



Review

Reverse genetics technology for Rift Valley fever virus: Current and future applications for the development of therapeutics and vaccines

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ABSTRACT

The advent of reverse genetics technology has revolutionized the study of RNA viruses, making it possible to manipulate their genomes and evaluate the effects of these changes on their biology and pathogenesis. The fundamental insights gleaned from reverse genetics-based studies over the last several years provide a new momentum for the development of designed therapies for the control and prevention of these viral pathogens. This review summarizes the successes and stumbling blocks in the development of reverse genetics technologies for Rift Valley fever virus and their application to the further dissection of its pathogenesis and the design of new therapeutics and safe and effective vaccines.

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1. Introduction

Several bunyaviruses including Rift Valley fever virus (RVFV), Crimean-Congo hemorrhagic fever and Hantaan viruses are responsible for potentially lethal hemorrhagic fevers and therefore are of significant medical and public health importance. Unfortunately, there are no FDA-approved vaccines available for any of these viruses. The real threat posed by RVFV, coupled with the fact that there currently is no effective licensed vaccine for human use, clearly illustrates the need for more RVFV vaccine research and development. A better understanding of bunyavirus-associated pathogenesis remains critical to the development of effective vaccines and antiviral compounds to combat hemorrhagic fevers. Many vaccine candidates and therapeutics developed through traditional methods either fail to provide protection or result in unacceptable adverse effects. This problem clearly requires the application of new technologies, such as reverse genetics systems, for the design and generation of safe and efficacious vaccine candidates and therapeutics based on the use of genetically manipulated viruses.

2. Bunyaviruses

Viruses within the *Bunyaviridae* family are classified into five genera: *Orthobunyavirus*, *Nairovirus*, *Phlebovirus*, *Hantavirus* and *Tospovirus* (Elliott, 1996; Schmaljohn and Hooper, 2001). Bunyaviruses are characterized by a tripartite negative-stranded RNA genome comprised of Large (L), Medium (M), and Small (S) segments (Schmaljohn and Hooper, 2001). The L segment encodes for the viral RNA-dependent RNA polymerase—RdRp (L), the M segment for a protein precursor which is post-translationally processed into the mature glycoproteins G_N and G_C and the S segment for the nucleoprotein (N). Some bunyaviruses express additional non-structural proteins from the S and M segments, NSs and NSm, respectively (see Fig. 1A). General features of viruses classified in the *Bunyaviridae* family are similar to other segmented negative-strand RNA virus families (Elliott, 1996).

Following envelope glycoprotein-mediated virus binding to a receptor on a permissive cell, viral and plasma membranes fuse and viral RNA is transferred into the cytoplasm. The immediate early primary transcription step then begins, allowing the viral genomic RNAs (vRNA), in association with N, to be transcribed into mRNAs by the RNP-associated viral RNA polymerase L (Bellocq and Kolakofsky, 1987; Ikegami et al., 2005b; Kolakofsky et al., 1987; Vialat et al., 2000). Genome replication takes place in the cytoplasm via a complementary full-length template RNA (cRNA), also called antigenomic RNA. The vRNAs generated from this repli-

cation step provide the template for additional mRNA synthesis (secondary transcription step) and serve as genome segments for progeny virions which are released by budding into Golgi vesicles (Elliott, 1996; Gerrard and Nichol, 2002, 2007). Maturation and budding of particles through the Golgi apparatus is a property of bunyaviruses. mRNA synthesis is initiated using capped oligonucleotides, captured from cellular mRNAs through a cap-snatching mechanism, similar to the one described for orthomyxoviruses except that all steps take place in the cytoplasm. In contrast with most mRNAs, bunyavirus mRNAs are not polyadenylated and represent an incomplete copy of the vRNA template.

3. Rift Valley fever virus

RVFV is an arthropod-borne member of the *Phlebovirus* genus that causes recurrent outbreaks affecting humans and ruminants predominantly in Sub-Saharan Africa, but spread to Egypt in 1977 and to the Arabian Peninsula, including Saudi Arabia and Yemen in 2000 (Al-Hazmi et al., 2003; Anonymous, 2000; Balkhy and Memish, 2003; Madani et al., 2003; Shoemaker et al., 2002), and re-emerged in four Egyptian Governorates in 2003 (Balkhy and Memish, 2003).

The largest RVFV epidemic-epizootic outbreak affected Egypt along the Nile River (1977–1979), afflicting approximately 200,000 persons with 594 deaths (Meegan, 1979; Meegan et al., 1979), followed by recurrent epidemics in 1993 and 1997 (Abd el-Rahim et al., 1999; Abu-Elyazeed et al., 1996; Arthur et al., 1993). Other severe outbreaks in the past twenty years have affected West African nations in Senegal-Mauritania (1987) where an outbreak affected an estimated 89,000 victims, resulting in 220 deaths

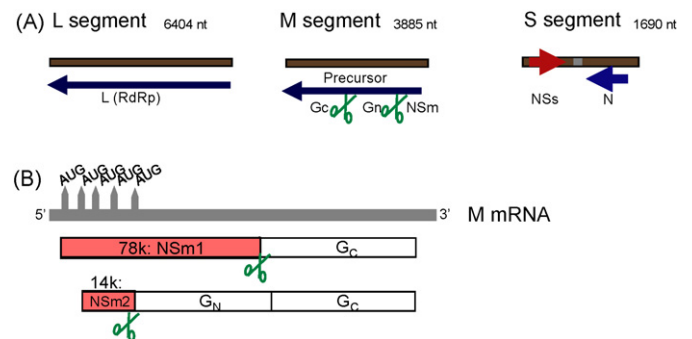


Fig. 1. RVFV genome organization. (A) Schematic representation of the three genomic segments and coding strategy. The arrows indicate the open reading frames in each segment with the cleavage sites generating the mature glycoproteins. (B) RVFV M segment-based mRNA with the five in frame AUG start codons at its 5' terminus. The proteins expressed from the first and the second AUG are displayed.

(Digoutte and Peters, 1989). An outbreak in East Africa occurred from 1997 to 1998 in the Garissa District of Northeastern Kenya, affecting an estimated 27,500 individuals with 170 hemorrhagic fever-associated deaths (Woods et al., 2002). RVFV has spread to Madagascar which experienced its own RVFV epidemics in 1990–1991 (Morvan et al., 1992, 1991) and 2008, resulting in only a few deaths in 1990–1991 but 476 cases and 19 deaths in 2008 (Reynes, 2009). Finally, as described above, RVFV spread to the Arabian peninsula in 2000 (Saudi Arabia and Yemen), where an estimated 2000 people were hospitalized or severely sickened with RVF-related symptoms, including acute hepatic necrosis, hepatitis, delayed-onset encephalitis and retinitis, resulting in at least 245 deaths (CFR 12%) (Al-Hazmi et al., 2003; Alrajhi et al., 2004; Madani et al., 2003).

In recent years (2007–2008) RVFV has circulated in East Africa causing serious epidemics in Kenya, Tanzania, Somalia and Sudan in 2007, and subsequently expanded to Madagascar and South Africa in 2008. RVFV activity was also reported in the Comoros Islands (LaBeaud et al., 2008; WHO, 2007).

Simple immunoglobulin (Ig) G testing through ELISA has shown that in endemic areas such as Kenya, RVFV seroprevalence has soared to 32% in high-risk regions (Woods et al., 2002). During interepidemic periods, RVFV seroprevalence rates can vary between 6% and 20% in the urban and rural settings, respectively, with an overall RVFV seropositivity rate of 13% (LaBeaud et al., 2008, 2007).

3.1. Disease

In ruminants, RVF is characterized by substantial die-offs of young animals (especially of lambs), fetal deformities and abortion (Flick and Bouloy, 2005; Gerdes, 2004; Swanepoel and Coetzer, 2003). In humans the disease is often associated with benign fever but can lead to more complicated cases such as retinal vasculitis, encephalitis, neurologic deficits, hepatic necrosis, or fatal hemorrhagic fever (Meegan, 1979).

Although the lethality for humans infected with RVFV has been reported to be below 2%, the case fatality rate in recent outbreaks, 23–45%, is substantially higher (LaBeaud et al., 2008; WHO, 2007). Although not addressed by these reports, it is likely that morbidity is also substantially increased. It has yet to be determined if new, more virulent strains is causing this sudden increase of fatal human cases.

3.2. Transmission

RVFV is readily transmitted through a broad range of mosquito genera including *Aedes*, *Anopheles*, *Culex*, *Eretmapoites* and *Mansonia*, and by other vectors including sand flies (Fontenille et al., 1998). Thus transmission could rapidly occur in many Western countries, including the United States (US) (Turell et al., 2008) or Europe (Moutailler et al., 2008). Recent studies have illustrated the ability of RVFV to utilize the dominant mosquito species of a given geographical location (Moutailler et al., 2008; Turell and Kay, 1998; Turell and Perkins, 1990), which indicates that there is no natural blockade to protect naive countries from the spread of the virus. By analogy, West Nile virus (WNV) was not considered a threat to the US prior to its emergence on the US mainland (New York) in 1999 (Hayes, 2001). However, within six years, WNV had become endemic across the US. Interestingly, while the WNV transmission route is limited to two mosquito genera (*Culex* and *Aedes*; Sardelis et al., 2001) and has a limited effective host range, RVFV can be transmitted by at least five mosquito species and has a large host range. RVFV has been shown to infect most livestock species (with young animals most susceptible) and reach sufficient titers to allow transmission from animal to insect vector (Turell et al., 2008),

insect vector to animal, animal-to-animal and animal-to-human (Easterday, 1965).

RVFV is only able to cause epizootics under the appropriate climatological conditions (e.g., wet seasons and resulting flooding required for *Aedes* mosquito breeding). While RVFV can appear to be absent in an arid region, the virus is capable of lying dormant in the eggs of infected mosquitoes.

