Rift Valley Fever - Development of diagnostics and vaccines.

Jonas Näslund
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Abstract

Rift Valley Fever virus (RVFV) causes an infection with severe impact on animal and human health. The disease is endemic throughout almost the entire African continent and large regions of the Arabian Peninsula. During epidemics, high mortality is observed in animals, especially among cattle, goats, and sheep. In humans, the symptoms vary from a benign influenza-like disease to a life-threatening hemorrhagic fever. Due to the devastating effect on communities in endemic regions and the possibility of further spread of this virus, there is an imperative need to improve and develop control measurements against this emerging disease. Therefore, this thesis focuses on diagnostics and vaccines against RVFV.

RVFV infection kinetics was studied in a mouse model system by detection and quantification of viral genomes, using a developed quantitative real-time PCR (QRT-PCR) method. This novel QRT-PCR method proved to be reliable and serves as a supplement to standard diagnostics, direct virus isolation and serological methods. High levels of viral RNA were found in blood and liver samples from experimentally infected mice during the first days post infection. Thereafter the levels declined rapidly and dropped below detection limit approximately seven days post infection. The QRT-PCR technique was also used in a study aimed to improve diagnosis of RVFV from field samples collected on filter strips.

Today, the available RVFV vaccines are only approved for animal use and these vaccines have several shortcomings. Since RVFV is a highly pathogenic organism requiring bio-safety level 3 laboratories, two different none-replicating vaccine approaches have been applied and evaluated using a mouse model. A DNA based vaccine, administered via gene-gun, and the use of virus-like particles (VLP), by the intra-peritoneal route. RVFV specific and neutralising antibodies were raised with both vaccine approaches. However, VLP vaccination against Rift valley Fever proved to be more promising as a future vaccine, since higher titres of neutralising antibodies and improved survival rate were found upon a lethal RVFV challenge in mice.

In conclusion, a sensitive and specific method for quantifying RVFV infection and a promising vaccine candidate against RVFV were developed.
List of papers

This thesis is based on the following papers, which are referred to in text by their roman numerals.


### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>BSL</td>
<td>bio-safety level</td>
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<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>G$_N$/G$_C$</td>
<td>Glycoprotein, N-/C-terminal of the precursor</td>
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<tr>
<td>HBsAg</td>
<td>Hepatitis B surface antigen</td>
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<tr>
<td>IFA</td>
<td>Immunofluorescence assay</td>
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<tr>
<td>ICTV</td>
<td>International Committee on Taxonomy of Viruses</td>
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<tr>
<td>kDa</td>
<td>Kilo dalton</td>
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<tr>
<td>N</td>
<td>Nucleocapsid</td>
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<td>NSm</td>
<td>Non structural m</td>
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<tr>
<td>NSs</td>
<td>Non structural s</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PKR</td>
<td>Protein kinase R</td>
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<tr>
<td>PRNT</td>
<td>Plaque reduction neutralization test</td>
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<tr>
<td>PUUV</td>
<td>Puumala virus</td>
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<tr>
<td>QRT-PCR</td>
<td>Quantitative real-time polymerase chain reaction</td>
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<tr>
<td>RdRp</td>
<td>RNA dependent RNA polymerase</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<td>RNP</td>
<td>Ribonucleoprotein</td>
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<td>RVF</td>
<td>Rift Valley Fever</td>
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<td>RVFV</td>
<td>Rift Valley Fever virus</td>
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<tr>
<td>SAP-30</td>
<td>Sin3A-Associated Protein 30</td>
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<tr>
<td>VLP</td>
<td>Virus-like particles</td>
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<td>WHO</td>
<td>World Health Organization</td>
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Introduction

Rift Valley Fever (RVF) is a zoonotic viral disease causing outbreaks on a regular basis in Africa, often with a devastating impact on the socio-economic situation (Meegan et al., 1979, Digoutte & Peters, 1989, Gerdes, 2002). These epidemics and epizootics are often preceded by heavy rain falls and flooding, which create good breeding conditions for mosquitoes, the main vector of this virus. RVF has a very broad host range, with symptoms ranging from benign to severe disease among the different susceptible species (Gerdes, 2004, Metwally, 2008). The virus can cause severe hemorrhagic fever in humans and is therefore considered as a high risk pathogen that requires BSL 3 (biosafety level) conditions for handling and manipulations. Rift Valley Fever virus (RVFV), like other members of the viral hemorrhagic fever group, is redeemed as a possible agent for bioterrorism (Sidwell & Smee, 2003). For this reason RVFV is listed as a bio terror agent by the “Centre for Disease Control and Prevention” and is classified as a “category A” high priority pathogen, by National Institute for Allergy and Infectious Diseases. Indeed, there have been intentions to weaponize RVFV, undertaken by the United States before their offensive biological warfare program was closed in 1969 (Bouloy & Flick, 2009).
**Bunyaviridae family**

The etiological agent responsible for this disease was isolated during an outbreak in 1930 in Rift Valley, Kenya, hence the name, Rift Valley Fever virus (Daubney *et al.*, 1931). RVFV belongs to the Phlebovirus genus within the *Bunyaviridae* family (Fig.1). This family consist of more than 300 viruses divided in to five genera; Hantavirus, Nairovirus, Orthobunyavirus, Phlebovirus, and Tospovirus, according to the “International Committee on Taxonomy of Viruses” (ICTV). Viruses included in this family are distributed all over the world and cause a variety of diseases in animals, insects, and plants. Most of them are arboviruses, i.e. transmitted by arthropods, except for the Hantaviruses that are transmitted by rodents. Several members of the *Bunyaviridae* family can cause disease in humans resulting in different symptoms, ranging from more benign illnesses to severe hemorrhagic fevers (Elliot, 1996, Swanepoel, 2009).

