Studies of mechanisms of virulence of viral hemorrhagic septicemia (VHS) – comparison between different genotypes of the virus

Philosophiae Doctor (PhD) Thesis

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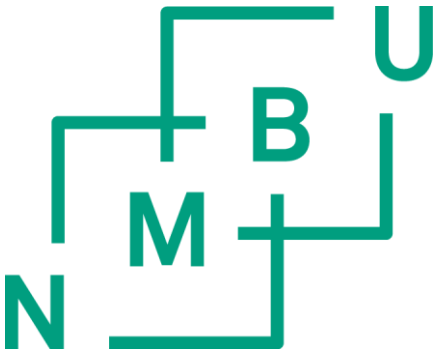

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SUMMARY

Viral hemorrhagic septicemia virus (VHSV) has four major genotypes (I, II, III, and IV) with different sub-lineages (Ia-e and IVa-c). VHSV infects a wide-host-range with a preference for low-temperature environments. The virus leads to a deadly disease and causes severe economic losses to rainbow trout (*Oncorhynchus mykiss*) and olive flounder (*Paralichthys olivaceus*) farming in Europe and East Asia. Infection also causes great losses in wild fish, particularly in the Great Lakes in North America.

The aim of this study (Paper I) was to identify virulence mechanisms of VHSV. It is known that virulence to rainbow trout vary between genotypes and on this basis the pathogenicity profile of different strains was tested for their ability to infect and translocate across polarized, rainbow trout gill epithelial cells (GECs). Infection of GECs *in vitro* mimic the initial infection process *in vivo* and four VHSV strains were tested for their ability to infect and translocate across GEC cells. On this basis the strains were separated into two virulence categories, one virulent and one avirulent group. The strains were (or had been tested) tested for *in vivo* virulence to rainbow trout. Next the different strains were subject to full genome sequencing and the obtained sequences were aligned and from this, single amino acid residues that were different between the two virulence categories were identified and selected as potential virulence residues. Among the differences detected between high and low virulence strains, eight amino acid residues spread across the G, NV, and L proteins were selected. An avirulent, genotype IVa strain (JF-09) was used as a template or backbone, and by use of reverse genetics the selected 8 residues were changed one by one, except for residues in the NV protein that were mutated as a block of 3 residues. The results showed that of those selected, only one residue was found to change the *in vitro* virulence profile of JF-09. When isoleucine (I) was exchanged with phenylalanine (F) in position 1012 of the L protein the mutated strain changed from an avirulent to an intermediate-virulent variant, using the GECs system *in vitro*. This residue is located in the conserved region (CR) IV of the L protein (RNA polymerase).

In the next study (Paper II), the importance of the terminal untranslated region (UTR) for *in vitro* viral replication and *in vivo* pathogenicity was studied. The conserved 3'-terminal sequence was identified as a potential promoter for initiation of RNA synthesis, and a specific sequence in 3'-terminus was then analyzed using an approach by point mutations at nucleotides A^4G^5 and A^7U^8. Through various mutations it was shown that the primary

sequence (A^4G^5) at the 3'-terminus is vital for replication and *in vitro* and *in vivo* virulence tested in a zebrafish model.

Finally, in the third study (Paper III), the importance of the L protein for temperature sensitivity was studied. This study indicated that VHSV strains have different temperature sensitivities. To assess the importance of the L protein as a determinant of temperature sensitivity, the L protein encoding region from two different strains within genotype IV were interchanged. This study showed that the L protein determines temperature sensitivity at elevated temperatures *in vitro*.

From our findings, we conclude that the RNA polymerase (L) is linked with the virulence of VHSV related to infectivity *in vitro* and *in vivo* virulence, and also temperature sensitivity. The conserved 3'-terminal promoter sequence is important for optimal replication in host cells and thus virulence, both *in vitro* and *in vivo*. Hence, the results obtained add to the understanding of virulence of VHS virus.

SAMMENDRAG

Viral hemoragisk septikemi virus (VHSV) har fire genotyper (I, II, III, og IV), med flere subgenotyper (Ia-e og IVa-c). VHSV har et bredt vertsspekter og gir sykdom ved lave vanntemperaturer. Viruset fører til en dødelig sykdom og gir spesielt store økonomiske tap i oppdrett av regnbueørret (*Oncorhynchus mykiss*) i Europa og i flyndreoppdrett (*Paralichthys olivaceus*) i Sør-Øst Asia.

Med det siktemål å bedre forstå virulensmekanismer hos VHSV for ulike genotyper, ble det i den første studien (Paper I) fokusert på å definere virulensmotivene hos regnbueørretpatogene VHSV stammer. Vi benyttet en *in vitro* modell som etterligner den naturlige infeksjonsbarrieren hos fisk. Dette ble gjort ved å dyrke gjelle-epitelceller fra regnbueørret (RBT-GEC) som så ble infisert med fire ulike stammer av VHSV, som igjen var inndelt i to forskjellige virulenskategorier (virulente og avirulente stammer). Ved å sammenligne aminosyresekvensen hos de ulike virusisolatene ble 8 utvalgte posisjoner i G, NV, og L-proteinene utvalgt for introduksjon av enkeltmutasjoner (på aminosyrenivå) hos en prototype av et marint VHSV isolat, kalt JF-09, som opprinnelig ikke er virulent overfor regnbueørret og heller ikke infiserer GEC i kultur. Av alle de introduserte mutasjoner så viste det seg at posisjon 1012 i polymerase-proteinet hadde betydning for *in vitro* virulens i GEC og når isoleucin (I) ble endret til fenylalanin (F), lokalisert i en konservert region (CR) IV i L protein (RNA-polymerase), ble virus ”omdannet” fra en avirulent variant til en middels virulent variant i GECs celler. Dette er første gang det er påvist at virulensegenskaper hos VHSV er knyttet til polymerasen.

I den neste studien (Paper II) så vi på betydningen av den 3`-terminalen ikke-translatert regionen (UTR) hos VHSV for *in vitro* replikasjon og *in vivo* patogenisitet. Den konservert 3'-terminale sekvensen ble karakterisert og ved hjelp av enkelt posisjon-mutagenese, og posisjonene 4/5 og 7/8 ble studert. Vi foretok en ombytting av nukleotidrekkefølgen i posisjon 4/5, A4G-G5A, og en mutasjon i posisjon 7/8, A7C-U8A. Gjennom *in vitro* studier fant vi at posisjon 4/5 er en mulig promotor for initiering av RNA-syntese, og at det er en spesifikk sekvens i 3'-enden som er av betydning. På samme måte fant vi at de effekter som ble observert *in vitro* også lot seg reprodusere *in vivo* når denne virusmutanten ble benyttet for å infisere zebrafisk. Studien viste at rekkefølgen av den primære sekvensen (A4G5) i 3'-enden er en viktig kode for replikasjon og *in vitro*/*in vivo*-virulens.

Til slutt, i den tredje studien (Paper III), ble betydningen av L proteinet for temperaturfølsomhet studert. For å vurdere om L proteinet fungere som en determinant for temperaturfølsomhet, ble genet som koder for L-proteinet fra to forskjellige stammer av genotype IV byttet om ved revers genetikk. Disse hydrid-stammene ble så testet ved ulike temperaturer fra 15 °C til 23 °C. Her ble det vist for det første, at ulike VHSV stammer har ulik temperaturfølsomhet og at man ved å bytte om polymerasen kan oppnå både en ”loss-of-function” og ”gain-of-function”. Denne studien viste at L- proteinet spiller en viktig rolle ved temperaturfølsomhet for vekst *in vitro*.

