predominant target cell in infected newborn lambs [92]. Hepatic necrosis is the major finding in infected animals, however, lesions in spleen, lymph nodes, kidney, lung, and the digestive tract are also commonly observed [93]. The lesions in infected target organs are considered to be directly virus-induced [94]. Within this pattern, there are age- and species-related variations in clinical manifestations and disease outcome.

3.1 ANIMAL

A classical hallmark of RVFV epizootics is the massive numbers of nearly simultaneous abortions in gestating ruminants, a scenario often described as "abortion storms" [54]. Pregnant animals may abort at any stage of gestation seemingly as a result of the direct effect of RVFV on the genital organs [95]. The abortion rates are typically 40-100% in herds and flocks [96] and they may vary between different epizootics. For instance, in Kenya in 2006-2007 the abortion rate in gestating goats was estimated to be comparable to the rates observed in cattle and sheep [97], whereas goats appeared resistant in Egypt 1977-78 [98]. Morbidity and mortality are greatly influenced by the age of the animal and the animal species [9]. Newborn lambs and kids are extremely susceptible and show fever and anorexia followed by death shortly after infection (within 24-36 hours) in 70-100% of the cases [9, 96]. In contrast, disease in older lambs and kids and adults varies from inapparent to acute and is manifested by fever, weakness, and bloody diarrhea and is less frequently associated with a deadly outcome [96, 99, 100]. In calves the course of illness resembles that of lambs and kids, but disease in adult cattle is most of often subclinical and less than 10% of the animals succumb to infection [96]. Spontaneous abortions and subclinical to severe disease and death have also been observed in camels [98, 101].

3.2 HUMAN

In the vast majority of human cases, RVFV infection is asymptomatic or displayed as mild self-limiting illness manifested by fever, headache, and malaise without any severe sequelae [33]. Severe manifestations *e.g.* meningoencephalitis, vision loss, or hemorrhagic fever are observed less frequently and can give permanent complications or even lead to death [102]. The fatality rate is estimated to 1-2%, and individuals who develop neurological disease or hemorrhagic complications are at increased risk for fatality [103].

The incubation period for Rift Valley fever (RVF) is 4-6 days [104]. Symptoms usually start abruptly with chills, muscle and joint pains, weakness, severe headache, and an elevated body temperature as the predominant manifestations [9, 68, 74]. These symptoms are followed by a broad clinical spectrum ranging from painful eyes, jaundice, rigor, constipation, diarrhea, vomiting, and anorexia to delirious and insomnia [11, 68, 74, 103, 105]. The elevated body temperature usually decreases to normal around day 4 after onset [104]. The fever sometimes shows a biphasic course

so patients may again experience a temporal increase in body temperature accompanied by the return of symptoms [11, 68, 73, 74]. Recovery is most often uneventful [9, 73, 74], but in a minor proportion of the cases the feverish phase is followed by severe manifestations.

The severe manifestations are usually divided into three categories; neurological disorder, hemorrhagic fever, and ocular complications [104]. Meningoencephalitis is characterized by the development of intense headache and delirium late in the course of fever [68] and when the central nervous system is involved the symptoms include irritation, confusion, stupor and coma, hyper salivation, and visual hallucinations [105]. The ocular complications, most commonly retinal lesions with associated hemorrhage and edema, have been reported to occur at various times after infection [68, 106, 107]. One or both eyes may be affected [68, 108, 109] and the visual defects may persist for months [11, 106, 108, 109], and in some cases complete recovery does not occur [110]. Patients who suffer from hemorrhagic manifestations initially experience the acute onset of fever, headache, and myalgia [68]. Shortly after disease onset, the patients develop signs of a severe hemorrhagic state followed by a fully developed state with profuse and fatal hemorrhages from the mucous membranes [68, 105]. Histopathological findings from postmortem livers show widespread necrosis [105, 111] and evidence of extensive submucosal hemorrhages has been found throughout the gastro-intestinal tract [105]. The mortality rates are high among patients who develop hemorrhagic fever and death often occurs 7-10 days after the onset of symptoms [102, 105, 112].

4 RIFT VALLEY FEVER VIRUS

Members of the *Bunyaviridae* family are roughly spherical, 80-120 nm in diameter and have a host cell-derived lipid bilayer spiked with virus-coded glycoproteins [113] (Fig. 2). The segmented RNA genome is of negative or, less commonly, ambisense polarity *i.e.* the segment contains both negative- and positive-sense sections [114]. The genome encodes few proteins, typically four structural proteins, and up to three nonstructural proteins, which only can be found in the virus-infected host cells [2]. RVFV was classified as a member of the *Phlebovirus* genus in 1980, when serological tests demonstrated the antigenic relationship of RVFV to the phlebotomus fever viruses [115] and is now considered the type species of this genus [1].

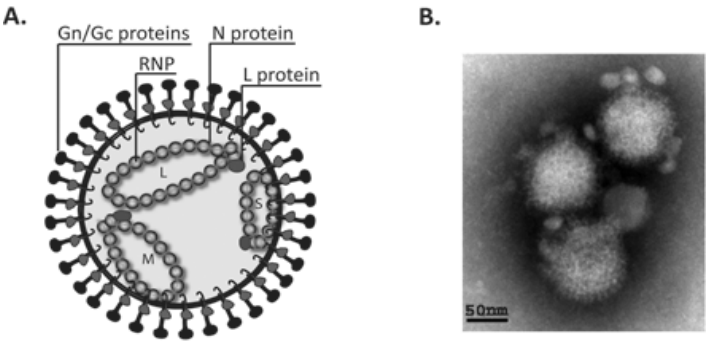

Figure 2. *The Rift Valley fever virus particle. A schematic illustration of the virion showing the viral RNA genome (L, M, and S segments), the RNP complexes, and the structural proteins (Gn, Gc, L, and N). Modified from [116]. Rift Valley fever virus particles fixated with glutaraldehyde and visualized by electron microscopy (B). Photograph; Kjell-Olof Hedlund.*

4.1 MORPHOLOGY

RVFV particles are spherical with an average diameter of 100 nm [117]. Previous studies suggested RVFV to be pleomorphic *i.e*. the virions vary in shape and size [118]. However, more recent studies have shown the virus particles to be more structurally homogenous than earlier recognized. The glycoproteins are organized at the surface as protruding hollow cylinders that cluster into distinct capsomers [117, 119]. The surface is covered with 122 capsomers arranged in an icosahedral symmetry with a triangulation number of 12 [117, 119, 120]. RVFV has a three-segmented single-stranded RNA genome and the segments are denoted after their size as large (L), medium (M), and small (S) [121]. The RNA genome is packed within the lipid envelope and numerous copies of the nucleocapsid (N) protein enwrap each of the genome segments resulting in ribonucleoproteins (RNPs) [122] (Fig. 2A). In contrast to other enveloped viruses, members of the *Bunyaviridae* family have no matrix protein that stabilizes the virion structure [2]. It has recently been suggested that the glycoprotein cytoplasmic tails interact directly with the RNP complexes [119] as has previously been proposed for other members in the *Bunyaviridae* family [123-125]. The L, M, and S segments contain non-coding regions (NCRs) at their 3' and 5' ends [121]. The termini of the NCRs have inverted complementary nucleotides (3'-UGUGUUUC) [126-128]. Base pairing of those complementary consensus sequences is believed to result in the formation of L, M and S RNP complexes with a panhandle-like structure [2] (Fig.

2A). The RNPs of RVFV are reported to have a string-like appearance, distinct from the helical symmetry of RNPs of other negative-sense RNA viruses [129]. The RNPs are suggested to associate with the L protein and together they play a central role in transcription and replication [130].

4.2 GENOME AND ENCODED PROTEINS

In general, the molecular composition of RVFV parallels that of other members of the *Bunyaviridae* family. While the L and M segments are of negative sense [126, 128] the genome of RVFV differs from that of most members, as an ambisense coding strategy is utilized by the S segment [131] (Fig. 3). An important feature of RVFV genomics is the low genetic diversity, indicating a relative recent common ancestor [132, 133]. A complete genome analysis of 33 ecologically diverse RVFV isolates showed that the L and S segments are highly conserved, differing 4% at nucleotide level and 1% at protein level. The M segment differs approximately 5% at nucleotide level and 2% at protein level [133].

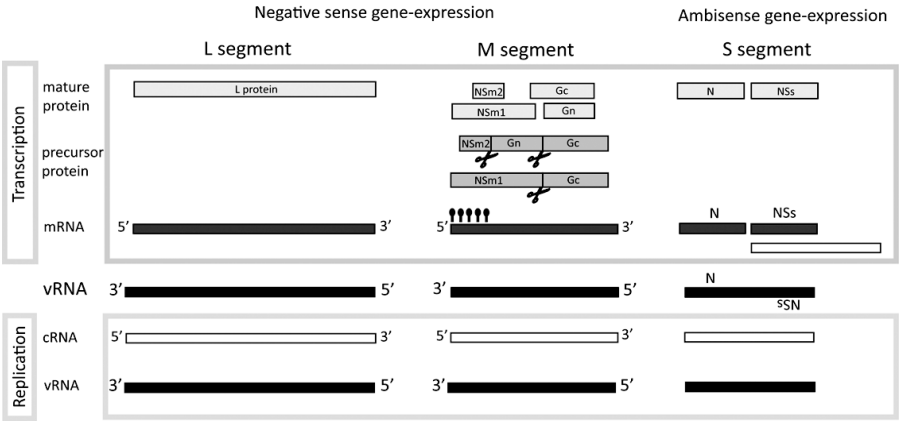

Figure 3. *Rift Valley fever virus genome segments and replication strategies. Precursor proteins expressed from the first and second AUG on the M segment are shown.*

4.2.1 L segment

The L segment with its 6404 nucleotides is, as the name states, the largest of the three genome segments [128] (Fig.3). It encodes the L protein, which is an RNA dependent RNA polymerase that catalyses the syntheses of RNA from RNA templates [134]. The L protein has a predicted molecular weight of 238 kDa and is encoded from a single open reading frame of 6275 nucleotides [134]. This protein is a multifunctional enzyme used for both transcription and replication [121].

