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REVIEW

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Countermeasure development for Rift Valley fever: deletion, modification or targeting of major virulence factor *NSs*

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ABSTRACT: Rift Valley fever (RVF) is a mosquito-borne zoonotic disease characterized by a high rate of abortion in ruminants, and febrile illness, hemorrhagic fever, retinitis and encephalitis in humans. RVF is caused by the RVF virus (RVFV), belonging to the genus *Phlebovirus* of the family *Bunyaviridae*. RVFV encodes a major virulence factor, *NSs*, which is dispensable for viral replication, yet required for evasion of host innate immune responses. RVFV *NSs* inhibits host gene upregulation at the transcriptional level, while promoting viral translation in the cytoplasm. In this article, we summarize the virology and pathology of RVF, and countermeasure development for RVF, with emphasis on *NSs* function and applications.

KEYWORDS

• MP-12 • *NSs* • *Phlebovirus*
• Rift Valley fever virus
• vaccine

Rift Valley fever

Rift Valley fever (RVF) is mosquito-borne zoonotic disease affecting both ruminants and humans. RVF was first reported in a farm near Lake Naivasha in Kenya in 1930 [1], and has been endemic to sub-Saharan Africa for more than 80 years. RVF is caused by the RVF virus (RVFV), belonging to the genus *Phlebovirus* of the family *Bunyaviridae* [2]. In 1977, a large RVF outbreak occurred in Egypt – 20,000–200,000 humans were infected, and approximately 600 patients died of RVF [3]. Furthermore, RVFV crossed the border of Africa, and caused a large outbreak in Saudi Arabia and Yemen in 2000 [4,5]. RVFV is primarily maintained in floodwater *Aedes* mosquitoes, by which RVFV is transmitted transovarially, while many other mosquitoes (e.g., *Culex spp.*) serve as amplifying vectors of RVFV [6,7]. Ruminants, such as sheep, cattle or goats, can be infected via mosquito bite, and pregnant animals show a high rate of abortion and fetal malformation, while newborn animals can die of acute hepatitis [8–11]. Most adult ruminants show subclinical or temporal febrile illness, and viremic animals may further feed mosquitoes, which can result in the amplification of RVFV. Human infection with RVF may occur by mosquito bite or contact with the body fluid of infected animals. Most human RVF patients develop self-limiting febrile illness lasting up to 1–2 weeks. In the convalescent phase of disease, some patients develop retinitis or uveitis, which may lead to blindness, while some develop neurological disorders [12]. Thrombosis in the convalescent phase is also reported in a few cases [13,14]. Importantly, some patients develop a lethal hemorrhagic fever-like illness at the early stage of disease, and the death may occur 3–6 days after the onset of symptoms [12]. Potential introduction of RVFV into nonendemic countries outside Africa may cause large RVF outbreaks, and persistence of RVFV in the area for long periods, which may negatively affect the economy of the country [15,16]. Due to the potentially serious consequences of this pathogen, RVFV is classified as Category A Priority Pathogen by the NIH in the USA. Furthermore, to regulate the possession of pathogens related to the use for bioterrorism in the USA, RVFV is included as a select

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agent by the CDC and the US Department of Agriculture [17].

Development of countermeasures against RVFV is important to minimize the impact of RVF. For example, early detection of RVFV or diagnosis of RVF can minimize the time for the government to take an action to prevent further spread of RVFV. Although postmortem examination, including immunohistochemistry, is still useful to identify RVFV [18], rapid detection of viral RNA from viremic samples can be done by conventional or real-time RT-PCR, loop-mediated isothermal amplification (LAMP) [19] or recombinase polymerase amplification assay (RPA) [20,21]. Alternatively, antigen-capture (or sandwich) ELISA is useful to detect RVFV antigens in the situation where viral RNA cannot be stably preserved in the field [22,23]. Since the viremic period is very short, the use of whole-blood samples rather than sera could increase the sensitivity of RVFV RNA detection [24]. Identification of animals or humans with IgM specific to RVFV by IgM-capture ELISA is also important, since the recent infection status (i.e., within 1 month) indicates the history of RVFV spread in the area [25–27]. After the detection of RVFV or IgM specific to RVFV, rapid containment of RVFV is required. In addition to the cull of infected animals, vaccination may be the most effective way to prevent animals from further amplifying RVFV in the area. In endemic countries, a live-attenuated Smithburn vaccine has been used since 1950s, which is derived from the mouse brain-adapted Entebbe strain [28–30]. The use of Smithburn vaccine in pregnant animals is prohibited because of the risk of abortion [31], and the vaccine is approved for veterinary purposes only in endemic countries. In the USA, a live-attenuated MP-12 vaccine, which was developed from the Egyptian ZH548 strain by serial plaque purification in the presence of chemical mutagen 5-fluorouracil, has been manufactured at the Salk Institute [32,33]. The MP-12 vaccine has been approved as a conditional (emergency) vaccine for veterinary use for a 2-year term. The MP-12 vaccine has also been tested in humans in a Phase II clinical trial, and no significant adverse effects have been reported [34]. Although MP-12 is highly efficacious and sufficiently safe, a lack of marker to differentiate infected from vaccinated animals (DIVA) might compromise the monitoring of animals upon the introduction of RVFV. In addition, a live-attenuated vaccine may not be the best choice for humans in terms of safety. In the USA, a highly safe formalin-inactivated vaccine for RVF is available (TSI-GSD-200). However, the dose is limited, and TSI-GSD-200 is less immunogenic compared with MP-12 [35–37]. An ideal vaccine for RVF might be the one that encompasses high efficacy, which rapidly induces protective immunity in both ruminants and humans; perfect safety, in particular, for humans; a DIVA marker for veterinary purposes; low-cost manufacturing; and no transmission in mosquito vectors. Although RVFV is still largely contained in Africa, the development of a better vaccine for RVF is an important task for the USA and other countries.

