RIFT VALLEY FEVER VIRUS: A REVIEW OF RECENT DATA

LE VIRUS DE LA FIÈVRE DE LA VALLÉE DU RIFT : UNE REVUE DES DONNÉES RÉCENTES

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(Communication présentée le 3 décembre 2009)

SUMMARY

Rift Valley fever virus (RVFV) causes large outbreaks of acute febrile and often fatal illness among humans and domesticated animals in sub-saharan Africa and the Arabian Peninsula. The RVFV is of the genus Phlebovirus, *within the family* Bunyaviridae*. Like all members of this large family, it contains a three-segmented RNA genome of negative/ambisense polarity, packaged into viral nucleocapsid protein, and finally surrounded by a lipid bilayer containing two viral glycoproteins. RVFV epidemiology, molecular biology, and virulence mechanisms have raised considerable interest over the past years. In the present article, we provide an overview of the basic features of this significant pathogen, and of the latest developments in this highly active research field.*

Key words: **Bunyaviridae*, genus* Phlebovirus*, pathogenesis, vaccines.***

RÉSUMÉ

Le virus de la fièvre de la vallée du Rift (RVFV) est à l'origine de grandes épidémies, caractérisées par un épisode fébrile, souvent mortel, chez les hommes et les animaux des régions sub-sahariennes et de la Péninsule Arabique. Le RVFV appartient à la famille *Bunyaviridae*, et au genre *Phlebovirus*. Comme tous les autres membres de cette vaste famille, ce virus est équipé d'un génome constitué d'un ARN négatif segmenté (trois segments) avec une polarité négatif/ambisens, empaqueté dans une nucléocapside protéinique et finalement enveloppé dans une membrane à deux épaisseurs comportant deux glycoprotéines virales. Au cours de ces dernières années, le RVFV a fait l'objet d'une attention accrue en ce qui concerne son épidémiologie, sa biologie moléculaire, et ses mécanismes de virulence. Dans notre revue, nous donnons un aperçu sur les caractéristiques essentielles de cet agent pathogène important, et nous rendons compte des derniers développements dans ce domaine très actif de recherche.

Mots-clés : *Bunyavirus*, *Phlebovirus*, pathogenèse, vaccin.

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INTRODUCTION

Viruses within the *Bunyaviridae* family are classified into five genera: *Orthobunyavirus*, *Nairovirus*, *Phlebovirus*, *Hantavirus* and *Tospovirus* (Nichol *et al.* 2005 ; Schmaljohn & Nichol, 2007 ; Schmaljohn 2007). Tospoviruses infect plants while the other bunyaviruses infect vertebrates. Several bunyaviruses including Rift Valley fever virus (RVFV), La Crosse, Crimean-Congo hemorrhagic fever, Hantaan and sin nombre viruses are responsible for potentially lethal hemorrhagic fevers and therefore are of significant medical and public health importance. Hantaviruses are transmitted by rodents whereas the other bunyaviruses are transmitted by arthropods: mosquitoes (orthobunyaviruses), phlebotomes (Phleboviruses), ticks (Nairoviruses) and thrips (Tospoviruses).

Rift Valley fever virus (RVFV) belongs to the genus *Phlebovirus*, family *Bunyaviridae*. It is a serious emerging pathogen affecting humans and livestock. Since the first description of an outbreak in Kenya 1931 (Daubney *et al.* 1931), recurrent epidemics have killed hundreds of thousands of animals, more than a thousand humans, and caused significant economic losses. During the last decades, serious outbreaks occurred in Egypt, West Africa, East and South Africa (Kenya, Tanzania and Somalia), Madagascar and in 2000 the virus spread to the Arabian Peninsula provoking two simultaneous outbreaks in Yemen and Saudi Arabia (Swanepoel & Coetzer, 2004). Recently, the virus was reported in the Comoros, including Mayotte. In East and South Africa, epidemics and epizootics are associated with periods of unusually heavy rains and the warm phase of El Nino. In spite of intense circulation during outbreaks and its ability to infect a wide range of hosts, the virus displays relatively low genetic diversity.