På bakgrunn av disse resultatene konkluderer vi med at RNA polymerase (L) protein er direkte knyttet til de virulensmekanismer hos VHS virus; dette har betydning for smittsomhet mellom fiskearter (*host-species specificity*) og temperaturfølsomhet. Den konservert 3'-terminale promotorsekvens er viktig for viruset virulens og for optimal replikasjon i vertsceller.

Resultatene i denne avhandlingen kan benyttes for å forstå virulensmekanismer og i siste instans også gi verdifulle bidrag til forebyggelse av utbrudd av VHS i oppdrettsnæringen.

PAPER LIST

Paper I

A single amino acid mutation (I1012F) of the RNA polymerase changes the in vitro virulence profile of marine viral hemorrhagic septicemia virus to rainbow trout gill epithelial cells.

Sung-Hyun Kim, Beate J. Thu, Helle F. Skall, Niccolò Vendramin, and Øystein Evensen.

J Virology, 88(13):7189-7198, 2014

Paper II

Specific nucleotides at the 3'-terminal promoter of viral hemorrhagic septicemia virus are important for virulence *in vitro* and *in vivo*.

Sung-Hyun Kim, Tz-Chun Guo, Vikram N. Vakharia, and Øystein Evensen.

Virology, 476:226-232, 2015

Paper III

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

Sung-Hyun Kim, Shamila Yusuff, Vikram N. Vakharia, and Øystein Evensen.

Virus research, 195:203-206, 2015

ACRONYMS/ABBREVIATIONS

cRNA	-	Complementary ribonucleic acid
CPM	-	Cumulative percent mortality
DNA	-	Deoxyribonucleic acid
HPIV-3	-	Human parainfluenza virus type 3
hRSV	-	Human respiratory syncytial virus
IFN	-	Interferon
IHNV	-	Infectious hematopoietic necrosis virus
G	-	Glycoprotein
L	-	RNA polymerase
M	-	Matrix
MOI	-	Multiplicity of infection
N	-	Nucleocapsid
NNS RNA	-	Non-segmented negative strand RNA
NV	-	Non-virion
NFκB	-	Nuclear factor kappa B
ORF	-	Open reading frame
P	-	Phosphoprotein
PB2	-	Subunit of influenza virus RNA polymerase
RBT GEC	-	Rainbow trout gill epithelial cell
RdRp	-	RNA-dependent-RNA-polymerase
RNA	-	Ribonucleic acid
RNP	-	Ribonucleoprotein complex
RT-PCR	-	Reverse transcriptase polymerase chain reaction
SHRV	-	Snakehead rhabdovirus
SVCV	-	Spring viremia of carp virus
TNF-α	-	Tumor necrosis factor alpha
UTR	-	Untranslated region
VHS	-	Viral hemorrhagic septicemia
VHSV	-	Viral hemorrhagic septicemia virus
vRNA	-	Viral ribonucleic acid
VSV	-	Vesicular stomatitis virus

INTRODUCTION

GENERAL BACKGROUND

A disease syndrome, with resemblance to viral hemorrhagic septicaemia (VHS), was first described by Schäperclaus in rainbow trout in 1938 [1]. He termed the disease "Nierenschwellung" (kidney swelling) and in 1946 Pliszka described a similar syndrome in Poland [2]. The same disease syndrome was later described in Denmark in the 1950s [3,4] and the first successful isolation of what turned out to be the causative agent, was done by Jensen (in Denmark) in 1963 [5]. He termed the virus Egtved virus and also called the disease *Egtved disease*. It was named after the Danish village from which the fish originated [6].

VHS virus was isolated from wild fish, Atlantic cod (*Gadus morhua*), for the first time from the coastal waters of Zealand in 1979 [7,8]. Despite so, up to the mid-90s VHS was considered primarily a disease of farmed fish in continental Europe [1,9–11]. The change came with detection of VHS virus in 1988/89 in healthy returning Chinook salmon and Coho salmon in the North American Pacific Northwest region [9,10,12]. Later it was also isolated from haddock (*Melanogrammus aeglefinus*) and cod in the Scottish waters (East coast) in 1993 and later in 1995 [13]. The first isolation in farmed marine fish in European waters occurred in 1991 in Germany [14], in Scotland in 1994 [15,16] and Ireland in 1997 [17] and on the basis of these findings, the virus and the disease attracted more attention from the legislators, particularly in the European Union. This resulted in the launch of the large screening project (FAIR CT 96-1594. *Rhabdoviruses in wild marine fish in European coastal waters: characterization and significance for aquaculture*) where Norwegian partners also joined in and this project is the basis for the research activities leading up to this thesis. The separation of the different virus strains into clades, aligns with previous categorization of strains of VHS virus into four major genotypes (I, II, III, and IV) with different sublineages (Ia-e and IVa-c). The different stains are found in different geographical regions but not related to host-specific infectivity [11,18].

Outbreaks of VHS have been observed in pen-reared rainbow trout in the marine coastal area off Gothenburg [19,20]. The isolates from these outbreaks were grouped in genotype Ib indicating that the virus causing the outbreak can originate from wild marine fish in the Kattegat. Outbreaks have also been observed in Finnish coastal waters off the Åland Island

[21]. Challenge studies using the Finnish and Swedish isolates show 20-40 % mortality in rainbow trout fingerlings (unpublished data in Skall et al. 2004). VHS virus is present at very low prevalence in marine fish species but despite so clinical outbreaks of VHS have occurred in rainbow trout farmed in seawater in the North-west coast of Norway (Storfjorden, Møre and Romsdal county) [22].

Through the referred project (FAIR CT 96-1594), which included a large screening program of wild caught marine fish species also in Norwegian waters [23], it became clear that VHS virus infects a vast diversity of species in the marine environment [17]. Also in Norwegian waters, VHS virus was detected in wild fish species, but at very low prevalence [23]. Over the last 25 years several VHSV strains have been isolated from freshwater and marine fish in the Northern hemisphere, altogether approximately 80 species [24,25]. On this basis, we now know there are VHSV strains circulating in wild fish in the North Pacific and the North Atlantic oceans, and in addition, VHS virus circulates in farmed fish in continental Europe (Fig. 1) [26]. From each of these "sources" of wild fish, inter-species transmission or host-switch has occurred several times, as indicated in Figure 1.