![Fig.1. Schematic overview of some selected viruses of the *Bunyaviridae* family.](image)

*Fig.1. Schematic overview of some selected viruses of the *Bunyaviridae* family.*
The virus particle

Like other members of the *Bunyaviridae* family the RVFV particle is small (80-120nm), enveloped and spherical in shape (Martin *et al.*, 1985, Elliot, 1996). The virion has been believed to have a pleomorphic shape, but more recent findings indicates a icosahedral structure (Freiberg *et al.*, 2008). The particle encloses three segmented negatively charged single stranded RNA genomes (Fig.2), tightly associated with the RNA dependent RNA polymerase (RdRp) and the nucleocapsid protein (N) subunits, that encapsidate and protect the RNA genomes (Elliot, 1996, Swanepoel, 2009). This ribonucleoprotein (RNP) complex has a helical shape surrounded by a lipid envelope of cellular origin with two viral encoded glycoproteins (G_N and G_C) protruding the surface (Sherman *et al.*, 2009), supposedly in a heterodimer formation (Gerrard & Nichol, 2007, Huiskonen *et al.*, 2009). The cytoplasmic tail of the glycoproteins is suggested to anchor the RNP complex (Overby *et al.*, 2007), and thereby mediates assembly of the particle (Shi *et al.*, 2007, Liu *et al.*, 2008, Sherman *et al.*, 2009). G_N and G_C are also responsible for the binding and uptake of the virions into the host cells upon infection, possibly by a class II fusion mechanism (Garry & Garry, 2004, Filone *et al.*, 2006, Liu *et al.*, 2008).
Fig.2. Schematic picture of a Rift Valley Fever virus particle, kindly provided by Nina Lagerqvist.

The genome

The three individual RNA genomes are denoted after their size small (S), medium (M), and large (L), and translates in the cytoplasm of the infected cells into unique polypeptides. The S-segment encodes, in an ambisense manner, the N protein and a non-structural protein (NSs). The M-segment codes for two glycoproteins (G_N and G_C) and a non-structural protein called NSm, yet there is also evidence of a 78 kDa protein. The L-segment codes for the RNA-dependent RNA polymerase (RdRp) (Elliot, 1996, Swanepoel, 2009).

The presence of genes encoding the non-structural proteins (NSs and NSm) varies between different viruses of the Bunyaviridae family. In the case of RVFV both genes are found. The NSs protein of RVFV is the major virulence factor and defects in the NSs gene leads often to a reduction in virulence. The NSs protein has been shown to inhibit the host immune system, both in general and specifically, to promote viral replication (Bouloy et al., 2001,
Madani et al., 2003, Billecocq et al., 2004, Le May et al., 2004, Le May et al., 2008, Habjan et al., 2009b, Ikegami et al., 2009a, Ikegami et al., 2009b). The more general inhibition occurs by the assembly of a filamentous structure in the nuclei that sequesters subunits of RNA polymerase II, and thereby interacts directly with the DNA (Bouloy et al., 2001, Le May et al., 2004, Mansuroglu et al., 2009). The NSs protein specifically targets activation of the interferon β promoter through an interaction with the Sin3A-Associated Protein 30 (SAP30), a suppressor of the promoter (Le May et al., 2008). To promote viral replication, NSs down-regulates protein kinase R (PKR), a host response regulator that interfere with the initiation of translation (Habjan et al., 2009b, Ikegami et al., 2009a, Ikegami et al., 2009b). The function of NSm is not yet fully understood, however, it is known that this protein disrupts apoptotic pathways of the cell (Won et al., 2007).
Transmission

The main route of RVFV infection is through haematophagous insects, including mosquitoes, ticks and sand fly species (Fontenille et al., 1998, Flick & Bouloy, 2005). Mosquitoes from different genera (*Aedes, Anopheles, Culex, Eretmapoites* and *Mansonina*) are known to act as vectors (Fig.3) (Flick & Bouloy, 2005). The *Aedes* species are especially important since RVFV is capable of transovarian transmission in *Aedes vexans* and *Aedes lineatopennis* (Linthicum et al., 1985, Zeller et al., 1997, Chevalier et al., 2009). The virus survives inside the mosquito eggs and spreads vertically through the offspring straight after hatching. This mechanism is believed to maintain the virus in the environment between outbreaks. This theory could also explain the explosion-like progress of some outbreaks that seems to start simultaneously all over a wide area.

![Aedes aegypti mosquito. Wikipedia Commons, public domain, Original author: US Department of Agriculture.](image)

Furthermore, there are assumptions of cryptic hosts for RVFV, harbouring the virus between outbreaks, where rats (Namaqua rock rat and *Rattus rattus*) or bats are the main suspects (Boiro et al., 1987, Pretorius et al., 1997, Youssef & Donia, 2002).
Although mosquitoes are the main vector of the virus, other routes of transmission can take place. Inhalation of infectious particles by handling infected animals or via body fluids may also lead to an infection by RVFV (Brown *et al.*, 1981, Sidwell & Smee, 2003, Bossi *et al.*, 2004, Gerdes, 2004). An interesting suggestion is that the main route for human transmission may not be through mosquito bites but rather direct contact with infected animals or body fluids (Flick & Bouloy, 2005, Swanepoel, 2009). The higher seroprevalence seen among abattoir workers in South Africa, in contrast to the general population, together with the fact that veterinarians, abattoir workers and farmers are high risk occupations favours this route of viral transmission (Gerdes, 2002, Flick & Bouloy, 2005). RVFV can also be transmitted through inhalation of aerosolised particles, hence increasing the risk for using RVFV in bioterrorism (Francis & Magill, 1935, Sidwell & Smee, 2003).
Outbreaks

