Virus Research 195 (2015) 203–206

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Short communication

# Interchange of L polymerase protein between two strains of viral hemorrhagic septicemia virus (VHSV) genotype IV alters temperature sensitivities *in vitro*

Sung-Hyun Kim[a], Shamila Yusuff[b], Vikram N. Vakharia[b], Øystein Evensen[a,*]

[a] Norwegian University of Life Sciences, PO Box 8146 Dep, N-0033 Oslo, Norway
[b] Institute of Marine & Environmental Technology, University of Maryland Baltimore County, Baltimore, MD 21202, USA

ARTICLE INFO

*Article history:*
Received 19 September 2014
Received in revised form 9 October 2014
Accepted 10 October 2014
Available online 18 October 2014

*Keywords:*
Viral hemorrhagic septicemia virus
Genotype IV
Temperature sensitivity
L protein
Reverse genetics

ABSTRACT

Viral hemorrhagic septicemia virus (VHSV) has four genotypes (I–IV) and sub-lineages within genotype I and IV. Using a reverse genetics approach, we explored the importance of the L gene for growth characteristics at different temperatures following interchange of the L gene within genotype IV (IVa and IVb) strains. VHSV strains harboring heterologous L gene were recovered and we show that the L gene determines growth characteristics at different temperatures in permissive cell lines.



Viral hemorrhagic septicemia virus (VHSV), an enveloped non-segmented negative-strand (NNS) RNA virus, belongs to the genus *Novirhabdovirus* in the family Rhabdoviridae (Tordo et al., 2004). The viral genome encodes five structural proteins (N, M, P, G, and L) and a non-structural protein (NV) between G and L protein (Ammayappan and Vakharia, 2009). VHSVs are classified into four major genotypes (I–IV) and further separated into different sub-lineages (Ia-e and IVa-c) based on geographical distribution (Einer-Jensen et al., 2004; Einer-Jensen et al., 2005; Pierce and Stepien, 2012). While various strains of VHSVs infect fish under different climate and temperature conditions, any difference in temperature sensitivity of VHSV genotypes (or strains) has been not studied.

The polymerase protein is a multifunctional catalytic component in RNA-dependent-RNA-polymerase (RdRp) complex required for viral replication, transcription, and gene expression (Galloway and Wertz, 2009). Earlier studies have shown that mutations of the RdRp are involved in temperature sensitivity of Sendai virus (*Paramyxoviridae*) (Feller et al., 2000a; Feller et al., 2000b), human parainfluenza virus type 3 (*Paramyxoviridae*) (Feller et al., 2000b), respiratory syncytial virus (*Paramyxoviridae*) (Luongo et al., 2012; Luongo et al., 2013), and vesicular stomatitis virus (VSV, *Rhabdoviridae*). For VSV, an insertion of EGFP in a hinge region or a point mutation in conserved region (CR) II in the L gene induces sensitivity to high temperature (Galloway and Wertz, 2009; Ruedas and Perrault, 2009). The functions of NNS RNA virus CRs are partly known: CR III is involved in the RNA polymerization, CR V for the cap addition, CR VI for the cap methylation, and CRs I, II and IV for the RdRp-containing ring domain composition (Rahmeh et al., 2010; Ogino and Banerjee, 2011). The implication of the L protein of novirhabdoviruses in temperature sensitivity still remains uncertain. Previously, it was shown that the G protein is a determinant for temperature sensitivity and it was suggested that the polymerase complex would be involved, but it was not studied (Biacchesi et al., 2002; Biacchesi, 2011). In this study, we interchanged the L protein gene between two strains of VHSV, JF-09 with an ability to grow at higher temperature (Kim et al., 2014) and MI03GL, a Great Lakes strain, (Ammayappan and Vakharia, 2009) sensitive to temperatures above 18 °C. These strains belong to genotype IVa and IVb, respectively. Here, we demonstrate the gain- and loss-of-function of VHSV titers at different temperatures using reverse genetics approach.