It has been suggested that between periods of epizootics, RVFV utilizes wild rats as a reservoir host, replicating to titers that are sufficient for transmission while disease is at subclinical levels (Pretorius et al., 1997). However, this is still a matter of debate and needs confirmation from the field. The spread of RVFV is not limited to vector-borne transmissions, as RVFV is readily passed via bodily fluids (Balkhy and Memish, 2003; Lutwick and Nierengarten, 2002) <http://www.cdc.gov/ncidod/dvrd/spb/mnpages/dispages/rvf.htm>, and by aerosol transmission (Hoogstraal et al., 1979; Lutwick and Nierengarten, 2002; Meadors et al., 1986). RVFV can be transmitted amongst slaughterhouse workers and animal handlers, and has been recently reported to have been inadvertently transmitted amongst veterinarians, veterinary students and laboratory staff at a South African veterinary college after performing necropsies on infected animals.

The recent expansion of RVFV's geographical range clearly indicates that RVFV is not solely an African problem, but rather is a geographically rapidly expanding disease that is a threat to other countries. It has been proposed that a single infected person or animal (live or dead) that enters a naive country is sufficient for the initiation of a major outbreak before RVFV would ever be detected. This transmission scenario is becoming increasingly more probable due to the expansion of worldwide trade and travel.

3.3. Bioterrorism threat

RVFV is a prototype of emerging/re-emerging pathogens and is classified as a BSL-3 agent in Europe and BSL-3Ag agent in the US. RVFV was weaponized by the US offensive biological warfare program prior to its termination in 1969 (Anonymous, 2009), illustrating that the real threat and utility of RVFV as a biological weapon is clearly recognized (Jones et al., 2002). The lack of prophylactic and therapeutic measures, the transmission by many species of mosquitoes and the significant threat to livestock associated with RVFV make this pathogen a serious public health concern not only for endemic, developing countries, but also for many non-endemic developed countries due to the possibility of bioterrorist attacks, based on a report released on December 4th, 2008 by the Commission on the Prevention of Weapons of Mass Destruction and Terrorism (Graham et al., 2008). Considered a potential bioterrorism agent (Sidwell and Smea, 2003), RVFV is on the Center for Disease Control (CDC) Bioterror Agent list (<http://www.bt.cdc.gov/agent/agentlist-category.asp#a>) and is classified as a Department of Health and Human Services (HHS), United States Department of Agriculture (USDA) overlap Select Agent (USDA, 2005). Rift Valley Fever Virus is also classified as a Category A High Priority Pathogen by the National Institute for Allergy and Infectious Diseases (NIAID) (<http://www3.niaid.nih.gov/topics/BiodefenseRelated/Biodefense/research/CatA.htm>). High-priority agents pose a risk to national security because they can be easily disseminated and can cause high mortality and morbidity, potentially resulting in a major impact on public health. Ultimately, this high-priority pathogen could lead to widespread public panic and social disruption.

4. RVFV genome organization and coding strategy

Over the past two decades, the ability to genetically manipulate viral genomes has facilitated both our understanding of viral

pathogenesis and studies of the interactions between viral and cellular components. The establishment of a reverse genetics system opened the door for many studies into the molecular biology of RVFV and mechanisms involved in pathogenicity that were unthinkable some years ago. The important role of non-structural proteins in viral pathogenesis has been gleaned from such investigations. The RVFV genome encodes three non-structural proteins, NSm1 (78k) and NSm2 (14k) expressed from the M segment-derived polyprotein precursor, and NSs expressed from the S segment (Elliott, 1996) (see Fig. 1A). The alternate utilization of the first two in-frame AUGs of the M segment (five different AUG start codons exist prior to the signal peptidase cleavage site of the mature G_N protein) generates NSm1 and NSm2, respectively (see Fig. 1B). Impaired cleavage of the G_N signal peptide insures that NSm1 contains the pre-glycoprotein sequence followed by G_N. NSs is encoded in positive sense orientation on the ambi-sense RVFV S segment, separated from the N-ORF by an intergenic region. Both NSm and NSs non-structural genes have been found to be dispensable in the replication cycle, but have been shown to exhibit properties of virulence factors.

5. Reverse genetics systems

Reverse genetics technology makes it possible to manipulate viral RNA molecules using complementary DNA (cDNA) copies so one can study the effects of genetic changes on the biology of the virus. This technology was applied to the modification of plus-stranded RNA virus genomes and to the recovery (rescue) of infectious virus from cDNA because these viruses are able to utilize the host cell DNA replication machinery to initiate their life cycles. Thus, plasmid-encoded or *in vitro* synthesized genomic RNA of these viruses generates infectious virions when introduced into permissive cells. On the other hand, recovery of negative-strand RNA viruses from either cDNA components or synthetic RNA was a substantial challenge because, unlike the plus-stranded RNA viruses, replication initiation requires *de novo* protein synthesis mediated by viral RNA-dependent RNA polymerase, and the input genomic or antigenomic RNA needs to be encapsidated with the viral nucleoprotein before it can serve as a functional template to initiate transcription/replication. The ultimately successful development and application of reverse genetics technology for the manipulation of negative-strand RNA virus genomes has considerably complemented the field of RNA virology. Much has been elucidated about the molecular characteristics and pathogenesis of these viruses, and the insights obtained from such studies has provided new impetus for the development of rationally designed vaccines and antiviral agents.

5.1. Basic strategies and technologies

The successful establishment of reverse genetics systems for a variety of different negative-strand RNA viruses (segmented and non-segmented) demonstrates that different technologies and strategies can be employed for the generation of recombinant viruses. The following briefly summarizes the common critical tools used for such reverse genetics endeavors.

The first step in the generation of recombinant virus using reverse genetics systems is the introduction of required viral components (expression plasmids: template for viral proteins to drive viral replication and transcription processes; transcription plasmids, PCR fragments or *in vitro* transcribed RNA: templates for the generation of viral genomes) into select mammalian cells. A variety of liposome-based transfection reagents as well as electroporation methodologies for a variety of different mammalian cells have been successfully demonstrated.

The most challenging task for the establishment of an infectious clone system is certainly the generation of viral RNA genomes/genome segments, due to the fact that many viruses require precise termini to provide suitable templates for their viral polymerase to perform replication and transcription steps. Both prokaryotic (e.g., phage T7 polymerase) and eukaryotic promoters (e.g., RNA polymerase I and II) are successfully employed to generate such full-length viral genome transcripts. Different eukaryotic promoters are described for the successful expression of structural (glycoproteins, nucleocapsid protein, matrix proteins, phosphoproteins, viral polymerase) and non-structural components of the infectious recombinant virions. Additionally, optimization of viral protein expression levels can be achieved by using translation initiation enhancers (e.g., Kozak sequence) and codon-optimized gene sequences.

Reporter genes are often used during the establishment of a reverse genetics system (e.g., minigenome rescue systems) and are often included in recombinant viruses to allow for ease of detection (e.g., monitoring intracellular or *in vivo* localization, plaque detection). The type and location of reporter genes for each respective system depends upon the questions to be assessed. For minigenome rescue systems, the viral ORF is replaced by a reporter gene that retains the flanking non-coding regions containing viral promoter, encapsidation and packaging signals and other regulatory *cis*-acting elements (Freiberg et al., 2008). The use of eGFP is preferred for system optimization, allowing the monitoring of reporter gene expression in live cells. However, other reporter genes including luciferase and chloramphenicol acetyltransferase have also been successfully employed, especially in systems in which highly sensitive detection techniques were required due to suboptimal expression levels. Reporter genes can be also used to generate viral fusion proteins allowing the intracellular tracking of proteins of interest.

One of the first applications of reverse genetics for a negative-strand virus was to influenza virus (Luytjes et al., 1989), followed by the successful rescue of recombinant rabies virus (Schnell et al., 1994). Rescue of other full-length RNA viruses soon followed, and included vesicular stomatitis Indiana virus (Lawson et al., 1995; Whelan et al., 1995), measles virus (Radecke et al., 1995), and many others (Kawaoka, 2005; Neumann and Kawaoka, 2004; Neumann et al., 2002; Walpita and Flick, 2005). A similar methodology was used to recover the bunyavirus *Bunyamwera*, which was also the first of the segmented negative-strand RNA viruses to be recovered entirely from cloned cDNA (Bridgen and Elliott, 1996). The key challenge in these studies was to generate the RNA genome/genome segments in eukaryotic cells while also providing required trans-acting factors including the viral RNA-dependent RNA polymerase, nucleoprotein, and a viral phosphoprotein specifically for non-segmented RNA viruses. The strong bacteriophage T7 promoter was initially used to generate the required components for subsequent assembly of recombinant infectious viruses. While the methodology based on the bacteriophage T7 polymerase has been used mostly for non-segmented negative-strand RNA viruses (e.g., filoviruses, rhabdoviruses, paramyxoviruses), an alternative technique employing the cellular DNA-dependent RNA polymerase I to transcribe transfected plasmids into viral RNA genome (segments) was initially developed for influenza viruses (for review, see Neumann et al., 1999; Walpita and Flick, 2005). The choice of method was mostly dictated by the intracellular location of viral replication: T7-based method was initially used for those viruses that replicate in the cytoplasm (e.g., bunyaviruses), and the Pol I-based methodology was used for those which have a nuclear replication strategy (e.g., orthomyxoviruses).

This paradigm was recently revisited when Flick and Pettersson (2001) demonstrated that a Pol I-driven approach is a viable technique for the successful rescue of Uukuniemi (a RVFV-related

phlebovirus) artificial genome segments (minigenomes) expressing reporter genes. Over the next several years, Dr. R. Flick's team demonstrated the ability of the Pol I system to generate functional viral genome segments for reverse genetics studies with other bunyaviruses including Hantaan virus (Flick et al., 2003a) and Crimean-Congo hemorrhagic fever virus (Flick et al., 2003b).

Recently, other studies have shown that both T7- and Pol I-based reverse genetic systems are equally efficient for minigenome rescue of Reston ebolavirus, a filovirus (Groseth et al., 2005) and the recovery of lymphocytic choriomeningitis (LCMV), an arenavirus (Flatz et al., 2006; Sanchez and de la Torre, 2006), both having a cytoplasmic strategy for transcription and replication. Furthermore, the T7 promoter-driven system, in addition to the originally established Pol I-driven rescue system (Fodor et al., 1999; Neumann et al., 1999), can also be successfully applied to the generation of influenza A virus which replicates in the nucleus (de Wit et al., 2007).