Since the first documented outbreak in Kenya in 1930 (Daubney et al., 1931), several have followed. The first recorded outbreak of RVF in South Africa, 1950-51, resulted in an estimated 100,000 dead sheep and an additional 500,000 aborted foetuses (Gerdes, 2002, Swanepoel, 2009). Before 1977, RVF was considered to be an exclusive animal disease, affecting mainly domesticated animals with a few recorded exceptions of human deaths. However, that view changed soon after the large outbreak in Egypt, that recorded 623 human deaths and between 18,000-200,000 infected persons (Meegan et al., 1979, Meegan, 1979, Abd el-Rahim et al., 1999). This was also the first time RVFV provoked disease outside the sub Saharan region of Africa. Today the virus has spread almost all over the African continent and into the Arabian Peninsula with Saudi Arabia and Yemen as new endemic regions (Shoemaker et al., 2002, Balkhy & Memish, 2003, Gerdes, 2004). Over the years, several human RVF epidemics have taken place. Mauritania, 1987, with 89,000 infected and 224 confirmed human fatal cases, Kenya, 1997-98, 27,000 infected and 170 deaths, Arabian Peninsula, 2000, with 245 confirmed deaths, and the recent outbreak in Kenya, Somalia, and Tanzania, 2006-07, 1,088 infected and 323 deaths (Digoutte & Peters, 1989, Woods et al., 2002, Al-Hazmi et al., 2003, Alrajhi et al., 2004, WHO, 2007).
Predictions of outbreaks

Epidemics and epizootics of RVF are closely linked to the climate and its variations. Especially, heavy rainfalls with stagnant water are closely associated with RVF outbreaks since flooding results in good breeding ground for the mosquito vectors (Davies et al., 1985, Anyamba et al., 2009). It is therefore possible to predict outbreaks and with good prediction models up to five months of preparations could be achieved (Linthicum et al., 1999). The El Nino phenomena is another factor that directly influence the weather and thereby the presence of RVFV. Hence, weather forecasts could therefore be a good way to predict outbreaks of this disease (Harvell et al., 2002, Anyamba et al., 2009).

RVF is an emerging disease with the potential to spread and become endemic in new regions of the world. In the case of RVF, this risk is elevated by the fact that RVFV is capable of using several host and arthropod species capable as reservoirs and vectors for the transmission, respectively (Gerdes, 2004). Several geographical regions around the world have been predicted to be high risk areas for establishment of RVFV, e.g. the river deltas of the Euphrates, Tigris and Indus River (Gerdes, 2004). Additionally, mosquitoes capable to hold and spread this virus are found around the Mediterranean basin, as well in North America (Moutailler et al., 2008, Turell et al., 2008).
**Pathogenesis**

The general idea of the kinetics of RVFV infections is that the virus first replicates in the lymph nodes close to the site of entry. Thereafter, spreads further using the bloodstream to the primary organs of virus replication, e.g. liver, spleen and brain, hence RVFV is able to pass through the blood-brain barrier. The virus replicates rapidly in these organs to high titers and causes viremia, which may lead to further spread of the virus by a second biting mosquito and thereby closing the circle of virus transmission. RVFV preferably replicates in hepatocytes of the liver and the necrotic livers often observed in severe cases are probably caused directly by the cytopathic virus amplification (Anderson *et al.*, 1987, Flick & Bouloy, 2005). On the other hand the meningoencephalitis and retinitis frequently observed during RVFV outbreaks are suggested to be associated with the immune response of the host (Laughlin *et al.*, 1979, Siam *et al.*, 1980, Gear, 1988, Gonzales-Scarano, 1996). RVFV cause abortions in cattle and the virus is present in the maternal placenta and foetal organs (Flick & Bouloy, 2005). Still, there is no clear evidence for abortions among humans, but vertical transmission of the virus is suspected (Arishi *et al.*, 2006).
Clinical picture

In animals, a broad host range of susceptible species develops RVF and the symptoms vary significantly. Less severe clinical signs, as nasal discharges and diarrhoea, to more severe ones, such as high fever, respiratory distress, haemorrhage and jaundice, are observed during RVFV outbreaks (Coetzer, 1977, Coetzer, 1982). A more severe form of the disease is found in cattle, goats and sheep where the case fatality rate varies between 10-30% and 40-100% for adult or young animals, respectively (Gerdes, 2002, Flick & Bouloy, 2005). A typical indication of RVF-infection in these species is sudden deaths among young animals, less than two weeks of age, and abortions. An “abortion storm” may involve the complete herd and almost all pregnant animals abort their foetuses (Abdel-Wahab et al., 1978). Other animals known to develop a more severe form of the disease (abortions, viremia and high mortality) are buffaloes, camels, hamsters, mice, monkeys, and rats. In contrast, RVFV cause less severe disease in cats, dogs, and horses to mention a few (Gerdes, 2004, Metwally, 2008). Likewise, common to all species is that the clinical picture and prognosis are worse for younger animals.

In humans, mild influenza-like symptoms are mostly observed upon infection, like fever, headache, nausea, and myalgia. Yet, more severe symptoms are frequently observed during outbreaks, such as retinitis, meningoencephalitis and hemorrhagic fever (Madani et al., 2003). The effect on the eye upon retinitis is mostly reversible with a complete recovery, however, some patients end up with seriously reduced sight or blindness as a
complication after a RVFV infection (Al-Hazmi et al., 2005). Hemorrhagic fever is the worst outcome of infection with a case fatality rate close to 50%. The onset of bleeding usually starts after the first week of illness with signs of persistent bleeding from needle wounds in the patients (Gear, 1988). The extent of bleedings varies from small petechial rashes on arms or legs to gastrointestinal bleeding (Isaacson, 2001). Patients with meningioencephalitis are also encountered during and after outbreaks and, the incubation time is usually between two and three weeks for this manifestation (Gonzales-Scarano, 1996). Common symptoms are severe headache, meningismus, and coma. Most patients recover but there may be residual brain damage and even fatal cases have been observed (McIntosh et al., 1980, Al-Hazmi et al., 2003). Although, the overall case fatality rate for this disease has been below 1%, a drastic increase have been reported recently with case fatality rates of 45% in the Somalia outbreak, 2006, 21% in Kenya, 2007, and 36% in Tanzania, 2007 (WHO, 2007). This recent increase is at the moment unexplained, however, several theories such as circulation of a new and more aggressive virus strain, change in diagnostic demands, or large numbers of unrecorded cases, are possible explanations.
Diagnostics

Even though heavy rainfall, high density of mosquitoes, and abortions might indicate RVFV transmission, laboratory confirmation must be performed. Specimen from blood, liver, spleen, or brain should be collected and analysed, either by isolation of the virus or antigens thereof, or by demonstrating the presence of RVFV specific antibodies.