RVFV & its lifecycle in infected cells

RVFV has a tripartite negative-stranded RNA genome, named small (S), medium (M) and large (L) segment **(Figure 1A)**. As with other phleboviruses, the S-segment (1690 nt) encodes the nucleocapsid (*N*) gene in the negative-sense (viral-sense) genome, and the nonstructural (*NSs*) gene in the positive-sense (antiviral-sense) genome in an ambisense manner. The N protein encapsidates viral genomic RNA, and is required for viral RNA synthesis and packaging of viral RNA into virions [38–40]. The NSs protein is a major virulence factor for RVFV [41–43], although it is dispensable for viral replication [44,45]. The M-segment (3885 nt) encodes a single open reading frame (ORF), while the M mRNA can make at least four different proteins (i.e., 78-kD, NSm, Gn and Gc) by leaky scanning of five different initiation codons as well as cotranslational cleavage of polypeptides [46–49]. The Gn and Gc are viral envelope proteins and form heterodimers on the surface of virion to form capsomers [50–54]. It is considered that the Gn plays a role in receptor binding, and the Gc serves as a class II fusion protein. The NSm protein (also described as NSm2) is dispensable for RVFV replication [55,56], localizes to the outer membrane of mitochondria [57], and delays the progress of apoptosis in infected cells [58]. The 78-kD protein (also described as NSm1) is less characterized, yet incorporation into the virion has been suggested [59]. The L-segment (6404 nt) encodes a single ORF for the RNA-dependent RNA polymerase (L protein) [60]. The L protein plays a role in genome RNA replication and viral mRNA transcription. L proteins snatch host mRNA to use it as a primer for transcription (cap snatching), and thus viral mRNAs contain heterogeneous sequences at the 5′-ends. The N-terminus of

