Available online at www.sciencedirect.com
ScienceDirect
Virology 362 (2007) 10–15
VIROLOGY
www.elsevier.com/locate/yviro

Rapid Communication

Rift Valley fever virus lacking NSm proteins retains high virulence *in vivo* and may provide a model of human delayed onset neurologic disease

Brian H. Bird [a,b], César G. Albariño [a], Stuart T. Nichol [a,*]

[a] *Special Pathogens Branch, Division of Viral and Rickettsial Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, 1600 Clifton Road MS G-14 Atlanta, GA 30329, USA*
[b] *University of California, Davis, School of Veterinary Medicine, Davis, CA 95616, USA*

Received 11 December 2006; returned to author for revision 5 January 2007; accepted 19 January 2007
Available online 6 April 2007

Abstract

Rift Valley fever virus is a significant human and veterinary pathogen responsible for explosive outbreaks throughout Africa and the Arabian Peninsula. Severe acute disease in humans includes rapid onset hepatic disease and hemorrhagic fever or delayed onset encephalitis. A highly efficient reverse genetics system was developed which allowed generation of recombinant RVF viruses to assess the role of NSm protein in virulence in a rat model in which wild-type RVF virus strain ZH501 (wt-ZH501) results in 100% lethal hepatic disease 2–3 days post infection. While extensive genomic analysis indicates conservation of the NSm coding capability of diverse RVF viruses, and viruses deficient in NSs proteins are completely attenuated *in vivo*, comparison of wt-ZH501, a reverse genetics generated wt-ZH501 virus (R-ZH501), and R-ZH501 virus lacking the NSm proteins (R-ΔNSm-ZH501) demonstrated that the NSm proteins were nonessential for *in vivo* virulence and lethality. Surprisingly, while 44% of R-ΔNSm-ZH501 infected animals quickly developed lethal hepatic disease similar to wt- and R-ZH501, 17% developed delayed onset neurologic disease (lethargy, head tremors, and ataxia) at 13 days post infection. Such infections may provide the basis for study of both RVF acute hepatic disease and delayed onset encephalitic disease in humans.
Published by Elsevier Inc.

Keywords: Rift Valley fever virus; NSm protein; Viral virulence factors; Bunyaviridae; Phlebovirus; Pathogenesis; Recombinant virus; Molecular pathogenesis; Reverse genetics; Hemorrhagic fever; Acute hepatic disease; Delayed neurologic disease; Rat model

Introduction

Rift Valley fever (RVF) virus (family *Bunyaviridae* genus *Phlebovirus*) is a mosquito-borne pathogen of humans and livestock historically capable of either low level endemic activity or large explosive epidemics/epizootics throughout Africa (Swanepoel and Coetzer, 1994; Nichol, 2001) and more recently in the Arabian peninsula (Madani et al., 2003). Severe hepatic destruction can be seen in livestock, leading to high mortality and abortion rates in susceptible species. Most human infections result in an acute febrile flu-like illness, but rapid onset hepatic disease with hemorrhagic symptoms and late onset severe encephalitis are seen in 1–2% of cases (Meegan et al., 1981; Swanepoel and Coetzer, 1994; Madani et al., 2003).

The negative sense tripartite RNA genome of RVF virus is comprised of the small ambisense (S) segment that encodes the viral nucleoprotein (NP) in genomic (−) orientation and in anti-genomic (+) orientation the non-structural (NSs) protein which is a major virulence factor functioning as a potent antagonist of host cell RNA Pol II transcription (Billecocq et al., 2004), and the large (L) segment encodes the virus polymerase (Schmaljohn and Hooper, 2001). A notable feature of the RVF virus M segment is the presence of 5 in-frame AUG-methionine start codons within the NSm protein coding region at antigenomic sense positions #21, 135, 174, 411, and 426 that occur prior to the signal peptidase cleaveage site of the mature Gn protein (Suzich et al., 1990; Gerrard and Nichol, 2007). These studies demonstrated that alternate utilization of the in-frame AUG's at position 21 and 135 were responsible for at least 2 forms of glycoprotein, a 14 kDa NSm protein and a 78 kDa NSm and Gn fusion polyprotein, respectively, in addition to the expected

* Corresponding author. Fax: +1 404 639 1118.
E-mail address: stn1@cdc.gov (S.T. Nichol).