Taken together, many different technologies and strategies have been employed to successfully establish reverse genetics systems for the generation of infectious recombinant viruses that can be used for basic research applications and as potential vaccine candidates.

5.2. Minigenome rescue systems for bunyaviruses

Rescue of minigenomes, which is a preliminary but recommended step before recovery of infectious virus, has been shown to be efficient with both T7- and Pol I-driven systems for bunyaviruses (Fig. 2). Minigenome transcription driven by T7 or Pol I promoters in combination with T7- or Pol II-driven expression plasmids of the corresponding bunyaviral nucleoprotein N and the polymerase L enable the reconstitution of RNP templates (Blakqori et al., 2003; Bridgen and Elliott, 1996; Dunn et al., 1995; Flick et al., 2003a,b; Flick and Bouloy, 2005; Flick and Pettersson, 2001; Gaulliard et al., 2006; Ikegami et al., 2005a; Kohl et al., 2004; Lopez et al., 1995; Walpita and Flick, 2005). Such minigenome rescue systems are used extensively to study viral *cis*-acting elements (e.g., promoter location and structure, packaging signals, transcription termina-

tion signals) and the function of *trans*-acting factors (polymerase, nucleoprotein, non-structural proteins) (Albarino et al., 2007; Barr et al., 2005; Barr and Wertz, 2005; Flick et al., 2004; Flick and Bouloy, 2005; Gaulliard et al., 2006; Haferkamp et al., 2005; Ikegami et al., 2007; Kinsella et al., 2004; Walpita and Flick, 2005). These systems are especially valuable for the effective study of negative-strand transcription and replication, as well as the identification of potential antiviral compounds for viruses classified for use in high containment facilities (BSL-3 and BSL-4) since they allow for experiments at a lower biosafety level (Flick and Bouloy, 2005; Flick and Whitehouse, 2005; Freiberg et al., 2008; Haferkamp et al., 2005; Walpita and Flick, 2005).

As a proof of concept, Uukuniemi virus (closely related to RVFV) and Nipah virus minigenome rescue systems were optimized to a 96-well high-throughput format in which potential antivirals are identified by their ability to interfere with viral replication and transcription in a time-efficient and cost-effective manner (Flick, 2009; Freiberg et al., 2008). Such high-throughput screening assays involve transfection of cells with minigenome replicons encoding a recombinant gene marker such as chloramphenical acetyl transferase (CAT), luciferase or GFP (Freiberg et al., 2008). Minigenome rescue systems enable the quantification of viral gene activity such that the level of reporter gene activity directly reflects the extent of viral transcription/replication activities (Freiberg et al., 2008). An ELISA-based format allows for a simple readout of differences in reporter gene activity. After distinguishing confirmatory hits from false positives, antiviral candidate compounds that show inhibitory effects through reduced reporter gene activities are considered as potential antiviral compounds that are then further discriminated for their antiviral effects. Both Pol I- and T7-driven minigenome rescue systems can be used to reduce false positive signals by eliminating antiviral activities against Pol I or T7. Finally, antiviral candidate compounds can be further analyzed in such minigenome rescue systems through dose-dependent titration assays to determine effective dose range. Future improvements will involve the use of stable expressing cell lines that constitutively express viral transcription/replication machinery and a minigenome of choice to eliminate the costly and cumbersome transfection step.

Another example in which minigenome rescue systems were used to identify antiviral compounds against the RVFV-related phlebovirus Uukuniemi virus was recently reported by Flick et al. (unpublished data, see below). Uukuniemi virus-specific antisense peptide-conjugated phosphorodiamidate morpholino oligomers (PPMOs) were used to identify the mechanism and optimal genome target for efficient antiviral activity. A clear dose-dependent reduction in minigenome expression levels and virus titer was observed using Uukuniemi-specific PPMOs (Dr. R. Flick, unpublished data). PPMOs have been shown to provide resistance *in vivo* and *in vitro* to several viruses including West Nile virus (Deas et al., 2007; Zhang et al., 2008), influenza A, dengue 2, and Zaire ebolavirus (Gabriel et al., 2008; Lupfer et al., 2008; Stein, 2008; Stein et al., 2008).

5.3. Infectious clone systems for bunyaviruses

Amongst the bunyaviruses, Bunyamwera virus was the first to be recovered from plasmids without the need of a helper virus (Bridgen and Elliott, 1996; Bridgen et al., 2001; Lowen et al., 2004), followed by the recovery of infectious LaCrosse and RVF viruses based on the T7 polymerase methodology (Bird et al., 2008, 2007a; Blakqori and Weber, 2005; Bridgen and Elliott, 1996; Bridgen et al., 2001; Gerrard et al., 2007; Ikegami et al., 2006; Lowen et al., 2004). More recently, Pol I-mediated transcription of viral cRNA was applied to the rescue of Akabane orthobunyavirus (Ogawa et al., 2007) and RVFV (Billecocq et al., 2008; Habjan et al., 2008b), indicating that the Pol I-based rescue system could be applied to bunyaviruses utilizing an ambisense

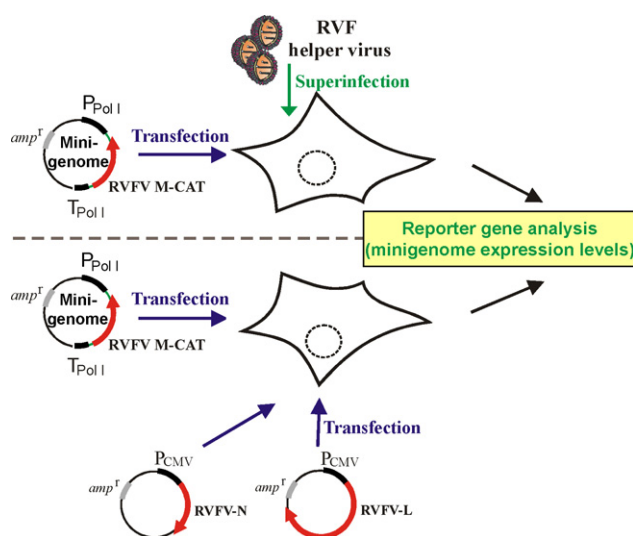


Fig. 2. Comparison of RVFV minigenome rescue systems. RVFV M segment-based minigenome plasmids under the control of the cellular RNA polymerase I are transfected into suitable mammalian cells (e.g., HEK293). The viral proteins needed for minigenome transcription and replication are provided by either superinfection with RVFV helpervirus (upper panel) or co-transfection of RVFV L and N expression plasmids (lower panel). Cells are harvested and reporter gene expression levels analyzed with suitable detection tools (e.g., CAT assays).

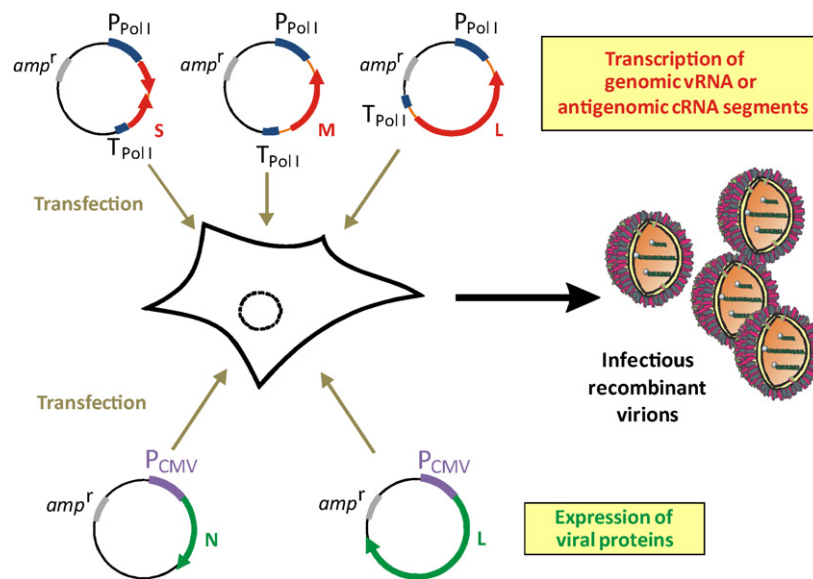


Fig. 3. RVFV infectious clone system. Plasmids containing the sequence of the three RVFV genome segments (L, M and S) under the control of the cellular RNA polymerase I are transfected into suitable mammalian cells (e.g., HEK293). Bunyaviral genome segments can be transcribed into genomic vRNA (as shown) or antigenomic cRNA, depending on the orientation within the plasmid. The viral proteins needed for RNA genome transcription and replication are provided by co-transfection of RVFV L and N expression plasmids. Supernatant is harvested and recombinant infectious RVFV can be used for subsequent applications (e.g., vaccine candidate).

coding strategy (Fig. 3). These two latter studies compared the efficiency of the T7- and the Pol I/Pol II-driven systems and concluded that both were similar. It should be noted that the Pol I system requires co-transfection of the bunyaviral N and L expression plasmids, whereas co-transfections are either not necessary or even inhibitory in the T7 system (Bird et al., 2008; Ikegami et al., 2006). Interestingly, NSs co-expression with the L and N proteins inhibits RVFV minigenome rescue and/or the generation of recombinant RVFV (N. Gaudiard, M. Bouloy, unpublished results), which is contradictory to the report by Ikegami et al. (2005a) which claims that NSs promotes minigenome viral RNA replication and transcription. However, Ikegami et al. (2006) also showed that NSs expression suppresses infectious RVFV rescue in a T7-driven reverse genetics system.

A disadvantage of T7- compared to Pol I-driven systems is that the T7 polymerase has to be provided *in trans*, using either eukaryotic expression plasmids, a recombinant vaccinia virus (MVAGKT7, Kovacs et al. (2003), or T7 polymerase stable-expressing cell lines (BHK21-T7 or BSR-T7/5) constitutively expressing the T7 RNA polymerase (Conzelmann et al., 1991; Kato et al., 2004) to drive the system, whereas Pol I does not have to be added *in trans*. Although the 5' triphosphate termini of T7 transcripts strongly activate the antiviral interferon response via the intracellular receptor RIG-I, T7 polymerase stable-expressing cell lines are suitable for the rescue of viruses of several families because they have a compromised RIG-I pathway (Habjan et al., 2008a).