Detection of virus or antigens

The golden standard of RVFV diagnostics is direct virus isolation. For that purpose blood, liver, or spleen samples are used for inoculation. Nowadays this test is performed in cell cultures, even though hamsters or mice are frequently used (Anderson et al., 1989, Digoutte et al., 1989, Flick & Bouloy, 2005). In addition, viral antigens can be readily detected from blood and tissue samples by capture ELISA (Enzyme-linked immunoassays), Complement fixation, gel diffusion test, immunofluorescence assays (IFA), or immunohistochemical assays (Swanepoel, 2009). These methods are efficient and reliable but time consuming and sometimes difficult to perform.

Detection of RVFV specific antibodies

Demonstrating the presence, or rising titers, of IgG or IgM antibodies specific for RVFV in serum samples are widely used in laboratories for the confirmation of a RVFV infection. Methods used are; complement fixation,
ELISA, IFA, haemagglutination inhibition tests, and plaque reduction neutralisation tests (Niklasson *et al.*, 1984, Swanepoel *et al.*, 1986, Pawska *et al.*, 2003, Swanepoel, 2009). However, it is important to note that specific antibodies are absent during the first days of infection.

**Treatment**

At the moment there exists no specific treatment for RVF. The therapy given is of supportive character and focuses mainly on the symptoms, i.e. administration of blood and coagulation factors in the case of hemorrhagic fever. The antiviral drug Ribavirin is a candidate for treatment, since it has shown to lower the viral replication in cell cultures and animal models. Also, plasma from recovered patients may be useful (Kende *et al.*, 1985, Peters *et al.*, 1986).
Viral Vaccines

The vaccine era started in 1796 when Dr. Edward Jenner, via human experiments, substantiated that inoculation of samples containing cowpox virus lead to cross protection against smallpox. Jenner based that conclusion on an old knowledge that inoculation of pus from smallpox patients could lead to protection from the corresponding disease and on the fact that cowpox infected people did not acquire smallpox. Later, Louis Pasteur took the vaccination trials even further when he succeeded to produce a chicken cholera vaccine by chemical or thermal attenuation of the infecting agent and some years later he produced a rabies vaccine. Modern vaccine science started sometime during the middle of the 20th century, when vaccines against several different diseases were introduced, e.g. measles, mumps, polio, rubella and Yellow fever. This progress in the history of vaccination came as a result of developments and establishments of new molecular tools; cell culture methods, bacterial chemistry, microbiology, and immunology. Since then, several techniques and methods to develop vaccines against a variety of different pathogens and diseases have been established. Nowadays, the list of vaccines grows each year and weakens the burden of these diseases (Plotkin, 2004b).
Traditional vaccine strategies

Over the years, killed, inactivated or live attenuated, and subunit based vaccines, are some approaches that have been used against viral diseases with success. Inactivation or killing of the causative agent through various treatments has produced vaccines against polio and hepatitis A (Salk, 1953, Peetermans, 1992). By lowering or abolishing the virulence through attenuation or, adaptation, of the microorganism to another host or tissue has lead to the development of vaccines e.g. measles, polio, and yellow fever (Katz et al., 1960, Sabin et al., 1960, Plotkin, 2004a). Another approach is subunit vaccines, where one or more proteins of the microorganism are used as a vaccine. As an example, the hepatitis B vaccine consists of only one component, the hepatitis B surface antigen (HBsAg). This protective HBsAg is an envelope protein that is produced in high amounts in patients during an infection (Blumberg et al., 1967, Hilleman et al., 1975, Zuckerman, 2006).

Novel vaccine approaches

The improvement of molecular techniques over the years has made it more and more possible to manipulate and genetically engineer microorganisms to produce vaccine candidates. Induction of the immune response towards viral proteins or peptides by the use of viral vectors for antigen delivery holds a huge possibility. Several different viruses and constructs thereof are available as vectors, e.g. adeno, herpes simplex, retroviruses, and vaccinia virus (Paoletti, 1996, Tatsis & Ertl, 2004, Marconi et al., 2008). These
vectors can be altered in several ways; to become replication incompetent or to, get altered tropism, stability, and expression levels of the construct (Bouard et al., 2009). Also, using genetic systems (mini genomes, reverse genetic systems) to produce tailor engineered progeny of infectious agents for vaccine purposes are very promising strategies. Indeed, for example the first reverse genetic system for the *Bunyaviridae* family was established in 1996 and for RVFV in 2005 (Bridgen & Elliott, 1996, Ikegami et al., 2005).

**DNA vaccines**

Vaccine development took a big leap in 1990 with the discovery that naked plasmid DNA injected intra-muscularly in mice induced expression of genetic material (Wolff et al., 1990). The vaccine properties of this strategy were shown in 1993, when protective immunity was conferred to mice in an influenza A study (Montgomery et al., 1993). This way to induce the immune response mimics a real infection of an intracellular microorganism perfectly. The gene product will be synthesised, altered and modified in the correct fashion by the host cell before it is presented to the immune system (Klinman et al., 1997). However, the DNA vaccine technique still waits for the “golden boy” to appear. In fact, when the first enthusiasm had settled several problems had emerged on the horizon that had to be unravelled. How to efficiently deliver the DNA, improving the immunogenicity, and the need for booster administrations are some issues that needs to be solved (Donnelly et al., 2005). Yet, there are three DNA vaccines approved for veterinarian usage; vaccine against West Nile in horses, Hematopoietic
necrosis virus in salmon, as well as melanoma in dogs (Traxler et al., 1999, Bergman et al., 2006, Martin et al., 2007).