0042-6822/$ - see front matter. Published by Elsevier Inc.
doi:10.1016/j.virol.2007.01.046

55 kDa Gn and 58 kDa Gc envelope glycoproteins. Recent complete genome sequencing of 33 naturally occurring RVF virus isolates indicated complete conservation of these 5 in frame AUG codons (Bird et al., 2007). Surprisingly, given their highly conserved nature, recent work in this laboratory and elsewhere, demonstrated that the NSm proteins of RVF virus were dispensable for efficient virus growth in cell culture (Won et al., 2006; Gerrard et al., 2007). In the present study, we describe modification and optimization of our reverse genetics system based on the virulent RVF virus ZH501 strain to produce a highly efficient system for the rapid generation of recombinant wild-type and mutant viruses lacking the entire NSm coding region. These recombinant viruses are used to examine of the role of the NSm 14 kDa and 78 kDa proteins in *in vivo* pathogenesis in the highly susceptible Wistar-furth rat model of lethal RVF virus infection (Anderson and Peters, 1988; Anderson et al., 1991). In this animal model we demonstrate that the NSm proteins (14 kDa and 78 kDa) are not essential for *in vivo* virulence or lethality.

Results

Rescue of recombinant RVF viruses, R-ZH501 and R-ΔNSm-ZH501

In a previous report (Gerrard et al., 2007), we described the successful generation of a recombinant RVF virus, R-ΔNSm-ZH501, lacking the NSm coding region. After careful examination by 3′RACE and Northern Blot techniques, we found that the original S plasmid produced low amounts of full length S-segment genomic sense replication products, yielding primarily short replication products encompassing the NSs gene region only (data not shown). This finding limited the overall efficiency of our initial attempts at the rescue of recombinant viruses.

In order to correct this problem, full length S RNA from the wild-type ZH501 virus strain was re-cloned into a version of the plasmid vector V(0.0) which was modified to remove the multiple cloning site. Within this backbone context, we observed greatly increased levels of N protein expression relative to the original construct. This allowed subsequent rescue of recombinant virus without the requirement of a support plasmid expressing the N protein as previously described.

In addition, although they did not affect rescue efficiency, two non-synonymous mutations in the M segment clone were corrected to match the wt sequence to allow exact *in vivo* comparisons to be made between recombinant and wild-type viruses. These were restored in both the full length M segment and Δ-NSm plasmids by site directed mutagenesis and restriction fragment exchange. These changes included removal of the *Xho*I restriction site (position 2136-9) we had previously introduced and which had resulted in an amino acid coding change from L to T, and an inadvertently introduced change at nt. position 847 which had resulted in a change from E to G. In order to maintain the ability to distinguish full-length recombinant virus from the wt virus, we preserved 2 silent mutations on the M segment at positions 1706 (C to T) and 2750 (C to T).

After incorporating the above corrections, virus rescue was accomplished by simultaneous liposome transfection of only the 3 full length antigenomic sense plasmids (S, M, L or S, Δ-NSm, L) in BSR-T7/5 cells. After complete lysis, cell supernatants containing rescued viruses were clarified, diluted and virus passaged twice on VERO E6 cells. All recombinant viruses were found to grow similarly to wt-ZH501 in VERO E6 cell culture (Gerrard et al., 2007; Bird et al., 2007; and data not shown). To ensure the exact molecular identity of all viruses used in this study complete genome sequence was obtained following techniques described elsewhere (Bird et al., 2007). As a measure of the robustness of the optimized reverse genetics system, we completed a total of 11 separate rescue attempts using a variety of plasmid concentrations ranging from 0.5 ug to 4 ug and achieved 100% success in rescuing recombinant RVF virus in each experimental replicate using only the combination of the 3 full length S, M and L antigenomic sense plasmids or full length S, ΔNSm, and L antigenomic sense plasmids. The efficiency of this system is similar to that of other highly efficient 3 plasmid systems developed earlier for other members of the family *Bunyaviridae*, including Bunyamwera and La Crosse viruses (Lowen et al., 2004; Blakqori and Weber, 2005).