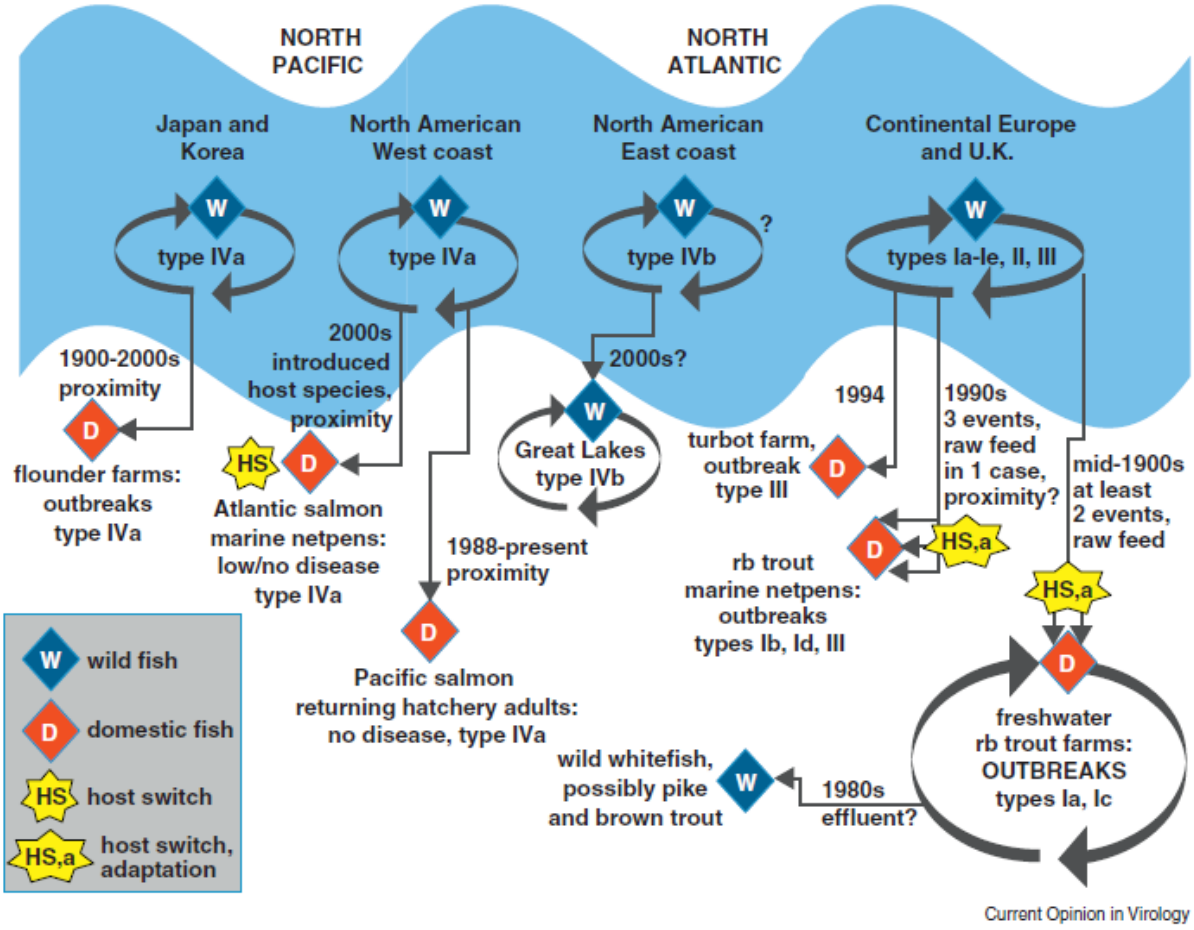

Figure 1. ***Continental Europe and UK.*** Until 1998 continental Europe was considered as the principal region of occurrence of VHS in rainbow trout farmed in freshwater. The disease was also found in rainbow trout held in the marine environment in Sweden (1998), and later in Finland (2000), and recently in Norway (2007) [17,21,22,27–29]. In Baltic Sea and North Sea *Clupidae* and Norway pout were the predominant host of VHSV [17,30]. In the North Sea VHSV was also isolated from cod with ulcus syndrome and haddock with skin haemorrhages [13]. Furthermore, VHSV was detected from wild marine fish without lesions [17,30].

North Atlantic Ocean – East Coast. The VHS disease also caused mass mortality of freshwater drum (*Aplodinotus grunniens*) and round goby (*Neogobius melanostomus*) in the Great Lakes in 2005 [31].

North West Coast. The first VHS virus isolates in North America originate from returning Chinook salmon (*Onchorhyncus tshawytscha*) and Coho salmon (*Onchorhyncus kisutch*) [9,10] The isolates were low or apathogenic for salmon and trout [32] but were highly pathogenic for marine fish species [33]. It clearly indicates that the virus isolates were originated from the marine environment.

North Pacific – Japan and Korea. Rhabdoviral disease was reported with mass mortality in olive/Japanese flounder (*Paralichthys olivaceus*) farms in the Seto inland Sea of Japan in 1996 and later the causative pathogen was identified as VHSV [34]. In East Asia VHSV was found widely distributed in both wild and farmed olive flounder [34,35].

Based on genome sequencing of the G-protein encoding gene [11] VHS virus strains have been divided into several different clades and the clades are shown below (Fig. 2). Subdivision includes one Eurasian clade with genotypes (strains) I, II and III and one New-World clade (strain) IV.

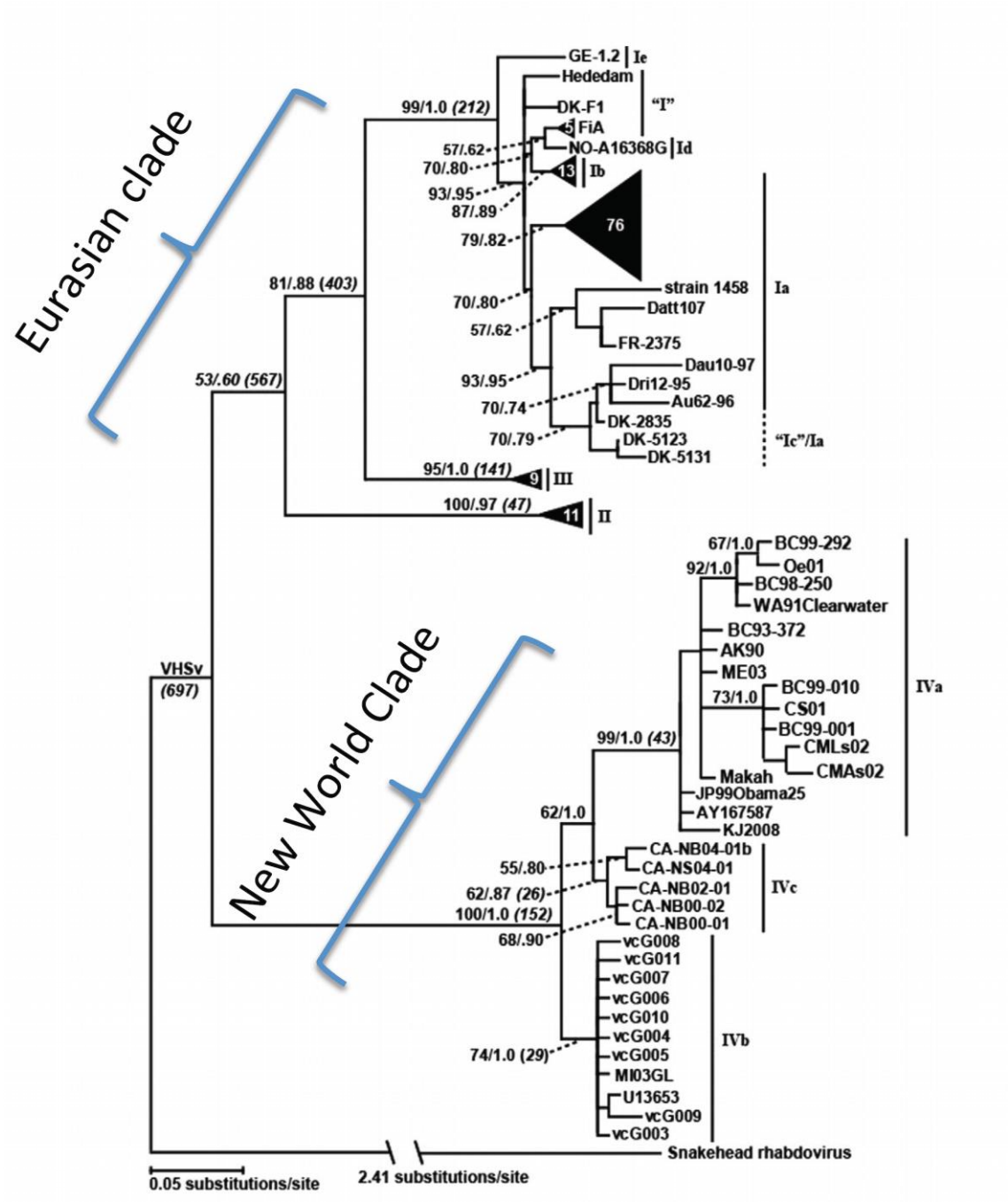

Figure 2. Compiled clade picture showing the *Eurasian clade* at the top equivalent to genotypes I, II and III, and the *New world clade* at the bottom, equivalent to Genotype IV and sublineages.