Virus-like particles

The idea of using this approach is to produce particles that resemble the authentic virus by over expressing one or several of the structural proteins of the virus in question. Production and assembly of the proteins and particles occurs via the normal expression and secretion route by the host cell (Johnson & Chiu, 2000). Since these particles lack the virus genome, virus replication is not possible, and thereby VLPs are considered to be safer than live attenuated vaccines (Mandell et al., 2009). The particles resemble the “authentic” virus both by shape and conformation and the virus display the protein epitopes in a similar way as a “real” virus particle (Noad & Roy, 2003). Since the proteins are correctly folded and modified they trigger the immune response and confer protection towards the corresponding virulent virus (Paliard et al., 2000, Warfield et al., 2003, Garcea & Gissmann, 2004, Grgacic & Anderson, 2006).
Evaluation of vaccine efficacy

The general concept with vaccination is to induce a long lasting protective immunity in the recipient. The immune system can be divided into the innate and the adaptive immunity (Roitt, 2001), where the innate immunity is characterized as a rapid unspecific response without memory. In contrast, the adaptive immunity is specific and has an immunological memory, but requires some time for activation. The adaptive immunity can be further divided to a cellular and humoral immune response. Vaccination aims at the adaptive immune response to give rise to a specific immune response and an immunological memory.

An important part of a functional vaccine is the capacity to induce the humoral response and generate production of antibodies with neutralising activity. In general, high titers of neutralising antibodies are associated with protection (Plotkin, 2004a). Furthermore, a high level of protection can also be achieved through induction of the cell mediated immune response. Though, activation of the innate response should not be overlooked, especially important to present antigens for, and activate the adaptive response.

Herd immunity is an additional positive effect of vaccination programs. If a sufficient percentage of individuals in a herd are vaccinated (or naturally immunised) the spread of the disease will be limited and thereby also protect the unvaccinated. This is a very beneficial effect, since it protects individuals
that cannot be vaccinated due to different reasons, for instance, immune compromised people and newborns (Anderson & May, 1985, Plotkin, 2004a, Goncalves, 2008).
Vaccines against Rift Valley Fever

Since the discovery of RVFV in 1930 several different approaches for vaccine development have been attempted. The results have varied from none to full protection, but various side effects have been observed post vaccination (Bouloy & Flick, 2009, Ikegami & Makino, 2009).

One of the earliest vaccine candidates was the Smithburn vaccine strain, produced through several passages of the Entebbe strain in the brain of suckling mice before sufficient attenuation were achieved (Smithburn, 1949). This neuroadapted RVFV strain was capable of inducing protective immunity, but was teratogenic in cattle and sheep (Barnard, 1979, Coetzer & Barnard, 1977, Botros et al., 2006). Moreover, the risk of reversion to wild type has to be considered.

Another early vaccine attempt towards RVFV was formalin inactivation of the virus (Randall et al., 1962). Several different batches have been tested and applied over the years. The levels of neutralising antibodies and conferred protection upon vaccination with these variants are low. These kind of vaccines require multiple inoculations for induction, and annual boosters to maintain the immune response (Barnard & Botha, 1977, Barnard, 1979, Lubroth et al., 2007). However, the only available vaccine for use in humans is a formalin inactivated variant (TSI-GSD-200) (Pittman et al., 1999, Metwally, 2008). This vaccine is produced for veterinarians and other personnel at high risk for RVF. Indeed, similar to the animal variants, it
requires several administrations and annual boosters to maintain its effect (Pittman et al., 1999, Frank-Peterside, 2000).

Clone 13 is a naturally attenuated RVFV strain which was isolated from a benign case in the Central African Republic in 1987. This strain has been tested as a vaccine, with promising results (Vialat et al., 2000, Lubroth et al., 2007). The reason for attenuation of Clone 13 is a large in frame deletion in the NSs gene (Muller et al., 1995).

MP12, another candidate vaccine is the product after chemically induced mutagenesis of the ZH548 strain upon several passages in cell culture (Caplen et al., 1985). MP12 has shown promising results in livestock (Morrill et al., 1987, Morrill et al., 1991, Morrill et al., 1997a, Morrill et al., 1997b, Morrill & Peters, 2003), but foetal malformations have been observed as a complication (Hunter et al., 2002). Yet, This candidate has recently been tested in a human phase II clinical trial, where a 95% seroconversion rate, production of neutralising antibodies and no adverse effects was observed (Bouloy & Flick, 2009).

Furthermore, R556, a reassortant of Clone 13 and MP12 has been produced (S-segment of Clone 13, L and M segment of MP12) and tested as a vaccine. The results are again promising, no adverse effects, production of neutralising antibodies in sheep, and capable to confer protection in mice (Bouloy & Flick, 2009).
The establishment of a reverse genetic system for RVFV in 2005 (Ikegami et al., 2005) has lead to production of yet another vaccine candidate (Bird et al., 2008). This candidate has the NSs and NSm gene removed from the genome and is thereby attenuated. The strain grows readily in cell cultures and is therefore easy to produce. Results from rat immunization experiments indicate good vaccine properties; high titers of neutralising antibodies, no side effects and conferred protection from a lethal dose of wild type RVFV (Bird et al., 2008). Still, one problem with this candidate is the need for safety facilities during production and the possible reversion to wild type.
Aims

The overall aim of this thesis was to apply and evaluate different vaccine strategies for RVFV.

In order to evaluate our vaccine candidates we have established a mouse model system and developed diagnostic tools.
Results and discussion

The goal is to develop a vaccine towards RVFV that would be a suitable candidate for both human and veterinarian usage. An animal vaccine is very desirable, especially for livestock, which are highly susceptible and develop severe forms of RVF with high mortality and abortions. Lowering the circulation of this virus in animal populations might reduce the numbers of human infections since most human transmission occurs via direct contact with infected animals or body fluids. Removing amplification hosts of RVFV would also decrease the numbers of mosquitoes carrying the virus and thereby further limit transmission.