4.2.2 M segment

The M segment consists of 3885 nucleotides [135] and has a complex coding strategy (Fig. 3). Two major glycoproteins Gn and Gc and two nonstructural proteins, the 78-kDa protein and the 14-kDa NSm protein (also referred to as NSm1 and NSm2, respectively), are all encoded from a single open reading frame [126, 136, 137] (Fig. 3). Five in-frame AUG-methionine start codons are present within the pre-glycoprotein coding region at anti-viral genomic sense positions 21, 136, 174, 411, and 426 [126] (Fig. 3). The resulting protein products, some with partially identical sequences,

depend on the in-frame AUG that is used and by post-translational proteolytic processing [138, 139]. The NSm1 protein is synthesized from the first AUG and includes the Gn sequence [136, 140] (Fig. 3). The NSm2 protein is terminated before the Gn sequence and is synthesized from the second AUG [136, 138] (Fig. 3). The polyproteins that are initiated either at the fourth or fifth AUG produce only Gn and Gc glycoproteins [136, 139]. The synthesis of mature Gc is suggested to include post-translational cleavage of a poly-protein precursor [139, 140]. Although a protein product has not been clearly defined from all in-frame methionine residues [140], all five remain conserved among 33 ecologically diverse RVFV strains [133]. The mature, glycosylated Gn (56 kDa) and Gc (65 kDa) proteins [126, 141] are essential for several steps in the virus lifecycle such as receptor recognition, entry, and budding [54, 142], whereas the NSm proteins are dispensable for viral replication in cell culture [143, 144].

4.2.3 S segment

The S genome segment is 1690 nucleotides and uses an ambisense strategy to encode for the N protein and a nonstructural protein (denoted NSs) [127] (Fig. 3). The N protein (25 kDa) consists of 245 amino acids [141] and is the most abundant protein within the virion [2]. The N protein binds the viral RNA (vRNA) [141] and provides structural stability to the genome [131]. As shown for other members of the *Bunyaviridae* family, the N protein is believed to possess additional functions important for replication and assembly [142, 145, 146]. The NSs protein (34 kDa, 265 amino acids) forms filamentous structures in the nucleus of the infected host cell [137, 147-149] and plays an important role in RVFV pathogenesis [150, 151].

4.3 LIFECYCLE

The replication cycle of RVFV can be divided into three major events *i)* attachment, entry, and fusion (steps 1-2, Fig. 4), *ii)* transcription, translation, and replication (steps 3-5, Fig. 4), and *iii)* assembly, budding, and release (steps 6-8, Fig.4) [131].

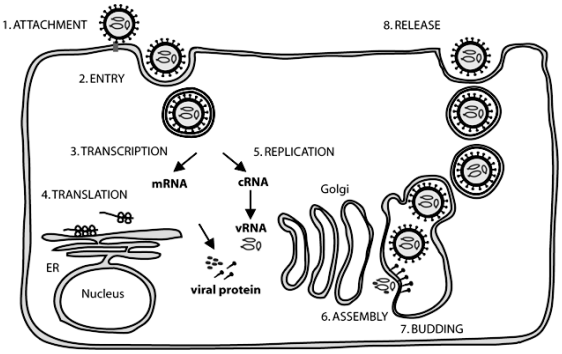

Figure 4. *A model of the replication cycle of Rift Valley fever virus. Modified from* [152].

The envelope glycoproteins mediate particle entry into cells through receptors, which remain to be identified for a majority of the *Bunyaviridae* family members [54]. The Gn protein has been suggested to be involved in receptor binding [153] and because RVFV infects a variety of cell types, it is likely that the virus uses a common cell surface

receptor or that there is no single receptor needed for virus infection [154]. However, there is some evidence that RVFV entry into dendritic cells (DCs) involves DC-specific intercellular adhesion molecule 3-grabbing nonintegrin [155], which is expressed on dermal DCs. Dermal DCs are among the first cells to encounter RVFV if transmission occurs through bites of infected mosquitoes [156]. Heparan sulphate, a commonly used attachment factor which serves to bind and concentrate the virus particles on the cell surface [157], is suggested to facilitate RVFV entry [158]. RVFV enters cells by endocytosis [159, 160] and delivers its genome into the cytoplasm of the host cell by fusion with endosomal membranes. Membrane fusion is suggested to be activated by the acidic environment in the endosomal compartments [159, 161] and analyses propose the Gc protein to employ a class II fusion mechanism [162, 163].

Transcription and replication occur in the cytoplasm of infected cells [121]. During the replication cycle, each of RVFV's genome segments is transcribed into messenger RNA (mRNA) (Fig.3). The synthesis of viral mRNA is initiated by a cap-snatching mechanism mediated by the endonuclease activity of the L protein [164]. The short primers for transcription are cleaved from the host's mRNAs [165]. The mRNAs are generally truncated at their 3' termini relative to the genome template and an unusual feature is that they lack a 3' poly(A) tail [131]. The switch mechanism between primary transcription and replication remains poorly characterized [121]. During replication, each segment is replicated through a process that involves a complementary RNA (cRNA), which serves as a template for the synthesis of new vRNA (Fig. 3). A small fraction of cRNAs have been identified in mature RVFV particles and the incoming S segment cRNA serves as a template for immediate synthesis of the NSs virulence factor [166].

The localization of the glycoproteins to the Golgi complex is essential for virus maturation and assembly [54, 167, 168]. RVFV usually bud into the lumen of the Golgi apparatus [121], but was also observed to bud at the plasma membrane in rat hepatocytes [169]. After budding, the progeny virions are transported within vacuoles to the plasma membrane where they are released from the cell [54, 131].

5 IMMUNOLOGY IN RIFT VALLEY FEVER VIRUS INFECTION

The immune system can be considered an umbrella term for all the biological systems and processes that are triggered within an organism in response to an invading pathogen with the sole purpose of eradicating the invader. The immune response includes a complex series of events and involves a variety of different actors and it is a simplified version presented here. Based on the time it takes for the response to initiate and the actors involved, the immune system can be divided into innate and adaptive immunity. Some aspects of the innate immune system is constitutively active, others need to be activated, but the innate system is generally poised to proceed rapidly in response to invading pathogens while the adaptive response, as indicated by the name, has to adapt to the situation and hence, develops more slowly [170].

5.1 INNATE IMMUNITY

The innate immune system provides the first line of defense against infections and comprises external barriers such as skin and mucous membranes as well as phagocytes and chemical mediators (*e.g.* complement and cytokines) [171]. The recognition molecules used by the innate immune system reacts upon conserved structures that are shared by various classes of pathogens [170], of which only viruses will be discussed here. These structures, known as pathogen-associated molecular patterns (PAMPs), can be RNA, DNA or other viral components and they are recognized by pattern recognition receptors (PRRs) located within or on the surface of a wide array of cells [172]. Binding of PAMPs to PRRs activates a cascade of events, mediated by chemokines and cytokines (including type I interferon (IFN)), which subsequently facilitate the eradication of the virus [172].

Innate immunity, especially type I IFN, has shown to play an essential role in RVFV infection [173]. During viral infection, type I IFN (IFN-α/β) is synthesized by infected cells for the purpose of inhibiting viral replication and, by priming neighboring cells to express antiviral factors, prevent the spread of the infection [174]. The IFN-induced antiviral factors are proteins with the ability to directly inhibit viral replication and trigger apoptosis of infected cells and they are important modulators of adaptive immunity [175].

A number of *in vitro* and *in vivo* studies suggest that RVFV is sensitive to IFN-α/β. RVFV replication in cell culture was impaired in the presence of type I IFN [94, 176, 177], whereas enhanced replication and accelerated death rate was observed in cells lacking an appropriate type I IFN response [178] and in IFN-α/β receptor-deficient mutant mice [179], respectively. A brisk IFN response has shown to be a major determinant of disease outcome in monkeys [180] and IFN and IFN inducers are effective prophylaxis and therapeutic treatment in RVFV infected monkeys and rodents [181, 182]. An intact type I IFN response is considered to be of utmost importance for limiting the viral spread during the first critical phase of RVFV infection [183].

5.2 ADAPTIVE IMMUNITY

The adaptive immune system is able to remember viruses it has fought in the past, and when it reencounters the enemy it strikes more quickly and more fiercely [170]. Depending on the molecules, proteins, and cells involved, the adaptive immunity is subdivided into cell-mediated and humoral immunity. A generalized partition is that

the cell-mediated immunity is mediated by T cells and acts against pathogens inside cells, whereas humoral immunity is mediated by antibody-producing B cells and is the arm of the adaptive immunity that takes action against pathogens outside the cell [170].