Farmed fish

VHS causes severe economic losses in fish farming, with higher incidences during the winter season [17,29,36,37]. All European marine VHSV strains (genotypes I, II, and III) were generally thought to be low- or nonpathogenic to rainbow trout, until VHS outbreaks were seen in rainbow trout farmed in the marine environment in Sweden (1998), Finland (2000), and Norway (2007), all caused by marine VHSV strains (genotype Ib-e and III) [17,21,22,27–29]. It is probable that low- or nonpathogenic marine VHSV strains, through mutations, cross the species barrier and infect farmed rainbow trout. Using waterborne infection (under experimental conditions) it was shown that a marine VHSV strain, genotype IVa could extend the host range from Pacific herring (*Clupea harengus pallasii*) to Atlantic salmon (*Salmo salar* L.) [38]. From these studies, it seems probable that low- or nonpathogenic marine VHSV strains can spread into salmonid aquaculture but very little is known about the underlying mechanisms of virulence differences between strains and genotypes.

Rainbow trout fresh water fish farms in continental Europe are highly affected by VHS disease outbreaks (fresh water VHSV genotype Ia). In Norway VHS has not been a problem in rainbow trout fish farms until 2007 when an outbreak of VHS (marine VHSV genotype III) occurred in seawater-reared rainbow trout located in Møre and Romsdal on the west coast of Norway [22]. Survey within the region identified VHSV in three other fish farms operated by the same company [22]. This was the first detection of VHSV in Norway since 1974, which at that time were caused by genotype Id [22]. The isolates in 2007 were tested in bath challenge trials in rainbow trout and showed high virulence to rainbow trout [22]. It was the first registration of a marine VHSV genotype III being virulent for rainbow trout [22] but luckily, Norwegian rainbow trout farms have not experienced disease outbreaks since 2007.

Olive flounder farming have been heavily affected by VHS disease outbreaks in Japan, South Korea, and China with mass mortality [34,35,39]. In Japan VHSV (Obama 25, genotype IVa) was first isolated from wild olive flounder caught in Wakasa Bay in 1999 [40]. Also a rhabdoviral disease was diagnosed in 1996 causing 50-70 % cumulative mortality in cultured olive flounder in the Inland Sea of Japan at water temperatures ranging from 8-15 °C. A later study identified the causative virus as VHSV (KRRV 9601, genotype Ib) [34]. According to *in vivo* challenge test with KRRV 9601, pathogenicity declined with increasing fish weight [34]. In South Korea VHSV was first detected in cultured juvenile olive flounder in East sea of Korea in 2001 [41]. VHSV was also detected in cultured market-sized olive flounder in South sea of Korea from winter to spring of 2005 with heavy mortalities, 40-60 % cumulative

mortality [35]. Until today, all VHS outbreaks in South Korea have been associated with genotype IVa [42]. In China a rhabdovirus was isolated from diseased olive flounder in 2005 and named *Paralichthys olivaceus* Rhabdovirus (PORV) and the virus has 98.3 % identity (nucleotide level) with a Korean VHSV strain (KJ2008, genotype IVa) [39].

GENOME ORGANIZATION OF VHSV

VHSV is an enveloped non-segmented negative strand (NNS) RNA virus and belongs to the genus *Novirhabdovirus* in the family *Rhabdoviridae* [43]. The viral genome is approximately 11.2 kb in size and encodes five structural proteins (Fig. 3); namely, (i) nucleocapsid protein (N), (ii) phosphoprotein (P), (iii) matrix protein (M), (iv) glycoprotein (G), and (v) RNA polymerase protein (L). The structural proteins consist of two major functional units: (i) a ribonucleoprotein complex (RNP) unit composed of N, P, and L for transcription and replication of genomic and anti-genomic viral RNA, and (ii) envelope components composed of G and M proteins to allow RNP in and out of the host cell [44]. In contrast to the rhabdoviruses, novirhabdoviruses have a nonstructural non-virion (NV) protein-coding gene placed in between G and L protein-coding genes [45]. In general, the NNS RNA viruses have highly conserved gene order, N, P, and M gene near 3'-end and L gene near 5'-end [46].

The VHSV RNA genome has (i) conserved UTR sequences in the 3'- and 5'-ends and (ii) conserved intergenic UTR sequences in between open reading frame (ORF) genes, similar to other rhabdoviruses.

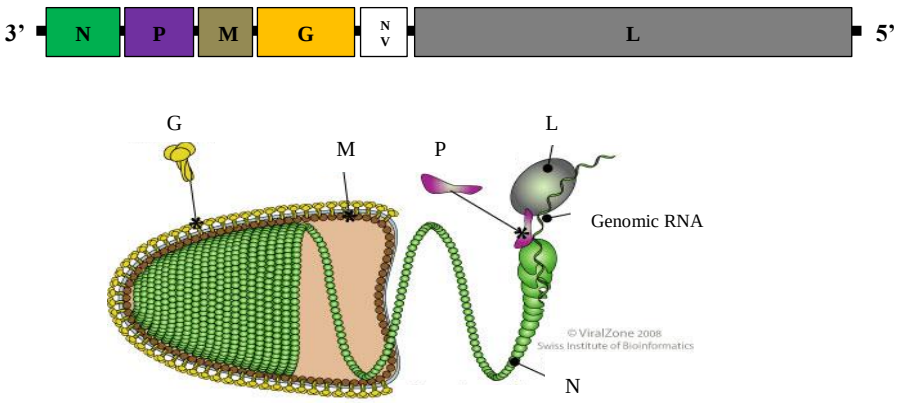

Figure 3. Genome organization of five structural proteins (N, P, M, G, and L) and a nonstructural protein (NV) of *Novirhabdovirus*. Pictures are from Viral Zone: www.expasy.org/viralzone, Swiss Institute of Bioinformatics.

Ribonucleoprotein complex

i. Nucleocapsid protein (404 amino acids) has five highly variable regions [45]. As a constituent of the RNP unit, the concentration of the N protein-encapsulated RNA genomic template is an important signal for switching from transcription (viral RNA to mRNA) to replication (cRNA to viral RNA) and viral genome copying (Fig. 4) [47,48].