Establishment of the RVF disease model using wt-ZH501 virus

In order to establish the Wistar-furth rat model of lethal RVF virus infection (Anderson and Peters, 1988; Anderson et al., 1991) in our laboratory, a total of 9 animals were infected in groups of 3 with 10^1, 10^2, or 10^3 PFU of wt-ZH501 virus (Table 1). A clinical feature of wt-ZH501 infection in these animals is peracute death occurring 2–3 days post infection (PI) due to fulminant hepatitis. Animals are reported to be clinically healthy until approximately 12 h prior to death, quickly followed by animals developing clinical signs of illness and rapidly succumbing due to hepatic injury. All animals infected with wt-ZH501 virus in our study remained clinically healthy until just prior (~12 h) to death. On day 2 PI 2 animals (1 each in 10^2 and 10^3 PFU dose groups) were found dead (Fig. 1). Gross pathology at necropsy indicated extensive diffuse hepatic necrosis with no other significant findings (data not shown). All other wt-ZH501 infected animals appeared

Table 1
Experimental design and clinical outcome of infection of WF rats with wt-ZH501, R-ZH501 or R-ΔNSm-ZH501 virus

Virus	Virus dose (PFU) and outcome (H/N/T)*					Time to death (days)
	0	10^1	10^2	10^3	10^4	
wt-ZH501	n.a.	3/0/3	3/0/3	3/0/3	n.d.	2–3
R-wt-ZH501	n.a.	3/0/3	n.d.	3/0/3	n.d.	3
R-ΔNSm-ZH501	n.a.	1/1/3	2/0/3	2/1/6	3/1/6	Hepatic 4 Neuro. 13
Negative control	0/0/3	–	–	–	–	n.a.

* *H*=number of animals with fatal hepatic disease, *N*=number of animals with fatal neurologic disease, *T*=total number of animals, ** n.a.=not applicable, *** n.d.=not done.

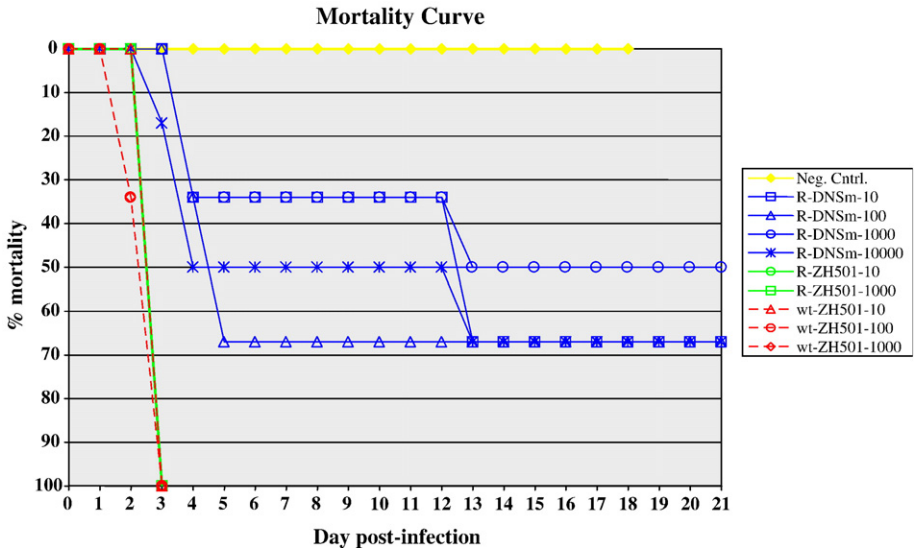

Fig. 1. Mortality curve of rats infected with wt-ZH501, R-ZH501 or R-ΔNSm-ZH501 viruses. Note that all wt-ZH501 and R-ZH501 virus infected animals succumbed to lethal hepatic disease at 2–3 days PI. R-ΔNSm-ZH501 virus infected animals experienced a biphasic mortality pattern with multiple deaths at days 3–5 PI due to hepatic disease and later at day 13 with clinically evident neurologic disease confirmed by RVF virus specific RT-PCR and virus isolation.

clinically healthy at day 2 PI and had either maintained or lost only 1–2 g of total body weight. On the morning of Day 3 post-infection all 7 remaining wt-ZH501 virus infected rats were found dead regardless of dose group (Fig. 1). Upon necropsy, gross pathology indicated extensive diffuse hepatic necrosis with no other significant findings. These findings demonstrate the successful establishment of the classic RVF disease model in our laboratory.