6. RVF pathogenesis

6.1. The role of the NSs protein as an interferon antagonist

NSs has been identified as a major virulence factor, primarily by acting as an interferon antagonist (Billecocq et al., 2004; Bouloy et al., 2001). Using the established reverse genetic system has been very instrumental to better understand RVFV pathogenesis. In an attempt to decipher the molecular mechanisms underlying this property, Dr. M. Bouloy's laboratory identified several cellular proteins that interact with NSs. One of them, SAP30, belongs to the Sin3A/NCOR/HDAC repressor complexes that intervene in the regulation of gene transcription. Moreover, it was shown that

SAP30 interacts directly with YY1, a transcription factor involved in regulating the expression of numerous genes, including IFN- β . Through different techniques such as co-immunoprecipitation, confocal microscopy and chromatin immunoprecipitations, Dr. M. Bouloy's group demonstrated that NSs, SAP30, YY1 and Sin3A-associated corepressor factors are recruited on the IFN- β promoter, inhibiting CBP recruitment, histone acetylation and transcriptional activation (Le May et al., 2008) (see Fig. 4). To ascertain the role of NSs interaction with SAP30 in this mechanism, Le May et al. (2008) used reverse genetics to produce a recombinant ZH548 in which the domain of NSs that interacts with SAP30 was deleted from the S segment. In contrast to the virulent ZH548 RVFV, this mutant, ZH548-NSs Δ 210-230, is able to induce IFN- β expression and is avirulent in the mouse model.

During the last few years NSs has been shown to be a multifunctional protein that enables RVFV to employ several strategies to evade the host antiviral response. One of these relies on its interaction with the p44 subunit of the TFIIF basal transcription factor which is sequestered by the NSs filamentous structure (see Fig. 4), characteristic of RVFV infection (Le May et al., 2004). As a consequence, TFIIF cannot assemble and its concentration drops rapidly, which explains the drastically reduced transcriptional activity of cells infected with NSs-expressing RVFV. Interestingly, the function of NSs as a general inhibitor of transcription (dependent on both Pol I and Pol II since TFIIF is involved in these two activities) is a relatively late event occurring >8 h p.i., whereas the specific inhibition of IFN- β gene transcription is in place as early as 3–4 h p.i.

Very recently, a novel function of NSs was described simultaneously by the groups of Drs. S. Makino (Ikegami et al., 2009) and Dr. F. Weber (Habjan et al., 2009b): NSs promotes post-transcriptional downregulation of protein kinase PKR and inhibits eIF2- α phosphorylation. This phenomenon occurs through the proteasome-dependent degradation of PKR in cells infected with NSs-expressing RVFV, whereas PKR is clearly detected and activated after infection with the NSs-deleted Clone 13. It was also observed that this activity is specific to RVFV NSs but not shared with other phleboviruses including Sandfly fever Sicilian virus or the orthobunyavirus LaCrosse, since a recombinant RVFV ZH548 in which the NSs sequence was replaced by the heterologous NSs from

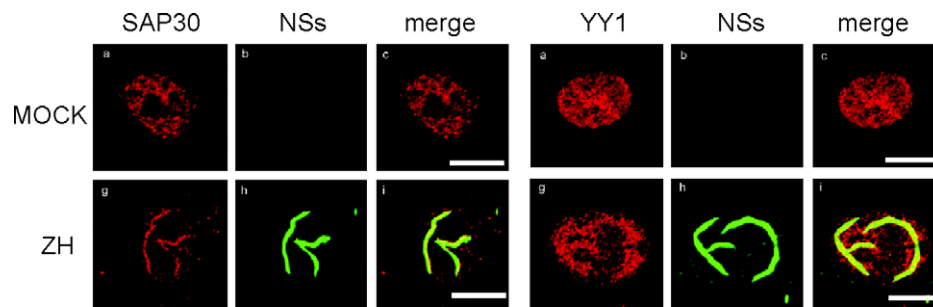


Fig. 4. Confocal microscopy of uninfected (MOCK) or infected L929 cells (ZH: with RVFV ZH548). Cells were fixed at 18 h p.i. and stained with antibodies against SAP30, NSs or YY1 and NSs.

the phlebovirus or the orthobunyavirus results in a virus lacking this ability.

Altogether, it appears that NSs has multiple functions to counteract the action of interferon, either at the transcriptional level or at the translational level, by degrading PKR which therefore facilitates translation of the viral products.

6.2. The role of the NSm protein as a suppressor of virus-induced apoptosis

To determine the biological function of NSm1 and NSm2, an M segment deletion mutant arMP12-del 21–384 was produced in which the first three initiating AUGs were deleted, resulting in a virus that is unable to synthesize the two non-structural NSm proteins. Although its growth was similar to that of the parental virus arMP12, arMP12-del 21–384 induced extensive cell death and produced larger plaques than the parent (Won et al., 2007). Further analyses indicated that the deletion mutant triggered apoptosis through the caspase 3, 8 and 9 pathways, thus revealing that NSm has an anti-apoptotic function and contributes to pathogenesis. Interestingly, a study by Pollitt et al. (2006) with Maguari virus showed that the NSm protein of this orthobunyavirus, which has no sequence homology with the NSm protein of phleboviruses (Nakitare and Elliott, 1993), could be deleted without affecting virus viability. It is not yet known if the NSm proteins from orthobunyaviruses and phleboviruses share the same anti-apoptotic function. However, if so, the same evolutionary pathway could have occurred to conserve the functions of both NSm and NSs amongst bunyavirus genera.

The function of the RVFV NSs and NSm proteins could not have been elucidated without the indispensable tools and methods of reverse genetics technology that allow the generation of recombinant viruses.

7. Historic and currently available RVF vaccines

As discussed above, aside from being endemic to many countries, RVFV is a rapidly expanding disease that may quickly become a worldwide threat. Unfortunately, the development of safe and efficacious RVFV vaccines has proven to be quite difficult (see Table 1). The urgent need for an effective vaccine for RVFV is illustrated by the statistics from recent outbreaks: From 13th January to 8th May 2007, a total of 290 cases including 117 deaths [case fatality ratio (CFR) 40%] of RVF were reported in Tanzania; from 30th November 2006 to 12th March 2007, a total of 684 cases including 155 deaths (CFR 23%) of RVF were reported in Kenya. From 19th December 2006 to 20th February 2007, a total of 114 cases including 51 deaths (CFR 45%) of RVF were reported in Somalia [World Health Organization (WHO), CSR, Disease Outbreak News, Wednesday 9, May 2007]. Moreover, the observed increase in RVFV-associated case fatality ratio, between 23% and 45% in naive populations, fur-

ther illustrates the continuing and emerging threat RVFV presents. Taken together, the significant increase in RVFV case fatality ratios and a broadening umbrella of epidemics beyond the traditional sub-Saharan African geographical range underlies the urgency for outbreak containment.

7.1. RVFV vaccines for livestock

7.1.1. Smithburn strain

The Smithburn RVFV strain utilized for vaccine development is a derivative of the Entebbe strain isolated in 1944 from a mosquito in Uganda which was mouse neuroadapted by 82 intracerebral passages in suckling mice (Smithburn et al., 1948). This Smithburn modified live virus (SMLV) was further passaged in mice and embryonated eggs before being utilized as a vaccine. Although extensively used for the immunization of livestock, the Smithburn vaccine is only partially attenuated and leads to high abortion rates or teratology in a significant proportion (8.8–28%) of pregnant animals (Botros et al., 2006; Coetzer and Barnard, 1977; Swanepoel, 2004b). Importantly, the concern that SMLV could revert to full virulence precludes its use in countries where RVFV is not known to be endemic (Botros et al., 2006; Swanepoel, 2004b).

7.1.2. Formalin-inactivated vaccine

Formalin-inactivated derivatives of the Smithburn vaccines, available commercially from Onderstepoort Biological Products Ltd. (South Africa) as a veterinary vaccine, have been extensively tested in livestock (Barnard, 1979; Barnard and Botha, 1977; Swanepoel, 2004a). These formalin-inactivated vaccines are more expensive to produce and require multiple inoculations and regular boosters to induce and maintain effective immunity (Barnard, 1979; Swanepoel, 2004a). Consequently, it is less useful in livestock than live-attenuated viruses, particularly when there is a need to attain rapid immunization in the face of an outbreak.

7.2. RVFV vaccines for human use

7.2.1. TSI-GSD-200

The only RVFV vaccine presently available for use in humans is the formalin-inactivated vaccine TSI-GSD-200 (Pittman et al., 1999). This vaccine is derived from RVF virus vaccine NDBR-103, originally developed by Randall et al. (1962) in African green monkey kidney cultures (Randall et al., 1964, 1962). While NDBR-103 has a stable phenotype and was used to protect laboratory personnel for over two decades, it is plagued with substantial inter-lot variation (Coetzer and Barnard, 1977). NDBR-103 was therefore further developed and standardized by the Salk Institute (1978–1979) under contract with the United States Army Medical Research Institute for Infectious Diseases (USAMRIID) (Pittman et al., 1999). This technology was improved and a more modern inactivated RVFV vaccine, TSI-GSD-200, is now produced in diploid

Table 1
RVFV vaccine candidates.