Antigen presenting cells (APCs) such as DCs trigger and initiate the specific adaptive immune response by engulfing viruses, process the viral antigens, transport the antigens to regional lymph nodes, and subsequently present them on major histocompatible complex (MHC) molecules to naïve CD4+ or CD8+ T cells. If the appropriate costimulatory factors are present and the T cell receptor is specific for the antigen the T cell differentiate into effector cells. CD4+ T cells recognize peptides bound to MHC class II molecules and differentiate into a subset of T helper (Th) cells that produce distinct sets of cytokines. The subset of Th cells includes Th1, Th2, and the more recently discovered Th17 cells [184]. The effector functions of Th17 cells are not yet completely understood but are suggested to include the clearance of pathogens not adequately handled by the Th1 and Th2 subsets [184]. Th1 cells are indispensible for clearing intracellular organism, while Th2 cells are largely involved in clearance of extracellular organisms by for instance supporting B cells in the induction of humoral immunity [171].

5.2.1 Cell-mediated immunity

A major challenge for the immune system is to identify and destroy cells that serve as factories for progeny virus [171]. When CD8+ T cells are activated by antigen presented on MHC class I molecules they differentiate into cytotoxic T lymphocytes (CTLs). The primary functions of CTLs are to identify and subsequently kill virus-infected cells [170]. In contrast to MHC class II molecules, which are primarily expressed on APCs, macrophages, and B cells, MHC class I molecules are expressed on a wide variety of cells [185]. Hence, most virus-infected cells are able to signal their status to CTLs by presenting MHC I/peptide complexes on their surface. Upon recognition, the effector CTL secretes proteins that creates pores in the membrane of the infected cell and induces signaling pathways that subsequently lead to cell lysis or apoptosis [170].

As of today, there is a remarkable paucity of data on RVFV cell-mediated immune responses. *In vitro* studies suggest that RVFV replication is not directly inhibited by the antiviral effects of IFN-γ (type II IFN) [186, 187]. However, monkeys administered IFN-γ prior to infection had reduced viremia in comparison to non-treated monkeys [188]. In contrast to IFN-α/β, which are produced by virtually all virus-infected cells, IFN-γ is mainly synthesized by activated T cells and natural killer cells in response to antigen recognition and is important in both innate and adaptive immunity [189]. For instance, IFN-γ activates macrophages, up-regulates MHC class I expression, hence increasing the potential of CTLs to detect foreign peptides, and up-regulates the MHC class II presenting pathway, thus promoting antigen specific activation of CD4+ T cells [174, 190].

Cell-mediated immunity has been suggested to be involved in the long-term protection against RVFV [186], since a high IFN-γ response is observed following restimulation of cells from convalescent goats and sheep [99, 186]. Experiments in mice have also indicated a role of cell-mediated immunity for post-vaccinal protection against RVFV (Paper III and [191]).

5.2.2 Humoral immunity

Humoral immunity is mediated by B cells that recognize antigen by their membrane-bound Ig receptor (IgD and IgM). Receptor-recognition of an antigen triggers B cell activation. The antigen-activated B cell starts to proliferate and may produce IgM in a secreted form. For the class switching and affinity maturation process to start, the antigen-activated B cell has to process the antigen to peptides and present the peptides, displayed on MHC class II molecules, to antigen-activated Th cells. In turn, the interacting Th cell delivers signals to the B cell that stimulate B cell proliferation, secretion of antibodies and switching of antibody isotype from IgM and IgD to IgA, IgE, or IgG (subclasses IgG1, IgG2, IgG3, and IgG4). [170]. Some B cells differentiate into memory B cells. The memory B cells do not secrete antibodies but are ready to respond if the antigen appears again. Prolonged or repeated exposure to a protein antigen results in the production of antibodies with higher affinity for that particular antigen. Secreted antibodies enter the blood and thus, are able to perform their functions throughout the body. Antibodies combat pathogens in many ways, they coat (opsonize) pathogens and target them for phagocytosis, activate the complement system, which promotes phagocytosis and destruction of the pathogen, and bind to pathogens to prevent them from infecting cells, thereby neutralizing the pathogen. Antibodies of different classes perform different effector functions. For instance, one function of the IgG class of antibodies is to bind and neutralize virus (so-called virus-neutralizing antibodies). For example, these antibodies can bind to specific structures on the surface of the virus and inhibit the receptor-virus interaction, and thereby prevent the virus from infecting the cell [192].

In experimentally infected sheep, RVFV was cleared from blood days before detection of virus-neutralizing antibodies [99], which bear witness of the importance of innate immunity. However, the disappearance of RVFV from the blood often coincides with the appearance of virus-neutralizing antibodies. The close temporal relation of serum neutralizing antibodies and viral clearance suggests that virus-neutralizing antibodies in addition to innate immunity play a pivotal role in the control of RVFV infection [180, 193]. Neutralizing antibodies are also considered the major mechanism responsible for the prevention of re-infection in convalescent or immunized hosts [54].

The glycoproteins are the most exposed viral component during infection and it was early determined that both the Gn and Gc proteins of RVFV are able to induce and interact with virus-neutralizing antibodies [194]. Passive immunizations *i.e.* transfer of blood components from immune animals or virus-neutralizing monoclonal antibodies to non-immune animals have repeatedly been shown to protect against disease [182, 193, 195-198]. Additionally, offspring of immune animals are protected for about five months after birth via suckling [199]. RVFV infection is considered to give lifelong protection against re-infection and neutralizing anti-RVFV antibodies have been detected in humans 25 years after exposure [200].

Although the RVFV N protein is a major immunogen and a strong antibody response generally is generated towards this protein, anti-N antibodies are not considered to prevent RVFV infectivity [54].

5.3 VIRAL COUNTERMEASURES

Virus-invasion triggers the host's immune system and for the virus to be able to establish an infection it has to counteract those mechanisms or escape [201]. The power of the IFN defense system has prompted many viruses to adopt strategies to

inhibit IFN production or block the actions of IFN [189], as has RVFV. The important role of the NSs protein in RVFV virulence was revealed when attenuation of a RVFV isolate was ascribed a large deletion in the NSs encoding gene [202]. Subsequent reports showed NSs to be a major virulence factor subverting the innate type I IFN system at transcriptional level and causing a general suppression of host cellular RNA synthesis [150, 151, 179, 203, 204]. In addition, the NSs protein has been shown to promote the degradation of double-stranded RNA-dependent protein kinase R [187, 205, 206], a highly efficient antiviral factor that is upregulated by IFN and promotes translation shut down in the host cell [190, 207]. These mechanisms allow RVFV infection to proceed with minimal involvement of the IFN defense system. Although, the NSs protein clearly has established its role as the major virulence factor, other viral proteins may also contribute to the virulence of RVFV [104]. Even though the NSm proteins were dispensable for viral replication *in vitro* [143, 208] and for viral pathogenesis *in vivo* [144], it was shown that cells infected with virus lacking the NSm proteins underwent apoptotic cell death earlier than cells infected with parental virus [209]. In addition to these counteractions that are specific for RVFV, more general mechanisms may also be of importance. The high mutation rate of the viral RNA polymerase (L protein) which lacks proofreading might result in antigenic drift and the exchange of genome segments between closely related viruses during a mixed infection may lead to antigenic shift [183]. Both antigenic drift and shift can, in the end, cause new variants of viral antigens to which the host is not adopted [183]. For instance, a single nucleotide mutation in the Gn protein was sufficient to alter the virulence of RVFV in mice [210].

6 HISTORICAL AND CURRENT RIFT VALLEY FEVER VIRUS VACCINES

Basically, vaccines can be categorized into three groups: live attenuated virus vaccines, inactivated (killed) virus vaccines, and subunit vaccines. Traditionally, vaccines have been based on either live attenuated strains of pathogenic virus, closely related non-pathogenic virus strains that elicit immunity against the pathogenic relative, or inactivated virus [211]. Live virus vaccines can replicate in the host, but are attenuated in their pathogenicity *i.e.* some of the viral components that causes disease has been altered. For the inactivation of viruses, chemicals such as formalin or β-propiolactone are employed to kill the pathogen, while most of its structure is intact [212]. Inactivated virus vaccines generally are less immunogenic than attenuated virus vaccines and are therefore normally administered in combination with an adjuvant [212]. Adjuvants (such as aluminum salts [213]) are compounds that are added to a vaccine in order to modulate the effect of the antigenic components. Adjuvants may exert their effects by different mechanisms, but what they all have in common is that they enhance the recipient's immune response to the delivered antigen [214].

6.1 LIVESTOCK

The control of RVFV requires several actions, from early warning systems and surveillance programs to vector monitoring, animal movement restrains, and farmer and people awareness [215]. The use of veterinary vaccines during inter-epizootic periods and as early countermeasures against nascent outbreaks is, however, the first line of defense [216, 217].

Currently, there are two vaccine strategies employed to control disease in livestock (Table 1), and 6 million vaccine doses were sold in South Africa during 2010 [218]. Vaccines based on the live attenuated Smithburn virus, which is attenuated by serial intra-cerebral (brain-to-brain) passages in mice [199], have low production costs, long-lasting immunity [218] and offspring of immunized animals are protected via suckling [199]. The Smithburn virus vaccine was reported to induce high antibody titers in sheep, but weaker antibody responses in cattle [219]. Studies have shown that this vaccine causes adverse effects in newborn kids and lambs and fetal malformation and abortion in gestating does, ewes and cows [95, 199, 220, 221]. Hence, its residual virulence renders its use unsuitable when the pregnancy status of the animals is unknown. Its use is also restricted in non-endemic areas and during outbreaks, due to the risk for revision to virulence and risk of reassortment. Reassortment events have been documented experimentally [222] as well as among natural RVFV isolates [223].