Pathogenesis of recombinant RVF viruses

R-ZH501 virus

To ensure that reverse genetics derived wt virus (R-ZH501) had the same lethality in rats as authentic wt virus, a total of 6 WF rats in groups of 3 were infected with R-ZH501 at doses of 10^1 or 10^3 PFU. These animals remained clinically healthy and maintained body weight until day 3 PI when 3 animals were found dead and the other 3 animals had signs of severe clinical illness (ruffled hair coat, hunched posture, moribund activity) and were euthanized. All moribund animals had lost significant weight (7 g–17 g) overnight. The mean time to death of these animals was identical to those infected with wt-ZH501 virus regardless of challenge dose (Table 1 and Fig. 1). Gross pathology at necropsy in all 6 animals indicated extensive diffuse hepatic necrosis consistent with that found in wt-ZH501 infection in WF rats.

R-ΔNSm-ZH501 virus

To assess the contribution of the NSm proteins to severe disease in the WF rat model, a total of 18 WF rats were infected with the R-ΔNSm-ZH501 virus at dosages varying from 10^1–10^4 PFU. Surprisingly, the R-ΔNSm-ZH501 virus was found to retain virulence for WF rats (Fig. 1 and Table 1). Although the virus retained lethality in this animal model it was found to be attenuated compared to wt-ZH501 or R-ZH501 controls. All animals regardless of dose began to show signs of clinical illness (ruffled hair coat, hunched posture, huddling behavior) at day 3 PI with 1 animal found dead in the 10^4 PFU dose group. On the subsequent day (day 4 PI) all animals in each dose group continued to show signs of clinical illness and weight loss ranging from 2 to 12 g and a total of 6 animals were found dead. These animals were found in each dose group with 1 death at 10^1 PFU, 1 at 10^2 PFU, 2 at 10^3 PFU, 2 at 10^4 PFU (Fig. 1 and Table 1). At day 5 PI one additional animal (dose group 10^2 PFU) was found dead and all surviving animals remained clinically ill. In all 8 early onset deaths, gross pathology at necropsy indicated extensive diffuse hepatic necrosis that was consistent with that observed in fatal infections in WF rats with wt-ZH501 virus. At necropsy, whole blood was collected, and virus S, M and L segment RNA was amplified by RT-PCR and sequenced to ensure that in the cases of lethal R-ΔNSm-ZH501 virus infection that no cross-contamination had inadvertently occurred with wild type virus (Fig. 2). By day 6 PI all surviving

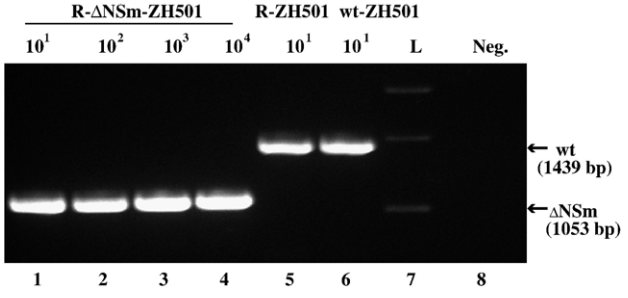

Fig. 2. RT-PCR detection of RVF virus RNA from early hepatic disease deaths. RT-PCR amplification of M RNA fragments from whole blood collected from infected animals. Lanes 1–4: animals from R-ΔNSm-ZH501 10^1, 10^2, 10^3 and 10^4 PFU dose groups respectively. Lane 5: R-ZH501. Lane 6: wt-ZH501. L: size markers. Note lack of contamination of R-ΔNSm-ZH501 infected animals with full length amplification products. Expected product sizes—R-ΔNSm-ZH501 1053 bp and wt and recombinant 1439 bp.

