Attenuation of pathogenic Rift Valley fever virus strain through the chimeric S-segment encoding sandfly fever phlebovirus NSs or a dominant-negative PKR

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ABSTRACT

Rift Valley fever is a mosquito-borne zoonotic disease affecting ruminants and humans. Rift Valley fever virus (RVFV; family Bunyaviridae, genus Phlebovirus) causes abortions and fetal malformations in ruminants, and hemorrhagic fever, encephalitis, or retinitis in humans. The live-attenuated MP-12 vaccine is conditionally licensed for veterinary use in the US. However, this vaccine lacks a marker for the differentiation of vaccinated from infected animals (DIVA). NSs gene is dispensable for RVFV replication, and thus, rMP-12 strains lacking NSs gene is applicable to monitor vaccinated animals. However, the immunogenicity of MP-12 lacking NSs was not as high as parental MP-12. Thus, chimeric MP-12 strains encoding NSs from either Toscana virus (TOSV), sandfly fever Sicilian virus (SFSV) or Punta Toro virus (Adames strain (PTA) were characterized previously. Although chimeric MP-12 strains are highly immunogenic, the attenuation through the S-segment remains unknown. Using pathogenic ZH501 strain, we aimed to demonstrate the attenuation of ZH501 strain through chimeric S-segment encoding either NSs of TOSV, SFSV, PTA, or Punta Toro virus (Balliet strain (PTB). In addition, we characterized rZH501 encoding a human dominant-negative PKR (PKRΔE7), which also enhances the immunogenicity of MP-12. Study done on mice revealed that attenuation of rZH501 occurred through the S-segment encoding either PKRΔE7 or SFSV NSs. However, rZH501 encoding either TOSV, PTA, or PTB NSs in the S-segment uniformly caused lethal encephalitis. Our results indicated that the S-segments encoding PKRΔE7 or SFSV NSs are attenuated and thus applicable toward next generation MP-12 vaccine candidates that encode a DIVA marker.

KEYWORDS

attenuation; NSs; phlebovirus; PKR; punta toro virus; Rift valley fever virus; sandfly fever Sicilian virus; Toscana virus; vaccine

Introduction

Rift Valley fever (RVF) is a mosquito-borne zoonotic disease found in Africa and the Arabian Peninsula, and is caused by the Rift Valley fever virus (RVFV; genus Phlebovirus, family Bunyaviridae). The disease is characterized by abortion and fetal malformation in ruminants (e.g., sheep, cattle or goats). 1 Humans can also be infected via direct contact with bodily fluids of infected animals or from the bite of infected mosquitoes. Infected individuals then go on to develop varying degrees of febrile illness, and some may progress toward a more severe state of the disease such as hemorrhagic fever, encephalitis or blindness. 2,3 RVFV is classified as a Category A Priority Pathogen by the National Institute of Health in the US, and an overlap Select Agent by the US. Department of Health and Human Services and the US. Department of Agriculture. 4–6 RVFV has a tripartite negative-stranded RNA genome: Large (L), Medium (M)-, and Small (S)-segments. 7 The L-segment encodes the RNA-dependent RNA polymerase (L protein). The M-segment encodes the envelope glycoproteins (Gn/Gc), a 78 kD protein, and a nonstructural NSm protein. The S-segment encodes a nucleoprotein (N) and a nonstructural protein (NSs) in an ami-sense manner. RVFV NSs
forms a filamentous structure in the nucleus, sequesters the transcription factor (TF) IIH subunit p44,8 and also promotes the post-translational degradation of the TFIIH p62 subunits.9,10 Thus, infected cells undergo shut-off of general host transcription due to the lack of a functional TFIIH. In addition, RVFV NSs inhibits the interferon (IFN)-β promoter by inhibiting the recruitment of the CREB-binding protein to the promoter, a process independent of TFIIH-mediated transcription shut-off.11,12 Lastly, RVFV NSs also promotes the post-translational degradation of dsRNA-dependent protein kinase (PKR).13-15