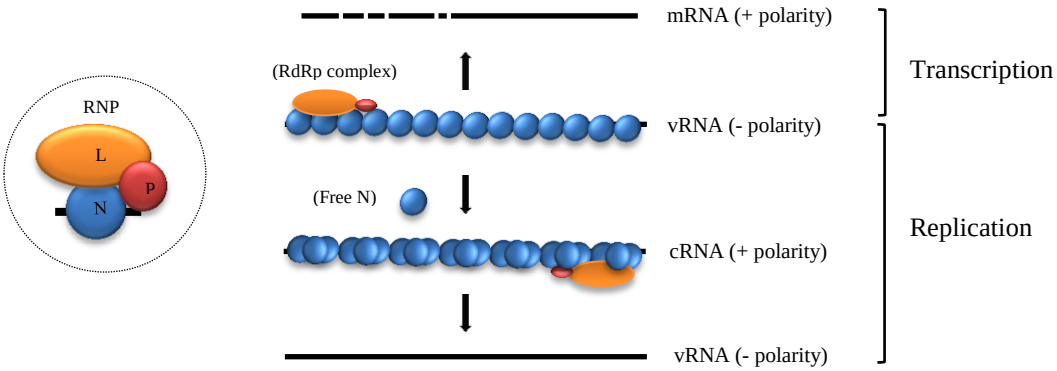

Figure 4. Transcription and replication cycle of negative strand RNA virus. In the absence of free nucleocapsid (N) protein, the RNA-dependent-RNA polymerase (RdRp) complex transcribes the genome to make mRNA at the early infection stage. After translation of the N proteins, the complex uses the highly N-coated complementary RNA (cRNA) genome as a copy to make more viral RNA (vRNA) and to produce more viral proteins, especially RNPs [48]. Figure prepared by S-H. Kim.

ii. Phosphoprotein (222 amino acids) has a highly variable N-terminal half and a conserved C-terminal half [45]. P, which is a noncatalytic polymerase-associated protein, has a putative conserved domain for binding with L protein to be a complete RNA-dependent-RNA polymerase (RdRp) complex [44,49]. Based on vesicular stomatitis virus (VSV, *Rhabdoviridae*), the P has three domains: an N-terminal domain to bind with the L protein, a central oligomerization domain, and a C-terminal domain to bind with the N-RNA template [50,51].

iii. RNA polymerase (1984 amino acids) which is a large catalytic RdRp subunit has six highly conserved regions (domains) in all NNS RNA viruses [52]. The L protein may be organized as a series of separated structural domains but the domains are functionally linked to influence other domains [50,53,54]. The conserved regions have been mapped to CR III, which is required for RNA polymerization on a template U tract; CR V, which is required for mRNA cap addition; and CR VI, which is required for cap methylation activities; whereas the

functions of CR I, CR II, and CR IV remain unclear [50,55,56]. However, based on VSV, CRs I, II, and IV seem to play an additional structural role for the integrity of the ring domain, which is required for facilitating RNA synthesis [50]. The NNS RNA viruses initiate viral copying in host cells by a specialized RNA synthesis machine that consists of RNP composed of the genomic RNA coated by N protein and the RdRp complex (Fig. 4) but the L protein alone is not capable of RNA synthesis [50,55,57]. During RNA synthesis, the N protein-encapsulated RNA is displaced for the L protein [50]. The enzymatic action of the RdRp complex (L-P complex) is important for the initiation and maintenance of viral RNA synthesis, but the RdRp complex of RNA viruses lacks the capacity for accurate proofreading, unlike DNA-dependent polymerases [48,58,59]. Hence, it may trigger the viral adaptation or evolution into new environments [48,58,59]. In general, the L proteins of RNA viruses have a similar shape, which resembles a right hand with conserved palm, thumb, and fingers domain [48].

Envelope component

i. The gene encoding the ***matrix protein*** (201 amino acids) is the most conserved among the six VHSV protein-coding genes across different isolates, with the protein itself being a structural component in between the viral outer membrane of the glycoprotein and RNP coil [45,60]. Studied in the family *Rhabdoviridae*, this protein works mainly for viral assembly, nucleocapsid condensation, bullet-shape formation, and viral budding processes [60–63]. It also induces host innate immune responses such as apoptosis (a programmed cell death process) and down-regulation of the host-directed gene expression, but it is unlikely to play a role in determining the host-specific virulence of VHSV [49,64].

ii. Glycoprotein (507 amino acids) constitutes viral spikes and is the viral attachment protein to cellular surface receptors and entry into host cells but only a few amino acids are involved in membrane fusion [65–67]. Unlike the M, G is not required for the viral budding process and it plays a key role in immunity and immune stimulation but lesser of a role in the host-specific virulence of VHSV [66,68–70].

Non-structural protein

i. The Non-virion protein (NV, 122 amino acids) is a unique entity in the genus *Novirhabdovirus*. The NV protein-coding gene is highly variable among novirhabdoviruses, has the ability to suppress host innate immune responses like VHSV and infectious

hematopoietic necrosis virus (IHNV) [71–73]. For VHSV the NV protein-coding gene is the most variable among the six protein-encoding genes (Fig. 5). It suppresses (i) apoptosis at the early infection stage, (ii) type I interferon (IFN) transcription, (iii) IFN-induced Mx expression, and (iv) tumor necrosis factor alpha (TNF-α)-mediated activation of nuclear factor kappa B (NFκB) [73–76]. Furthermore, the NV protein is important for pathogenicity, but it does not determine host-specific virulence in rainbow trout [68,73,74,77]. In contrast to low-temperature novirhabdoviruses, the NV protein of snakehead rhabdovirus (SHRV), which is a high-temperature novirhabdovirus, is not required for viral pathogenicity [78].

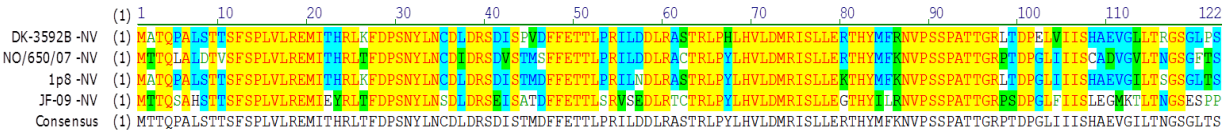

Figure 5. Variation of the non-virion (NV) protein-coding gene among viral hemorrhagic septicemia virus (VHSV) strains (DK-3592B: Ia; NO/650/07: III; 1p8: Ib; and JF-09: IVa).

Conserved UTR RNA sequence

A summary of the conserved UTR sequences and the patterns are given in Table 1.

i. 3'- and 5'-end UTRs of VHSV have conserved sequences, and the end sequences are complementary (Table 1). The end-UTRs have an unusual A/U-rich content. In the NNS RNA viruses, the end-UTRs have been shown to be multifunctional, which include control of RNA synthesis processes (transcription and replication), encapsidation, and assembly of newly synthesized RNAs [46].

ii. Intergenic UTRs of VHSV have a conserved gene-start (-CG(A)UG-), and gene-end sequence (UCUAU(A)CUUUUUUU). The poly-A^7 is a putative transcription stop signal of the shuttering mechanism [45,79]. The UTRs also have two conserved intergenic nucleotides (G(A)C), which serve as a spacer between genes for subsequently moving the RdRp complex to the next gene-start sequence [45,79]. The gene starting point of the L protein has a Kozak context (-ACCATGG-), which is unique among the six protein-coding genes [45].

Table 1. Conserved intergenic, untranslated regions (UTRs) and 3'- and 5'-end UTRs from the complete genomic sequence of JF-09 strain (GenBank: KM926343.1).

	Gene-end	Intergene	Gene-start	Position
3'- / N		GC	CGUG	54-59
N / P	UCUAUCUUUUUUU	GC	CGUG	1415-1433
P / M	UCUAUCUUUUUUU	GC	CGUG	2177-2195
M / G	UCUAUCUUUUUUU	AC	CAUG	2920-2938
G / NV	UCUAUCUUUUUUU	AC	CGUG	4530-4548
NV / L	UCUAUCUUUUUUU	AC	CGUG	4953-4971
L /- 5'	UCUAACUUUUUUU			11041-11053
3'-end	CAUAGUAUUUU			1-11
5'-end	TAAAAGAUAUG			11158-11168
Complementary	GUA 3'- CAUA UUUU 5'- GUAU AAAA AG_			

VIRULENCE MECHANISM OF VHSV

Virus virulence is defined by the capacity of the viral infection to cause tissue damage, ailment, clinical signs in infected host fish, and it correlates with viral replication ability [80]. The virulence of a VHSV is critically dependent on the viral strain, host fish species, fish size, and water temperature [80]. The clinical signs and gross pathology of two representative cultured fish that are highly susceptible to VHSV infection are presented as follows (Fig. 6).