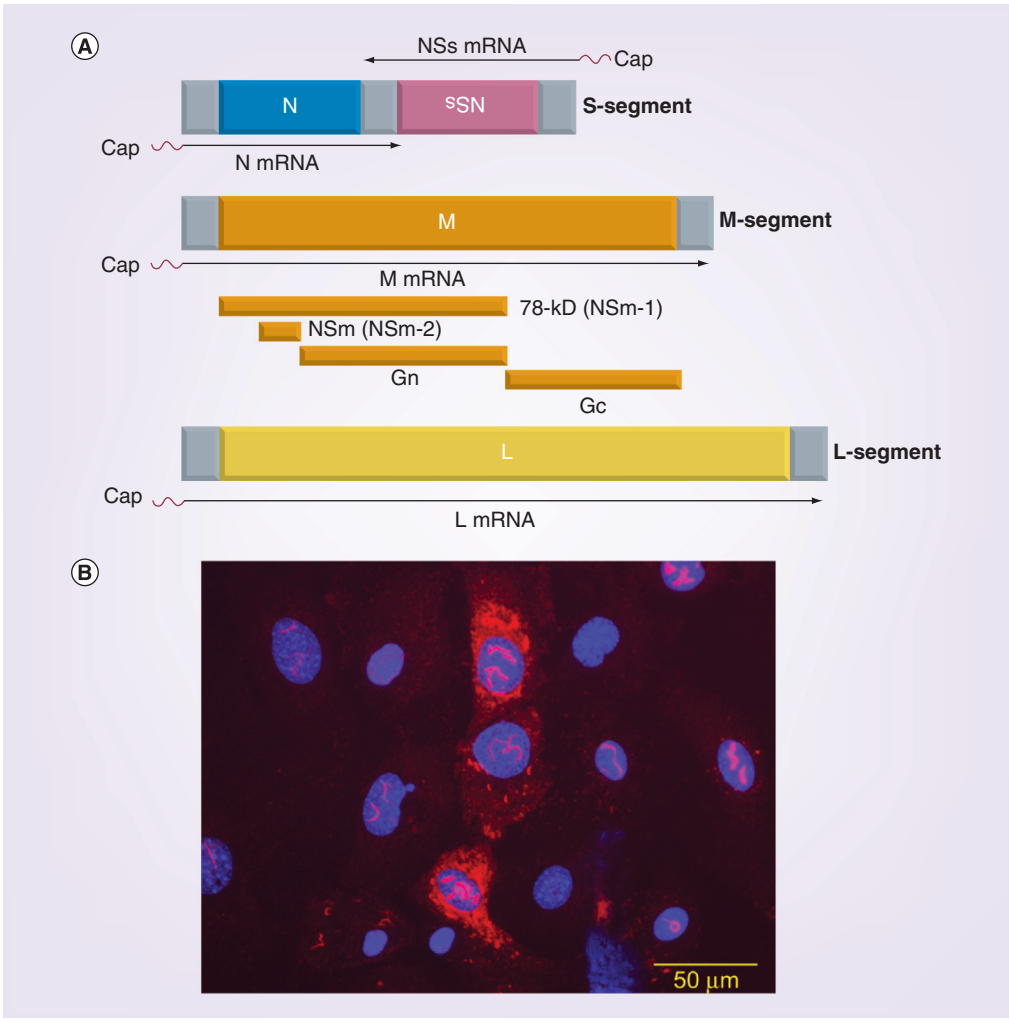

Figure 1. Rift Valley fever virus genome structure and the NSs protein. (A) Rift Valley fever virus (RVFV) genome structure. RVFV has tripartite negative-stranded RNA genome named the S-, M- and L-segments. The S-segment encodes N and NSs mRNA in an ambisense manner. The M- and L-segments encode M and L mRNA, respectively. M mRNA will be translated into at least four different proteins – 78-kD, NSm, Gn and Gc by leaky scanning of several in-frame initiation codons, as well as cotranslational cleavage of translated polypeptides. The 5′-end terminus of each mRNA is derived from host mRNA. **(B)** The NSs protein. Mouse embryonic fibroblast cells lacking PKR were transfected with *in vitro*-synthesized RNA encoding MP-12 NSs. RVFV NSs accumulates in the nucleus and cytoplasm, while nuclear NSs forms a filament structure.
Please see color figure online at www.futuremedicine.com/doi/pdf/10.2217/fvl.13.11.

the L protein encodes a NL1 domain conserved between bunyaviruses and arenaviruses, which may serve as an endonuclease to cleave off host mRNA for the cap-snatching process [61,62].

Viral attachment occurs between Gn/Gc and DC-SIGN [63,64]. Alternatively, the virus can bind to heparansulfate for entry [65]. Upon attachment to a viral receptor, RVFV enters cells by dynamin-dependent caveolae-mediated endocytosis [66]. Like other phleboviruses, RVFV may also cause membrane fusion to cells in the late endosome via exposure of the Gc fusion loop [50,64]. Since the viral L protein is attached to the viral ribonucleocapsid (RNP; viral genomic RNA encapsidated with N proteins) [67] in virions, the incoming genomic RNA immediately becomes a template for mRNA synthesis [68]. Theoretically, N mRNA, M mRNA and L mRNA can be transcribed from the negative-stranded S-, M- and L-segments, respectively

(primary transcription), and NSs mRNA can be transcribed from the positive-stranded S-segment only after viral genome RNA replication. However, NSs mRNA was also found to be transcribed at the primary transcription stage [68]. It was found that both negative-sense and positive-sense S-, M- and L-segments are packaged into virions, which allows a head-start synthesis of NSs mRNA in infected cells [68]. Subsequently, viral RNA genome replication occurs, and further accumulation of viral mRNA also occurs (secondary transcription). Then, the viral assembly process starts at the Golgi region. The Gn encodes a Golgi retention signal at the transmembrane domain and the cytoplasmic domain, while the Gc encodes an ER retention signal [69]. The coexpression of Gn and Gc allows the accumulation of Gn/Gc at the Golgi region [69]. Since there are no matrix proteins, the cytoplasmic domains of Gn/Gc are considered to be the viral components that interact with the RNP, although there has been little research regarding this so far. Gn and Gc encode one (N438) and four N-glycosylation sites (N794, N829, N1035 and N1077), respectively [47], which may play a role in the maturation of viral virions, as well as attachment to the DC-SIGN receptor in next infection cycle.