Vaccine platform	Species	Vaccine dose	Vaccination route	Challenge dose/virus	Challenge route	Protection levels	PRNT80	Reference
Recombinant baculovirus/Sf9 cell lysate TSI GSD-200	Mice	1×10^6 disrupted cells (2–10 μ g RVFV protein)	SC	1×10^3 LD ₅₀ /ZH501	IP	100%	PRNT ₅₀ : 1:40–640	Schmaljohn et al. (1989)
	Human	Incremental doses (0.1–1 ml), 3 doses	SC	N/A	N/A	N/A	Low dose (0.1 ml): 1:48, high dose (1 ml): 1:436	Kark et al. (1982)
	Human	Incremental doses (0.1–1 ml)	SC	N/A	N/A	Only localized erythema at the site of injection	1:1280 (1 month), >1:40 (12 months)	Meadors et al. (1986)
	Human	0.5 ml, 3 doses, 6 month booster	SC	N/A	N/A	90.3% show titer, 9.7% show no titer	Responders: \geq 1:40, non-responders: <1:40	Pittman et al. (1999)
	Pregnant ewes	1×10^5 pfu ZH548	SC	N/A	SC	Abortion rate: ZH548: 100%	Ewes: 1:80–1:320	Morrill et al. (1987)
	Lambs	1×10^5 pfu MP12				MP12: 0%	Lambs (post-parturition): \geq 1:80	
MP12	Ewes	Ewes: 5×10^3 pfu	Ewes: SC	1.6×10^5 pfu/ZH501	SC	Abortion rate (ewes): unvaccinated: 100%	Ewes: 1:320–1:10,240	Morrill et al. (1991)
	Lambs	Lambs: 5×10^3 pfu or 5×10^5 pfu	Lambs: SC	5×10^5 pfu/ZH501		Vaccinated: 0%; lambs: 100% survival	Lambs: 1:160–1:2560	
	Lactating cows	Lactating cows: 1×10^5 pfu				Abortion rate (dams): unvaccinated: 100%	Lactating cows: 1:80–1:2560	Morrill et al. (1997a)
	Dams	Dams: 1×10^5 pfu	SC	$1 \times 10^{5.7}$ pfu/ZH501	SC	Vaccinated: 0%	Dams' serum: 1:80–1:2560, colostral: 1:320–10,240	
	Yearling steers	Yearling steers: 1, 2, 3 and 4 log ₁₀ pfu				26% of vaccinated dams developed transient viremia	Steers: 1:160–1:5120	
	Dams	1×10^5 pfu	<i>in utero</i> , IM	$1 \times 10^{5.7}$ pfu/ZH501	SC	100% survival (vaccine safety)	<i>In utero</i> vaccinated calves: \geq 1:20–1:40	Morrill et al. (1997b)
	Bovine fetus						Dams' serum: >1:80, colostral: >1:320, calves (post-parturition): 1:80–10,240	
	Pregnant ewes	1×10^6 pfu	SC	N/A	N/A	Teratogenic effects	N/A	Hunter et al. (2002)
	Rhesus Macaques	1×10^7 pfu	IM, IT, IS	N/A	N/A	N/A	\geq 1:640	Morrill and Peters (2003)
	Human	1×10^5 pfu	IM	N/A	N/A	No viremic side effects	>1:100	Bettinger et al. (2009)
Clone 13	Mice	1×10^{-2} to 1×10^7 pfu	IP	1×10^4 LD ₅₀ /ZH501	IP	100%	1:1613–2280	Muller et al. (1995)
	Hamsters		By mosquitoes	1×10^4 LD ₅₀ /ZH501	IP	100%	N/A	Muller et al. (1995)
R566	Pregnant ewes	1×10^2 to 1×10^5 pfu	IM	1×10^6 MICLD ₅₀ /buffalo 1998	IM	Abortion rate: unvaccinated: 100%, vaccinated: 12.5%	N/A	Hunter (2001)
Recombinant lumpy skin disease virus	Mice (6 weeks)	1st vacc.: 1×10^7 pfu, 2nd vacc.: 1×10^7 pfu	SC/SC and IM	100 MLD ₅₀ /AR20368	IM	100%	1:12	Wallace et al. (2006)
DNA vaccine	Mice (4–6 weeks)	5 μ g	Gene Gun	100 LD ₅₀ /ZH501	IP	Unvaccinated: 10%, vaccinated: 100%	PRNT ₅₀ 1:160–11,280	Spik et al. (2006)
	Mice (6–8 weeks)	50 μ g	Gene Gun	2.4×10^3 pfu or 2.4×10^4 pfu/ZH548	IP	Vaccinated (N): 50% asymptomatic, vaccinated (G _N /G _C): 62.5% asymptomatic	N: <1:25, G _N /G _C : 1:25–75	Lagerqvist et al. (2009)

Recombinant RVFV (Δ NSs and/or Δ NSm)	Rats	1 × 10 ³ pfu, 1 × 10 ⁴ pfu	SC	1 × 10 ³ pfu/ZH501	SC	Unvaccinated: 40%, vaccinated: 100%	PRNT ₅₀ : 1 × 10 ³ ; 1:640–1120, 1 × 10 ⁴ ; 1:640–7040 1:8–1:128	Bird et al. (2008)
Sindbis virus replicon	Mice (6 weeks)	1st vacc.: 1 × 10 ⁵ IU; 2nd vacc.: 1 × 10 ⁵ IU	footpad	100 MLD ₅₀ /VRL688/78	IP or IN	Vaccinated: 100%		Heise et al. (2009)
	Sheep	1st vacc.: 2.5 × 10 ⁵ IU; 2nd vacc.: 2.5 × 10 ⁵ IU	SC	N/A	N/A	Neutralizing antibodies	1:32 – 1:128	Heise et al. (2009)
Virus-like particles (VLP)	Mice	1 × 10 ⁵ or 1 × 10 ⁶	IP	2.4 × 10 ⁴ pfu/ZH 548	IP	Unvaccinated: 8%, vaccinated: 1 × 10 ⁵ : 50%; 1 × 10 ⁶ : 92%	1 × 10 ⁵ : <1:50, 1 × 10 ⁶ : 1:250–1,250	Naslund et al. (2009)
	Mice	3 × 6 µg (total protein)	SC	1 × 10 ³	IP	70%	>1:640	Mandell et al. (2009)
	Rats	60 µg (total protein)	SC	1 × 10 ⁵	SC	100%	N/A	Mandell et al. (2009)

SC: Subcutaneous, IM: intramuscular, IP: intraperitoneal, IN: intranasal, IT: intrathalamic, IS: intraspinal, IV: intravenous, pfu: plaque forming units, PRNT_{50/80}: plaque reduction neutralization titer by 50% or 80%, ZH501: virulent Zagazig Hospital RVFV strain 501, ZH548: Zagazig strain 548, NA: not applicable, IU: international units, LD₅₀: 50% lethal dose, MICLD₅₀: 50% mouse intracerebral lethal dose, MLD₅₀: 50% mouse lethal dose.

fetal rhesus lung cells and is available for veterinarians working in endemic areas, high containment laboratory workers and others at high risk for contracting RVFV (Pittman et al., 1999). Unfortunately, this vaccine is: (i) expensive, (ii) difficult to produce, (iii) in short supply, (iv) requires a larger dose relative to an attenuated vaccine and three initial inoculations followed by a 6-month booster and (v) requires continued annual boosters (Frank-Peterside, 2000; Kark et al., 1982, 1985; Niklasson et al., 1985; Pittman et al., 1999).

7.2.2. MP12

Derived by mutagenesis (5-fluorouracil) of a RVFV strain (ZH548) that was isolated during the 1977 outbreak in Egypt and grown in MRC5 human diploid fibroblast cells, MP12 is efficacious in livestock (Caplen et al., 1985; Hunter et al., 2002; Morrill et al., 1991, 1987, 1997a,b; Morrill and Peters, 2003). MP12 neutralizing antibodies are passed to unvaccinated neonates after suckling colostrum, giving rise to detectable titers.

Recently, MP12 was tested in a Phase II clinical trial to determine adverse effects in humans using a single injection dose escalation protocol (Bettinger et al., 2009). MP12 responders injected with 1 × 10⁵ pfu were asymptomatic, had a 95% antibody seroconversion rate and developed plaque reduction neutralization test antibody titers (PRNT₈₀) greater than 1:100. Those injected with formalin-inactivated TSI-GSD-200 (as a control) showed 90% seroconversion but PRNT₈₀ titers of only ≤1:28. Genetic analysis of MP12 isolated from vaccinated individuals showed no reversions of vaccine virus (in attenuated regions) to wild-type RVFV.

7.2.3. Clone 13/R566

To counteract the undesirable side effects of live-attenuated RVFV vaccine candidates described above (e.g., SMLV strain), two alternative live-attenuated RVFV vaccines are being developed: (i) the natural isolate Clone 13 (Muller et al., 1995) and (ii) the reassortant R566 (Bouloy et al., unpublished data). RVFV Clone 13 is characterized by a large deletion of the NSs gene from the S segment which prevents the virus from hijacking the type 1 IFN pathway (Billecocq et al., 2004; Bouloy et al., 2001). As described above, NSs is an interferon antagonist as well as a general transcription inhibitor (Le May et al., 2004), and its deletion thus allows the vaccinated host to launch an effective immune response (Bouloy et al., 2001). R566 is a reassortant of the L and M segments of MP12 and the S segment of Clone 13 that combines attenuation markers from both strains.

Clone 13 has been tested in sheep and cattle (Hunter, 2001), and all vaccinated animals elicit immune responses protective against challenge with virulent RVFV. Concerning R566, a vaccination trial was carried out in sheep and pregnant ewes in Senegal using a single dose of R566 ranging from 10² to 10⁶ pfu. None of the vaccinated animals exhibited signs of illness and none of the pregnant ewes aborted. All animals inoculated with 1 × 10⁵ pfu developed antibody responses including the generation of neutralizing antibodies (Y. Thionganne, personal communication). While a challenge experiment was not possible, preliminary experiments in mice show that all animals vaccinated with a dose of 100 pfu are protected against challenge with virulent virus (M. Bouloy et al., unpublished data). Because the deletion of NSs is responsible for attenuation, both Clone 13 and R566 can be considered safe from reversion to a replication-competent, fully infectious virus.

7.2.4. Lumpy skin disease vector

Wallace et al. (2006) expressed RVFV G_N and G_C in a lumpy skin disease virus (LSDV) and found that mice vaccinated twice with 1 × 10⁷ pfu recombinant LSDV develop neutralizing antibodies and are fully protected from a 100 LD₅₀ lethal viral challenge (Wallace et al., 2006).