RVFV is transmitted to animals by many mosquito species (*Aedes*, *Anopheles*, *Culex*, *Eretmapoites*, *Mansonia*), but has also been shown to be transmitted by other vectors, e.g. sand flies or by contact with infected material. Infected animals develop necrotic hepatitis, hemorrhage and abortion, with death rates up to 100% among newborn animals. Lambs and kids are less susceptible but develop acute disease associated with high fever, weakness and anorexia. Some animals may exhibit melaena, foetid diarrhoea, nasal discharge and icterus. In adults animals RVFV infection is often asymptomatic, but the virus manifests itself by high rates of abortions which may vary from 15 to 100%.

Humans are often infected by close contact with sick animals which may occur during herding, slaughtering and butchering, milking or birthing livestock and through veterinary manipulations. The disease in human is associated with symptoms ranging from uncomplicated acute febrile illness to retinitis, hepatitis, renal failure, meningoencephalitis, severe hemorrhagic disease, and death. Platelet dysfunction, thrombocytopenia and depletion of coagulation factors are observed in many cases. Elevated levels of serum aspartate and alanine aminotransferases are recorded even in the mild clinical forms. The hemorrhagic syndrome probably results from an increased endothelium permeability due to capillary injuries, as for other viral hemorrhagic fevers. Although the mortality rate for humans was reported to be approximately 2%, in recent outbreaks it went up to 35% (LaBeaud *et al.* 2008).

The severity of RVFV zoonosis, its capability to cause major epidemics among livestock and humans, and the lack of efficient prophylactic and therapeutic measures make infection with this pathogen a serious public health concern not only in endemic, developing countries, but also in many non-endemic industrial countries (Morrill & McClain, 1996). Authorities have therefore listed RVF as a notifiable disease and classified RVFV as a BSL-3 or BSL-3+/4 agent respectively, in Europe and in the US, and a potential biological weapon.

During the past years, there was an increased interest in RVFV epidemiology, molecular biology, and virulence mechanisms. Here, we will try to provide an overview over the basic features of this significant pathogen, and review the latest developments in this highly active research field.

VIRION STRUCTURE

Early ultrastructural studies by electron microscopy and negative staining (Ellis *et al.* 1979) described RVFV particles measuring 90-110 nm in diameter. The envelope is composed of a lipid bilayer containing the Gn and Gc glycoproteins forming surface subunits, 5-8 nm in length, regularly arranged on its surface. The viral ribonucleoproteins (RNPs) composed of the three genomic segments associated with numerous copies of the nucleoprotein N and the RNA dependent RNA polymerase L are packaged into the virion. More recent studies by cryo-electron microscopy (Freiberg *et al.* 2008; Huiskonen *et al.* 2009; Sherman *et al.* 2009) have changed the former view that phleboviruses are pleiomorphic. Instead, these studies indicated that the virion is likely to have an icosahedral symmetry: the structure of RVFV is surprisingly ordered and the surface covered by a shell of 122 glycoprotein capsomers arranged in an icosahedral lattice with T=12.

THE VIRAL GENOME

Bunyaviruses are enveloped and have a tri-segmented single-stranded RNA genome of negative or ambisense polarity (Elliott 1996). The genome segments of bunyaviruses encode four structural proteins: the viral polymerase (L) on the large (L) segment, two glycoproteins (Gn and Gc) on the medium (M) segment, and the viral nucleocapsid protein (N) on the smallest (S) segment. RVFV additionally expresses two nonstructural proteins encoded on the M segment, a 78kDa protein and a 14kDa protein also called NSm1 and NSm2 (Gerrard & Nichol, 2007), and one on the S segment, termed NSs. These nonstructural proteins are dispensable for viral multiplication in cell culture, but play important roles for pathogenesis *in vivo* (Vialat *et al.* 2000; Won *et al.* 2006 ; Bird *et al.* 2007 ; Gerrard *et al.* 2007).

The three genomic RNA segments of bunyaviruses contain untranslated regions (UTRs) at the 3' and 5' ends which are segment-specific and serve as promoters for transcription and replication by the viral polymerase. The terminal sequences of the UTRs are conserved and form panhandle structures which give the RNPs a circular appearance when observed by electron microscopy for review, (Elliott 1996). For RVFV, the L and M segments are of negative polarity while the S segment utilizes an ambisense strategy to code for both N and NSs (Giorgi *et al.* 1991). The coding capacity of the genome is depicted in ***figure 1A***. The general view that only the viral, negative-sense genome is incorporated into the mature particle must be revised as a small but significant fraction of the antigenomes i.e. replicative intermediates have been detected in purified RVFV particles (Ikegami *et al.* 2005).