Development of an animal model and diagnostic tools

To be able to evaluate our vaccine candidates we have established an animal model. We chose to work with mice and have used two different mice strains, BALB/c and C57BL/6 (Paper I, III and IV). Mice are a lethal animal model for RVF and they develop a severe form of the disease, which makes them suitable for infection studies (Easterday, 1965, Flick & Bouloy, 2005). Mice are easy to handle and smaller than for instance, rats, otherwise also considered to be a good animal model for RVF (Anderson & Peters, 1988, Anderson et al., 1991, Bird et al., 2007). We have successfully infected and induced RVF in mice with different doses of the wild type strain ZH548, an Egyptian isolate (Paper I).
Quantitative real-time PCR

We had already established several serological assays (e.g. ELISA, IFA, PRNT and western blot) that could be used to evaluate our immunisation attempts. However, we wanted to be able to directly quantify the viral load, hence, we aimed to set up a QRT-PCR analysis (Paper I). At this time, two other publications regarding QRT-PCR for RVFV were found in public databases, both using probe based assays. On the contrary, they targeted different sites of the genome; the S-segment (NSs gene) and the M-segment (GC gene) (Garcia et al., 2001, Drosten et al., 2002). The idea with our QRT-PCR was to develop a cheap and broad assay. Therefore, we choose SYBR-green as fluorescing dye, cheaper than probes and not target specific. Furthermore, we designed the primers to, at least in theory, pick up many isolates of RVFV. We evaluated our QRT-PCR in several steps, first by using in vitro produced RNA at different dilutions, and second by titrated numbers of viruses added to various samples, e.g. serum, blood and homogenized organs. Upon optimization the QRT-PCR proved to be sensitive, reliable and accurate (Paper I).

Viral kinetics in Rift Valley fever

To further evaluate the possibility to use a QRT-PCR as a diagnostic tool in the case of RVF we infected mice and collected blood samples at several different time-points. In addition, we also included brain, kidney, liver, lung,
and spleen samples, since RVFV is known to replicate and cause extensive liver damage and a high level of viremia. A previous study in mice indicated high numbers of viable particles in those organs during the first days of infection (Brown et al., 1981). Interestingly, we could detect high levels of RVFV RNA in the blood of our mice during the first days of infection, proving the usefulness of a QRT-PCR for RVFV. We also observed high levels of viral RNA in brain and liver samples during this time, and lower levels from kidney, lung, and spleen. However, the initially high levels of RVFV RNA were followed by a harsh decline and at the end of the first week post infection none or very low levels of RNA were found.

Field sampling

The presence of high amounts of viral RNA in blood during the first week of infection led us to try filter paper strips, a novel sampling procedure for RVFV. We also included the Puumala virus (PUUV), a locally endemic hemorrhagic fever (Hantavirus, Bunyaviridae) (Juto et al., 1997, Olsson et al., 2003, Johansson et al., 2004, Pettersson et al., 2008). PUUV was included to test clinical samples of a hemorrhagic fever virus. Filter strips have been used earlier for detection of other viruses (Cassol et al., 1992, Prado et al., 2005). Our results indicated that this method is possible to use for PUUV but not for RVFV. We could easily detect PUUV RNA with PCR after weeks of storage, still, it was not possible to accurately quantify the RNA copy numbers. Even though we successfully eluted viable RVFV particles from filters stored up to 48 h, none or only very low levels of RNA
were detected by the PCR. The reason for this difference could be due to their lifecycles, as speculated in a previous study including three other Bunyaviruses; Crimean-Congo Hemorrhagic fever virus (Nairovirus), Hantaan virus (Hantavirus), and the Sandfly fever Sicilian virus (Phlebovirus) (Hardestam et al., 2007), have all been shown to differ in viability in wet conditions. The Crimean-Congo Hemorrhagic fever virus, which lifecycle resembles RVFV the most by being spread by ticks or by direct contact with patients and infected blood (Whitehouse, 2004, Ergonul, 2006), was found to be the least stable one (Hardestam et al., 2007). Hantaan virus on the other hand, which has a similar lifecycle as PUUV, tolerated these conditions better. Notable, Hardestam with colleagues observed no differences in tolerance towards dry conditions (Hardestam et al., 2007).
Development of new vaccines

Since the discovery of RVFV, several efforts to produce a vaccine have been launched (Bouloy & Flick, 2009, Ikegami & Makino, 2009). At the moment there exist two RVFV vaccines, however, for veterinarian usage only and either poorly immunogenic or accompanied by side effects. This renders the use of these two vaccines and since RVFV is considered as an emerging disease there is a huge need to develop practical and useful vaccines. RVFV is a highly pathogenic virus and no treatment is available at the moment. For these reasons we have applied and evaluated two none replicating vaccine candidates.

Genetic immunisation

In the first study, we attempted to induce protection against RVF in experimentally infected mice via cDNA immunisation (Paper III). We immunised mice with four different constructs, encoding different structural genes; the nucleocapsid (N) gene or the two glycoproteins G_N and G_C, separately or together.

We included the N gene based on the fact that this protein is very immunogenic and high levels of antibodies directed towards the N protein are observed after infection. In addition, there are indications that the N protein can induce protective immunity in mice, which has been documented both in previous study with RVFV, as well as in a study involving the closely related Toscana virus (Phlebovirus, Bunyaviridae) (Wallace et al., 2006,
Valentini et al., 2008). The authors speculate that the protection found might be through a cellular immune response. The N protein is an internal protein of the virion, and should therefore not induce production of neutralising antibodies. Our results upon immunising mice with the N gene substantiated the immunogenic properties of this protein. Antibodies specific for the N protein was observed already after the first immunisation and upon the final booster very high levels were reached (Paper III). We did also observe a 50% protection from symptoms upon a challenge with the wild type virus (Paper III). This protection, were not, as we expected, due to neutralising antibodies since we did not detected any after immunisation (Paper III). Instead, we observed a dose dependent proliferative response in spleen cells upon specific stimulation (Paper III). This cellular immune response could be responsible for the protection we observed, even though further evaluation is needed.

If the protection induced by the N protein was unexpected, the low level of protection and neutralising antibodies observed after immunisation with the glycoprotein constructs were equally unexpected. The G\textsubscript{N}/G\textsubscript{C} proteins are known to induce production of neutralising antibodies and have been shown to induce protective immune response, even though at various percentages (Schmaljohn et al., 1989, Besselaar & Blackburn, 1991, Besselaar & Blackburn, 1994). Another DNA vaccine study, using similar glycoprotein construct and gene-gun for administration succeeded to confer full protection in mice (Spik et al., 2006). On the contrary, two other studies using glycoprotein DNA immunisations have not conferred protection (Wallace et al., 2006, Lorenzo et al., 2008).
Interestingly, we noticed that the $G_N$ gene was a necessity for production of neutralising antibodies in our animal model. However, the levels of neutralisation detected were very low and the protection in mice did not extend the N gene immunisation (Paper III).