7.2.5. Alphavirus replicon

Alphavirus replicon vectors that express the RVFV glycoproteins have been developed by USAMRIID (Heise et al., 2009). These vectors elicit RVFV-specific immune responses and are 100% protective against a 100 LD₅₀ intraperitoneal and intranasal RVFV lethal challenge in a mouse model. Furthermore, this vaccine candidate also elicits a RVFV neutralizing antibody response in vaccinated sheep (Heise et al., 2009). These encouraging data suggest that the use of alphavirus replicon particles (Roy and Noad, 2008) presents a promising vaccine platform for the development of RVFV vaccine candidates.

7.2.6. Baculovirus/insect cells

Recombinant baculovirus systems present another promising platform for RVFV vaccine development (Roy and Noad, 2008). Sf9 (*Spodoptera frugiperda*) insect cells have been used to produce RVFV glycoproteins after infection of recombinant baculoviruses encoding the RVFV M segment ORF (Liu et al., 2008; Noad and Roy, 2003; Schmaljohn et al., 1989). One of the key advantages of this system is the potential for ease of scale-up. Sf9 lysates containing expressed RVFV glycoproteins used for a single subcutaneous inoculation protect mice from lethal (100 LD₅₀) challenge (Schmaljohn et al., 1989). However, 100% protection requires the use of Freund's Complete Adjuvant in the vaccine formulation and only 50% protection was achieved when Sf9 cell lysates were delivered in PBS alone (Schmaljohn et al., 1989).

7.2.7. Virus-like particles

The use of virus-like particles (VLPs) is another promising alternative approach for the development of a safe and efficient RVFV vaccine. VLPs may be more immunogenic than recombinant proteins as they present a more natural format that maintains conformational epitopes that can induce neutralizing antibodies (Grgacic and Anderson, 2006). It has been previously shown that the expression of recombinant G_N and G_C glycoproteins of a phlebovirus closely related to RVFV, Uukuniemi, leads to the assembly and budding of VLPs from transfected eukaryotic cells (human embryonic kidney 293 cells) (Overby et al., 2007a,b; Overby et al., 2006). The incorporation of reporter gene-encoding minigenomes into VLPs enables the monitoring of VLP production and also allows the study of packaging signals located on these minigenomes (Flick et al., unpublished data).

Efficient generation of RVF VLPs has been demonstrated by Naslund et al. (2009) and Habjan et al. (2009a,b) in human embryonic kidney 293 cells using transfected DNA encoding the complete RVFV M segment and both the RNA polymerase L and a reporter minireplicon construct (Habjan et al., 2009a; Naslund et al., 2009). The addition of a GFP-expressing minigenome to the VLP producing cells allows minigenome packaging into the budding VLPs and subsequent measurement of VLP titers by quantifying GFP expression in susceptible cell lines expressing the required RVFV transcription machinery (L and N) (Habjan et al., 2009a). Naslund et al. (2009) showed that these RVF VLPs can be used as vaccines. Three i.p. injections of 1×10^6 RVF VLPs in mice induces antibody titers from 1:300 to 1:900 against G_N and G_C proteins but does not result in the development of N-specific antibodies. Importantly, these VLPs protect 11 of 12 vaccinated mice from lethal virus challenge (2.4×10^4 pfu) whereas only 1 of 12 survived in the unvaccinated control group (Naslund et al., 2009).

Recombinant baculovirus system-derived VLPs are an attractive and potentially cost-effective approach for the development of an effective vaccine strategy against RVFV infection because of the potential for high-yield production of VLPs in insect cells using the recombinant baculovirus expression system and the ease of scaleup

for manufacturing under GMP conditions. Liu et al. (2008) demonstrated the efficient generation of VLPs in insect cells using a single recombinant baculovirus that expresses the RVFV glycoproteins together with the N protein using a dual expression baculovirus vector (Liu et al., 2008). The purified VLPs exhibit enveloped structures that are similar to wild-type RVFV.

Using two separate animal models, Mandell et al. (2009) showed that RVF VLPs generated from 293 cells induce RVFV-specific humoral and cellular immune responses and protect mice from lethal challenge with RVFV. Splenocytes from mice injected with RVF VLPs secrete IL-2, IL-4, IL-5 and IFN- γ in response to RVFV antigens, consistent with both humoral (T_H2) and cellular (T_H1) responses. Additionally, vaccination with RVF VLPs elicits RVFV neutralizing antibody titers of $>1:640$ based on PRNT₈₀ assays even 6 months post-vaccination.

Interestingly, Mandell et al. (2009) were able to generate RVF VLPs lacking N, demonstrating that the glycoproteins can form VLPs without the need of any other viral proteins (Mandell et al., 2009). This is opposed to findings from others claiming that N is absolutely required for RVF VLP generation (Habjan et al., 2009a).

Differences in survival rates were observed when RVF VLPs containing or lacking the N protein are used in mouse and rat lethal challenge studies. While mice vaccinated with RVF VLPs survived lethal challenge, RVF VLPs containing N provide substantially higher protection rates ($\sim 70\%$ vs. $\sim 20\%$ without N) (Mandell et al., 2009), consistent with results seen for other vaccine platforms (e.g., DNA, see below), suggesting that RVFV N plays an important role in eliciting a protective immune response. Since rats are often considered more relevant than mice as a model for RVFV disease (Anderson et al., 1991; Bird et al., 2007b) a subsequent rat challenge study was performed. 100% of RVF VLP-vaccinated animals survived a high-dose (10^5 pfu ZH501) lethal challenge, while all unvaccinated animals died within the first 5 days, post-infection (Mandell et al., 2009).

7.2.8. DNA vaccines

DNA-based vaccine platforms have been used to induce immunity against RVFV. Spik et al. (2006) showed that a DNA vaccine encoding the RVFV M segment (with NSm), administered by gene gun technology, displays poor immunogenicity and is only marginally efficacious ($<50\%$ survival) in vaccinated mice upon lethal RVFV challenge (Spik et al., 2006). However, DNA vaccines encoding the M segment without the NSm is very immunogenic, and three gene gun-mediated vaccines delivered in 3-week intervals ($5 \mu\text{g}$ total DNA each) leads to 100% survival from a 100 LD₅₀ lethal challenge (Spik et al., 2006).

Three inoculations of sheep with $400 \mu\text{g}$ of a RVFV glycoprotein-expressing DNA vaccine using cationic-liposomes has also shown utility in stimulating RVFV-specific antibodies and boosting proliferating memory cell populations (Lorenzo et al., 2008). The immune response was improved by vaccination with a combination of the above described DNA vaccine with an N-expressing DNA vaccine (Lorenzo et al., 2008).

An alternative DNA vaccine approach was reported by Lagerqvist et al. (2009), who assessed the possible contribution of the RVFV N protein to vaccine immunogenicity. Using a non-lethal challenge of 2.4×10^3 and 2.4×10^4 pfu of RVFV ZH548, four of eight and five of eight mice vaccinated with DNA constructs encoding RVFV N or G_N/G_C, respectively, were aviremic (Lagerqvist et al., 2009). In contrast, all unvaccinated control mice showed clinical manifestations of RVFV. Analysis of neutralizing antibody titers revealed that mice vaccinated with constructs expressing both G_N/G_C show higher neutralizing levels (PRNT₅₀: 25–75) than those encoding the N protein (PRNT₅₀: <25). These findings suggest that antibody-mediated *in vitro* neutralization of RVFV might

not specifically correlate with vaccine efficacy, and RVFV N might indeed be an important vaccine antigen.

7.2.9. Immune modulation

Since a balanced immune response (humoral and cellular) is required for life long protection (Andrew et al., 1989; Guiso et al., 2007; Petrovsky, 2006; Petrovsky and Aguilar, 2004; Silva et al., 2004; Tangy and Naim, 2005), the use of immune modulators such as the HyperAcute® α Gal Technology (Flick et al., 2009; Hellrung et al., 2007, 2006; Turell and Bailey, 1987; Unfer et al., 2003) presents an interesting concept for the improvement of RVFV vaccines based on VLPs or other inactivated and/or replication-incompetent platforms. The HyperAcute® α Gal Technology exploits the α Gal-mediated barrier against pathogens from lower mammals that have incorporated α Gal epitopes (Gal- α (1–3) Gal- β (1,4) GlcNAc-R) on their cell surfaces (Rossi et al., 2005a,b; Squinto, 1996). A continual priming of this endogenous antigen is provided by α Gal epitopes on bacteria that reside in the intestine. With up to 2% of the entire circulating IgG antibodies targeting the α Gal epitope (Galili, 1999) and more than 1% of B lymphocytes producing anti- α Gal antibodies (Galili, 1993), vaccine antigens modified with α Gal epitopes are naturally primed to elicit a more robust and protective immune response than vaccines displaying the antigen alone (Abdel-Motal et al., 2005, 2007, 2009; Flick et al., 2009; Galili et al., 1985a,b,c, 2007; Slavin et al., 1985; Thall and Galili, 1990). Such technology could be a major step forward in the improvement of existing vaccines or for the development of efficacious new vaccine candidates (Mandell et al., 2009).

8. Historic and currently available therapies for RVF

While vaccines are the primary defense against viral diseases (Plotkin, 2005), the development of antiviral compounds with therapeutic efficacy against highly pathogenic RNA viruses is also important (Beigel and Bray, 2008; Bray, 2008). Licensed antiviral influenza compounds such as amantadine (Symmetrel, generic) and the neuraminidase inhibitors oseltamivir (Tamiflu, Roche Laboratories, Inc.) and zanamivir (Relenza, GlaxoSmithKline) are available. However, with the exception of ribavirin (Rebetol, Schering Corporation), few compounds are licensed for treatment of hemorrhagic fever viruses (Gowen and Holbrook, 2008). Furthermore, ribavirin has limited utility because of adverse side effects and lack of specificity (Kilgore et al., 1997; McCormick et al., 1986; Monath, 2008). Pyrazinecarboxamides (Toyama Chemical Co., Ltd.) are new antiviral compounds that have been shown to be especially useful for post-exposure antiviral therapy as broad spectrum antiviral inhibitors (Furuta et al., 2009) (see Table 2).