VIRAL REPLICATION CYCLE AND THE ROLE OF VIRAL GENE PRODUCTS

Transcription and replication

The general features of RVFV transcription and replication are similar to those of other negative stranded RNA viruses (Elliott 1996; Schmaljohn & Nichol, 2007). Transcription and replication take place in the cytoplasm. During the replication cycle, each segment is transcribed into a mRNA and is replicated through a process which involves the synthesis of the exact copy of the genome, called complementary RNA (cRNA) or antigenome. For Phleboviruses, and RVFV in particular, the cRNA representing the copy of the S ambisense segment serves as template for the synthesis of the NSs mRNA. Since some cRNA is also present in the input virus, the virulence factor NSs is expressed immediately after the virus has entered the host cell. Bunyaviral mRNA synthesis in general is initiated through a cap-snatching mechanism by which host mRNAs are cleaved through an endonuclease activity of the L protein to obtain 10 to 18 nucleotide-long primers for transcription. Synthesis of cRNA and vRNA does not require an oligonucleotide primer and is directly initiated with 5' nucleoside triphosphates. cRNA represents a complete copy of the vRNA, whereas mRNAs terminate before the 5' end of the template. The mechanism of the switch between transcription and replication remains unknown. Moreover, although RNA-dependent polymerase consensus motifs are present in the RVFV L protein (Muller *et al.* 1994), the domains responsible for the different activities have not been defined precisely.

Minigenome systems have been helpful to analyze some steps in RNA synthesis, i.e. transcription, replication, transcription termination, and packaging. Minigenomes are similar to viral genome segments but the viral ORF is replaced by a reporter gene. Minigenomes are usually expressed either from T7- or from Pol I-based promoter plasmids, or transcribed *in vitro* and transfected as RNA. Early on, studies with minigenomes have established that transcription and replication requires the N and L proteins (Lopez *et al.* 1995) confirming that naked vRNA cannot be transcribed. Analysis of the RVFV RNPs showed that the N protein is able to establish intermolecular interactions through several amino-acids located in the N terminal region of the protein which are conserved among phlebovirus N sequences (Le May *et al.* 2005). The ability of N to form oligomers may be a conserved feature in bunyaviruses. The L protein, probably in association with N, is able to perform both transcription and replication, excluding the possibility that the L protein is modified by a third viral factor to function as a replicase.

When compared to each other, the L, M and S segment-based minigenomes do not express identical levels of reporter gene. This indicates differential promoter activities associated with the UTRs (Gauliard *et al.* 2006). Although the mutagenesis analysis was not as extensive as the one carried out for Uukuniemi virus (Flick *et al.* 2002), it appeared that some of the conserved UTR nucleotides play an important role in promoter activity and the regulation of gene expression (Prehaud *et al.* 1997).

RVFV mRNAs are not polyadenylated at their 3'end. They have a shortened 3' end relative to the full-length cRNA, suggesting that a specific termination signal is recognized during transcription. Preliminary data had indicated that a conserved motif is present in the intergenic region of the S segment of the phleboviruses Toscana (TOSV), Sandfly fever Sicilian (SFSV), and RVFV (Giorgi *et al.* 1991; Gro *et al.* 1992). Albarino *et al.* mapped precisely the 3' ends of the L, M, N and NSs mRNAs of TOSV, SFSV and RVFV, and identified a sequence motif 3'-$C_{1\text{-}3}$GUCG/A-5' which is conserved on the M and S segments of phleboviruses (Albarino *et al.* 2007). With regard to the L segment mRNA, conflicting data were reported, showing either that it is a full-length 3' end (Albarino *et al.* 2007) or that a short sequence at the 3' end is missing (Ikegami *et al.* 2007).

Glycoproteins and particle formation

The RVFV envelope glycoproteins mediate particle entry into cells through receptors which, remain to be identified. RVFV entry is predicted to employ a class II fusion mechanism that is activated by low pH following endocytosis of the virion (Filone *et al.* 2006). The RVFV M segment encodes Gn (encoded by amino-terminal sequences) and Gc (encoded by carboxy-terminal sequences) as well as two nonstructural proteins NSm1 (78kDa) and NSm2 (14kDa). The NSm1 and NSm2 proteins are produced by alternative use of the first and second of the 5 in-frame AUG codons, respectively, which are present at the 5' end of the M mRNA, i.e. upstream of the Gn sequence ***(figure 1B)***. The role of these proteins will be discussed further below.