Sheep, cattle, and goats are highly susceptible to RVFV, and are thus provided sources for viral transmission to mosquitoes or humans.3 Thus, veterinary vaccination plays a key role in the prevention of RVF outbreak. In Africa, a live-attenuated Smithburn vaccine has been used since the 1950s.16 However, this vaccine still retains residual virulence that causes abortion in pregnant animals.17,18 In the US, the live-attenuated MP-12 vaccine has been conditionally licensed for veterinary use.19 The MP-12 vaccine is attenuated by a combination of mutations within the L-, M- and S-segments, and complete attenuation happens with the combination of all attenuated segments.20 Moreover, each L- and M-segment encode 2 independent temperature-sensitive mutations, which limit MP-12 replication above 38°C.21 A potential disadvantage of the MP-12 vaccine is a lack of DIVA marker (Differentiation of Infected from Vaccinated Animals).22 Without a DIVA marker, vaccinated animals elicit antibodies toward the MP-12 vaccine strain, which is a similar response to pathogenic RVFV. Since the MP-12 vaccine encodes the NSs gene, a major virulence factor in the S-segment,22 the MP-12 NSs was artificially truncated by a reverse genetic approach to introduce additional attenuation, as well as a DIVA marker, into the S-segment (rMP12-ΔNSs16/198, alternatively named as rMP12-C13type).24 The rMP12-ΔNSs16/198 lacks a functional NSs, and infected animals induce type-I IFNs immediately after viral replication.25 While the rMP12-ΔNSs16/198 is efficacious in a mouse model,25 immunogenicity was not as high as the MP-12 vaccine in sheep.26 Probably, complete disruption of NSs functions affects viral replication in vivo, due to a lack of ability to counteract with host type-I IFN responses, and lead to the reduced vaccine immunogenicity. On the other hand, MP-12 encoding a truncation of the 78 kD and NSm gene on the M-segment (rMP12-ΔNSm21/384) still encodes intact NSs, and retained strong immunogenicity in ruminant, and is thus considered an alternative MP-12 vaccine with a DIVA marker.26,27

To increase the immunogenicity of MP-12 lacking NSs, our group previously developed several chimeric MP-12 strains encoding a foreign gene in the place of the NSs gene. Sandfly fever phlebovirus NSs genes were selected to create chimeric MP-12 vaccine candidates, because those NSs inhibit induction of host IFN-β through different mechanisms from that of RVFV NSs, and support the replication of RVFV in type-I IFN-competent cells. Toscana virus (TOSV), sandfly fever Sicilian virus (SFSV), or Punta Toro virus (PTV) Adames strain (PTA) are known to inhibit the upregulation of IFN-β gene.24-31 In addition, TOSV NSs also promotes the degradation of PKR.32 The MP-12 encoding TOSV NSs (rMP12-TOSNSs), however, showed increased viral encephalitis in vaccinated mice (30% or more), compared to parental MP-12, which causes encephalitis in up to 5% of mice.33 The MP-12 encoding SFSV NSs (rMP12-SFSNSs) or PTA NSs (rMP12-PTANSs) displayed strong immunogenicity similar to that of the parental MP-12 strain, while no increased virulence has been detected in vaccinated mice.30 On the other hand, a dominant-negative PKR (human PKRN167) showed 100% efficacy against pathogenic RVFV challenge in mice.25 PKRΔE7 is endogenously expressed in human tissues,35 and thus, rMP12-PKRΔE7 will be useful for human RVF vaccine, as it can be more efficacious than rMP12-ΔNSs16/198.

The contribution of chimeric S-segment to the attenuation has not been demonstrated, because the attenuation of MP-12 also occurs through the mutations in L- and M-segments.20 Safety of chimeric S-segment encoding functional sandfly fever NSs proteins or dominant-negative PKR should be evaluated for further development of such vaccines. A definitive approach to demonstrate the attenuation through chimeric S-segment is to analyze the virulence of pathogenic RVFV strain (e.g., ZH501 strain) encoding the chimeric S-segment. In this study, we analyzed the attenuation of pathogenic recombinant ZH501 strain (rZH501) through the chimeric S-segment, encoding either PKRΔE7 (rZH501-PKRΔE7), or the NSs of SFSV (rZH501-SFSNSs), PTA (rZH501-PTANSs), or PTV Balliet strain (rZH501-PTBNSs), in a mouse model. We also characterized rZH501 encoding in-frame truncation of NSs (rZH501-ΔNSs16/198), rZH501 encoding in-frame truncation of 78 kD/NSm (rZH501-ΔNSm21/384), or rZH501 encoding TOSV NSs (rZH501-TOSNSs) to determine those relative attenuation levels. The study showed that
PKRΔE7 and SFSV NSs contribute to the attenuation of ZH501 through the chimeric S-segment, and thus supports further development of safe and immunogenic MP-12 vaccine candidates.