8.1. MxA

Although RVFV is a strong antagonist of IFN production, it is very sensitive to the action of IFN or to IFN inducers (Bouloy et al., 2001). The infection cycle of bunyaviruses is significantly retarded by human interferon-induced protein MxA by blocking the immediate early primary viral transcription step (Kochs et al., 2002; Reichelt et al., 2004). MxA, a large dynamin-like GTPase, exhibits antiviral capabilities against a wide array of RNA viruses that are beyond the scope of this review (Kochs et al., 2002; Pavlovic et al., 1992; Reichelt et al., 2004).

8.2. Ribavirin and polyriboinosinic acid

As a preliminary assessment of antivirals, Peters et al. (1986) showed that ribavirin and polyriboinosinic acid complexed with poly-L-lysine and carboxymethylcellulose (poly(ICLC)) prevents

clinical symptoms of RVFV in mice and hamsters (Peters et al., 1986). Furthermore, RVFV-infected rhesus macaques injected 50 mg/kg of ribavirin followed by daily 10 mg/kg injections at 8 h intervals over a 9-day period showed no RVFV-induced viremia (Peters et al., 1986).

In a more recent study, real-time RT-PCR was used to quantify RVFV titers in cell culture in the presence of the antiviral compounds ribavirin, IFN- α , 6-azauridine and glycyrrhizin (Garcia et al., 2001), all of which are commercially available. The maximal tolerated dose of each drug inhibited viral replication by >4 log RNA copies/ml. The minimal doses that showed antiviral activity were tested with titration of RVFV by TCID₅₀ and RT-PCR. At the lowest tested concentrations, 62.5 μ g/ml ribavirin, 1 IU/ml interferon alpha (IFN- α) and 0.3 μ g/ml 6-azauridine were still inhibitory, while glycyrrhizin showed no antiviral activity with viral replication at the lowest tested concentration (156 μ g/ μ l), with inhibition only at high concentrations (1250 and 2500 μ g/ml). IFN- α and ribavirin showed dose-dependent reduction in viral replication.

Although ribavirin was shown to have prophylactic RVFV activity *in vitro* (Peters et al., 1986), it might have unexpected/undesired effects. Ribavirin was used in Saudi Arabia to treat patients suffering from the hemorrhagic icterus form of RVF. Although ribavirin seemed active in treating these hemorrhagic forms, it did not prevent the development of severe meningo-encephalic complications, including hallucinations, lethargy and coma (B. Swanepoel, personal communication). The ribavirin molecule is too large to pass through the meningeal barrier. New molecules that mimic ribavirin activity but are small enough to pass the meningeal barrier are in development for RVF treatment (P. Formenty, personal communication).

8.3. T-705

Compound T-705, a pyrazinecarboxamide, inhibits RNA-dependent RNA polymerase activity in a dose-dependent manner. It has been suggested that host cell enzymes (cellular kinases) convert T-705 into T-705 ribofuranosyl phosphate (T-705RTP), a form that inhibits virus polymerase without affecting host cellular RNA or DNA synthesis. T-705 protects mice and hamsters against a lethal challenge with Punta Toro virus (PTV) when administered orally 4 h pre- to 24 h post-challenge. 10 mg/kg administered twice daily over a 5-day post-infection period was sufficient to protect mice, while initiation of T-705 treatment 24 h post-infection requires 30 mg/kg twice daily (Gowen et al., 2007). Hamsters given 30 mg/kg of T-705 orally twice daily beginning 4 h prior to PTV infection followed by treatment over 6 days showed significant protection (90%). In fact, T-705 was shown to be efficacious *in vitro* for several viruses in the bunyavirus family including LaCrosse, Punta Toro, Rift Valley fever, sandfly fever Sicilian virus, and in the arenavirus family including Junín, Pichinde, and Tacaribe viruses (Gowen et al., 2007). T-705 was also shown to be less toxic (less of a reduction animals' body weight and shorter clinical recovery times) compared to ribavirin (Gowen et al., 2008). Antiviral therapeutics such as T-705 may therefore present a more viable choice than ribavirin for combating post-exposure treatment of RNA viral infections (Furuta et al., 2009; Julander et al., 2009).

8.4. PPMO

Reverse genetics-driven minigenome rescue systems were used recently in Dr. R. Flick's group to identify antiviral compounds against the RVFV-related phlebovirus Uukuniemi virus (unpublished data). Uukuniemi virus-specific antisense peptide-conjugated phosphorodiamidate morpholino oligomers (PPMOs)

Table 2
Bunyaviral therapeutic candidates.

Therapeutic	Species	Vaccine dose	Vaccination route	Challenge dose/virus	Challenge route	Protection levels	PRNT ₈₀	Reference
Ribavirin	Rhesus macaques (Adult)	Rhesus: 50 mg/kg, followed by 10 mg/kg at 8 h intervals for 9 days	Rhesus	Rhesus NHP: $1 \times 10^{4.2}$ pfu/ZH 501	Rhesus: IV	Rhesus: Unvaccinated: viremia: 100%, vaccinated: 25%	Rhesus: 1:640–1:1280	Peters et al. (1986)
	Hamsters (Syrian golden)	Hamsters: 60 mg/kg twice daily, followed by 20 mg/kg twice daily for 9 days	Hamsters: SC	Hamsters: 10 pfu/ZH 501	Hamsters: SC	Hamsters: 80%		
	C57BL/6N mice (male, 6 weeks old)	Mice: 75 mg/kg at 8 h post-infection and 25 mg/kg twice daily for 10 days	Mice: SC	Mice: 200 pfu/ZH 501	Mice: SC	Mice: 75%, 30% after rechallenge with 5×10^3 pfu		
Interferon α	Rhesus macaques	$5 \times$ at 5×10^5 U/kg at 24 h before or 6 h post-challenge	IM	1×10^5 pfu/ZH501	IV	Neutralizing antibodies	1:320–1:5120	Morrill et al. (1989)
T-705 pyrazine-carboxamide	Mice	$2 \times$ for 5 days beginning 4 h prior to challenge: 3–30 mg/kg/day	Oral	500 pfu/PTV	SC	4 h prior to infection: 30 mg/kg/ $2 \times$ day: 100%, 10 mg/kg/ $2 \times$ day: 100%, 3 mg/kg/ $2 \times$ day: 10%	N/A	Gowen et al. (2007)
		$2 \times$ for 5 days beginning 24 h after challenge: 10–30 mg/kg/day		5000 pfu/PTV		24 h after infection: 30 mg/kg/ $2 \times$ day: 90%, 10 mg/kg/ $2 \times$ day: 30%		
				500 pfu/PTV		30 mg/kg/ $2 \times$ day: 90%, 10 mg/kg/ $2 \times$ day: 40%		
				50 pfu/PTV		30 mg/kg/ $2 \times$ day: 100%, 10 mg/kg/ $2 \times$ day: 0%		
	Hamsters	$2 \times$ for 6 days beginning 4 h prior to challenge: 15–60 mg/kg/day		5 pfu/PTV		60 mg/kg/ $2 \times$ day: 90%, 30 mg/kg/ $2 \times$ day: 90%, 15 mg/kg/ $2 \times$ day: 50%		

SC: Subcutaneous, IM: intramuscular, IV: intravenous, pfu: plaque forming units, PRNT_{50/80}: plaque reduction neutralization titer by 50% or 80%, ZH501: virulent Zagazig Hospital RVFV strain 501, PTV: Punta Toro virus, NA: not applicable.

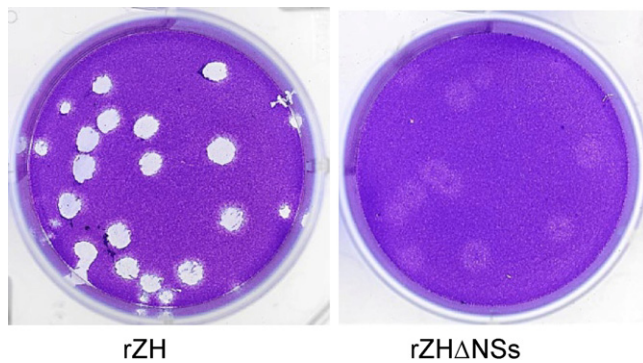


Fig. 5. Plaques generated by RVFV rescued from plasmids using reverse genetics. rZH: rescued from wild-type ZH548-based plasmids; rZHΔNSs: rescued with a mutated S segment, lacking the NSs coding sequence.

were used to identify the mechanism and optimal genome target for efficient antiviral activity. A clear dose-dependent reduction in minigenome expression levels and virus titer was observed using Uukuniemi-specific PPMOs (unpublished data). PPMOs have been shown to provide resistance *in vivo* and *in vitro* to several viruses including West Nile virus (Deas et al., 2007; Zhang et al., 2008), Influenza A, Dengue 2, and Zaire ebolavirus (Berghaler et al., 2006; Buchholz et al., 2000; Flanagan et al., 2001; Gabriel et al., 2008; Lupfer et al., 2008; Stein, 2008; Stein et al., 2008).

9. Reverse genetics-derived RVF vaccine candidates

Until recently, vaccine trials and basic science investigations have been hampered by the biosafety restrictions surrounding RVFV, but new technology utilizing a minigenome rescue system has facilitated studies of the molecular biology of RVFV (see above) (Flick and Bouloy, 2005; Walpita and Flick, 2005). In addition, established reverse genetics systems (infectious clone systems) have allowed for the generation of attenuated recombinant RVFV vaccine candidates (Billecocq et al., 2008; Gerrard et al., 2007; Habjan et al., 2008b; Ikegami et al., 2006).

RVFV has only one serotype which makes vaccine development relatively straightforward in design. Unlike previous live-attenuated vaccines developed empirically by adapting the virus to a new species (e.g., the attenuated RVFV Smithburn strain; see above), new vaccines are expected to be devoid of toxicity or pathogenic effects. Studies carried out in many laboratories on the immune-evading (e.g., interferon antagonist) role of some viral proteins (mostly non-structural) have paved the way for the creation of viruses deficient for these proteins using reverse genetics methodologies. In the case of RVFV, it became possible to produce an avirulent virus like Clone 13 but in which NSs is entirely deleted (see Fig. 5) (Billecocq et al., 2004; Bird et al., 2008; Habjan et al., 2009b; Ikegami et al., 2005b).