Evasion of host innate immune responses by RVFV

RVFV causes unique pathology in different animal species [8–10,12,70]. The susceptibility of host species is as follows: mouse/hamster/lamb > rat/sheep > human/monkey/cow/goat, while no symptomatic disease can occur in rabbits, pigs, horses, guinea pigs or birds [70]. The mouse is one of the most susceptible animal species to RVFV and dies in 7–10 days postinfection by either fulminant hepatitis or viral spread in the CNS [71]. Upon entry of RVFV into a mouse, dendritic cells, macrophages and granulocytes may be the primary target cells [72,73]. In BALB/c mice infected with the RVFV ZH501 strain (1000 PFU subcutaneously), the virus can spread systematically by 3–4 days postinfection (dpi; e.g., liver, spleen, kidney, heart and adrenal gland), while viral antigens can be efficiently cleared in those tissues by 6–7 dpi [71]. At the same time, RVFV spreads throughout the CNS around 6–7 dpi, which leads to the death of mice [71,74]. To control the virulence of RVF, it is important to know how the clearance of RVFV can occur. It was reported that passive transfer of neutralizing antibodies (1:10) was sufficient to protect hamsters from virulent RVFV challenge [75]. This result indicates that neutralizing antibodies are sufficient to protect the host from the progression of disease. Viral evasion in the host occurs by the NSs protein, since RVFV lacking *NSs* induces abundant type-I interferon (IFN) in mice and cannot establish viremia [41]. On the other hand, mice lacking IFNAR succumb to the infection with RVFV lacking *NSs* [41], indicating that host innate immunity plays an important role in preventing RVFV replication, while viral replication can induce the death of mice even without *NSs* if the host does not have a competent innate immune system. Using C57BL/6 mouse models, the requirement for mice to be protected from disease due to RVFV lacking *NSs* (RVFVΔ*NSs*) was studied [76]. Although B-cell-deficient mice were largely resistant to infection with RVFVΔ*NSs*, such mice were all dead after challenge with wild-type RVFV [76]. On the other hand, mice with depleted $CD4^+$ T cells, but not with depleted $CD8^+$ T cells, succumbed to lethal encephalitis caused by the infection [76]. Therefore, $CD4^+$ T cells (and maybe neutralizing antibodies) but not $CD8^+$ T cells should play an important role in viral clearance and prevention of the progression of disease in this model. This result is also consistent with the finding that mice with neutralizing antibodies can be always protected from wild-type RVFV challenge [73,77,78]. Other studies indicate that cellular immune responses toward N protein can also alter the pathology of the diseases [79]. Currently, it remains unknown how viral clearance occurs by immune cells in the liver or other organs.

Multiple functions of RVFV NSs

As described above, RVFV NSs plays a role in evasion from host innate immunity. NSs protein (265 amino acids) forms a unique filamentous structure in the nucleus, although it also accumulates in the cytoplasm **(Figure 1B)** [80]. The 17 C-terminal amino acids are required for the formation of the NSs filament [80]. There is no typical nuclear localization signal in NSs, while there are four proline-rich motifs (PXXP motif 1–4) [81] and two phosphorylation sites by casein kinase II at Ser 252 and Ser 256 [82]. Mutations of the PXXP motif 1 and 2, but not 3 and 4, could largely abolish the accumulation and filament formation of NSs in the nucleus, while mutations of Ser 252 or 256 did not affect

filament formation [81]. NSs is responsible for the suppression of *IFN-β* gene expression, and the intact PXXP motif 1 and 2 appears to be important for NSs function [81]. Inhibition of the *IFN-β* gene occurs at the transcription level: that is, the inhibition of IFN-β mRNA synthesis occurs in the presence of the activation of three important transcription factors for *IFN-β* promoter activation – IRF-3, NF-κB and AP-1 in infected cells [81]. Subsequently, it was found that RVFV NSs could bind to and sequester the subunit protein p44 of TFIIH, which is an essential transcription factor for host RNA polymerase I and II, and inhibits general host transcription [83]. RVFV NSs also binds to SAP30, and it has been proposed that the recruitment of CBP/p300 is interrupted when the NSs filament is bound to the *IFN-β* gene promoter via SAP30 [84]. It is still unclear how the two distinct mechanisms affect *IFN-β* promoter suppression, since there are no available mutants lacking either function.

RVFV NSs also promotes the post-translational degradation of dsRNA-dependent PKR [43,85]. The degradation of PKR does not require the replication of RVFV, and PKR could be stabilized in the presence of the proteasome inhibitor MG132 [43,85]. A point mutation at R173 into alanine could abolish this PKR degradation function due to a lack of specific interactions with PKR [86]. Interestingly, this mutant NSs could form filament structures with a mosaic-like pattern in the nucleus, which is distinct from the intact NSs filament, and allows early accumulation of eIF2α phosphorylation in infected cells, which dramatically decreases the accumulation of viral proteins [86]. Those findings indicate that RVFV regulates host transcription suppression and PKR degradation independently. Although the mechanism of early accumulation of phosphorylated eIF2α remains unclear, a similar phenomenon was also observed in cells infected with RVFV lacking *NSs* in the presence of transcription inhibitor, actinomycin D [85]. Thus, it is proposed that NSs-mediated PKR degradation plays an important role in active viral translation in the presence of host transcription suppression induced by NSs itself.