Safe alternatives based on formalin-inactivation of whole virus formulated with an adjuvant (aluminum hydroxide) were produced and were shown to induce virus-neutralizing antibodies in cattle and sheep (Paper I and [219, 224]). Production of formalin-inactivated RVFV vaccines are labor intensive, time consuming, and requires biosafety level (BSL)-3 laboratories and the need of annual booster vaccination to remain protective immunity makes their use expensive and inconvenient [218, 225].

More recently, a naturally attenuated virus vaccine named Clone 13 was marketed in South Africa [226]. The NSs gene of Clone 13 contains a large internal deletion of 549 nucleotides [227], which later was proven to be the cause of the attenuation [202]. Although still highly immunogenic, Clone 13 was found basically avirulent in mice [227]. Further evaluation in pregnant ewes and their offspring showed that Clone 13 was efficient and safe [228] and equally potent as the

Smithburn virus vaccine in protecting calves against infection [229]. However, Vialat *et al.* reported neurological disorders and paralysis in a small proportion of Clone 13-vaccinated mice, suggesting that the isolate is not completely harmless [202].

6.2 HUMAN

As of today, no commercial RVFV vaccine is available for human use. Initially, a killed vaccine (NDBR-103) was prepared from the 180th mouse passage of the Entebbe strain and propagated in African green monkey kidney cells prior to formalin inactivation [76, 230]. This vaccine was evaluated in human volunteers including approximately 1000 Swedish United Nations soldiers deployed to the Sinai Peninsula in Egypt [231]. One of those soldiers developed neurologic complications diagnosed as Guillain-Barrés syndrome after vaccination [232]. This outcome has not, however, been conclusively linked to the vaccine.

A new vaccine lot (TSI-GSD 200) was prepared under rigorous safety restrictions [233]. The TSI-GSD 200 vaccine has been extensively evaluated in man and was shown to induce virus-neutralizing antibodies in 90% of human volunteers after three primary immunizations [234], but booster administrations are necessary to maintain protective immunity [235, 236]. The side effects were reported mostly to be mild transient local reactions [234, 237-239]. The TSI-GSD 200 vaccine is in a limited supply and is currently only available to at-risk individuals including laboratory workers (Table 1) [240].

Table 1. Available Rift Valley fever virus vaccines

Vaccine strategy	Vaccine (species)	Pros	Cons	References
Live attenuated virus	Smithburn (livestock)	Highly immunogenic; life-long protection	Severe adverse effects; restricted use in non-endemic countries	[199, 220, 221]
	Clone 13 [1,2] (livestock)	Highly immunogenic		[228, 229]
Formalin-inactivated virus	Formalin-inactivated virus vaccines [2] (livestock)	No adverse effects	Yearly booster doses required	Paper I and [219, 224]
	TSI-GSD 200 [3] (human)	No severe adverse effects	Limited supply; booster doses required	[238, 241]

[1]Marketed recently

[2]For example, produced by Onderstepoort Biological Products in South Africa

[3]Available for personnel in high risk occupations

7 RIFT VALLEY FEVER VIRUS VACCINE CANDIDATES

The general consensus about the need to improve the first generation RVFV vaccines (Table 1) has resulted in tremendous efforts to develop and evaluate novel vaccine strategies (Table 2). RVFV is uniquely suited for a one health approach, reducing the risk for disease in human and animal through vaccination of livestock, the amplifying hosts [217]. Consequently, most candidate vaccines are aimed for use in animals. The advantages and drawbacks of each of the different vaccine regimens employed against RVFV are outlined in Table 2.

Table 2. Rift Valley fever virus vaccine strategies and their pros and cons

Vaccine strategy	Pros	Cons	References
Live attenuated virus (*e.g.* MP12 and R566)	Can induce life-long protection after a single dose; low production costs	Can cause adverse effects; risk of revision to virulence; manufacturing may require BSL-3 facilities; risk for spread to unintended individuals	[242, 243]
Recombinant virus	Highly immunogenic; possible to produce viruses with targeted alterations	Manufacturing may require BSL-3 facilities; can cause adverse effects in immuno-compromised individuals	[244-246]
Plasmid DNA	Safe and rapid production; easy to manipulate; long shelf life	Low immunogenicity; may require adjuvant	Paper III and [247, 248]
Viral-vectors	Easy to manipulate; rapid production	Dependent on vector, suitable for different species and areas; may cause adverse effects in immuno-compromised recipients	[249, 250]
Virus-like particles	Safe in immunocompromised individuals; safe manufacturing process; immunogenic	Expensive manufacturing, relatively difficult to manufacture large quantities; may require adjuvant	Paper IV and [251, 252]
Protein subunit	Safe in immunocompromised individuals; safe manufacturing process	Low immunogenicity; usually requires adjuvant	Paper V and [248, 253, 254]

7.1 LIVE-ATTENUATED VIRUS VACCINES

Of all the current RVFV vaccine candidates, MP12 is the one that has been most extensively evaluated. MP12 was obtained by 12 serial passages of RVFV (strain ZH548) in 5-fluorouracil. The growth in presence of this mutagen, which masquerades as uracil during RNA synthesis [255], resulted in a virus that was attenuated in mice [256] and hamsters [257]. Analysis of the MP12 strain showed a total of 25 nucleotide changes compared to the parental strain [258, 259] and further characterization indicated that the determinants of attenuation were present in each genome segment [260]. Comprehensive animal trials have shown MP12 to be safe and efficacious in newborns and in pregnant cows and ewes [242, 243, 261-263] and immunization protected lambs against RVF [264]. However, MP12 caused malformation in sheep fetuses when administered during the first trimester (at days 35 to 56) [265]. In an attempt to find a new candidate for a human vaccine, MP12 was further evaluated in rhesus macaques, which serve as models for human RVF disease [195]. MP12 was

markedly attenuated compared to the parental ZH548 strain in those animals [266], immunogenic, and protected against viremia and disease upon challenge [79, 267]. However, viral shedding has been documented in vaccinated rhesus macaques [79] and can increase the risk of spread to unintended individuals.

More recently, strain R566 has been proposed as an alternative candidate. R566 is a reassortant of the L and M segment of MP12 and the S segment of Clone 13. R566 protected mice against virulent challenge and did not cause adverse effects in pregnant ewes [268].

7.2 RECOMBINANT VIRUS VACCINES

The availability of reverse genetic systems enables scientists to abrogate RVFV pathogenicity by producing viruses with targeted alterations *i.e.* mutations or deletions [244, 269, 270]. This system is based on the rescue of infectious particles from complementary DNA. Plasmids encoding vRNA and viral proteins are co-transfected into mammalian cells after which it is possible to recover infectious virus particles [268]. Several advantages arise from using this system to create recombinant virus vaccines. The recombinant virus is genetically highly defined because it originates from DNA and the possibility to introduce large deletions or mutations in all gene segments further adheres to its safety [268]. To overcome some of the inherent limitations with previous live attenuated vaccines, *e.g.* fetal malformations and abortion, a recombinant RVFV was created lacking both the NSs and NSm proteins [244]. This vaccine was evaluated for its immunogenicity in rats [244] and was found to be safe and effective in pregnant and non-pregnant sheep [245]. However, the BSL-3 constraint remains until the recombinant RVFV has proven completely attenuated.

7.3 PLASMID DNA VACCINES

Since the discovery in 1990 that naked DNA could be directly transferred into tissue and express antigen *in situ* [271] and that humoral and cell-mediated immune responses could be raised towards such an antigen [272, 273], DNA vaccines have been employed against a number of pathogens [274]. A DNA vaccine comprises a plasmid encoding the antigen of interest along with a strong promoter that is used to drive protein expression [275]. Plasmid DNA vaccines are commonly administered via the intradermal, intramuscular, or mucosal route and uptake into host cells is thought to occur via endocytic vesicles, transient membrane pores or be receptor-mediated [276]. Once inside the cell, the foreign DNA is transported into the nucleus, is processed by the host-cell machinery and is presented to the immune system [274], a process that mimics the production of viral antigens during natural infection [277]. Consequently, genetic vaccines gain access to immune mechanisms not generally utilized by subunit or inactivated virus vaccines [278]. The use of genetic vaccines is of special interest against highly pathogenic viruses since the safety concerns during manufacturing of traditional vaccines are circumvented [279]. Additional advantages include beneficial production costs and the relative simplicity of construction [280].

DNA vaccines against RVFV have shown divergent results in early animal trials. Some authors report no immunogenicity while some reports that DNA vaccines confer partial or complete protection against lethal challenge (Paper III and [247, 248]). The rather low immunogenicity of DNA vaccines has led to the use of prime-boost strategies, which includes a primary immunization using DNA vaccines followed by a booster with virally-vectored [281] or attenuated virus vaccine [282]. Other groups

have sought to further enhance the effect of RVFV DNA vaccines by including immune modulators in their preparations [191, 198].

7.4 VIRALLY-VECTORED VACCINES

Viruses have evolved efficient mechanisms to infect cells and for hijacking the cellular machinery for the production of self-proteins. Those intrinsic features make viral vectors optimal as vehicles for heterogeneous gene delivery [283]. Members of the *Poxviridae* family (*e.g.* vaccinia virus, lumpy skin disease virus (LSDV), and sheep pox virus (SPV)) and Newcastle disease virus (NDV) are widely used for such applications [284, 285].