First we propagated five different VHSV strains (DK-3592B; Ia, 1p8; Ib, NO/650/07; III, JF-09; IVa, and MI03GL; IVb) in *Epithelioma papulosum cyprini* (EPC) cells at 15 °C, in line with previous studies (Ammayappan and Vakharia, 2009; Kim et al., 2014). All strains were also used to infect EPC cells at 23 °C for 72 h post-infection (MOI = 1) to test temperature sensitivities of the viruses. The culture

* Corresponding author. Tel.: +47 47400119.
*E-mail address:* oystein.evensen@nmbu.no (Ø. Evensen).

http://dx.doi.org/10.1016/j.virusres.2014.10.013
0168-1702/

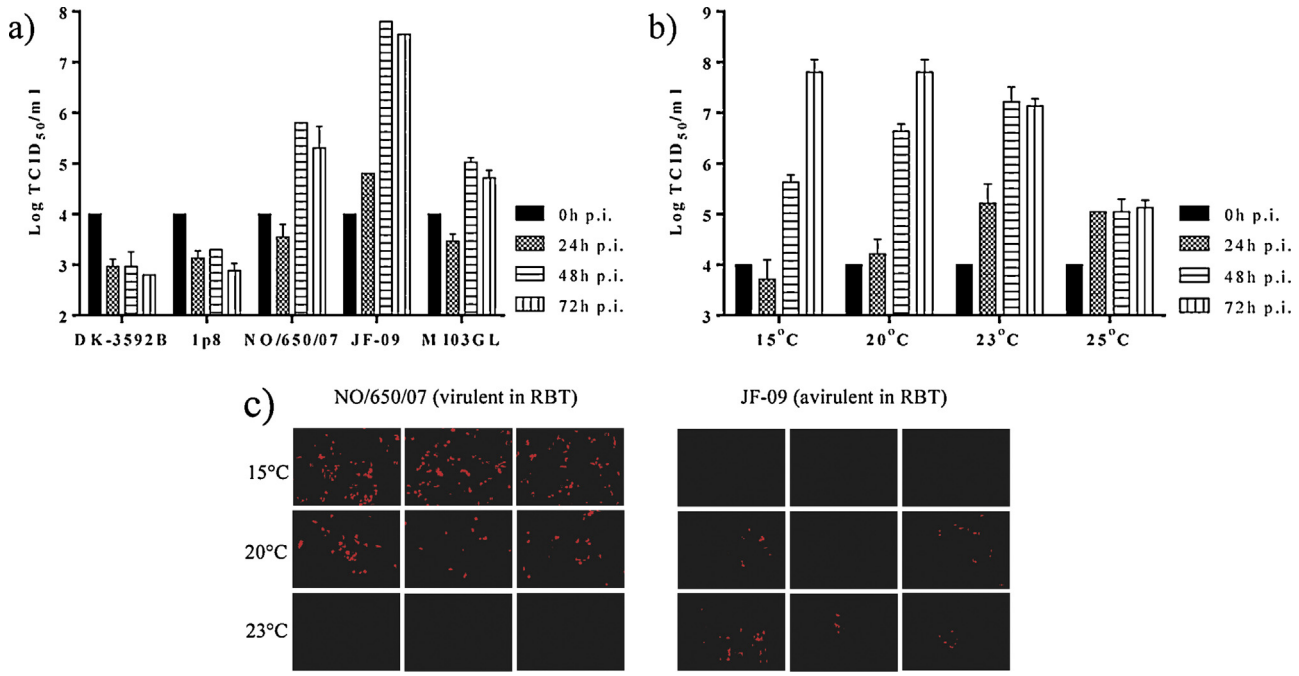


**Fig. 1.** ***In vitro*** temperature sensitivity of VHSV strains. (a) Different temperature sensitivities for five VHSV strains tested in permissive cells (EPC) at 23 °C. Virus titers are given ($TCID_{50}$/ml) over a 72 h incubation period (MOI = 1). (b) Temperature sensitivity of JF-09 in permissive cells (EPC) shown viral titration ($TCID_{50}$/ml) over a 72 h incubation period (MOI = 1; 15 °C, 20 °C, 23 °C, and 25 °C). The viral titers in supernatants are presented as a mean ± standard deviation (SD) from three parallels. (c) Temperature sensitivity of JF-09 in non-permissive cells (primary cultured RBT GECs) at 24 h post-infection (MOI = 1; 15 °C, 20 °C, and 23 °C) was shown by IFAT (viral proteins in red color) under an Olympus IX81 fluorescence microscope. NO/650/07 was used for a positive (virulent) strain in RBT GECs.

supernatants were collected and titrated on EPC cells (Karber, 1931). The results showed that JF-09 grew to significantly higher titers than any of the other strains at 23 °C, and results were in the order JF-09 > NO/650/07 > MI03GL > 1p8 > DK-3592B (Fig. 1a).