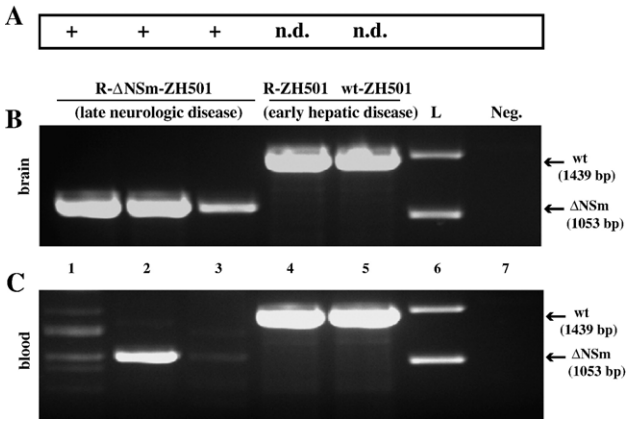

Fig. 3. Virus isolation and RT-PCR detection of RVF virus from brain homogenates and whole blood. (A) Virus isolation from RVF virus infected animal brain homogenates collected at time of death. n.d. = not done. (B) M segment amplification products from brain homogenates. (C) M segment amplification products from RVF virus infected animal whole blood. In all panels: lanes 1–3: R-ΔNSm-ZH501 animals with lethal neurologic disease, lane 4: R-ZH501 (hepatic disease) and lane 5: wt-ZH501 (hepatic disease) infected animal brain (panel B) or blood (panel C) tissue included for size comparisons. L: size markers. Note lack of contamination of R-ΔNSm-ZH501 infected animals with full-length amplification products and the greater amount of R-ΔNSm-ZH501 amplification product in brain tissue compared to whole blood. Expected product size R-ΔNSm-ZH501: 1053 bp; wt and recombinant: 1439 bp.

animals had returned to clinical health and began to demonstrate consistent daily weight gains. Unexpectedly, at day 13 one rat was found dead and 2 others were euthanized while displaying clinical signs of severe neurologic disease (head tilt, head tremors, lethargy, ataxia). In contrast to R-ZH501 and wt-ZH501 infected controls, at necropsy these animals had no evidence of hepatic necrosis and showed no other gross pathology. In order to confirm that these delayed onset neurologic deaths were attributable to R-ΔNSm-ZH501 virus infection, tissues were harvested for RNA extraction (blood and brain) and virus isolation (brain). In all 3 animals, brain tissue was strongly positive by both RT-PCR and virus isolation for R-ΔNSm-ZH501 virus (Fig. 3, Panels A and B). In 2 of the 3 animals results of RT-PCR completed on RNA extracted from blood samples demonstrated little or no detectable R-ΔNSm-ZH501 virus RNA (Fig. 3, Panel C). In the remaining animal, a faint R-ΔNSm-ZH501 virus amplification product was detected. These results taken together indicate that the virus detected in brain tissue was primarily due to specific R-ΔNSm-ZH501 virus replication in brain tissue rather than contamination from viremic blood. Two additional R-ΔNSm-ZH501 infected animals showed signs of mild neurologic illness (head tremors) on days 14 and 15 which resolved by day 16. All remaining animals appeared clinically healthy and continued to gain weight and the experiment was terminated by euthanizing all surviving animals on day 21.

Discussion

We report here the development of a highly efficient reverse genetics system for RVF using only 3 plasmids expressing antigenomic copies of the S, M and L segments, similar to successful highly efficient systems developed for Bunyamwera and La Crosse viruses (Lowen et al., 2004; Blakqori and Weber, 2005). This system allowed the rapid generation of both full length recombinant RVF virus and a mutant virus containing a deletion of the entire coding region of the NSm gene on the M segment. We and others have reported the ability of recombinant RVF virus lacking the NSm 14 kDa and 78 kDa proteins to exhibit growth in cell culture comparable to that of wild type virus (Gerrard et al., 2007; Won et al., 2006). Having based our reverse genetics system on the fully virulent ZH501 strain (isolated in Egypt in 1977 from a fatal human case), we were able to directly begin *in vivo* pathogenesis studies in a Wistar-furth rat model of infection and lethal disease. Central to this ability was the robustness of the reverse genetics system after optimization and correction of several inherent difficulties with the original system, which included lack of efficient full length S segment virus RNA replication, the requirement of an expression plasmid for NP, and 2 amino acid coding changes on the M segment (data not shown).