Viruses within the *Poxviridae* family have a large DNA genome that is able to stably maintain multiple foreign genes [285]. The closely related LSDV for cattle and SPV have been evaluated as vaccine vectors for RVFV [248, 286, 287]. LSDV was genetically modified to express the RVFV glycoproteins Gn and Gc and this vaccine protected mice against lethal challenge [286] and induced virus-neutralizing antibodies in sheep subsequent immunization [248]. A SPV vector expressing the glycoproteins conferred protection in mice and suppressed viremia in sheep after infection [287]. An important rationale for using those vaccine designs is that the habitat of RVFV overlaps those of LSDV and SPV [288]. This would give the opportunity to prevent two important health problems for livestock by a single vaccine. However, the use of these vectors can constrain the use of the vaccines to LSDV and SPV endemic countries. Thus, Papin *et al.* used a vaccinia virus vector expressing the RVFV glycoproteins and showed that this approach was safe and partly efficacious in mice and baboon [249].

NDV has a negative stranded RNA genome and is exclusively pathogenic for avian species, however, when artificially inoculated the virus can replicate in mammalian species [289]. A recombinant NDV expressing the Gn glycoprotein was created and evaluated in calves [290]. The immunogenicity was rather low, consequently a new candidate expressing both the Gn and Gc glycoproteins was constructed [250]. Initial studies in mice were promising [250] and the vaccine induced virus-neutralizing antibodies in sheep after two doses [291].

7.5 VIRUS-LIKE PARTICLE-BASED VACCINES

Virus-like particles (VLPs) are a promising platform for the development of safe and effective vaccines. VLPs are formed by the self-assembly of viral structural proteins and because the viral genome is not incorporated into the particles they are replication deficient [292]. Expression of the Gn and Gc glycoproteins in mammalian or insect cells, with or without the presence of N protein, results in the assembly and release of RVF VLPs [251-253, 293-295]. The production of VLPs is described in more detail in section 9.4.

VLPs are usually more immunogenic than protein subunit vaccines. The morphological similarity to the virulent virus is likely to enhance the production of virus-neutralizing antibodies, since the conformational epitopes are displayed authentically [292]. Indeed RVF VLPs were shown to have similar morphology as the wild-type virus [293, 295] and when evaluated in animal models, RVF VLPs were found to induce strong virus-neutralizing antibody responses and immunizations protected against RVF (Paper IV and [251, 252]). It may be difficult to scale-up the manufacturing process [296], hence, the major challenge using this approach is to produce cost-effective vaccines.

7.6 PROTEIN SUBUNIT VACCINES

The strategy of protein subunit vaccine takes advantage of the possibility of using viral proteins or parts of viral proteins to raise protective immune responses. To elicit a strong response, protein subunit vaccines often require multiple doses and the use of an adjuvant [297]. Thus far, three neutralizing epitopes have been mapped in the Gn protein [298]. Accordingly, truncated variants of the Gn protein, all possessing those three epitopes, have been found to be immunogenic in mice (Paper V and [248, 253, 299]. When comparing truncated Gn protein administered with or without adjuvant to VLPs and Smithburn virus vaccine, the protein subunit vaccine induced lower virus-neutralizing antibodies in mice, but immunized mice performed equally good in challenge [248, 253]. Beeselaar *et al.* showed that monoclonal antibodies raised towards the Gn and Gc proteins were able to neutralize virus infectivity *in vitro* [194]. Mice immunized with truncated Gn protein survived, whereas animals administered truncated variants of the Gc protein succumbed to infection [299]. N protein preparations administered with or without adjuvant have shown to be highly immunogenic in mice and immunization conferred partial protection against infection [248, 254].

8 LABORATORY DIAGNOSIS

In the case of RVFV infection in livestock, the clinical signs vary and even though the characteristic “abortion storms” usually give an indication of what disease to suspect, there are a number of differential diagnoses to preclude such as Nairobi sheep disease, Wesselsbron disease, and bovine ephemeral fever [300]. Similarly in humans, due to the broad clinical picture often resembling influenza or dengue fever [57], the clinical diagnosis may be complicated or often impossible without accompanying laboratory confirmation. As can be exemplified by the schematic illustration in Fig. 5, the appropriate laboratory test to choose is dependent on the elapsed time after infection. During the first few days, viral genomes or proteins can readily be detected in blood. However, later during the course of disease, the virus is cleared from the circulation and only detection of anti-RVFV antibodies is possible [301].

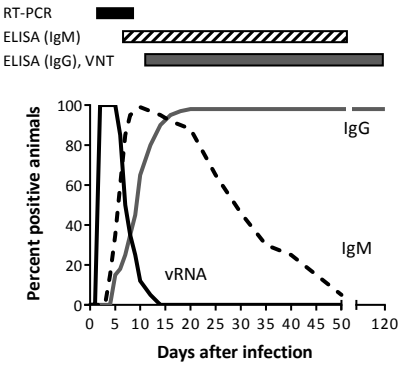

Figure 5. *Generalized time course of viremia, antibody responses, and useful diagnostic tools in Rift Valley fever virus infected animals. Modified from [54, 301].*

8.1 DETECTION OF VIRUS

Viremia in RVFV-infected individuals often reaches very high titers, in many animal species 10^6 to 10^9 [301, 302] and in humans 10^1 to 10^8 infectious particles/mL serum [303]. Consequently, it is possible to detect virus (vRNA or proteins) that circulates in the blood shortly after infection. For this purpose, a number of reverse-transcriptase polymerase chain reactions (RT-PCRs) and enzyme-linked immunosorbent assays (ELISAs) have been developed, respectively. Most RT-PCRs target the S [304, 305] or the L segment [306, 307], since these segments have least inter-strain variability [133]. The RT-PCRs are fast and have high sensitivity (as few as five vRNA copies can be detected in a sample) [306]. ELISAs detecting circulating viral antigens are other methods used to confirm diagnosis during the early phase of disease [308, 309]. However, ever since RT-PCR became a standard method in most laboratories, detection of antigen has increasingly been replaced by detection of vRNA. Definitive confirmation can be performed by virus isolation at reference laboratories with BSL-3 facilities [301]. RVFV is easily isolated from blood [310], but the requirement of BSL-3 laboratories renders this approach useless in most endemic countries.

8.2 DETECTION OF ANTIBODY

Because of the relatively short viremia, several methods for measuring anti-RVFV antibodies (IgM or IgG) have been developed during the years. Notably, those

methods are unusable during the first few days after infection [100] (Fig. 5). Hemagglutination inhibition and complement fixation assays were early developed [311], but members of the *Phlebovirus* genus show considerable cross-reactivity in those assays [312]. Virus-neutralization test (VNT) is highly specific and sensitive and is the most frequently used reference method when BSL-3 laboratories are accessible [313]. The VNT is the only method that accurately detects functional antibodies *i.e.* virus-neutralizing antibodies and is therefore commonly applied in studies evaluating vaccines. However, low-level cross-reaction of RVFV with a few phlebovirus immune sera has been observed when using VNTs [115, 314]. The plaque-reduction neutralization test (PRNT) is one commonly applied VNT for RVFV. PRNT is performed by co-incubating virus and heat-inactivated serum and thereafter allowing the virus to infect cell monolayers. To prevent virus spread in the cell culture, the cells are overlaid with agar and incubated for 4-6 days. Due to the cytopathic effects of RVFV, the formation of plaques in the cell monolayer reveals if, and to which extent, the serum has neutralized the virus. Normally, a serum that reduces the number of plaques by 80% ($PRNT_{80}$) compared to untreated virus control is regarded as positive. Immunofluorescence assays have shown to be less sensitive and less specific than VNT and ELISA, but they are still frequently applied [314, 315]. Early ELISAs were based on the use of purified RVFV particles as detecting antigen [316-318]. A number of ELISAs based on recombinant RVFV proteins have been developed to circumvent the requirement of BSL-3 laboratories [319, 320]. Those ELISAs, most of which detect anti-N IgM or IgG antibodies, have been extensively evaluated for a number of species *e.g.* human, buffalo, sheep, and cattle [321-323].

9 RESULTS AND DISCUSSION

9.1 STABILITY OF A FORMALIN-INACTIVATED VIRUS VACCINE (PAPER I)

In Paper I we evaluated the influence of transport conditions on the efficacy of a commercial formalin-inactivated RVFV vaccine, here referred to as the OBP vaccine. By using 23 monoclonal antibodies, Blackburn *et. al.* showed that treatment with formalin had a detrimental effect on the majority of the epitopes on the RVFV glycoproteins [324]. The antigenic changes combined with the lack of replication makes formalin-inactivated RVFV vaccines less immunogenic than live attenuated virus vaccine preparations.

In line with the manufacturer's instructions (Onderstepoort Biological Products) livestock in Mozambique are given a primary series of two vaccinations followed by yearly boosters. However, it is difficult to comply with the manufacturer's guidelines regarding the vaccine storage temperature, which should not exceed 8°C. The OBP vaccine has to be transported a distance of approximately 2000 km within Mozambique before administered to livestock in Zambezia Province (Fig. 6), which is the only province with a continuous RVFV immunization program. The transport takes more than a week and it is extremely difficult to maintain low storage temperatures during that time. Although it is known that inactivated virus vaccines are more stable than attenuated virus vaccines, storage of formalin-inactivated RVFV for six months at 4°C resulted in changes in the glycoprotein epitopes [324]. Hence, we evaluated if the OBP vaccine was still immunogenic when administered to livestock. For that purpose, vaccine stored one week at different temperatures (4°C, 25°C, and alternating 4°C and 25°C) were administered to livestock in the Namaacha district in Maputo Province (Fig. 6) and the induced antibody responses were monitored (Fig. 7). Yet another group of animals was given vaccine that had been transported from Maputo City to Zambezia Province (Fig. 6) under the conditions ordinarily occurring during transit ("customary", Fig. 7).