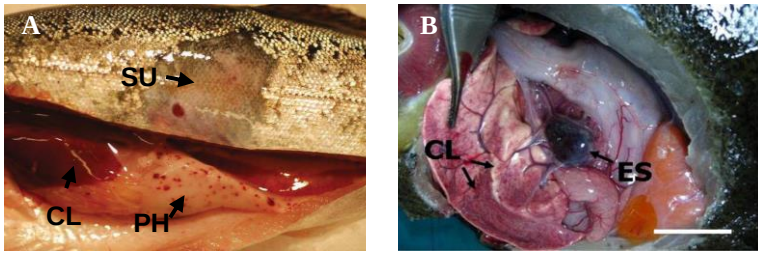

Figure 6. Gross pathology of viral hemorrhagic septicemia virus (VHSV)-infected fish. A) Infected rainbow trout from [81]: skin ulcer (SU), congested liver (CL), and petechial hemorrhage (PH). B) Infected olive flounder from [35]: congested liver (CL) and enlarged spleen (ES).

i. Rainbow trout swim in circles (or spiral) near the water surface. VHSV infection results in loss of scales, skin ulcers, pale gills, severe anemia, slight pericarditis, necrosis of the

cardiomyocytes in the heart ventricle, congested liver, enlarged dark spleen, muscle bleeding (petecchiae) and petecchiae over the pyloric caeca (Fig. 6A) [22,81].

ii. Olive flounder show dark body coloration, expanded abdomen due to ascites, pale gills, clear fluid in the peritoneal (or pericardial cavities), congested liver, enlarged spleen, swollen kidney, and multiple petecchiae (Fig. 6B) [34,35].

Sequence of viral infection and spread

In general virus infections of fish include a port of entry, often with some local replication, spread to primary replication sites/organs, dissemination and often secondary replication in target organs, and then shedding of the virus to the external environment and spread to new hosts (simplified in Fig. 7) [82].

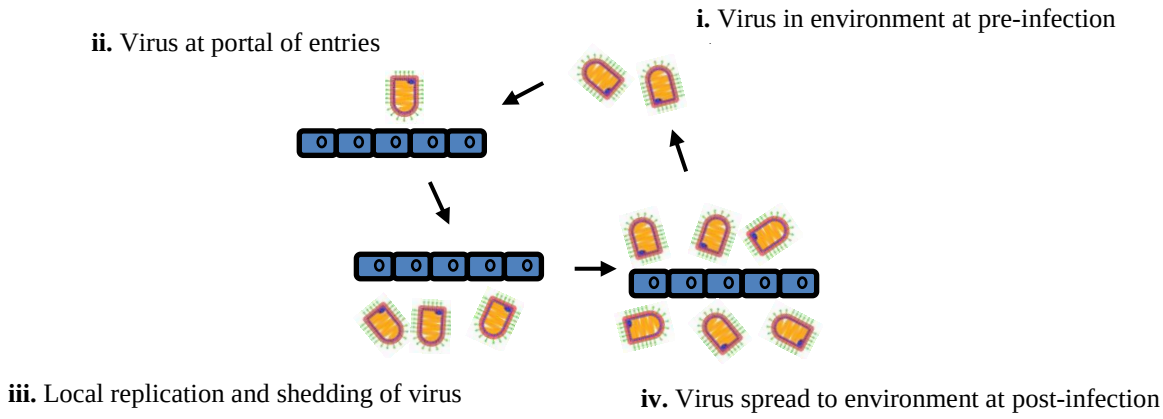

Figure 7. Infection and spread of virus.

i. Port of entry: VHSV enters the host circulation system via three main epithelial cell barriers of fish, namely the gill, skin, and gut [80]. In the gill, virulent strains (for rainbow trout) can translocate across gill epithelial cells (GECs) and also replicate at early post-infection time in the cells [25,80]. Fin cells and tissues can be entry sites for viral infection and formation of progeny [83–85]. Studies of VHSV and IHNV infections have shown that the fin bases are important ports of entry, likely more important than the gills in rainbow trout [86,87]. Vaccination studies show that virus can have an entry route through the gut epithelial layer with systemic host immune responses being induced by the oral delivery of attenuated VHSV strains [88,89].

ii. Local replication and systemic dissemination: At the fin base which is considered a key port of entry, major chemokine components of mucosal immunity and IFN-induced Mx transcripts are up-regulated at early time of infection [87]. The virus is actively replicating in dermis cells with translation being interfered by chemokine production in epidermal cells [87]. Most virus infections have a viral incubation period with no disease signs or symptoms during the dissemination period [82]. Following entry through the primary barriers, the virus particles spread through the host circulation system to hematopoietic organs (primary replication sites) such as the kidney and spleen [23,25,80].

iii. Shedding and transmission: Infected fish transmit VHSV horizontally to naïve fish by direct contact, spread of virus to the water or mucosal uptake from the water, but vertical transmission from brood-fish to egg has not yet been reported [36,90,91]. For interspecies transmission, there is a requirement that the virus conditions in different hosts provide a similar environment for a successful viral infection cycle to take place (entry, replication, translation, assembly, and release) [36]. Viral reproduction and release/shedding from the infected host are most likely essential factors determining transmission rate [36]. Under natural conditions, VHSV is more stable in freshwater than in seawater and prefers lower water temperatures (5-25 °C) in the aquatic environment [34,92].

Viral host specificity: species and size

i. Host species: VHSV has a broad host-range infecting approximately 80 fish species in fresh water and marine environments. However only a few VHSV strains cause disease in rainbow trout and there are no reports on clinical outbreaks in farmed Atlantic salmon under field conditions (Table 2) [17,18,21,24,25,28,29,36,37,68,81,91,93–95]. Marine VHSV genotype III strains have been found pathogenic in both turbot (*Scophthalmus maximus*) and rainbow trout, and marine VHSV genotype I strains cause severe economic losses in rainbow trout farming [17,21,22,27–29,36,81]. While VHSV has a broad host-range, cross-species infection mechanisms are still not well understood. By use of reverse genetics entire genes have been transferred from virulent VHSV strains to IHNV backbones and a recent study showed that G or NV proteins are less likely to be determinants of host-specific virulence for rainbow trout [68]. In avian influenza virus (*Orthomyxoviridae*), a single amino acid in polymerase (residue 627 in PB2) changes cross-species infectivity and the avian isolate H5N1 with the single amino acid mutation is able to invade extrapulmonary tissues [96–98]. In VHSV, the host-specific role of the RdRp complex has not yet been investigated in any detail.

ii. Host size/age: VHS in fry has a typical acute progression compared with that in adult fish [80]. For example, challenge of small-sized (14 g) and a medium-sized (124 g) groups of olive flounder resulted in close to 100 % cumulative percent mortality (CPM) but the small fish reached 100 % CPM 9 days earlier than the medium-sized fish, following challenge with wild-type VHSV, genotype IVa [34]. In larger fish (1059 g), CPM reached 60 % by 60 days post-infection [34]. Olive flounder of 9.8 cm in size had higher up-regulation of Mx immune gene expression and viral-specific antibody against VHSV antigen than smaller fish (less than 9.8 cm) where Mx up-regulation was low and specific antibodies were undetectable [99].