Our finding of the non-essential nature of the NSm proteins in mammalian host pathogenesis was surprising. The apparent importance of NSm proteins was highlighted in a recent comparative phylogenetic analysis of 33 isolates of diverse RVF virus from throughout the natural geographic range and spanning 56 years (1944–2000). The complete conservation of the 5 in-frame AUG codons found in the NSm coding region that are utilized to generate either the NSm 14 kDa or NSm + Gn fusion of 78 kDa suggested an evolutionarily conserved role for these proteins (Bird et al., 2007). In addition, reverse genetics attempts to generate Bunyamwera virus similarly completely lacking its NSm protein failed to even generate infectious virus (Shi et al., 2006). However, it should be mentioned that the orthobunyavirus NSm is sandwiched between Gn and Gc whereas the RVFV NSm is upstream of Gn. Consequently, although having similar names, the NSm proteins may be functionally distinct. In addition, the Uukuniemi-like phleboviruses do not have an NSm protein. Also, it had previously been shown that RVF virus deficient in the expression of the NSs non-structural protein encoded by the S segment was completely attenuated in the WF rat model despite its ability to grow well in tissue culture (Vialat et al., 2000). While the NSm deficient RVF virus (R-ΔNSm-ZH501) was attenuated, it retained the ability to cause clinical illness and remained lethal in 61% of rats infected regardless of challenge dose. The finding of clinical illness and death at all challenge doses suggests that within the population of WF rats some animals are more susceptible to infection with R-ΔNsm-ZH501 virus. This finding may allow for the elucidation of the precise role of the NSm proteins in these animals by comparisons of survivor versus lethal outcomes in future serial bleed and serial sacrifice studies to address issues of virus load, tissue specific replication and inherent host factors responsible for survival.

It is surprising that in cell culture both NSs and NSm non-structural proteins are individually dispensable for RVF virus replication, assembly and maturation (Gerrard et al., 2007; Won et al., 2006; Ikegami et al., 2006; Muller et al., 1995).

The complete genome of RVF virus encompasses a total of 11.9 kB and is likely under evolutionary pressure to remove non-essential genes. The finding that a total of 1.2 kB or approximately 10% of the total genome is devoted to non-structural proteins not required for virus growth in cell culture suggests that strong evolutionary selection operates to maintain their function. While the NSs coding region, which functions to antagonize host cell RNA Pol II transcription and indirectly allow the virus to circumvent the innate host antiviral response, is essential for virus virulence *in vivo*, the role of the NSm coding region and the 2 resultant proteins remains unknown (Billecocq et al., 2004). Our hypothesis that R-ΔNSm-ZH501 virus would be rendered highly attenuated and non-lethal *in vivo*, similar to a Δ-NSs virus such as the naturally occurring mutant strain Clone 13 (Vialat et al., 2000), was proven false by the development of clinical disease in all animals challenged with 61% progressing to lethal outcomes. The unexpected finding of delayed onset RVF neurologic disease ~2 weeks post-infection without accompanying high level viremia mimics late stage encephalitis observed in a proportion of human or livestock infections (Meegan et al., 1981; Rippy et al., 1992; Swanepoel and Coetzer, 1994; Madani et al., 2003). This surprising result suggests that the use of reverse genetics generated wt or mutant RVF viruses in WF rats provides a useful model system to investigate the pathogenesis of both severe rapid onset hepatic disease and delayed onset neurologic disease in humans and livestock.

It is clear that while the NSm proteins contribute to *in vivo* mammalian pathogenesis they are not essential for lethal disease. Our finding of the dispensability of the NSm proteins in mammalian pathogenesis leads towards speculation of perhaps a more significant role for NSm proteins in arthropod vectors than in mammalian hosts. Infection and subsequent transmission of Rift Valley fever virus by arthropod vectors requires penetration of several cellular membranes including the mid-gut barrier and avoidance of the innate insect immune response (Romoser et al., 2005) Perhaps the NSm 14 kDa or the NSm+Gn 78 kDa fusion protein play a role *in vivo* in vector species. Further comparisons using larger numbers of animals are needed, and may provide insights into the differential innate and adaptive immune response of survivor animals infected with R-ΔNSM-ZH501 virus with those succumbing to either lethal hepatic or neurologic disease and may help elucidate the as yet undefined role of these evolutionarily conserved NSm non-structural proteins.