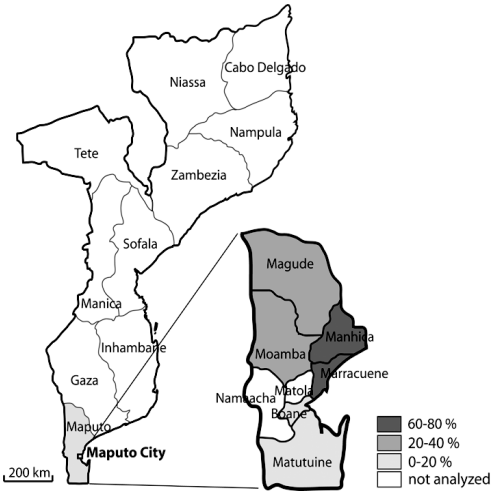

Figure 6. *Map of Mozambique showing the provinces (Paper I) and the percentage of seropositive bovines at district level in Maputo Province (Paper II).*

A total of 71 animals were included in this study and virus-neutralizing antibody titers in the range of 1:40 to 1:320 were detected in 99% of cattle after a primary and a booster vaccination. It can be mentioned that a comparable response rate (90%) was observed in human volunteers after a primary series of three immunizations with TSI-GSD-200 [234]. Barnard *et al.* showed that a single vaccination resulted in seroconversion in two out of five cattle and that all of those animals exhibited virus-neutralizing antibody responses after two immunizations [219].

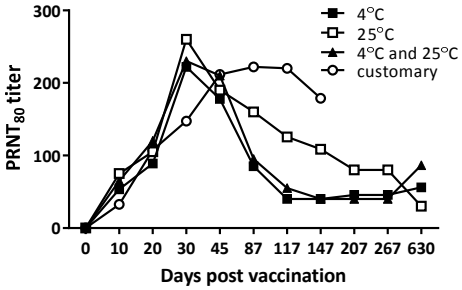

Figure 7. *Virus-neutralizing antibody titers in cattle administered the OBP vaccine. The storage temperature of the administered vaccine is indicated in the graph (4°C, 25°C, alternating 4°C and 25°C, or vaccine transported as customary). The animals were immunized at days 0 and 21 and the curves correspond to the mean titer in each vaccine group. Animals receiving vaccine that had been transported as customary were sampled for 147 days.*

A general concern regarding the use of formalin-inactivated RVFV vaccines is the extent and duration of the vaccine-induced immune responses. Consequently, annual booster immunizations are given to cattle to restore immunity. We found that cattle exhibited antibody responses that were more long-lasting than expected (Fig. 7). Fifteen animals were sampled after 21 months (630 days), of those animals had 11 detectable virus-neutralizing antibodies titers in the range of 1:40 to 1:360. It can be mentioned that cattle vaccinated with a comparable vaccine had detectable levels of virus-neutralizing antibodies nine and 12 months after the primary vaccination [219, 224]. When the formalin-inactivated TSI-GSD-200 vaccine was evaluated in humans, it was shown that 85% of the responders had a virus-neutralizing antibody titer of ≥1:40 six months after vaccination [234]. However, only 35% of the recipients had maintained that titer one year after vaccination [234].

Due to technical reasons, we did not evaluate the protection induced by the OBP vaccine. Virus-neutralizing antibody titers in the range of 1:20 to 1:40 is generally accepted as protective against disease in rodents [80, 196], sheep [325], and rhesus macaques [195], and human vaccinees are classified as responders if a titer of ≥1:40 is obtained using the TSI-GSD-200 vaccine [234, 238].

Live virus vaccines, such as the Smithburn virus vaccine, are often heat-labile [326]. The ability of the OBP vaccine to induce antibody responses in cattle was not adversely affected by storage at ambient temperatures (Fig. 7). Despite its poor immunogenicity, the OBP vaccine might be preferable to the Smithburn virus vaccine, not only in non-endemic countries and during outbreaks which is accepted practice, but also in situations when it is difficult to maintain a cold-chain.

9.2 SEROPREVALENCE AMONG CATTLE IN MAPUTO PROVINCE (PAPER II)

We found evidence of previous RVFV infections in 17% of the animals raised in the Naamacha district (Paper I), indicating that the virus circulates in Maputo Province but is either not causing manifest clinical disease or is not being diagnosed. We explored this in Paper II, in which we report the findings from a seroprevalence study performed in livestock. Of 404 bovine sera collected throughout Maputo Province, 37% were found to contain RVFV-neutralizing antibodies. An overall seroprevalence of 37% is unexpectedly high for an area in which no RVF disease activity has been reported since 1969 [13]. By comparison, seroprevalences of 26% and 11% were observed in cattle on the islands of Madagascar and Mayotte (Fig. 1), respectively [327, 328], and 21% of wildlife buffaloes in Kruger National park, which border to Maputo province, were positive for anti-RVFV antibodies [46]. Further, our study revealed difference in seropositivity at district level (Fig. 6).

To assess the seropositivity, we used $PRNT_{80}$. One could always speculate that the high seroprevalence observed in Paper II partly could be explained by cross-reactions to closely related viruses. A study performed using immune serum from four African phleboviruses (Gordil, Arumowot, Saint-Floris, and Gabek-forest virus) showed no cross-reactivity [312], which renders that scenario unlikely. A possible scenario is that RVFV circulates sub-clinically in cattle, as also described recently for goats and sheep in Zambezia Province [329]. It should be noted that abortions are more common in small domesticated ruminants than in cattle and they are also more prone to display severe RVF manifestations [96].

The high rate of seropositive cattle in Maputo Province suggests that it is necessary to extend the current vaccination program to include Maputo Province and we show in Paper I that the formalin-inactivated vaccine is well suited for that application.

9.3 IMMUNOGENICITY OF DNA VACCINES (PAPER III)

In Paper III we evaluated the immunogenicity and protective efficacy of plasmid DNA vaccines encoding structural RVFV proteins. The encoding sequences of the N, Gn and Gc proteins, and the GnGc polyprotein were cloned into an eukaryotic expression vector downstream a cytomegalovirus promoter and the resulting constructs are hereafter referred to as pcDNA3.1/N, pcDNA3.1/Gn, pcDNA3.1/Gc, and pcDNA3.1/GnGc, respectively. The plasmid DNA was precipitated onto gold microparticles and was delivered intradermally to mice by a gene gun (Fig. 8).

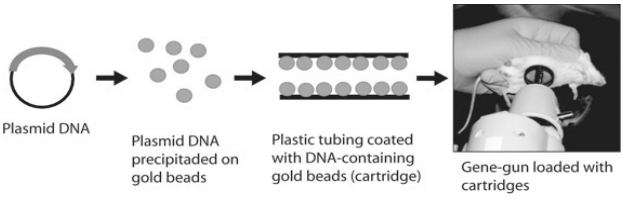

Figure 8. *Gene gun-mediated DNA vaccination. Photograph; Göran Bucht.*

Although antibodies directed towards the N protein repeatedly have failed to neutralize virus infectivity *in vitro* (Paper III and [254], partial protection against RVF was observed in mice immunized with bacterially expressed N protein [248]. In an attempt to explore the immune factors involved in protection, we analyzed both the

humoral and to some extent the cellular immune response induced by the pcDNA3.1/N vaccine. Mice immunized with pcDNA3.1/N exhibited uniform anti-N antibody responses with titers ranging from $3x10^4$-$6x10^4$ after one primary and three booster doses. Interestingly, we found that spleenocytes of immunized mice proliferated in response to antigen stimuli (purified N protein) in a dose-dependent manner. To the best of our knowledge, this was the first report showing that vaccination with N protein induces cellular immunity, which has more recently been confirmed by other groups [191, 281].

In an attempt to determine the most effective glycoprotein-based DNA vaccine, we compared the three variants (pcDNA3.1/Gn, pcDNA3.1/Gc, and pcDNA3.1/GnGc) for their ability to induce specific antibodies in mice. Half of the animals immunized with pcDNA3.1/Gc seroconverted, whereas all mice immunized with pcDNA3.1/Gn or pcDNA3.1/GnGc did. Our results are in accordance with previous studies showing the Gn protein to be more immunogenic than the Gc protein [299, 330]. Since both Gn and Gc harbor epitopes for virus-neutralizing antibodies [194, 331] it was expected that pcDNA3.1/Gn would induce a weaker response than pcDNA3.1/GnGc. Bhardwaj *et al.* showed that it is possible to increase the weak antibody responses observed after Gn-based DNA vaccinations by fusing C3d to the Gn encoding sequence [198]. The C3d complement factor helps to maintain immunological B cell memory and synergizes to activate B cells [332].

From the initial immunogenicity study we deduced that pcDNA3.1/N and pcDNA3.1/GnGc induced the strongest antibody responses. Hence, these vaccines were evaluated for their ability to confer protection in mice against RVF. Mice, including the BALB/c strain, have been shown to be highly susceptible to RVFV. Infection most often results in a moribund condition followed by death shortly after [333] and as few as 10-100 infectious particles are sufficient for establishing infection and disease in the BALB/c mouse model (unpublished data). Thus, it was highly unexpected that the majority of the control mice, immunized with empty pcDNA3.1 vector or pcDNA3.1 containing an irrelevant gene, survived inoculation with $2.4x10^3$ or $2.4x10^4$ infectious virus particles. In light of this, we evaluated the challenge study based on lack of clinical signs instead of survival. We hypothesize that this outcome was an unfortunate combination of the RVFV strain used (ZH548, isolated from a mild human case [334]) and the extensive immunization scheme, which resulted in that the animals were 15-17 weeks old when they were infected.