TFIIH consists of ten subunit proteins: XPD (a gene defective in xeroderma pigmentosum patient complementation group D), p8, p34, p44, p52, p62, XPB (a gene defective in xeroderma pigmentosum patient complementation group B), MAT1, cyclin H and cdk7. RVFV also promotes the post-translational degradation of TFIIH p62 subunit protein [87]. Similarly to PKR degradation by NSs, the NSs can bind to p62, and p62 was stabilized in the presence of MG132. On the other hand, the degradation of p62 occurs even in the presence of leptomycin B (nuclear export inhibitor), indicating that degradation occurs inside the nucleus [87]. Thus, there are at least two distinct substrates for RVFV NSs-mediated degradation: PKR and TFIIH p62. Currently, there is no evidence of increased polyubiquitination of these proteins in the presence of NSs. It is also unclear whether the sequestration of p44 and the degradation of p62 are redundant functions to inhibit host transcription, or whether they are just a snapshot of more dynamic NSs functions that inhibit host transcription machinery.

RVFV NSs also affects cell cycle progression. Cells usually undergo interphase – G1 phase (growth), S phase (DNA replication), G2 phase (growth) and M phase (preparation for mitosis) – before mitosis. Chromosomal segregation is driven by the centromere, which binds to the mitotic spindles [88]. RVFV NSs filaments physically exclude chromatin DNA and interact with the γ-satellite sequence (pericentromere; a region flanking the centromere), but not with the α-satellite sequence (centromere) [89]. Cells infected with RVFV exhibit nuclear abnormality and a reduced rate of mitosis, which are most likely the results of chromosome cohesion and segregation defect [89]. RVFV NSs also activates the classical DNA damage signaling pathway via ATM (phosphorylation of p53 [Ser 1]), Chk.2 [Thr 68] and H2A.X [Ser 139]) and induces cell cycle arrest at the S phase (MP-12 strain) or the G0/G1 phase (ZH548 strain) [90]. Another study also showed that RVFV NSs can induce the phosphorylation of p53 at Ser 9, 15, 20, 37, 46 and 392 in cells infected with RVFV, and a lack of p53 reduction could result in reduced viral replication and a subsequent decrease in cell death [91].

Subsequently, genome-wide identification of cellular DNA bound to RVFV NSs was performed by chromatin immunoprecipitation (IP) combined with a promoter array chip. Among the 33 NSs-interacting genes, the expression of ten genes (*Mapk8ip3*, *Fbxo3*, *Stat2*, *Il3*, *Il10rb*, *Tyk2*, *Casp9*, *Phf21*, *Ncoa3* and *Notch4*) at 8 hpi was significantly downregulated in RVFV-infected cells [92]. Those genes are involved with protein phosphorylation, ubiquitination, immune responses, apoptosis, transcription or

development. In addition, the upregulation (FX, FII, FVIII, Tfpi) or downregulation (FIII) of coagulation factors occurs in cells infected with RVFV, indicating the potential involvement of NSs with coagulation disorders during hemorrhagic fever [92]. These results also indicate that RVFV NSs selectively inhibits host genes.

RVFV accumulates a lower level of NSs proteins in C6/36 cells derived from *Aedes albopictus* or LL-5 cells derived from *Lutzomyia longipalpis* (sandfly) than that in mammalian cells [93]. A recent study showed that U4.4 cells (*Aedes aegypti*) barely allow the expression of NSs filament formation, and Aag2 cells (*Aedes aegypti*) allow NSs filament formation only at the early stage of infection (~24 hpi), whereas C6/36 cells (*A. albopictus*) can express NSs filaments for a longer period [94]. In mosquito cells infected with RVFV, the RNAi silencing pathway can potentially inhibit the replication of RVFV. Namely, RNA secondary structures or viral replicative intermediates become the template to form a small virus-derived RNA duplex (viRNA) by Dicer-2, which allows sequence-specific degradation of target mRNA via RISC [94]. The viRNAs specific to S (mostly antiviral-sense), M (both viral and antiviral-sense) and L (both viral and antiviral-sense) appears as 21–28 nt in size, while the signature for Piwi-interacting RNA (piRNA; 5′-terminal uridine and an adenosine at position 10) is clearly visible for large viRNA such as 27–28 nt in size [94]. Although C6/36 cells are known to be Dicer-2 deficient, the presence of viRNA with a piRNA signature indicates that there is also an antiviral mechanism in C6/36 cells [94]. The attenuated expression of the *NSs* gene in mosquitoes may contribute to the persistent infection of RVFV.

Significance of RVFV NSs in live-attenuated vaccine development

The first RVFV strain lacking NSs (Clone 13) was isolated from the RVFV 74HB59 strain (in the Central African Republic), and the NSs encodes an in-frame 69% truncation, which results in the abolishment of all known NSs functions [44]. The Clone 13 strain has been extensively used as a control virus to evaluate the functions of NSs. Clone 13 is completely attenuated in mice, and its safety and efficacy has been demonstrated in sheep and cattle [41,95,96]. Due to a lack of functional NSs, infected animals can mount innate immune responses, including IFN-α/β, and no viremia can be detected in immunocompetent animals. On the other hand, Clone 13 can cause lethal disease in IFNAR$^{-/-}$ mice or *PKR* knockout mice [41,43], indicating that the limiting factor is the competent host innate immune system. The reassortment of Clone 13 with the ZH548 S-segment; that is, reassortant consisting of ZH548 S and Clone 13 M and L, resulted in a virulent RVFV strain [41]. On the other hand, the reassortant consisting of Clone 13 S and ZH548 13 M and L was completely attenuated [41]. A reassortant virus encoding the Clone 13 S-segment and MP-12 M- and L-segments is also under development as the R566 strain [97], which might decrease the risk of the potential creation of virulent reassortant RVFV.