Table 2. Susceptibility of viral hemorrhagic septicemia viruses in cultured fish tested by artificial waterborne infection trials (based on genotype).

	Susceptibility	from different genotypes (region of origin)
Atlantic salmon [22,38]	Yes	IVa (Pacific Ocean)
	No	III (North East Norway)
Rainbow trout [22,29]	Yes	Ia (Freshwater) and III (North East Norway)
	No	I, II, and III (Baltic sea, North sea, English Channel, Kattegat, and Skagerrak)
Turbot [100]	Yes	Ib (Baltic sea) and III (North sea)
	No	Ia (Freshwater) and II (Baltic sea)
Olive Flounder [35,90]	Yes	IVa (Pacific Ocean)
Atlantic Halibut [37]	No	Ia (Freshwater), Ib, II (Baltic sea), III (North sea), and IV (US)
Atlantic Cod [37]	No	Ia (Freshwater), Ib, II (Baltic sea), III (North sea), and IV (US)

Viral RNA synthesis in host cell

Viral transcription or replication of NNS RNA virus involve the RdRp complex where the complex interacts with encapsulated N protein to form RNP [101]. In general, an RNA virus is rather "self-reliant", with a virally encoded preexisting "machinery" (RNP: N, P, and L) to support viral RNA-dependent RNA synthesis [101]. RNA virus take over the normal cellular RNA processing machineries for its own viral RNA synthesis [101]. The RdRp complex is essential for viral copying of RNA from RNA, and thus the L protein has a well-defined positively charged template tunnel, holding the RNA template to initiate RNA synthesis [48,59,102,103]. From VSV studies, a two-entry sites model was suggested with a standpoint that transcription is initiated at gene-start and replication likely occurs at end of 3'-UTR resulting in RNA synthesis [104–106]. The L-P complex (RdRp complex) is essential for

RNA synthesis but it is not sufficient for viral copying [59] and here the template-associated N protein is required [59]. The L protein is in charge of the recognition template or nucleotide polymerization, but P and N proteins are not essential for this to occur [59]. For example, influenza virus, which is a segmented negative strand RNA virus, has 3'- and 5'-ends conserved and complementary sequences forming a panhandle structure that guides the RdRp complex for RNA synthesis while no such mechanisms have been shown for NNS RNA virus species. For negative strand RNA viruses, the conserved terminal sequences are essential for control of transcription and replication, possibly functioning as a promoter for the RdRp complex [46]. In the genus *Novirhabdovirus*, VHSV also has the conserved end terminal UTR sequences at the 3'- and 5'-ends, which are complementary, also found in other NNS RNA viruses [45,46]. The promoter region might be acting as a catalytic core domain for RNA synthesis [48,107] but there are no previous studies showing the importance of the UTR-sequence for the initiation of transcription or/and replication in the genus *Novirhabdovirus*.

Temperature sensitivity

VHSV has a preference for lower temperatures and its virus replication and formation of progeny is very limited (or not occurring) at temperatures above 25 ºC [108–111]. For poikilothermic animals the immune responses are obviously influenced by the water temperature, and temperature directly affects the susceptibility of VHSV infection in several fish species [92,109,112,113]. For example, VHSV replicates well in olive flounder at temperatures below 21 °C, but it does not cause VHS at 21 °C or above [109].

The L protein is a multifunctional component in the RdRp complex [114]. Studies carried out in VSV (*Rhabdoviridae*) show that the L protein determines temperature sensitivity at high-temperature: (i) Insertion of a fluorescence gene into a hinge region (in front of CR VI) changes the virus to a temperature-sensitive variant at high temperature (37 °C) [55] and (ii) an L protein mutant ((G)114) triggers the temperature sensitivity at high temperature (39 °C) and interestingly the mutant ((G)114) affects mRNA transcription but not viral RNA replication [114].

The L protein of human parainfluenza virus type 3 (HPIV-3, *Paramyxoviridae*) is also functionally influenced by temperature, which has been used for the development of a candidate vaccine strain (cp45) [115]. Viral RNA accumulation of influenza virus was also significantly decreased as a consequence of dissociation of the L protein-RNA template

(promoter) at elevated temperatures (41 ºC) [116]. Furthermore, a non-structural NS1 gene in the influenza virus is required for optimal growth at high temperature (39 °C) [117,118].

There are few studies addressing the underlying mechanisms of temperature sensitivity in novirhabdoviruses. Using a reverse genetics approach, it was shown that the functional aspects of the G protein are not enough to determine temperature sensitivity, and the polymerase complex is likely involved in defining temperature optimum for replication [62,119]. The determinant for the temperature sensitivity of novirhabdoviruses, however, has not yet been documented. The difference of temperature sensitivity (or permissibility) among different VHSV strains is also not understood.

REVERSE GENETICS OF VHSV

Since the first recovery of an entire negative strand RNA virus from cDNA in 1994, the reverse genetics system has been found to be a powerful manipulation tool to study all aspects of viral RNA synthetic mechanisms, virus-host interaction, pathogenicity, etc. [44,120]. A principle of the reverse genetics method is that the genome-manipulated viral DNA sequences are inserted into heterologous recombinant plasmids that are subsequently transfected into cells [120–123]. In strike contrast to the positive strand RNA virus (mRNA sense (+)), the negative stand RNA virus (anti-mRNA sense (-)) is more difficult to manipulate by reverse genetics, for the following three reasons: (i) precise 3'- and 5'- end UTR sequences are required; (ii) RNA polymerase is essentially transcribing both viral RNA sense (-) and complementary mRNA sense (+) sequences; and (iii) RNPs, which preexist in virus particles under natural conditions, are required to drive the initial viral RNA synthesis as helpers (RNP: N, P, and L) [120,124].

For negative strand RNA viruses infecting fish, reverse genetics systems have been developed for VHSV, IHNV, SHRV, and spring viremia of carp virus (SVCV) with the purpose to develop live attenuated viral vaccines, to characterize unknown functions of viral proteins, and to better understand viral infection routes (or patterns) in host fish [62]. In contrast to positive strand RNA viruses, the genomes of negative strand RNA viruses alone are not infectious [62]. Negative strand RNA viruses need a minimal infection unit, such as RNP containing RNA polymerase, to transcribe and replicate the full-length RNA genome [62]. To fulfill the complete recovery of recombinant viruses, helper plasmids harboring RNPs (N, P,

and L) are co-transfected into permissive cells together with a complete genomic plasmid harboring the full-length viral genome [62]. The plasmids are under the controlled of T7 or cytomegalovirus (CMV) promoters, using a RNA polymerase II system driving the transcription of the viral genomes in the plasmids [62]. In this system, a self-cleaving hepatitis delta virus ribozyme (HdvRz) sequence is precisely fused to the 3'-end of anti-genome without any additional nucleotides inhibiting recombinant virus rescue [62]. By use of reverse genetics, the following viral gene functions of VHSV have been elucidated:

i. The NV protein is a determinant of pathogenicity [74].
ii. The NV protein suppresses host apoptosis [73].
iii. The NV protein suppresses type I IFN induced Mx gene expression [75].
iv. The NV protein suppresses early activation of TNF-α-mediated NFκB expression [76].
v. The structural protein G of VHSV can be exchanged with IHNV G [62].
vi. Both G and NV protein are unlikely to be host-specific virulence determinants [68].
vii. As a vector system, the non-coding regions in between N-P or P-M are optimal places for insertion of "foreign genes" for heterologous expression [125].
viii. As a vaccine delivery system, recombinant VHSV presents as the surface E glycoprotein from West Nile virus (*Flaviviridae*), which is a non-segmented positive strand RNA virus, and it provides immune protection [126].