Half of the mice immunized with pcDNA3.1/N were protected from disease, a similar proportion as observed after immunization with recombinant N protein [248]. A more recent study confirmed that the sole expression of N protein is enough to induce partial protection (43%) in mice [281]. Boshra *et al*. tried to enhance the efficacy of N-based DNA vaccines by incorporating immune modulators into the plasmid DNA construct [191]. Incorporation of ubiquitin, which causes rapid proteasome-dependent degradation and thereby facilitates MHC class I presentation to T-cells [335, 336], resulted in that 72% of the mice were protected against RVF [191].

In our study, the mice exhibited virus-neutralizing titers in the range of 1:25 to 1:75 after immunization with pcDNA3.1/GnGc, and 63% or the animals were protected against disease. Three doses of a comparable vaccine, delivered by gene-gun, conferred complete protection in mice [247], as did intramuscular delivery [281]. On the other hand, Wallace *et al.* reported that mice failed to seroconvert after intramuscular immunization with constructs encoding the Gn and Gc proteins [248].

Interestingly, DNA vaccines encoding the glycoprotein precursor (NSm, Gn, and Gc) repeatedly have been shown to be poorly immunogenic in mice and sheep [247, 281, 282].

Expression of the N protein and the glycoproteins in recombinant baculovirus-infected insect cells resulted in spontaneous assembly of the viral components [295]. We speculated that a combined administration of DNA encoding those proteins might generate VLPs, and thereby enhance the immune response observed after DNA vaccination. However, Lorenzo *et al.* showed that co-delivery of N- and GnGc-encoding plasmids did not have synergistic effects, rather the contrary [281]. The combined vaccine induced weaker virus-neutralizing antibody responses as compared to the individual vaccines and was less protective than glycoprotein-based DNA vaccine [281].

The DNA vaccine platform has been extensively evaluated during the last decade. As of yet, only a handful DNA vaccines has been approved for use in animals and none has been licensed for human use [337]. The benefits of DNA vaccines (see Table 2) do not compensate for the low immunogenicity observed for RVFV-based DNA vaccines today. However, further studies including immune modulators and construct-optimization might in the end lead to an effective DNA vaccine that either can be used alone, or more likely, in combination with other vaccine platforms.

9.4 IMMUNOGENICITY OF RIFT VALLEY FEVER VIRUS-LIKE PARTICLES (PAPER IV)

RVF VLPs can be divided into VLPs and infectious VLPs (iVLPs). While the VLPs do not pack any genetic material within their particle, the iVLPs are produced by transient expression of the structural proteins in the presence of a minigenome. Habjan *et al*. describe the production of iVLPs from human 293T cells by transfection of plasmids encoding recombinant glycoproteins, L and N proteins and a minireplicon consisting of the antisense gene for Renilla-Luciferase flanked by M segment NCRs [293] (Fig. 9). The expression of the reporter gene is under the control of a promoter for the cellular RNA polymerase II [293]. The minireplicon is subsequently packed within the VLPs resulting in iVLPs, which are able to transport the RNA to receiving cells. Whereas primary transcription *i.e.* synthesis of the reporter gene occurs in these cells, transfection of plasmids encoding L and N proteins allows replication of the minigenome and secondary transcription *i.e.* high-level reporter gene expression [293] (Fig. 9). These iVLPs are therefore unable to replicate and produce progeny in the vaccinated individual.

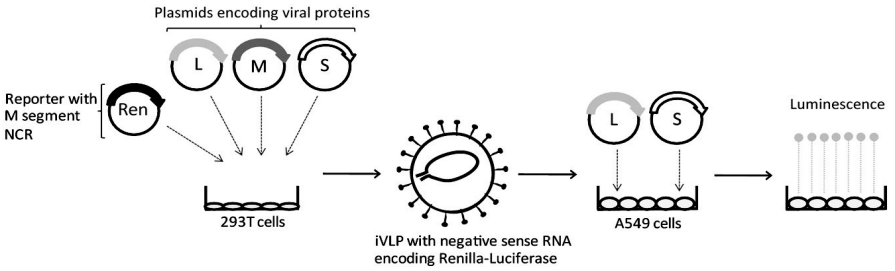

Figure 9. *The production of iVLP. Ren, renilla-luciferase.*

In Paper IV we evaluated the immunogenicity of these iVLPs in mice. Since BALB/c mice were shown to be relatively resistant to challenge (Paper III), we decided to use another commonly applied laboratory mouse strain, C57/black mice, in the present study. Mice were either administered a low dose (10^5 iVLPs/dose), a high dose (10^6 iVLPs/dose), supernatant of cells transfected with all constructs except the glycoprotein encoding plasmid, or PBS. The two latter groups constituted the negative controls. The iVLPs induced strong virus-neutralizing antibody responses in mice after three subsequent immunizations (Fig 10).

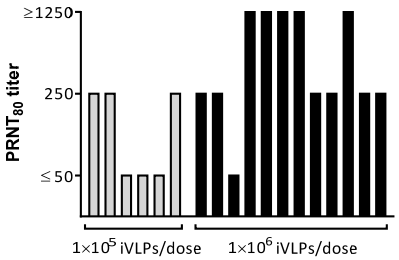

Figure 10. *Virus-neutralizing antibody titers in mice vaccinated with infectious virus-like particles (iVLPs). Mice were vaccinated three times two weeks apart with $1x10^5$ iVLPs/dose (n=6) or $1x10^6$ iVLPs/dose (n=12). Two weeks after the third and last immunization, the virus-neutralizing antibody titers were determined by plaque reduction neutralization test ($PRNT_{80}$).*

We were unable to house the animals for a longer period due to technical reasons, hence the durability of the iVLP-induced immune responses were not evaluated. However, a more extensive study using RVF VLPs showed that high titers of virus-neutralizing antibodies could be detected 161 days after the third and last immunization [251], indicating the robustness and durability of VLP-induced immune responses.

Two weeks after the third immunization the animals were challenged with RVFV. The survival proportions were significantly higher in mice vaccinated with the high dose compared to control animals, 92% and 8%, respectively, whereas only 50% of the animals in the low-dose group were protected. Animals with a virus-neutralizing titer of 250 or above (Fig. 10) were protected from a lethal challenge with 10^4 infectious particles, pointing to the importance of neutralizing antibodies in protection against RVFV infection. Another study compared the efficacy of VLPs containing N protein to those lacking N protein and it was found that 56% of the mice survived challenge compared to 19% in the latter group [251]. These results show that protection against RVFV infection is dependent on the immune responses raised towards the glycoproteins, but is enhanced by the presence of N protein. Indeed, when Pichlmair *et al*. modified the iVLPs used in our study [293] so that the minireplicon expressed N protein instead of Renilla-Luciferase, they observed complete protection in mice after a single vaccine dose [252]. More recently, a modified version of VLPs was produced [338]. These particles, called viral replicon particles (VRPs), resembles VLPs in that they are unable to spread within the immunized host, but they have some features in common with recombinant viruses as they are able to replicate and synthesis N and L protein [338]. VRPs are produced by transfection of plasmids encoding the L and S genome segments (fully deleted NSs gene), in cell lines constitutively expressing the glycoproteins [338]. The VRPs were found to be more immunogenic and conferred

better protection against infection compared to irradiated VRPs (non-replicating) [339].

Since RVFV repeatedly has shown the ability to cross international and geographical borders, differentiating infected from vaccinated animals (DIVA) is essential for adopting RVFV vaccination programs in non-endemic areas. Hence, a DIVA-compatible vaccine platform is of high interest. Vaccination with iVLPs did not generate detectable anti-N antibody levels and since most novel ELISAs are based on recombinant N protein [320, 322], these already validated and commercially available assays would be useful in differentiating infected from iVLP vaccinated individuals.

The fact that iVLP-vaccination did not induce detectable levels of anti-N antibodies was used in the post challenge analysis combined with RT-PCR analysis on the viral load in blood. In comparison to the unvaccinated controls, mice vaccinated with iVLPs had lower anti-N antibody titers and less circulating virus after infection. The immune responses induced by vaccination suppressed viral replication, an event which was more pronounced in the high-dose group. Similarly, de Boer *et al.* reported that immunization with VLPs produced by transient expression of RVFV glycoproteins in insect cells induced sterile immunity in a proportion of the animals [253].

Mandell *et al.* showed the promising aspects of chimeric RVF VLPs [251]. The chimeric VLPs were produced by transient transfection of cells, which constitutively express Moloney murine leukemia virus gag protein, with expression vectors encoding the glycoproteins and the N protein [251]. Inclusion of retroviral gag is suggested to increase the stability of VLPs [340]. The chimeric VLPs were found to be more efficient in mice than VLPs exclusively composed of RVFV components and they conferred protection in 100% of the rats challenged 67 days after the third and last immunization [251].

Paper IV was the first report describing the potential of RVF VLP-based vaccines, and more recent studies have all verified that iVLPs, or variants thereof, might be good alternatives to the available vaccines [251-253]. However, studies in larger mammalians are needed before the true prospects of RVF VLPs are revealed.