Methods

RVF virus and bio-safety

All work with live virus and infected animals was conducted within the CDC bio-safety level 4 (BSL-4) laboratory. Working stocks of low passage of ZH501 wild-type or recombinant RVF viruses were prepared by passage on Vero E6 monolayers. The Genbank entries for the RVF virus strain ZH501 S, M and L genome segment sequences are DQ380149, DQ380200 and DQ375406, respectively. Animal whole blood or 10% brain homogenates were added to Tripure reagent (Roche) at a ratio of 10:1 for virus inactivation and subsequent RNA extraction in a BSL-3 laboratory.

Generation of recombinant viruses

Rescue of recombinant viruses was accomplished by simultaneous liposome mediated transfection of the 3 full length antigenomic sense plasmids in 1 ug quantities with LT-1 lipofectant (Mirus) at a ratio of 5:1 and transferred onto subconfluent (~60%) monolayers of BSR-T7/5 cells stably expressing the T7 polymerase. BSR-T7/5 cells were a generous gift from Dr. K. Conzelmann (Max-von Pettenkofer-Institut, Munchen, Germany). Beginning at 48–72 h post transfection (DPT) cytopathic effect in the BSR-T7 cells was observed and after complete monolayer lysis, typically days 4–5 post-transfection, transfected cell supernatants were clarified by low speed centrifugation, and passaged twice (dilution 1:500) onto fresh confluent monolayers of VERO E6 cells. The exact molecular sequence identity of each rescued virus was confirmed by complete genome sequencing following techniques described previously (Bird et al., 2007).

Animal infections

A total of 36 female Wistar-furth (WF/NSd) (Harlan) rats aged 12 weeks old (average=175 g) were used in this study and were housed in micro-isolator pans and provided food and water *ad libitum*. All pans were kept in HEPA filtration rack units following standard barrier care techniques within a BSL-4 laboratory. All animals were inoculated subcutaneously (SQ) in the right hind flank. A total of 3 animals served as sham inoculated (sterile PBS only) controls. Animals were monitored daily for signs of clinical illness and weight loss. Any animals found either in severe clinical illness or moribund were immediately euthanized (isoflurane/sodium pentobarbital). Whole blood and tissue samples were collected at the time of euthanasia or from animals found dead, and frozen at −80 C for later total RNA extraction and virus isolation following standard techniques. All procedures completed with animals during this study were approved by the CDC Institutional Animal Care and Use Committee and the CDC Institutional Bio-Safety committee.

Acknowledgments

The authors would like to thank Bobbie Rae Erickson, Darcy Bawiec, Tara Sealy and Amy Hartman for their expert technical assistance and Archer Miller for animal husbandry support during completion of these studies. We are grateful to Tom Ksiazek and Pierre Rollin for their support and encouragement. The plasmid backbone V (0.0) was a generous gift from Dr. Andrew Ball, University of Alabama, Birmingham. BB would like to thank Dr. N. James MacLachlan for his steadfast support during the completion of this work. BB was supported by funding from the Veterinary Scientist Training Program and the

Students Training in Advanced Research programs of the University of California, Davis, School of Veterinary Medicine.

References

Anderson Jr., G.W., Peters, C.J., 1988. Viral determinants of virulence for Rift Valley fever (RVF) in rats. Microb. Pathog. 5, 241–250.

Anderson Jr., G.W., Rosebrock, J.A., Johnson, A.J., Jennings, G.B., Peters, C.J., 1991. Infection of inbred rat strains with Rift Valley fever virus: development of a congenic resistant strain and observations on age-dependence of resistance. Am. J. Trop. Med. Hyg. 44, 475–480.

Blakqori, G., Weber, F., 2005. Efficient cDNA-based rescue of La Crosse bunyaviruses expressing or lacking the nonstructural protein NSs. J. Virol. 79, 10420–10428.

Billecocq, A., Spiegel, M., Vialat, P., Kohl, A., Weber, F., Bouloy, M., Haller, O., 2004. NSs protein of Rift Valley fever virus blocks interferon production by inhibiting host gene transcription. J. Virol. 78, 9798–9806.

Bird, B.H., Khristova, M.L., Rollin, P.E., Ksiazek, T.G., Nichol, S.T., 2007. Complete genome analysis of 33 ecologically and biologically diverse Rift Valley fever virus strains reveals widespread virus movement and low genetic diversity due to recent common ancestry. J. Virol. 81 (6), 2805–2816.