To further investigate the role of RVFV nonstructural protein NSm, recombinant viruses lacking the gene were created by reverse genetics, either in the context of the virulent ZH501 strain or the attenuated MP12 strain (Won et al., 2006). The recombinant ΔNSm ZH501 (rZH501) virus lacks the sequence from the first AUG start codon to the sequence immediately upstream of the fourth AUG, whereas the recombinant MP12 viruses (rMP12) contain mutations in the first and/or the second AUG to generate RVFV lacking the 14k or 78k NSm protein (or both). These recombinant viruses grow in various cell types, indicating that the NSm proteins are dispensable for the viral replication cycle. Furthermore, studies with highly susceptible Wistar-Furth rats which, like mice, are suitable animal models for RVFV vaccine testing in challenge studies, show that

ΔNSm-rZH501 virus retains lethality in these animals but is attenuated compared to ZH501 (Anderson et al., 1991; Anderson and Peters, 1988; Bird et al., 2007a).

More recently, using an established infectious clone system, Bird et al. (2008) employed reverse genetics to generate live-attenuated recombinant ZH501 RVFV vaccine candidates by replacing NSs with the reporter gene GFP and by eliminating the non-structural protein NSm (rZH501-ΔNSs:GFP, rZH501-ΔNSs:GFP-ΔNSm). These highly attenuated viruses proved to be asymptomatic and protective, after a single 1×10^3 or 1×10^4 pfu vaccination, against viral challenge (1×10^3 pfu ZH501) with robust seroconversion in a lethal rat model. The use of reverse genetics to develop vaccine prototypes, such as these with precisely engineered disruptions to the NSs and NSm genes, clearly enables the study of fundamental questions regarding the molecular biology and pathogenesis of bunyaviruses.

Interestingly, these viruses, similar to Clone 13, lack some molecular markers as indicated by the absence of anti-NSs antibodies in vaccinated animals. These gene deletions therefore allow for serological differentiation of naturally infected animals from vaccinated animals based upon NSs antibody titers. By assessing the presence or absence of anti-NSs specific antibodies, Bird et al. (2008) demonstrate the applicability of reverse genetics for the development of vaccine candidates providing the ability for differentiating infected from vaccinated animals (DIVA) (see below) (Bird et al., 2008).

10. Future applications of reverse genetics systems

Reverse genetics has been successfully used to study the molecular biology of many negative-strand RNA viruses. Important discoveries in regard to pathogenicity mechanisms, the analysis of genome packaging and studies of the entry mechanism of viruses into their host cells are a few examples of the power of reverse genetics. Recent successes in the generation of improved (safety and efficacy) vaccine candidates for RVFV by means of reverse genetics further illustrate the utility and importance of such systems.

The use of minigenome rescue systems allows for the determination of the role of individual proteins in the viral replication cycle (Billecocq et al., 2008; Gerrard and Nichol, 2007; Habjan et al., 2008b, 2009a; Ikegami et al., 2006). These systems are also ideal for the identification of *cis*-acting elements responsible for the regulation of viral genome encapsidation, transcription, replication and packaging (Albarino et al., 2007; Billecocq et al., 2008; Habjan et al., 2009a; Ikegami et al., 2007). As identified through reverse genetics, the non-structural proteins of RVFV (NSs and NSm), while accessory to replication, can be fully characterized for their virulence properties and compared to non-structural proteins of other bunyaviruses. Using reverse genetics systems or classical genetic reassortment, NSs type I IFN antagonistic activity has also been described for another phlebovirus, Punta Toro virus (Perrone et al., 2007), as well as for the orthobunyaviruses Bunyamwera virus (Bridgen et al., 2001) and LaCrosse virus (Blakqori et al., 2007).

Importantly, these systems also facilitate the identification of pathogenic factors and viral mechanisms used to evade the innate immune system that are crucial for targeted vaccine development (Bird et al., 2007a; Ikegami et al., 2006). Similar approaches to eliminate viral proteins involved in immune evasion via reverse genetics could result in additional promising vaccine candidates. Furthermore, such minigenome rescue systems can also be used for the identification of potential antiviral agents in an high-throughput screening assay (Flick, 2009; Freiberg et al., 2008), especially considering the recent progress in the development of sensitive minigenome rescue systems and the establishment of a reverse genetics-driven infectious clone system for RVFV.

Minigenome rescue systems were also used to identify other potential antivirals (Flick, 2009; Freiberg et al., 2008). Based on successful reduction of viral titer using virus-specific PPMO's for several virus families, a similar approach can be applied to the identification of PPMOs with RVFV-specific inhibitory activities. Especially for viruses classified as high-containment agents, minigenome rescue systems eliminate the cumbersome work required under biosafety levels 3 and 4 conditions.

The establishment of an infectious clone system for RVFV via reverse genetics allowed the generation of recombinant RVFV which were successfully used as promising RVFV vaccines. Reverse genetics allows the change of a single nucleotide in the RVFV genome to generate new live-attenuated vaccine candidates. But it also enables the exchange or deletion of a whole viral protein, e.g., NSs or NSm, resulting in safe vaccine candidates.

A major drawback for commercially available RVFV vaccines for livestock, which are based on live-attenuated vaccine, is the inability to differentiate infected from vaccinated animals. Vaccines that allow for the differentiating infected from vaccinated animals (DIVA) can be applied to livestock in enzootic regions of countries listed by the World Organization for Animal Health (WOAH) (IOE, 2007). The use of live-attenuated vaccine strains, such as genetically engineered RVFV recombinants without NSm or NSs genes, or naturally derived Clone 13 lacking most of its NSs ORF, shown to protect vaccinated animals, allows for the serologic differentiation of naturally or experimentally infected subjects by the absence of anti-NSs antibodies (Bird et al., 2008; Muller et al., 1995). Another RVFV vaccine candidate following the DIVA concept is based on VLPs (Mandell et al., 2009; Naslund et al., 2009). Since RVF VLPs do not induce a detectable anti-N response in contrast to natural infection, vaccinated animals can be easily differentiated from naturally infected individuals by the presence or absence of anti-N antibodies.

Reverse genetics may also enable a broad expansion of tissue specific detection of non-segmented and segmented minus strand RNA viruses including those that infect specific target organs. Generating chimeric proteins comprised of the viral protein of interest fused to a reporter gene, e.g., GFP, replacing a viral gene with a GFP gene, or inserting such reporter gene into a viral genome using reverse genetics technology can generate useful tools to monitor virus replication and protein trafficking.

For example, the intracellular localization of the CCHFV glycoproteins G_N and G_C have been clearly defined by expression of GFP fusion proteins. Haferkamp et al. (2005) used recombinant glycoprotein-GFP chimeras to identify Golgi and endoplasmic reticulum retention signals in the cytoplasmic and ectodomains of the G_N and G_C glycoproteins, respectively (Haferkamp et al., 2005). Similar intracellular trafficking signals have been identified for other bunyaviruses including Uukuniemi (Andersson and Pettersson, 1998), Punta Toro (Chen and Compans, 1991; Chen et al., 1991; Matsuoka et al., 1996), RVFV (Gerrard and Nichol, 2002), Bunyamwera (Lappin et al., 1994) and Sin Nombre (Spiropoulou et al., 2003).

Replication-incompetent viral vectors including retroviral, adeno- and adeno-associated vectors that encode reporter genes, including GFP, have been extensively used to show tissue-specific expression of heterologous transgenes. GFP is the reporter gene of choice for tracking tissue specific expression including the pancreas, cornea, liver, kidney, small intestine, and peripheral blood in mouse models (Cheng et al., 2004; Fu et al., 2003; Thalmeier and Huss, 2001; Thalmeier et al., 2001). Flow cytometry, immunofluorescence, direct tissue fluorescence and immunohistochemical staining are all options for GFP detection if the proper controls are implemented (Guo et al., 2007; Swenson et al., 2007). These *in vivo* models are of particular relevance for studies on tissue specific tropism for RVFV-induced hepatic necrosis in the liver,

hemorrhagic damage in the peripheral blood system, and neurogenic disorders in the brain (e.g. delayed-onset encephalitis) and eye (severe retinitis).

Additionally, reverse genetic technologies can be applied to the development of sensitive diagnostic tools. For example, a recombinant rabies virus encoding the GFP gene is the key component of a new high-throughput, rapid fluorescent focus inhibition test (RFFIT) that eliminates the need for direct immunofluorescence with anti-rabies antibodies to measure infection/neutralization (Khawplod et al., 2005, 2006).

An example of a GFP-tagged replication-competent RVFV is described by Bird et al. (2008) who replaced NSs with a GFP gene. This construct allows for tracking of the tissue distribution of RVFV load, *in vivo*, without the need for invasive procedures. This live-attenuated vaccine candidate can also serve as an immunological indicator of vaccination because it is easily distinguished from infection by wild-type virus since immunized animals do not generate antibodies against NSs. In contrast, animals infected with RVFV generate anti-NSs antibodies. Therefore, this recombinant vaccine provides a platform that differentiates naturally infected and vaccinated animals (DIVA).

Thus, recombinant viruses developed by reverse genetics encoding the GFP gene or other reporters can be used for intra- and extracellular, tissue-specific tracking of viruses and/or specific viral proteins, as an efficient diagnostic for antiviral compounds and as tools for the development of highly sensitive, cost-effective assays for diagnostic screening.

The importance of reverse genetics systems cannot be overstated. The established reverse genetics systems for RVFV opened the doors to previously unreachable research areas, including (i) RVFV-specific pathogenicity, (ii) innate immune system evasion mechanisms, (iii) a detailed dissection of the molecular biology of the virus and (iv) rational design of safe and efficient vaccine candidates. Importantly, the tools developed by reverse genetics for RVFV are readily applicable to other human pathogenic viruses.

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