Results

RVFV ZH501 strains encoding TOSV, SFSV, PTA, or PTB NSs inhibit upregulation of IFN-β mRNA

A definitive approach to determine the attenuation of RVFV, through the replacement of NSs, is to characterize the virulence of pathogenic RVFV strain (e.g., ZH501 strain) encoding the S-segment with such foreign gene in the place of NSs. To understand the attenuation of RVFV S-segments encoding PKRΔE7, TOSV NSs, SFSV NSs, PTA NSs, or PTB NSs, we generated recombinant ZH501 (rZH501) encoding human dominant-negative PKR (rZH501-PKRΔE7), TOSV NSs (rZH501-TOSNSs), SFSV NSs (rZH501-SFSNSs), PTA NSs (rZH501-PTANSs), or PTB NSs (rZH501-PTBNSs). We also generated rZH501 encoding a 69% in-frame truncation of the NSs gene (rZH501-ΔNSs16/198) or rZH501 encoding an in-frame truncation of the 78 kD/NSm gene (rZH501-ΔNSm21/384). Overall, all viruses replicated efficiently in Vero and mouse Hepa-1-6 cells (Fig. 1a and b). The rZH501-PKRΔE7 and rZH501-TOSNSs replicated more efficiently than parental rZH501, and the titers were 30-fold (p=0.036, unpaired t-test, vs. rZH501) and 21-fold (p=0.07, unpaired t-test, vs. rZH501) higher than the rZH501 titer at 72 hpi in Hepa-1-6 cells, respectively (Fig. 1b). Unexpectedly, rZH501-ΔNSs16/198 replicated efficiently in type-I IFN-competent Hepa-1-6 cells, indicating that rZH501-ΔNSs16/198 can replicate more robustly in type-I IFN-competent cells than rMP12-ΔNSs16/198 (or rMP12-C13type). Next, we analyzed the upregulation of IFN-β mRNA in infected Hepa-1-6 cells (3 MOI). As expected, rZH501-ΔNSs16/198 and rZH501-PKRΔE7 induced accumulation of IFN-β mRNA at 8 hpi, though IFN-β mRNA was getting less visible in cells infected with rZH501-PKRΔE7 (Fig. 1c). Also, mouse IFN stimulated gene (ISG) 56 mRNA accumulated in cells infected with rZH501-ΔNSs16/198 and rZH501-PKRΔE7. The rZH501-TOSNSs, rZH501-SFSNSs, rZH501-PTANSs, and rZH501-PTBNSs induced neither IFN-β mRNA nor ISG56 mRNA, confirming that NSs of TOSV, SFSV, PTA, and PTB are antagonists for IFN-β induction.
The rZH501-PKRΔE7 and rZH501-SFSNSs are more attenuated than rZH501-TOSNSs, rZH501-PTANSs or rZH501-PTBNSs in mice

Outbred CD1 mice were infected with $1 \times 10^3$ pfu of rZH501, rZH501-ΔNss16/198, rZH501-ΔNSm21/384, rZH501-TOSNSs, rZH501-PTANSs, or rZH501-PTBNSs via i.p. inoculation (Fig. 2a). We used outbred mice to evaluate the attenuation of virus occurring regardless of host genetic background. To be consistent with previous mouse experimental conditions with various rZH501 mutants,20 a $1 \times 10^3$ pfu dosage was used. All mice infected with parental rZH501, rZH501-ΔNSs16/198, rZH501-ΔNSm21/384, rZH501-TOSNSs, rZH501-PTANSs, or rZH501-PTBNSs succumbed to infection. On the other hand, 50% of mice infected with rZH501-SFSNSs survived infection. While all mice infected with rZH501 showed weight loss from 2 dpi to 3 dpi, 7 out of 10 mice infected with rZH501-ΔNSm21/384 did not show weight loss during the same time frame (Fig. 2b). These results indicated that disease caused by rZH501-ΔNSm21/384 is not as severe as that caused by parental rZH501, at 3 dpi (Fig. 2c). Though mice infected with either rZH501-TOSNSs, rZH501-SFSNSs, rZH501-PTANSs, or rZH501-PTBNSs did not show body weight loss at 3 dpi, 70%, 20%, 50%, or 50% of mice infected with rZH501-TOSNSs, rZH501-SFSNSs, rZH501-PTANSs, or rZH501-PTBNSs had temporal weight loss by 5% or more at 6 dpi, compared to the body weight at 5 dpi, respectively (Fig. S1). The survival curve of rZH501-TOSNSs and rZH501-SFSNSs were significantly different (Log-rank test: $p = 0.0001$). The survival curve difference of rZH501-PTANSs and rZH501-PTBNSs was not statistically significant using the Log-rank test ($p = 0.085$). The mean survival time was 6 d (rZH501), 8 d (rZH501-TOSNSs), 7 d (rZH501-PTANSs) and 7.5 d (rZH501-PTBNSs). In postmortem