Like all bunyaviruses, RVFV particles usually bud into the lumen of the Golgi apparatus (Elliott 1996). Both RVFV Gn and Gc localize to the Golgi apparatus when expressed together from polyprotein-expressing plasmids (Wasmoen *et al.* 1988) When expressed alone, Gn is transported to the Golgi apparatus due

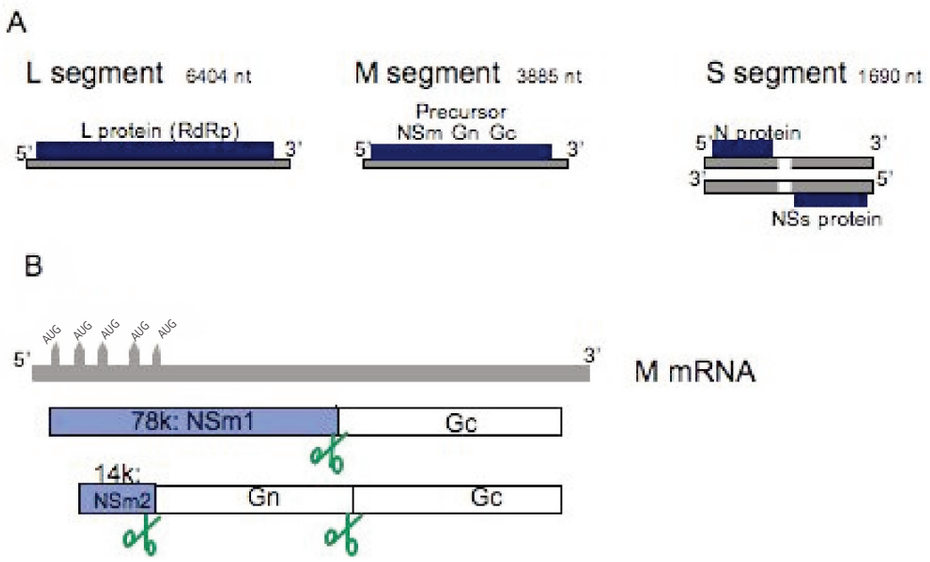

Figure 1: *Genome and expression strategies of RVFV.*
(A) *Schematic representation of the three genomic segments L (large), M (medium) and S (small) and the proteins encoded on them.*
(B) *The mRNA transcribed from the M segment contains five in-frame start codons which gove rise to the 78kDa and the 14 kDa proteins, as well as some minor products (Gerrard & Nichol, 2007). Scissors indicate host cell protease sites for cleavage of the polyproteins.*

to a specific Golgi localization motif, whereas Gc localized to the endoplasmic reticulum (ER) due to the presence of a specific ER retention signal (Gerrard & Nichol, 2002). This suggests that Gc moves to the Golgi apparatus via its physical association with Gn.

The minigenome systems expressing a reporter gene were also useful to investigate viral packaging activity. In that case, the glycoproteins were co-expressed with the transcription machinery to allow formation of viral like particles (VLPs) released into the medium (Habjan *et al.* 2009a). When observed by electron microscopy and negative staining, these particles resembled RVFV particles in size and morphology. They were able to infect naïve cells and to undergo the first step of the replication cycle, i. e. primary transcription. If both L and N were provided in trans, replication of minigenomes also occurred.

Reverse genetics

Reverse genetics systems allow to freely manipulate the viral genome and to dissect individual steps in the viral multiplication cycle. Fully infectious, recombinant virus particles can be recovered from cloned cDNA plasmids if the minireplicon is replaced by constructs for the full-length viral RNA segments. This technique was first established for Bunyamwera virus, the prototype of the *Bunyaviridae* (Bridgen & Elliott, 1996). These rescue systems were based on T7-transgenic cells to express the constructs for the viral genome segments and the helper proteins N and L (Ikegami *et al.* 2006 ; Gerrard *et al.* 2007), but promoters for the cellular pol I could also be used to express the genome segments (Billecocq *et al.* 2008; Habjan *et al.* 2008b). The cell lines (BSR-T7 or BHK-T7) used for T7 expression are deficient in the RIG-I pathway (Habjan *et al.* 2008a) which would normally respond to the 5' triphosphorylated T7 transcripts with the production of the antiviral IFNs.