Then we examined the temperature sensitivity of JF-09 over a temperature in permissive EPC cells between 15 and 25 °C with 72 h incubation and titration as above (MOI = 1). JF-09 grew between 15 and 23 °C but not at 25 °C (Fig. 1b, 1 log increase at 25 °C). We also examined the temperature sensitivity of the JF-09 strain in non-permissive cells using primary cultured rainbow trout gill epithelial cells (RBT GECs) using a virulent strain (NO/650/07) as positive control (Kim et al., 2014). Viral proteins were detected at 24 h post-infection (MOI = 1) by indirect fluorescent-antibody test (IFAT) (Kim et al., 2014). Interestingly, JF-09 showed increased staining at 20–23 °C in GECs while NO/650/07 staining declined with increasing temperature (Fig. 1c).

Mutations of the RdRp of other non-segmented, negative-strand viruses have shown to influence temperature sensitivity (Feller et al., 2000a,b; Galloway and Wertz, 2009; Luongo et al., 2012, 2013; Ruedas and Perrault, 2009). With this in mind, we used a previously made recombinant VHSV genotype IVa construct of rJF-09 (genotype IVa, Genbank no. KM926343) (Kim et al., 2014) and one of rMI03GL (genotype IVb) (Ammayappan et al., 2011) with the purpose to swap the two L protein encoding genes after introducing a silent mutation at a rear KpnI restriction site, as described (Ammayappan et al., 2011) (Fig. 2a). The parental plasmids were double digested with KpnI and NotI restriction sites and the L protein genes were then cross-inserted into two backbones by ligation (T4 DNA ligase) (Fig. 2a). Digestion with KpnI restriction enzyme did not result in any substitution of first 44 amino acids in the N-terminus sequence (Fig. 2a). However due to L gene exchange, 31 amino acids in the L protein (1984 amino acids) are different between JF-09 (IVa) and MI03GL (IVb), which are depicted in Fig. 3. Substitutions of amino acid residues are indicated on a linear map, with the six CRs of VHSV, based on a previous NNS virus L protein mapping study (Poch et al., 1990) (Fig. 3). All four recombinant viruses were rescued from EPC cells transfected as described (Kim et al., 2014) and the viruses were partially sequenced to clearly identify the L protein interchange. The viruses (MOI = 0.1) were used to infect EPC cells at 15 °C, 21.5 °C, and 23 °C, incubated for 72 h post-infection and then supernatants were titrated. We found that L protein interchange between two strains of VHSV genotype IV results in change of temperature sensitivities at the high-temperature (21.5 and 23 °C). First, substitution of the L gene in rJF-09 virus with L-IVb ($IVa + L_{IVb}$) resulted in reduced viral titers when compared with the parental rJF-09 strain. Titers were down $10^{2.25}$ at 21.5 °C and $10^{2.67}$ at 23 °C (Fig. 2b). When IVa L gene was substituted in rMI03GL virus ($IVb + L_{IVa}$), the viral titers were up compared to the parental rMI03GL (increased by $10^{2.33}$ at 21.5 °C and $10^{1.08}$ at 23 °C; Fig. 2b). For both "daughter strains" the viral titers were slightly lower at 15 °C ($10^{0.17}$ difference; $p > 0.05$) compared the parental strain (Fig. 2b). Furthermore, the titer of '$IVb + L_{IVa}$' still was down $10^{1.42}$ at 23 °C compared to the titer at 15 °C. The titers obtained for the parental strains at MOI = 1 and MOI = 0.1 were the same (Fig. 1a compared to Fig. 2b). Together, the MOI range used did not seem to influence the titers obtained at the two temperatures tested and the data is also indicative of the L protein not being the only determinant of temperature sensitivity. The L protein could be involved in other viral replication mechanisms such as the functionality of the RNP (Ribonucleoprotein) complex.

This study is first report to show that the L protein gene of *Novirhabdovirus* plays a role in sensitivity to growth at different temperatures, and we find a gain- and loss-of-function effect from L protein interchange. The observation that the JF-09 strain also shows improved growth at higher temperature in a non-permissive cell line (GEC) is also an interesting observation that warrants further studies. Amino acid differences between IVa and IVb for the L protein is shown in Fig. 3, and among the 31 amino acids positions that are different, there are no differences in CR II, an important region for the temperature-sensitive VSV strain ((G)114) (Galloway and Wertz, 2009). Furthermore, the 31 amino acids could be important in determining temperature sensitivity of VHSV strains and