The recent development of reverse genetics for RVFV allowed the creation of recombinant RVFV lacking *NSs* [42,45,56,98]. The recombinant ZH501 strain lacking both *NSs* and *NSm* (rZH501Δ*NSs*Δ*NSm*) has been tested in pregnant ewes and has been demonstrated to be efficacious and safe for veterinary vaccine use [99,100]. Vaccination with rZH501Δ*NSs*Δ*NSm* does not cause detectable viremia, does not induce abortion or fetal malformation in pregnant ewes, and protects ewes from wild-type RVFV challenge [100]. The *NSm* truncation partly attenuates the ZH501 M-segment [101], and, thus, the risk of reversion to virulence upon reassortment with wild-type RVFV may be reduced compared with the single deletion of the *NSs* gene. Another study also indicated that RVFV lacking *NSm*, but not that lacking NSs, is less efficient in mosquito transmission [102]. Thus, this may be an advantage for use in the field. Due to demonstrated safety, rZH501Δ*NSs*Δ*NSm* is excluded from the select agent rule, and can be handled at biosafety level (BSL) 2 in the USA upon approval by each institutional biosafety committee. The absence of anti-NSs antibodies in vaccinated animals is useful for DIVA [100,103], although not all animals naturally infected with RVFV raise against NSs sufficiently [104]. On the other hand, the sensitive detection of anti-NSm antibody in ruminant sera infected with RVFV appears to be more difficult than that of anti-NSs [100,105].

A live-attenuated MP-12 vaccine is highly attenuated in both animals and humans, and it is considered as a conditional vaccine for veterinary use in the USA, as well as a potential vaccine candidate for human use [34]. MP-12 is also excluded from the select agent rule, and handling can be done at BSL2 level in the USA. MP-12

is derived from the wild-type RVFV ZH548 strain, and encodes 23 mutations in the genome (four in the S-segment, nine in the M-segment and ten in the L-segment) [33]. The attenuation largely occurs by the M- and/or L-segment, but not in the S-segment [42], while the attenuation mutation(s) are still not identified among them. Importantly, the NSs of MP-12 is still fully functional, and the recombinant ZH548 strain encoding MP-12 NSs has a virulent phenotype [42]. Since the NSs is a major virulence factor for RVFV [41], the deletion of the *NSs* gene from the vaccine can greatly improve the safety profile of the vaccine. On the other hand, the reduction in efficacy due to a lack of *NSs* may also need to be considered. Therefore, a recombinant MP-12 lacking a functional *NSs* (rMP12-C13type) was generated by a reverse genetics system [45], and efficacy was compared with parental MP-12 in a mouse model. MP-12 vaccination induced viremia in 30% of vaccinated mice without detectable induction of IFN-α, and 72% of vaccinated mice were protected from wild-type RVFV challenge at 44 days postvaccination [73]. On the other hand, the rMP12-C13type failed to develop detectable viremia, induced detectable IFN-α (~100 pg/ml) in sera at day 1, and 80% of vaccinated mice were protected from the wild-type RVFV challenge [73]. The mean neutralizing antibody titers of mice vaccinated with MP-12 or rMP12-C13type (1×10^5 PFU subcutaneously, one dose) are 1:641 or 1:549 on day 30, 1:2,987 or 1:1,056 on day 90, and 1:1,067 or 1:216 on day 180, respectively. Thus, some reduction of neutralizing antibody titer might occur due to a lack of the *NSs* gene in the MP-12 vaccine. On the other hand, rMP12-C13type is attenuated in the S-, M- and L-segment, and encodes a DIVA marker for agricultural use [106]. To further develop rMP12-C13type as a vaccine, a dose–response analysis should be carried out in ruminants.