![Figure 2. Attenuation of rZH501-ΔNss16/198, rZH501-ΔNSm21/384, rZH501-PKRΔE7, rZH501-TOSNSs, rZH501-SFSNSs, rZH501-PTANSs, and rZH501-PTBNSs in mice. Outbred CD1 mice were infected with $1 \times 10^3$ pfu (i.p) of parental rZH501 (control) (n = 10), rZH501-ΔNss16/198 (ΔNss) (n = 20), rZH501-PKRΔE7 (PKRΔE7) (n = 10), or rZH501-ΔNSm21/384 (Δ78 kD/NSm) (n = 10), rZH501-TOSNSs (TOSNSs) (n = 10), rZH501-SFSNSs (SFSNSs) (n = 10), rZH501-PTANSs (PTANSs) (n = 10), or rZH501-PTBNSs (PTBNSs) (n = 10). (a) Survival curves of infected mice are shown. (b) Body weight change at 3 dpi from 2 dpi in individual mice. Statistical differences were analyzed by Mann-Whitney U-test (vs. rZH501, *p < 0.05, **p < 0.01).]
Thus, the rZH501-PTANSs or rZH501-PTBNSs showed viral antigens in the central nervous system (CNS) centered in the brainstem area, and viral antigens were also detected in the hippocampus, caudate putamen, cerebral cortex, and cerebellum (Fig. S2). Viral antigens were not detectable in the liver and spleen of moribund mice sampled 7 dpi or later. Taken together, results indicated that rZH501-TOSNSs, rZH501-SFSNSs, rZH501-PTANSs, and rZH501-PTBNSs induce less severe diseases at the early stage of infection, while still causing lethal viral encephalitis at late stage of infection. Furthermore, mice infected with rZH501-SFSNSs showed delayed onset of disease, and 50% of them survived for 21 d. Thus, rZH501-SFSNSs are more attenuated than parental rZH501 strain due to the S-segment.

Most mice infected with rZH501-ΔNsS16/198, or those infected with rZH501-PKRΔE7 survived for 21 d period. However, 15% of mice infected with rZH501-ΔNsS16/198, and 30% of those infected with rZH501-PKRΔE7 showed temporal weight loss by 5% or more at 8 d post infection (dpi), compared to the body weight at 7 dpi (Fig. S1). Due to clinical signs of severe diseases (scruffy coat, hunched posture, and weight loss), 2 mice infected with either rZH501-ΔNsS16/198 (n = 1) or rZH501-PKRΔE7 (n = 1) were euthanized at 11 dpi or 8 dpi, respectively. Both mice showed viral encephalitis affecting the midbrain, hypothalamus and surrounding areas, with no findings of viral hepatitis (Fig. S2). A log-rank test showed statistically significant differences between rZH501 and rZH501-ΔNsS16/198 (p < 0.0001), or between rZH501 and rZH501-PKRΔE7 (p < 0.0001). Thus, the rZH501-ΔNsS16/198 and rZH501-PKRΔE7 are more attenuated than parental rZH501 strain due to the S-segment.