Yet, one important finding during this study was that the BALB/c mice were rather resilient against RVFV in general. This was probably due to the age of our mice, caused by the long immunisation schedule, as older animals in general are more resilient to RVFV infections (Gerdes, 2002). Thus, we have evaluated the induced protection based on signs of symptoms.
Virus like particles

The second approach tested was to use RVFV VLPs for immunisation (Paper IV), produced by Professor Friedemann Weber and co-workers at Freiburg University, Germany (Habjan et al., 2009a). Basically, the VLPs consist of four RVFV specific proteins, the two glycoproteins (G\textsubscript{N} and G\textsubscript{C}), the N protein and the RdRp. These VLPs resemble in shape and structure RVF virions, with the glycoproteins protruding the membrane envelope of cellular origin. This kind of vaccination strategy has been applied on several viruses, with good results (McAleer et al., 1984, Andre & Safary, 1987, Harro et al., 2001, Tacket et al., 2003, Cox et al., 2008, Perrone et al., 2009).

To evaluate the effectiveness of this RVFV VLP vaccine, we immunised groups of mice with two different doses at three time-points with two weeks intermission (Paper IV). We could, already after the second immunisation, detect antibodies specific for the glycoproteins of RVFV. Two weeks post the third and final booster, antibodies with neutralising capacity were observed in 14 out of 18 mice, titers ranging from 1:250 to 1:1250 (Paper IV).

To further investigate the protection induced by the RVFV VLPs, we challenged the immunised mice with lethal doses of RVFV. The result of this experiment is truly encouraging, since all 14 mice that developed neutralising antibodies survived the challenge with wild type virus, while the remaining four mice did not. In comparison, for the control group (administered PBS), only one out 12 mice survived. The 92.5% survival rate among mice that were given the high VLP dose clearly indicates the good
vaccine potential of the RVFV VLPs. Interestingly, another RVFV study, also using VLPs as a vaccine has shown equally promising result, 68 % and 100 % protection were observed in mice and rats upon challenge, respectively (Mandell et al., 2009). These results indicate a huge vaccine potential in this VLP approach for RVF.

The VLPs used for immunisation were not capable to induce antibodies specific for the N protein, even though small amounts of this immunogenic protein is included in the particles (Habjan et al., 2009a). The lack of N specific antibodies makes it really easy to distinguish infected animals from vaccinated ones. A so called marker vaccine (van Oirschot, 1999, Henderson, 2005), would be very beneficial for RVFV, since outbreaks results in restrictions in export of animals and related products.

Even though our result from the VLP vaccination is encouraging, several problems need to be addressed before such a vaccine can be available on the market. How to administer efficiently, produce sufficient amount and the need for boosters are some issues that need to be solved.
Future approaches

The promising results from our VLP vaccine study (Paper IV), and the knowledge that simultaneous expression of the glycoproteins together with the N protein is sufficient to produce RVFV VLPs (Liu et al., 2008, Habjan et al., 2009a, Mandell et al., 2009), might be useful to enhance genetic immunisation for RVFV. Introducing both the G\textsubscript{N}/G\textsubscript{C} and the N gene constructs to cells at the same time could result in production and budding of VLPs from host cells of the recipient and hopefully improving the induction of the immune response.

There are several ideas on how to improve vaccination strategies. One way could be by mixing two different approaches e.g. prime boost techniques (Woodland, 2004, Lu, 2009, Radosevic et al., 2009). By, priming with VLP immunisation and then boosting through genetic immunisation might be a good approach. This strategy could lower the costs since it is more expensive to produce VLPs than genetic vaccines. Another disadvantage is that VLPs need storage facilities and a cold chain for transportation, whereas DNA vaccines do not. Additionally, this would reduce the total amounts of VLPs needed to obtain a protective immune response.

At the moment, there are several vaccine candidates in the pipeline for RVFV (Bouloy & Flick, 2009, Ikegami & Makino, 2009). The recently established reverse genetic system (Ikegami et al., 2005) has lead to production of an attenuated RVFV strain (Bird et al., 2007). The complete NSs and NSm genes have been removed, rendering this candidate vaccine and highly
attenuated virus strain incapable to induce RVF in rats. This strain has shown promising results in rat immunisation experiments where it conferred a 100% protection from lethal challenge. Furthermore, like with our VLPs, it is possible to distinguish between vaccinated and infected animals, due to the lack of NSs specific antibodies upon vaccination (McElroy et al., 2009). It is relatively easy to produce high amounts of this attenuated virus and there is a low risk for reversion to a wild type virus, since the genes of interest have been completely deleted. However, a high safety level is still necessary for production as there is always a risk when handling viable viruses. Likewise, over-attenuation of the virus may result in a too low induction of the immune response in higher animals (Ikegami & Makino, 2009).

Another approach under evaluation is an Alphavirus (Sindbis virus) replicon candidate, expressing Gn, Gc, and NSm of RVFV. This vector-based vaccination conferred full protection in mice after challenge and induced production of RVFV neutralising antibodies in sheep (Heise et al., 2009). This approach has shown promising results for other viruses as well (Hevey et al., 1998, Pushko et al., 2001) and it is possible to distinguish infected animals from vaccinated ones. However, the recombinant virus vector replicates in host cells and this might be of concern for the safety as a vaccine.

Finally, there are also an Adenovirus vector construct (CAdVax-RVF), expressing a RVFV glycoprotein construct (Gn and Gc). This approach showed promising results in mice experiments (Holman et al., 2009). This non replicative approach has earlier been proven to be good vaccine strategy
(Swenson et al., 2008) and like the above mentioned vaccine candidates it is possible to distinguish immunised animals immunised from infected. One inherent problem with adenovirus vectors has been pre-immunity, however this problem can be overcame by increased vaccine dose. Still, full protection was not conferred in pre-immune mice (Holman et al., 2009).