Another strategy to make the MP-12 vaccine capable of DIVA is to replace the *NSs* gene with functional NSs derived from other phlebovirus species. Using this approach, MP-12 may still escape host innate immune responses, while being more attenuated than original MP-12. *Phlebovirus* genera consist of approximately 70 named viruses, which are largely divided into three major phylogenetic groups: the mosquito/sandfly-borne phlebovirus group, the Uukuniemi group, and the severe fever with thrombocytopenia syndrome virus (SFTSV)/Heartland/Bhanja group [107,108]. The mosquito/sandfly-borne phlebovirus consists of Naples serocomplex (e.g., Toscana virus [TOSV]), Sicilian serocomplex (e.g., Sandfly fever Sicilian virus [SFSV]), Punta Toro serocomplex (e.g., Punta Toro virus Adames strain [PTV]), RVF serocomplex, Icoaraci serocomplex (e.g., Icoaraci virus) and Frijoles serocomplex (e.g., Frijoles virus) [109]. Recombinant MP-12 encoding the *NSs* of TOSV (rMP12-TOSNSs), SFSV (rMP12-SFSNSs) or PTV (rMP12-PTNSs), which are phylogenetically close to RVFV *NSs*, were generated by reverse genetics, and efficacy in mice was tested. Surprisingly, the rMP12-TOSNSs induced viral encephalitis in up to 30% of vaccinated mice [77]. Since parental MP-12 infrequently induces viral encephalitis in mice, the high rate of neuroinvasion might be caused by a unique TOSV NSs function. All mice vaccinated with rMP12-TOSNSs could be protected from wild-type RVFV challenge. On the other hand, the efficacy of rMP12-PTNSs or rMP12-SFSNSs was 78 or 89%, respectively [78]. Thus, these chimeric MP-12s showed similar or better efficacy in the mouse model than MP-12. To further understand the phenomenon, the NSs functions of TOSV, SFSV and PTV were analyzed. TOSV NSs does not induce host general transcription suppression [110], but inhibits the *IFN-β* gene [77,111], and promotes the degradation of PKR [110]. PTV NSs induces host general transcription suppression and inhibits the *IFN-β* gene, but no degradation of PKR is induced [78,112]. SFSV NSs induces neither host general transcription suppression nor PKR degradation, but inhibits the *IFN-β* gene [43,78]. It is of note that TOSV NSs retains similar functions with RVFV NSs, except for host general transcription suppression. If infected cells play a role in the neuroinvasion of the virus, rMP12-TOSNSs-infected cells might trigger neuroinvasion at the blood–brain barrier by active host gene expression. On the other hand, SFSV or PTV NSs appear to be more suitable for vaccine development than TOSV NSs. The attenuation levels of SFSV or PTV NSs compared with RVFV NSs should be further evaluated in a virulent RVFV background to understand the risk of reversion to virulence of those chimeric viruses. Furthermore, the efficacy of these vaccine candidates in ruminants should be determined in the future. Although we only described live-attenuated vaccines, the status of other type of vaccines may be found elsewhere [34,113–116].

RVFV *NSs* & therapeutics for RVF

Upon exposure to wild-type RVFV, either effective antiviral or postexposure vaccination might be required to improve the prognosis of RVF. Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) was found to be effective to RVF alone or combination with poly-L-lysine and carboxymethylcellulose (poly[ICLC]) [117,118] with or without the liposome-encapsulated form in mice or rhesus monkeys [119]. In the USA, ribavirin is approved for the treatment of hepatitis C (oral ribavirin) [120] and respiratory syncytial virus (ribavirin with aerosol form) [121], whereas the adverse effects such as hemolytic anemia or teratogenicity hamper the development of ribavirin for intravenous use to treat many viral diseases [122]. Currently, there are no approved therapeutics for RVFV infection. RVFV MP-12 and rMP12-C13type were evaluated for potential post-exposure vaccine candidates in a mouse model. When mice were exposed to wild-type RVFV, subcutaneous administration of 1×10^5 PFU of rMP12-C13type, but not MP-12, significantly attenuated the replication of wild-type RVFV in the liver and spleen, and improved the survival of the mice, although administration should be carried out within 20–30 min postexposure [123]. If a lack of NSs is important for protection, the postexposure vaccination regimen may be further improved by the stimulating host's innate immune response.

On the other hand, coexpression of a nonfunctional NSs in RVFV-infected cells might inhibit the functions of an intact NSs. To test this, 293 cells were infected with MP-12 (functional *NSs* gene is encoded) and separately transfected with 11 different *in vitro*-synthesized RNA encoding a partially truncated *NSs* (lacking all known NSs functions). Although the truncated NSs could be coexpressed abundantly, the intact MP-12 NSs could still inhibit the host *IFN-β* gene and promote the degradation of PKR [124]. Although NSs can self-assemble via the C-terminus and form filament structures **(Figure 1B)**, coexpression of truncated NSs does not allow the dominant-negative suppression of NSs, potentially due to the change of NSs structure required for correct protein localization. Thus, other approaches will be required to target NSs functions to attenuate RVFV replication.

It was found that RVFV NSs could be phosphorylated by a unique form of IKK-β, called IKK-β2, which forms in cells infected with RVFV [125]. IKK-β2 can form a complex (IKK complex) with IKK-α and IKK-γ, and phosphorylate IkBα, which leads to the degradation of IkBα and nuclear translocation of p65. The phosphorylated NSs can bind to mSin3A better than nonphosphorylated NSs [125]. Curcumin, which is known as the major active component of turmeric, can inhibit both conventional IKK-β and IKK-β2 complexes [125]. Cells treated with curcumin fail to arrest the cell cycle at the S phase upon MP-12 infection and reduce RVFV replication, indicating attenuation of NSs function by curcumin [125]. Furthermore, $IFNAR^{-/-}$ mice that were pretreated with curcumin (sc.) showed 60% survival upon MP-12 infection and up to a 90% decrease in the virus titer in the liver [125]. Thus, the attenuation of NSs effector functions may be an important concept to develop therapeutics for RVF.