Depending on the laboratory which developed the rescue system, the genetic backbone of the recombinant virus was based either on the attenuated strain MP12 (Ikegami *et al.* 2006 ; Billecocq *et al.* 2008), or the virulent strains ZH501 (Gerrard *et al.* 2007) or ZH548 (Billecocq *et al.* 2008; Habjan *et al.* 2008b).

ROLE OF THE NON-STRUCTURAL PROTEINS

The establishment of reverse genetics systems has opened the door for studies of RVFV molecular biology and pathogenesis which were unthinkable a few years ago.

The role of NSs in virulence

NSs of RVFV forms a ribbon-like filament in the nucleus (Struthers & Swanepoel, 1982; Swanepoel & Blackburn, 1977) ***(figure 2)***. This feature is unexpected for a virus replicating in the cytoplasm. Moreover, it is particular to RVFV and not shared with NSs proteins of other bunyaviruses. NSs was characterized as a major virulence factor counteracting the antiviral IFN system (Vialat *et al.* 2000 ; Bouloy *et al.* 2001; Billecocq *et al.* 2004). It has become clear meanwhile that NSs is a multifunctional protein inhibiting cellular basic transcription and suppressing two different arms of the IFN response, namely

(i) IFN induction by a specific, early, mechanism

(ii) the antiviral protein PKR.

With respect to specific suppression of IFN induction, the cellular protein SAP30 was identified as interacting with NSs. SAP30 belongs to the Sin3A/NCoR/HDAC repressor com-

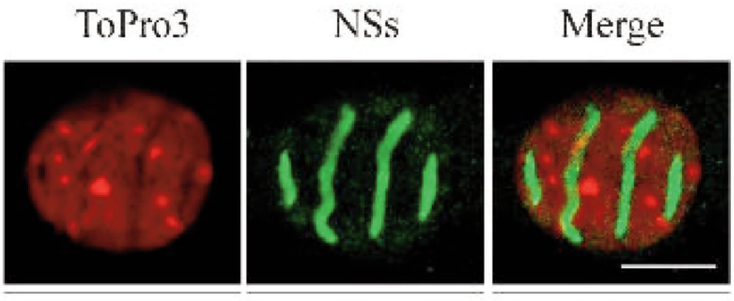

Figure 2: *RVFV NSs forms a filamentous structure in the nucleus*
Confocal microscopy picture showing a section of a L929 cell nucleus infected by ZH548 and stained with the DNA intercalating dye ToPro3 (red), with anti-NSs antibodies (green) and merge. The cellular DNA is predominantly excluded from the filament. (From Mansuroglu et al. 2009*)*

plexes intervening in gene transcription regulation (Le May *et al.* 2008). Moreover, SAP30 interacts directly with YY1, a transcription factor involved in the regulation of expression of numerous genes, including IFN-β. Through a series of co-immunoprecipitation, confocal microscopy and chromatin immunoprecipitations, it was demonstrated that NSs, SAP30, YY1 and Sin3A-associated corepressors are recruited on the IFN-β promoter inhibiting CBP recruitment, histone acetylation and transcriptional activation. Using reverse genetics, a recombinant ZH548 in which NSs was deleted of the SAP30 interaction domain was produced. Still located in the nucleus, this mutant protein was not able to form filaments. In contrast with the wt virus, this mutant, ZH548-NSsΔ210-230, induced IFN-β expression and was unable to kill mice. Further analyses on the wt NSs filament indicated that, even though cellular DNA is mostly excluded from the filament, heterochromatin clusters of pericentromeric gamma satellite sequences appeared intimately associated with NSs (Mansuroglu *et al.* 2009). This results in a high incidence of nuclear anomalies, translating chromosome cohesion and segregation defects which could contribute to RVFV pathology.

NSs also suppresses cellular gene expression by a more general mechanism which relies on the interaction with the host cell protein p44. p44 is a subunit of the TFIIH basal transcription factor and becomes sequestered into the NSs filamentous structure characteristic of RVFV infection (Le May *et al.* 2004). As a consequence, TFIIH cannot assemble and its concentration drops rapidly, explaining the drastically reduced transcriptional activity of cells infected with RVFV. Interestingly, NSs functions as a general inhibitor of Pol II as well as Pol I, since TFIIH is involved in transcription by both these host polymerases. Inhibition of TFIIH and hence general transcription is a relatively late event occurring after 8 h of infection, whereas the specific inhibition of IFN-β transcription (mediated by SAP30 recruitment) is in place as early as 3-4 h after infection.