Hopefully, one or several of these novel approaches or improved older candidates will be successful and lead to the development and production of a fully working vaccine against RVFV in the future.
Conclusion

We have established an animal infection model and developed diagnostic tools for RVFV.

Mice proved to be a good animal model for RVFV infection studies. High amounts of viral RNA were detected in blood, brain and liver samples, shortly after infection.

The established QRT-PCR analysis proved to be accurate, reliable and sensitive. The high amounts of RVFV RNA in circulation early post infection make the QRT-PCR a very good compliment to the more traditional diagnostic tools since RVFV specific antibodies need around 4-7 days to develop.

Filter paper could make blood sampling for PUUV under field conditions easier, since PUUV RNA was readily detected in our experiments. However, this was not the case for RVFV, though viable RVFV particles could be eluted.

We have developed and evaluated two none replicating RVFV vaccine candidates.

The genetic vaccination did induce production of RVFV specific antibodies but did not confer a high level of protection.

On the other hand, immunization with RVF VLPs show very promising results and could after further evaluations probably become a good future vaccine candidate.
Rift Valley Feber (RVF) är en myggburen blödarfeber. Sjukdomen anses vara allmänt förekommande över hela den afrikanska kontinenten och sedan 2000 talets början inkluderas även den arabiska halvön. Ett stort antal djurarter kan insjukna i RVF med varierande sjukdomsbild. Kor, får och getter drabbas hårdast, med en dödlighet upp till 30 % för vuxna djur och upp mot 100 % hos ungdjur. Ett karaktäristiskt drag för ett RVF utbrott är aborter, något som beskrivs som att en ”abortstorm” sveper genom hjordar där ca 100 % av de havande djuren aborterar. Till skillnad från djur, insjuknar människor oftast i en mild influensaliknande sjukdom, även om det förekommer svårare fall med blödarfeber och hjärnhinneinflammation. Dödligheten har generellt varit låg vid epidemic, under 1 %, men vid de senaste utbrottet har betydligt högre siffror rapporterats.


Viruset överlever transovariellt (inuti äggen) hos vissa myggarter (Aedes) och bibehålls på så sätt i naturen. Aedes myggor är bärare av RVF och
sprider i sin tur smittan vidare till olika værdjur, vilka sprider viruset till andra myggor. Det är ett flertal olika myggarter som kan sprida RVF viruset; *Anopheles, Culex, Eretmapoites* and *Mansonina*. Intressant nog finns flera av dessa myggarter i Europa och övriga världen vilket gör det möjligt för RVF att spridas utanför Afrika.

RVF-utbrott har en stor påverkan på drabbade samhällen då utbrott inte enbart påverkar djur och allmän folkhälsa utan även matproduktion och ekonomi. Animaliska produkter måste förstöras och exportförbud införs för att förhindra smittspridning.

Vid diagnos av RVF är den huvudsakliga metoden direkt isolering av viruset även om påvisandet av antikroppar mot RVF också används i stor utsträckning. Kvantitativ real-tids PCR, är en metod som visat sig vara pålitlig och användbar för andra sjukdomar eftersom den möjliggör påvisning och kvantifiering av virusets arvsmassa. Vi har etablerat denna metod för RVF och utvärderat dess användbarhet med hjälp av prover från experimentellt infekterade möss. Denna metod har visat sig vara stabil och pålitlig för RVF och ett bra komplement till de befintliga metoderna. Den ger svar tidigare än de andra metoderna, och väldigt höga nivåer av RVF arvsmassa påvisades i prover från blod och lever. Emellertid klingar de höga nivåerna relativt snabbt av och efter en vecka finns inte virus kvar.

Vid fältstudier och i låginkomstländer kan det vara problem med att samla in och behandla prover på ett korrekt sätt. Vi har därför undersökt möjligheterna att använda Nobuto filterpapper för att samla in och förvara
blod och serum prover. Filterpapper tar upp liten plats och kan förvaras i rumstemperatur. Våra resultat tyder på att denna metod fungerar bra för Puumala viruset (PUUV), där vi kunde påvisa förekomsten av arvsmassa från filterpapper. PUUV är en nära släkting till RVFV som finns i norra Sverige där det orsaker sorkfeber (Nephropathia epidemica). Däremot var det inte möjligt att applicera filterpapper-metoden på RVFV. De två virusen har dock olika livscykel, vilket möjligtvis kan vara en avgörande faktor i stabilitetsskillnaden.

För tillfället är behandlingen vid RVF enbart av livsuppehållande karaktär, då det inte existerar några specifika läkemedel, och de vacciner som existerar är för veterinärbruk. Där med finns det ett stort behov av utveckling av nya behandlingsmetoder, antivirala medel och vaccin mot denna sjukdom.

Vi har fokuserat på vaccinering och använt oss av två olika tekniker, genetisk vaccinering och virus-lika partiklar. I den första studien använde vi oss av genetisk vaccinering, och immuniserade möss med DNA som innehåller olika RVFV proteiner. Enligt teorin ska proteinerna i fråga tillverkas, processas och modifieras på samma sätt som vid en riktig infektion och därmed även stimulera immunförsvar på ett korrekt sätt. Mössen immuniserades med gener för två olika RVFV proteiner. Vi kunde visa att antikroppar bildades mot de två RVFV proteinerna men skyddseffekten var inte fullständig, bara hälften av djuren var skyddade mot RVF.

I den andra studien använde vi oss av RVF virus-lika partiklar. Dessa partiklar kan enkelt beskrivas som ”tomma virus”, som är uppbyggda av

Sammanfattningsvis kan vi säga att vi har utvecklat en metod (kvantitativ real-tids PCR) som har stor potential som diagnostiskt verktyg för RVFV, framför allt som komplement till de traditionella metoderna.

Vidare har vi visat att RVF virus-lika partiklar har mycket goda vaccinegenskaper och har stor möjlighet att utvecklas till ett framtida, framgångsrikt och säkert vaccin.
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References