Gerrard, S.R., Nichol, S.T., 2007. Synthesis, proteolytic processing and complex formation of N-terminally nested precursor proteins of the Rift Valley fever virus glycoproteins. Virology 357, 124–133.

Gerrard, S.R., Bird, B.H., Albarino, C.G., Nichol, S.T., 2007. The NSm proteins of Rift Valley fever virus are dispensable for maturation, replication and infection. Virology 359 (2), 459–465.

Ikegami, T., Won, S., Peters, C.J., Makino, S., 2006. Rescue of infectious rift valley fever virus entirely from cDNA, analysis of virus lacking the NSs gene, and expression of a foreign gene. J. Virol. 80, 2933–2940.

Lowen, A.C., Noonan, C., McLees, A., Elliott, R.M., 2004. Efficient bunyavirus rescue from cloned cDNA. Virology 330, 493–500.

Madani, T.A., Al-Mazrou, Y.Y., Al-Jeffri, M.H., Mishkhas, A.A., Al-Rabeah, A.M., Turkistani, A.M., Al-Sayed, M.O., Abodahish, A.A., Khan, A.S., Ksiazek, T.G., Shobokshi, O., 2003. Rift Valley fever epidemic in Saudi Arabia: epidemiological, clinical, and laboratory characteristics. Clin. Infect. Dis. 37, 1084–1092.

Meegan, J.M., Watten, R.H., Laughlin, L.W., 1981. Clinical experience with Rift Valley fever in humans during the 1977 Egyptian epizootic. Contrib. Epidemiol. Biostat. 3, 114–123.

Muller, R., Saluzzo, J.-F., Lopez, N., Dreier, T., Turell, M., Smith, J., Bouloy, M., 1995. Characterization of clone 13, a naturally attenuated avirulent isolate of Rift Valley fever virus, which is altered in the small segment. Am. J. Trop. Med. Hyg. 53, 405–411.

Nichol, S.T., 2001. Bunyaviruses. In: Fields, B.N., Knipe, D.M., Howley, P.M., Griffin, D.E. (Eds.), Fields Virology, vol. 2. Lippincott Williams and Wilkins, Philadelphia, pp. 1603–1633.

Rippy, M.K., Topper, M.J., Mebus, C.A., Morrill, J.C., 1992. Rift Valley fever virus-induced encephalomyelitis and hepatitis in calves. Vet. Pathol. 29, 495–502.

Romoser, W.S., Turell, M.J., Lerdthusnee, K., Neira, M., Dohm, D., Ludwig, G., Wasieloski, L., 2005. Pathogenesis of Rift Valley fever virus in mosquitoes-tracheal conduits and the basal lamina as an extra-cellular barrier. Arch. Virol. Suppl. 89–100.

Schmaljohn, C.S., Hooper, J.W., 2001. *Bunyaviridae*: the viruses and their replication. In: Fields, B.N., Knipe, D.M., Howley, P.M., Griffin, D.E. (Eds.), Fields Virology, vol. 2. Lippincott Williams and Wilkins, Philadelphia, pp. 1581–1602.

Shi, X., Kohl, A., Leonard, V.H., Li, P., McLees, A., Elliott, R.M., 2006. Requirement of the N-terminal region of orthobunyavirus nonstructural protein NSm for virus assembly and morphogenesis. J. Virol. 80, 8089–8099.

Suzich, J.A., Kakach, L.T., Collett, M.S., 1990. Expression strategy of a Phlebovirus: biogenesis of proteins from the Rift Valley fever virus M segment. J. Virol. 64, 1549–1555.

Swanepoel, R., Coetzer, J.A.W., 1994. Rift Valley fever. In: Coetzer, J.A.W., Thomson, G.R., Tustin, R.C. (Eds.), Infectious Disease of Livestock with Special Reference to Southern Africa. Oxford Univ. Press, Cape Town, South Africa, pp. 688–717.

Vialat, P., Billecocq, A., Kohl, A., Bouloy, M., 2000. The S segment of Rift Valley fever phlebovirus (Bunyaviridae) carries determinants for attenuation and virulence in mice. J. Virol. 74, 1538–1543.

Won, S., Ikegami, T., Peters, C.J., Makino, S., 2006. NSm and 78-kilodalton proteins of Rift Valley fever virus are nonessential for viral replication in cell culture. J. Virol. 80, 8274–8278.