The rZH501-PTANSs and rZH501-PTBNSs, but not rZH501-TOSNSs and rZH501-SFSNSs, cause detectable viral hepatitis

The rZH501-TOSNSs, rZH501-SFSNSs, rZH501-PTANSs or rZH501-PTBNSs showed neurovirulence in mice, but did not show any clinical signs of disease at 3 and 4 dpi. Since parental rZH501 causes fulminant liver necrosis as early as 3 dpi, we analyzed the differences in viral replication and pathological changes at this early stage of infection (3 dpi) on the liver in a separate mouse experiment (n = 3 per group). Outbred CD1 mice were infected with 1 × 10^3 pfu of rZH501, rZH501-ΔNsS16/198, rZH501-ΔNsSm21/384, rZH501-TOSNSs, rZH501-SFSNSs, rZH501-PTANSs, or rZH501-PTBNSs (i.p.). Mice inoculated with PBS served as controls. At 3 dpi, viremia was detected in mice infected with rZH501, rZH501-ΔNsS16/198 (1 of 3), or rZH501-ΔNsSm21/384, whereas mice infected with rZH501-TOSNSs, rZH501-SFSNSs, rZH501-PTANSs, or rZH501-PTBNSs did not have detectable viremia (Fig. 3a). Infectious RVFV was detected in liver from mice infected with rZH501, rZH501-ΔNsSm21/384, rZH501-PTANSs, or rZH501-PTBNSs, whereas virus was not detectable from liver samples infected with rZH501-ΔNsS16/198, rZH501-TOSNSs or rZH501-SFSNSs (Fig. 3b). The mean serum virus titers were 3.5 × 10^5 pfu/ml (rZH501) or 8 × 10 pfu/ml (rZH501-ΔNsSm21/384). The mean virus titers in liver among positive samples were 5.9 × 10^5 pfu/ml (rZH501), 1.1 × 10^4 pfu/ml (rZH501-ΔNsSm21/384), 2.8 × 10^4 pfu/ml (rZH501-PTANSs), or 2.1 × 10^3 pfu/ml (rZH501-PTBNSs). Viral loads in livers and spleens were also analyzed by droplet digital PCR (ddPCR) using Taqman probe specific to RVFV S-segment and N mRNA.20,21 The mean RNA copy numbers in liver samples were 3.0 × 10^5 (rZH501), 7.3 × 10^4 (rZH501-TOSNSs), 2.0 × 10^4 (rZH501-PTANSs), 2.7 × 10^4 (rZH501-PTBNSs), and 2.8 × 10^4 (rZH501-ΔNsSm21/384) copies per µg total RNA, whereas viral RNA was not detected in liver samples infected with either rZH501-ΔNsS16/198 or rZH501-SFSNSs (Fig. 3c). The mean RNA copy numbers in spleen samples were 3.0 × 10^4 (rZH501), 3.2 × 10^3 (rZH501-PTBNSs), and 8.9 × 10^3 (rZH501-ΔNsSm21/384) copies per µg total RNA, whereas viral RNA was not detected in spleen samples infected with rZH501-ΔNsS16/198, rZH501-TOSNSs, rZH501-SFSNSs, and rZH501-PTANSs (Fig. 3d). We performed a blood chemistry analysis using the VetScan Comprehensive Diagnostic Profile (Abaxis). Among various parameters shown in Methods, blood samples from mice infected with rZH501-ΔNsS16/198, rZH501-TOSNSs, or rZH501-SFSNSs did not show any increase in those parameters. Blood samples from mice infected with rZH501-PTANSs or rZH501-PTBNSs had an increase in alanine aminotransferase (ALT): mean values for rZH501-infected mice or rZH501-PKRΔE7 (n = 1) were 5,467 or 683 unit/L, respectively (Fig. 3e). Mean ALT values for rZH501-infected mice or rZH501-ΔNsSm21/384-infected mice were 5,467 or 683 unit/L, respectively. On the other hand, none of rZH501-PTANSs, rZH501-PTBNSs, or rZH501-ΔNsSm21/384 samples showed an increase in alkaline phosphatase (ALP). An increase in alkaline phosphatase (ALP)
hepatocytes (Fig. S3). Mice infected with rZH501-DNSs16/198 did not have any lesions or viral antigens in livers (Fig. S3). Liver sections from mice infected with rZH501-TOSNSs or rZH501-SFSNSs did not have notable infiltration of inflammatory cells at 3 dpi, while necrotic hepatocytes with karyorrhexis were found infrequently in rZH501-TOSNSs-infected mice. Livers infected with rZH501-PTANSs and rZH501-PTBNSs had a mild infiltration of inflammatory cells, and necrotic hepatocytes with karyorrhexis or pyknosis were sporadically found (Fig. S4). Viral N antigens were detected in hepatocytes sporadically in mice infected with rZH501-TOSNSs, rZH501-PTANSs, rZH501-PTBNSs, but not those infected with rZH501-SFSNSs (Fig. S4). Altogether, these results showed that rZH501-PTANSs, rZH501-PTBNSs, and rZH501-ΔNSm21/384, cause hepatitis at 3 dpi in the mouse model. The pathological findings for rZH501 S-segment mutants are summarized in Table S1.