9.5 IMMUNOGENICITY OF PLANT-DERIVED SUBUNIT VACCINES (PAPER V)

The reuse of needles resulted in unnecessary spread of RVFV during a vaccination campaign in livestock in northeast Africa at times when virulent virus was circulating [341]. Clearly, such vaccination practices have counterproductive effects. Needle-free immunizations would be ideal in those situations, when vaccine has to be distributed during ongoing outbreaks and to large groups, both for the safety of the veterinarian administering the vaccine as well as for the recipient animal [342].

In Paper V we produced transgenic *Arabidopsis thaliana* plants. The encoding sequences of the N protein and a truncated Gn protein (denoted tGn) were integrated into the plant genome by *Agrobacterium*-mediated gene transfer (Fig. 11). These transgenic plants were given to mice and evaluated for their immunogenicity after oral intake.

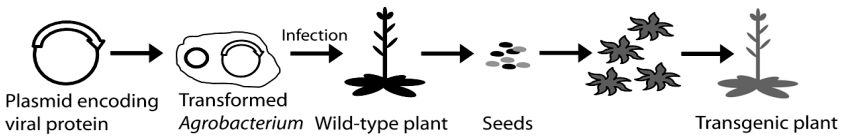

Figure 11. *An illustration of the production of transgenic Arabidopsis thaliana.*

The RVFV antigens chosen for this study were the N protein, due to reasons previously stated in section 9.3, and the Gn protein, since antibodies to Gn alone have been shown to be sufficient for neutralizing virus infectivity [197, 299, 330]. The N protein accumulated to high extent in tissue (root, stem, and leaf) of transgenic plants despite that our constructs were not codon-optimized for expression in plant systems. The glycoproteins contain transmembrane regions [139] and efficient production of recombinant membrane proteins is known to be difficult to achieve [343]. For that reason, our construct was designed to contain a part of the Gn ectodomain, harboring three epitopes that have shown to bind important virus-neutralizing antibodies [298]. Immunization with comparable Gn subunits expressed in bacteria has been shown to induce low levels of virus-neutralizing antibodies in mice and confer protection against lethal infection [248, 299]. Similar results were observed using an insect-derived Gn ectodomain [253]. Even though we were unable to detect protein expression in tGn transgenic plant lines by Western blotting, a proportion of the mice that were fed tGn transgenic plant material seroconverted after three feedings, as did mice fed N transgenic plants (Fig. 12).

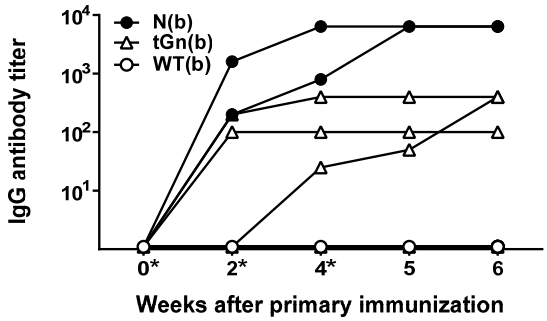

Figure 12. *Specific antibody responses in mice orally immunized with transgenic plants. Groups of mice were fed plants expressing the N protein (N), plants positive for tGn specific mRNA (tGn), or wild-type plans (WT). Each curve corresponds to one animal. The asterisks under the x-axis indicate weeks when immunization was performed.*

Despite that the N protein is highly immunogenic, oral intake resulted in relatively low antibody titers (10^3-10^4). Roughly estimated, each group of four animals had access to 60-80 µg plant-derived N protein during the free feeding procedure. In comparison, 15 µg bacterial expressed and purified recombinant N protein delivered once by the subcutaneous route induced antibody titers ranging from 10^4 to 10^6 in all mice (unpublished data). Since the source of the N protein differ between the two experiments the data is not directly comparable, but it indicates the limited antibody response generated towards orally delivered N protein. Similarly, plant-generated Puumala virus (*Hantavirus*, *Bunyaviridae* family) N protein was immunogenic in rabbits when delivered intramuscularly and intraperitoneally [344], but not when delivered orally to mice [345]. The poor immunogenicity of the transgenic plants, as indicated by low antibody titers and lack of virus-neutralizing antibodies (mice fed tGn transgenic plants), might be improved by modifying the delivery mode, adjusting the dose, and by determining the most favorable frequency of which booster doses should be distributed.

The intestinal immune system is the largest part of the immune system and in many ways the most complex. It has the ability to classify the massive amount of antigens daily passing as either harmful or harmless. Induction of a local and systemic immunological tolerance (oral tolerance) is the result of a harmless classification (*e.g.* antigen generated by food), while harmful antigens stimulates an immune response [346]. It is possible to induce both mucosal and systemic immune responses after oral vaccination [347], whereas parenteral vaccines generally are ineffective against mucosal infections [346]. The indirect evidence of aerosol transmission to humans (see section 2.3.3) and records of horizontal transmission in animals [99], illustrate that the ability to induce mucosal immunity might be an important characteristic for future RVFV vaccines. Formalin-inactivated RVFV vaccine administered subcutaneously was unable to protect mice against aerosol infection [348]. Yet another study was undertaken to determine if the TSI-GSD-200 vaccine, used to vaccinate at risk personnel against contact and aerosol infection, could protect rats against aerosolized RVFV. The rats were given the vaccine subcutaneously according to the immunization schedule used in humans and six months later, 68% of the rats survived challenge, whereas the survival proportion was 3% in unvaccinated controls [80]. After intramuscular vaccination with live attenuated virus vaccine (MP12), rhesus macaques (used extensively as a surrogate model for RVF in humans [180, 195]) were protected against a small aerosol infection [79]. Recently, Morrill and Peters addressed the question of oral immunizations and evaluated if mucosal immunization (aerosol, intranasal drops, or orally) using the MP12 vaccine candidate protected against aerosol virus challenge in rhesus macaques [267]. All animals given aerosol or intranasal vaccine developed virus-neutralizing antibodies, whereas only two out of four animals given oral instillation seroconverted [267]. These results indicate that it is possible to protect animals against aerosol infection using the traditional RVFV vaccine strategies.

Taken together, the production of RVFV antigens in plants for the purpose of producing a highly immunogenic edible vaccine may not be feasible. In addition, there are a few practical obstacles for such a vaccine delivery, the primary and maybe most important being the dose. It is almost impossible to ensure that the receiver get the exact amount of antigen by this approach. Additionally, the frequency of vaccine intake has to be regulated otherwise raising the possibility of oral tolerance [349]. It is more realistic to think of plants as factories for antigens that later are injected. Production of antigens in plants is cost-efficient [350] and for that reason is a plant-based production platform for RVFV vaccines of high interest.

The animal research included within this thesis was carried out in strict accordance with the provisions and general guidelines of the Swedish Animal Welfare Agency and the Ministry of Agriculture in Mozambique. The protocols were approved by the Committee on the Ethics of Animal Experiments (Umeå or Stockholm north) or the National Directorate of Veterinary Services in Maputo, Mozambique. All procedures using viable RVFV were carried out in BSL-3 containment laboratories.

10 CONCLUDING REMARKS

The development and effective distribution of safe and efficacious vaccines are the most realistic hope to limit the impact RVFV has on animal and human health. During recent years, the efforts from the scientific community to evaluate new vaccine strategies have been extensive. From a vaccine-development perspective, RVFV has several advantageous characteristics *i)* the close antigenic relationship between natural isolates, and between natural isolates and laboratory adapted strains, *ii)* virus-neutralizing antibodies seem to be sufficient to protect against infection, and *iii)* if a robust immune response is generated, protection is considered life-long. Thus, it appears to be a straightforward process to develop a RVFV vaccine. In spite of all efforts, only one new vaccine (Clone13) has so far been implemented as an alternative to the Smithburn attenuated and formalin-inactivated virus vaccines.

There are several important aspects to take into consideration *i)* the broad range of mammalian hosts to vaccinate, *ii)* the use of replicating viruses in non-endemic areas is not optimal because of RVFV's ability to maintain in newly conquered habitats, *iii)* the adverse effects often observed in live-attenuated virus vaccine preparations, and *iv)* RVFV circulates in resource-poor areas and in regions where it might be complicated to maintain cold-chains. Thus, the route to an effective and safe RVFV vaccine is more complex than appeared at a first glance. It might be necessary to have vaccines adapted for different species and for different areas and there are several promising RVFV vaccine candidates available for all of those applications.

To further complicate the decision on what candidate to bet on, different vaccine candidates have been evaluated in different animal species. Hence, direct comparisons between the candidates based on the available studies are almost impossible. A number of different animal models are available for RVF [66] and it would be beneficial if the scientific community could come to a consensus on which animals to use in future vaccine studies (especially during the early trials) and the appropriate methods for evaluating such comparable studies.

Of the three candidates we have evaluated, the most promising vaccine strategy was RVF iVLPs. Several VLP-based vaccines are in human clinical trials or are approved by the U.S. Food and Drug Administration including those for hepatitis B virus [351] and human papilloma virus [352, 353]. VLP-based vaccines also have important agricultural applications as can be exemplified by the available candidates for livestock diseases such as bluetongue [354] and foot-and-mouth disease [355, 356]. The most pronounced limitations for VLP-based vaccines are the complicated and costly production. It is therefore quite unlikely that VLP-based vaccines will be used to prevent RVF in herds of livestock over the next few years, even if the VLP-vaccine platform shows potency in larger mammalians. Until more cost-, time-, and labor-efficient production strategies are at hand the most realistic use for RVF VLPs are to vaccinate individuals at risk.

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