Very recently, a novel function of NSs was described, the targeting of the antiviral, IFN-induced protein kinase PKR (Habjan *et al.* 2009b; Ikegami *et al.* 2009). PKR is a serine-threonine kinase activated by viral RNAs and mediating a stop of translation. NSs-dependent degradation is specific for PKR and occurs through the proteasome. Hence, cells infected with RVFV are devoid of PKR, whereas PKR was clearly detected and activated by infection with NSs-deleted virus strains, e.g. Clone 13. Interestingly, it was also observed that this activity is specific to RVFV NSs and not shared with other Phleboviruses like Sandfly fever Sicilian virus or the orthobunyavirus LaCrosse.

Altogether, it appears that NSs has multiple functions to counteract the IFN system, either at the transcriptional level or at the translational level by degrading PKR.

The role of the NSm protein as a virulence factor and a suppressor of virus-induced apoptosis

The biological function of the NSm1 and NSm2 proteins was addressed by producing RVFV mutants with either a deletion in the pre-Gn region or point mutations in the start codons of the two respective reading frames (Gerrard *et al.* 2007; Won *et al.*, 2006). In all cases, virus growth in cell culture was similar to the parental viruses. However, when the pre-Gn deletion was introduced into the MP12 strain, plaques were larger than for the parental MP12 strain and a more extensive apoptosis due to elevated caspase activity was observed (Won *et al.*, 2007).

NEW INSIGHTS FOR THE DEVELOPMENT OF VACCINES

The only vaccine commercially available, was developed by Smithburn (Smithburn 1949) who passaged the Entebbe isolate in mice and eggs to attenuate the virulence However, the passaged virus, called the Smithburn strain, had lost only partially its virulence: it induces abortions and teratogenesis in ewes, so that its use is restricted to periods of intense RVFV circulation and pregnant animals cannot be vaccinated.

A more recent attenuated virus MP12 obtained after 12 passages in the presence of a mutagen was immunogenic but is still found abortigenic during a vaccination trial in ewes (Hunter *et al.* 2002). An alternative candidate is the natural virus, Clone 13, which lacks more than 70% of the coding sequence for the IFN antagonist NSs and is avirulent for mice, sheep and cattle, but highly immunogenic (Muller *et al.* 1995).

While the deletion in Clone 13 was obtained naturally, it is now possible to manipulate the viral genome via reverse genetics, opening ways to abrogate its pathogenicity. The NSs and NSm sequences are obvious targets to produce live attenuated RVFV. Several attenuated recombinant RVFV were created: rMP12 in which the NSs sequence was deleted partially as in Clone 13 or completely (Ikegami *et al.* 2006) and rZH548△NSs (Billecocq *et al.* 2008; Habjan *et al.* 2008b), rZH501△NSs or rZH501△NSs/NSm (Bird *et al.*, 2007 ; Bird *et al.*, 2008) characterized by the complete deletion of NSs and/or NSm produced in the ZH548 or ZH501 genetic context, respectively. RVFV mutant lacking NSm are somewhat attenuated compared to wt RVFV but can still cause either acute lethal hepatic necrosis or delayed lethal neurologic disease (Bird *et al.* 2007). The double deletion mutants induced robust anti-RVF virus antibody responses and 100% protection from a lethal challenge (Bird *et al.* 2008).

Sub-unit recombinant vaccine candidates expressing the RVFV glycoproteins have been described for reviews (Bouloy & Flick, 2009; Ikegami & Makino, 2009): they used different vectors such as the lumpy skin disease vector or the alphavirus or adenovirus vectors. Other groups have developed another type of sub-unit vaccine by expressing the RVFV glycoproteins which can assemble into VLPs. For efficient production, recombinant baculovirus were constructed expressing the glycoproteins alone or in association with N (Liu *et al.* 2008). DNA-based vaccine administrated by gene gun has also been described but required several immunizations.

Thus, the recently gained knowledge on RVFV molecular biology already proved useful for the understanding and development of modern vaccine candidates (Bouloy & Flick, 2009; Ikegami & Makino, 2009), and future findings will certainly help to develop antivirals and strategies to combat this important pathogen.

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