Figure 3. Early viral replication in mice infected with rZH501 and the mutants. Outbred CD1 mice (n = 3 per group) were mock-infected (PBS), or infected with 1 × 10^4 pfu (i.p) of rZH501, rZH501-ΔNSs16/198 (ΔNSs), rZH501-TOSNSs (TOSNSs), rZH501-SFSNSs (SFSNSs), rZH501-PTANSs (PTANSs), rZH501-PTBNSs (PTBNSs), or rZH501-ΔNSm21/384 (Δ78 kD/NSm). Virus titers in sera (a) or liver tissue (b) were measured by plaque assay. Total RNA was extracted from liver and spleen at 3 dpi. RVFV S-RNA copies per 1 μg total RNA in liver (c) or spleen (d) were measured by droplet digital PCR. Heparinized blood was used for VetScan Comprehensive Diagnostic Profile test. Alanine aminotransferase (ALT) (e) or alkaline phosphatase (ALP) (f) levels in blood are shown. Graphs represent the mean and standard error. Asterisk (*) indicates that one sample was lost during the assay.
Discussion

In this study, we analyzed the attenuation of RVFV rZH501 through the S-segment encoding sandfly fever phlebovirus NSs (TOSV, SFSV, PTA, or PTB) or a dominant-negative human PKR (PKRΔE7) in an outbred mouse model. We previously used the same mouse model to evaluate the attenuation of individual MP-12 mutations using rZH501 backbone.20 Mice are highly susceptible to RVFV strain ZH501 infection (100% lethality), and the disease is characterized by an acute onset of liver and spleen disease and a delayed onset of encephalitis.20,36 According to blood biochemistry and histopathology results, rZH501-PTANSs or rZH501-PTBNSs, but not rZH501-SFSNSs, showed evidence of viral hepatitis in mice at 3 dpi. Viral encephalitis still occurred in mice infected with rZH501-SFSNSs (50%), but was less frequent than those infected with rZH501-TOSNSs (100%), rZH501-PTANSs (100%), or rZH501-PTBNSs (100%). Thus, rZH501 were partially attenuated through the S-segment encoding SFSV NSs. Our result indicated that the S-segments encoding SFSV NSs are attenuated and applicable toward MP-12 vaccine candidates that encode a DIVA marker (rMP12-SFSNSs). Though we did not find attenuation of rZH501 through the chimeric S-segment encoding PTA or PTB NSs, we do not exclude the potential as RVF vaccine candidates since those chimeric S-segments do not increase the virulence of RVFV. The safety and efficacy of chimeric MP-12 vaccines encoding either the NSs of SFSV, PTA, or PTB should be further tested in ruminants for safety and immunogenicity.

Human RVF vaccines will be important to prevent viral transmission from infected animals or mosquitoes into human, during outbreaks. The PKRΔE7 is an alternatively spliced form of human PKR (N-terminal 174 amino acids), lacking the exon 7, which is broadly expressed at low levels (~5%) in human tissues including the heart, liver, and skeletal muscle.35 PKRΔE7 binds to dsRNA and interferes with the activation of intact PKR in human cells.13 The PKRΔE7 does not completely inhibit the activation of mouse PKR, even though it binds to dsRNA.35 To know the effect of dominant-negative PKR expression in the vaccine immunogenicity, we previously generated rMP-12 encoding mouse N-terminal PKR (167 amino acids: mPKRN167) in the place of MP-12.25 The expression of mPKRN167 increased the accumulation of viral N protein in MP-12-infected cells, and mice vaccinated with recombinant MP-12 encoding mPKRN167 showed 100% protection against pathogenic rZH501 challenge.25 For vaccine development, the PKRΔE7 is more desirable than mPKRN167, because PKRΔE7 is endogenously expressed in human tissues. Our study showed that rZH501 was significantly attenuated through the chimeric S-segment encoding the PKRΔE7 in mice. Although this is still early in the stage of development, rMP12-PKRΔE7 will be a unique candidate vaccine, attenuated through all L-, M-, and S-segment, and more efficacious than rMP12-ΔNSs16/198.