Conclusion

RVFV NSs has multiple functions to allow the virus to replicate efficiently by shutting down host antiviral responses. Live-attenuated vaccine candidates with the deletion of *NSs* are highly attenuated in the S-segment, and a lack of the *NSs* gene may be useful for DIVA, while the RVFV MP-12 strain encoding other phlebovirus species exerts NSs functions partly similar to those of RVFV NSs, and can be applicable to DIVA. Furthermore, MP-12 lacking *NSs* is efficacious in a postexposure vaccination regimen, and further optimization can be expected. Finally, inhibition of the NSs function is an effective approach to improve the prognosis of RVFV-infected animals.

Future perspective

Since 1930s, sporadic RVF outbreaks have been a concern for public health and agriculture in Africa. During the past 80 years, scientific techniques in multidisciplinary fields have been greatly advanced, and the best approaches, to date, to detect and prevent this disease have also changed. On the other hand, RVFV continues to spread throughout Africa, and has even invaded the Arabian Peninsula. A recent study indicates that the live-attenuated Smithburn vaccine potentially reassorted with wild-type RVFV during a RVF outbreak [30]. Thus, a future live-attenuated RVF vaccine should have reduced environmental risks; complete attenuation of all the S-, M- and L-segments, or modifications of the virus to be incapable of mosquito-mediated transmission. The S-segment can be largely attenuated by the deletion or replacement of the *NSs* gene with little concern of reversion to virulence. This is an advantage for phleboviruses, which encode the

NSs gene separately from *N* gene by an ambisense coding strategy. On the other hand, attenuation of the M- and L-segments is not very easy. MP-12 is attenuated by the M- and L-segments, although attenuation of the M- or L-segment is not complete by itself [Ikegami T, Freiberg AN, Unpublished Data]. Further studies to establish nonreversible attenuation for bunyaviruses will support the improvement of RVF vaccine safety. On the other hand, the development of treatment

EXECUTIVE SUMMARY

Rift Valley fever

- Rift Valley fever (RVF) is a mosquito-borne zoonotic disease affecting Africa and the Arabian Peninsula.
- High rates of abortion in ruminants and human diseases are public health concerns.
- Effective vaccines or therapeutics for animal and human for RVF are required.

RVF virus & its lifecycle in infected cells

- RVF virus (RVFV; family *Bunyaviridae*, genus *Phlebovirus*) has a negative-stranded RNA genome with an S-, M- and L-segment.
- RVFV attaches to DC-SIGN for entry via caveola-mediated endocytosis, transcribes viral mRNA by cap-snatching, replicates viral genomic RNA, and assembles viral genomic RNA encapsidated with nucleocapside (N) into Gn/Gc at Golgi.
- RVFV encodes two nonstructural proteins: NSs and NSm.

Evasion of host innate immune responses by RVFV

- RVFV lacking *NSs* does not cause disease in mice.
- $CD4^+$ T cells and B cells play key roles in host immune responses against RVFV.

Multiple functions of RVFV NSs

- RVFV NSs sequesters TFIIH p44, while promoting the degradation of TFIIH p62 and PKR.
- RVFV interacts with chromatin DNA, induces cell cycle arrest and affects various host gene expressions, including the *IFN-β* gene.
- RVFV NSs accumulation is restricted in mosquito cells by the RNAi mechanism.

Significance of RVFV NSs in live-attenuated vaccine development

- RVFV lacking *NSs* (e.g., Clone 13, R566, rZH501Δ*NSs*Δ*NSm* and rMP12-C13type) is highly attenuated in animals and may be useful to differentiate infected from vaccinated animals.
- Replacement of MP-12 *NSs* with that of other phleboviruses does not decrease the efficacy of MP-12.

RVFV NSs & therapeutics for RVF

- MP-12 lacking NSs, but not parental MP-12, is efficacious as a postexposure vaccine.
- Coexpression of a nonfunctional NSs does not exhibit a dominant-negative phenotype.
- Curcumin inhibits the phosphorylation of NSs, and may attenuate NSs functions.

Future perspective

- A live-attenuated vaccine for RVF with complete nonreversible attenuation in S-, M- and L-segments will solve the safety concerns of vaccination use in the environment.
- Postexposure vaccination regimens can be further optimized.
- RVFV therapeutics may be developed by the inhibition of NSs functions.

regimens for RVF patients should also be established. The approaches to inhibit the functions of RVFV NSs will be further developed in the near future.

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- •• of considerable interest

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