The NSs protein is a major virulence factor for RVFV and plays a key role in the development of disease.23 Thus, a lack of NSs leads to a major attenuation of RVFV.37 Though MP-12 lacking a functional NSs (rMP12-ΔNSs16/198) is fully attenuated in the mouse model,25 the ZH501 lacking NSs still retains residual virulence. Dodd et al. showed that RVFV ZH501 strain lacking NSs can still cause lethal encephalitis in C57BL/6 mice via intranasal, but not subcutaneous injection.38 Furthermore, the present study showed a 5% mortality of rZH501-ΔNSs16/198-infected mice via i.p. inoculation. Though a lack of NSs can highly attenuate pathogenic RVFV strain (e.g., ZH501) by itself, additional attenuation mutations (e.g., MP-12 mutations) other than NSs will be important for the RVF vaccine safety.

Authentic PTA infection, but not PTB infection, causes coagulative necrosis in liver in hamsters or mice.31,39-41 The hepatitis caused by PTA is similar to that is caused by RVFV, and PTA-infected hamsters or mice are considered as a model for acute phleboviral hepatitis. Authentic PTA infection induces higher levels of IFN-β than PTA infection in hamsters.31 The S-segment is considered as the determinant of PTA virulence,31 and thus, we initially expected that rZH501-PTBNSs would be more attenuated in mice than rZH501-PTANSs. Unexpectedly, the rZH501-PTANSs and rZH501-PTBNSs had similar pathogenic phenotype in mice, in terms of viral hepatitis and neurovirulence. Both viruses also inhibited the induction of endogenous IFN-β mRNA in Hepa1-6 cells. Thus, we concluded that PTA and PTB NSs, expressed from rZH501, equally contribute to the pathogenesis of rZH501 in mice.

Neither viral RNA, N antigens, nor infectious viruses could be detected in liver samples infected with rZH501-SFSNSs. On the other hand, viral RNA and N antigens were detected in liver samples infected with rZH501-TOSNSs, without detectable infectious viruses. The rZH501-TOSNSs had a capability to replicate in mouse Hepa1-6 cells more efficiently than rZH501-SFSNSs (Fig. 1), indicating that TOSV NSs supports RVFV replication better than SFSV NSs, in mouse hepatocytes. However, even with the expression of TOSV NSs, rZH501-TOSNSs did not reproduce severe necrotic hepatitis caused by parental rZH501. ALT values were normal in mice infected with rZH501-TOSNSs or rZH501-SFSNSs, and thus, those NSs expressions probably did not support RVFV replication sufficient enough
to induce hepatic necrosis. Different from RVFV NSs, neither TOSV NSs nor SFSV NSs suppress host general transcription. SFSV NSs does not promote the degradation of PKR, while suppressing the induction of host IFN-β gene activation. TOSV NSs not only inhibits the induction of IFN-β gene upregulation, but also promotes the degradation of PKR. We assume that RVFV NSs functions, such as general transcription suppression, PKR degradation, or IFN-β suppression, coordinately support efficient RVFV replication and spread in liver tissues, and thus, the spreads of rZH501-TOSNSs and rZH501-SFSNSs were more restricted than parental rZH501 in mouse livers.

The deletion of 78 kD/NSm from the M-segment is known to partially attenuate RVFV in a rat model. In this study, we also tested the attenuation of rZH501 through an in-frame deletion of 78 kD/NSm region (ΔNSm21/384) in the M-segment. All the mice infected with rZH501-ΔNSm21/384 succumbed to infection by 7 dpi. The rZH501-ΔNSm21/384 was only weakly attenuated in the ability to induce viral hepatitis at 3 dpi. Since the deletion of 78 kD/NSm does not confer sufficient attenuation to rZH501, additional attenuations (e.g., NSs deletion and/or MP-12 attenuation mutations) will be required to fully attenuate pathogenic RVFV for vaccine purposes.

Methods

Media, Cells, and Viruses. Mouse hepatoma Hepa1-6 cells (ATCC CRL-1830), African green monkey kidney (Vero) cells (ATCC CCL-81) or Vero E6 cells (ATCC CRL-1586) were maintained in Dulbecco’s modified minimum essential medium (DMEM) containing 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 μg/ml). BHK/T7-9 cells stably expressing T7 RNA polymerase were maintained in MEM-α supplemented with 10% FBS, penicillin/streptomycin and 600 μg/ml of hygromycin. We used a recombinant RVFV ZH501 strain (rZH501) or that encoding ZH501 S-segment in the pProT7-wS(+) plasmid were replaced with NSs open-reading frame (ORF) of TOSV (ISS. Phl.3), SFSV (Sabin), PTA (TVP10208), PTB (TVP13993), or PKRAE7, as described previously. To rescue rZH501-TOSNSs, rZH501-SFSNSs, rZH501-PTANSs, rZH501-PTBNs, or rZH501-PKRAE7, respectively. To generate rZH501-ΔNSs16/198 or rZH501-ΔNSm21/384, corresponding regions in the S- or M-segment were deleted by site-directed mutagenesis using pProT7-wS(+) or pProT7-wM(+) plasmids, respectively.

Northern blot analysis. Northern blot using mouse IFN-β or ISG56 probes were described previously. Mouse experiments with RVFV. 5-week-old female CD1 mice (Charles River) were intraperitoneally (i.p.) inoculated with 1 × 10^3 pfu of rZH501 or the mutants (n = 10 per group). Clinical signs of disease were observed daily, and individual body weights were measured daily for 1 week, and then, every third day until 21 dpi. Moribund mice (more than 20% body weight loss, and/or clinical signs such as paralysis, encephalitis, or severe lethargy) were humanely euthanized. All surviving mice were euthanized at 21 dpi. Tissues were fixed in 10% buffered formalin for postmortem histopathological analysis. Subsequently, the experiment was repeated to analyze disease which occurred at 3 dpi. Mice were infected with 1 × 10^3 pfu of rZH501 or the mutants (n = 3 per group), and humanely euthanized at 3 dpi. Heparinized blood, liver, and spleen were analyzed for viral load, blood chemistry, or histopathology.

Droplet digital PCR analysis. RVFV S-segment RNA was quantitatively measured by droplet digital PCR (ddPCR) using the QX100 droplet generator and reader (Bio-Rad) as described previously.

Blood chemistry. Heparinized blood from mice at 3 dpi were analyzed with the VetScan Comprehensive Diagnostic Profile (Abaxis) according to the manufacturer’s instructions. The test measured alanine aminotransferase (ALT), albumin, alkaline phosphatase (ALP), amylase, total Calcium, creatinine, globulin, glucose, phosphorus, K^+^, Na^+^, total bilirubin, total protein, and blood urea nitrogen.

Statistical analysis. GraphPad Prism 6 for Windows version 6.05 program (GraphPad Software Inc.) was used for the survival curve analysis by Log-Rank test or for statistical analysis of 2 weight groups by Mann-Whitney U-test.

Ethics statement. All experiments using recombinant DNA or biologicals have been performed upon the approval of the Notification of Use by the Institutional...
Biosafety Committee at UTMB. Mouse studies were performed at the UTMB Robert E. Shope BSL-4 laboratory accredited by the Association for Assessment and Accreditation of Laboratory Animal Care in accordance to the Animal Welfare Act, NIH guidelines, and US federal law.

**Abbreviations**

- ALP: alkaline phosphatase
- ALT: alanine aminotransferase
- DIVA: Differentiation of Vaccinated from Infected Animals
- ddPCR: droplet digital PCR
- hpi: hours post infection
- IFN: Interferon
- moi: multiplicity of infection
- PKR: dsRNA-dependent protein kinase
- PKRΔE7: dominant-negative human PKR
- PTA: Punta Toro virus Adames strain
- PTB: Punta Toro virus Balliet strain
- PTV: Punta Toro virus
- RVFV: Rift Valley fever virus
- SFSV: Sandfly fever Sicilian virus
- TFIIH: transcription factor IIH
- TOSV: Toscana virus

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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