OBJECTIVES

The aim of this study was to elucidate some of the virulence mechanisms employed by VHS virus and particularly, the role of the polymerase in virus virulence. The work was subdivided into the following subtasks:

i. Importance of the polymerase for infectivity:
   a. Study the *in vitro* virulence between different genotypes of VHSV using primary cultures of rainbow trout gill epithelial cells (GECs).
   b. Identify virulence residues or motifs through comparison of high and low-virulent strains.
   c. Evaluate the importance of individual residues for virulence by site-directed mutations using the backbone of a low-virulence marine VHSV strain (JF-09) using a gain-of-virulence approach.

ii. Importance of 3'-end UTR for transcription and replication and *in vivo* infection:
   a. Study the conserved sequences in 3'-end UTR as a potential promoter region.
   b. Study the functions of 3'-end UTR sequence as a primary, specific viral promoter code.
   c. Study the importance of the 3'-UTR region for virus virulence *in vitro* and *in vivo.*

iii. Importance of the L protein for temperature sensitivity of genotype IV strains:
   a. Study the difference of temperature sensitivity between different VHSV strains.
   b. Study the importance of the L protein for VHSV temperature sensitivity for genotype IV strains particularly.

SUMMARY OF PAPERS

Paper I

A single amino acid mutation (I1012F) of the RNA polymerase changes the *in vitro* virulence profile of marine viral hemorrhagic septicemia virus to rainbow trout gill epithelial cells

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Viral hemorrhagic septicemia virus (VHSV) is separated into four different genotypes (I, II, III, and IV) with different sub-lineages. European marine VHSV strains (of genotypes I, II, and III) are in general nonpathogenic or have very low pathogenicity to rainbow trout after waterborne challenge, and here we also show that genotype IVa is non-pathogenic to trout. Despite several attempts it has not been possible to link genomic variation to *in vivo* virulence. *In vitro* virulence to gill epithelial cells (GECs) has been used as a proxy for *in vivo* virulence and here we extend these studies further with the purpose to identify residues associated with *in vitro* virulence. Genotype Ia (DK-3592B) and genotype III (NO/650/07) isolates, pathogenic to rainbow trout, were compared to two marine strains nonpathogenic to trout, genotype Ib [29] and genotype IVa (JF-09). DK-3592 and NO/650/07 were pathogenic to gill epithelial cells (GECs), while marine strains 1p8 and JF-09 were non-pathogenic to GECs. Eight conserved amino acid substitutions contrasting high and low virulent strains were identified and reverse genetics was used for a gain-of-virulence approach based on the JF-09 backbone. Mutations were introduced in the G, NV and L genes and 7 different virus clones were obtained. For the first time we show that a single amino acid mutation of the L protein close to the RNA dependent RNA polymerase domain, I1012F, rendered the virus able to replicate and induce cytopathic effect in trout GECs. The other six mutated variants remained nonpathogenic.

Paper II

Specific nucleotides at the 3'-terminal promoter of viral hemorrhagic septicemia virus are important for virulence *in vitro* and *in vivo*

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Viral hemorrhagic septicemia virus (VHSV), a member of the *Novirhabdovirus* genus, contains an 11-nucleotide conserved sequence at the terminal 3'- and 5'-untranslation regions (UTRs) that are complementary. To study the importance of the 3'-UTR terminal sequence as a potential promoter for virus replication of novirhabdoviruses, we performed site-directed mutagenesis of selected residues at the 3'-terminus and generated mutant viruses using a reverse genetics approach. Assessment of growth kinetics and *in vitro* real-time cytopathogenicity studies showed that the two nucleotides order (A^4G^5) of the 3'-terminus of VHSV directly affects growth kinetics *in vitro* as the mutant A4G-G5A virus has reduced positive-strand RNA synthesis efficiency at 48 hrs post-transfection (51 % of wild-type; WT-VHSV) and 70 hrs delay in causing complete cytopathic effect in susceptible fish cells, as compared to the WT-VHSV. Furthermore, when the A4G-G5A virus was used to challenge zebrafish, it exhibited reduced pathogenicity by 54 % difference, as compared to the WT-VHSV. From these studies, we infer that specific residues in the 3'-UTR terminal sequence of VHSV are essential to modulate the virulence in cells and pathogenicity in fish.

Paper III

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

Sung-Hyun Kim[1], Shamila Yusuff [2], Vikram N. Vakharia[2], and Øystein Evensen[1]

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Viral hemorrhagic septicemia virus (VHSV) has four major genotypes (I-IV) with sub-lineages (Ia-e and IVa-c). First of all, we compared different temperature sensitivities among five VHSV strains (or genotypes) on growth *in vitro*. Using reverse genetics, we show L polymerase protein interchanged between two strains of VHSV genotype IV growing at different temperatures results in exchanged temperature sensitivities *in vitro*.

METHODOLOGY

OUTLINE OF THE STUDY

This thesis includes five different VHSV strains (DK-3592B - genotype Ia; 1p8 - genotype Ib; NO/650/07 - genotype III; JF-09 - genotype IVa; and MI03GL - genotype IVb) used in three different studies represented by Papers I, II, and III. The purpose was to elucidate parts of the virulence mechanisms of VHSV.

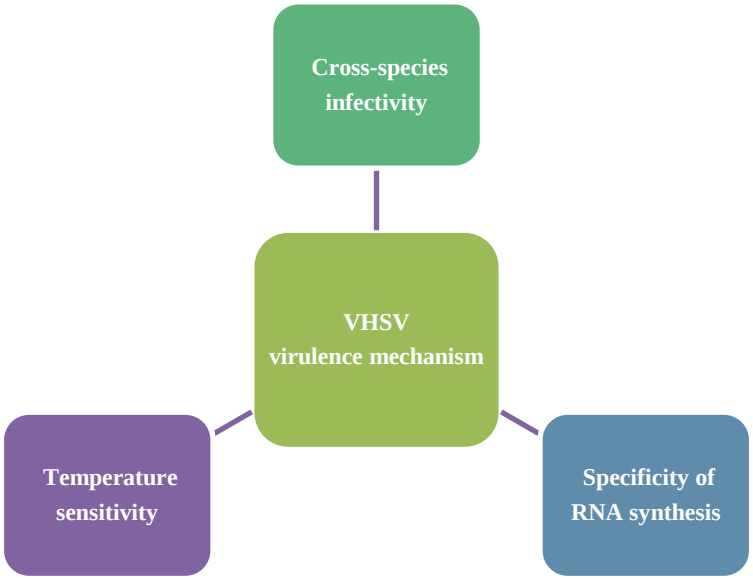


Figure 8. Schematic layout of the studies performed as part of this thesis..

Three knowledge gaps in VHS virus research were addressed in this study (Fig. 8):

i. VHSV is a wide-host-range virus (approximately 80 species) [24,25]; therefore, the first question is "What determines fish species infectivity or cross-species infectivity?" (Paper I).

ii. Negative strand RNA virus has a unique transcription and replication cycle; the RdRp complex is a crucial factor combining with viral promoter sequence in the 3'-end UTR and 3'- and 5'-end UTR sequences are conserved among VHSV strains and are complementary. Hence, the second question is "What is the function of the UTR end sequences in the transcription and replication of the virus ?" (Paper II).

iii. VHSV growth is restricted at high temperatures; thus, the third question based on other previous NNS RNA virus studies is "Is the L protein important for temperature sensitivity?" (Paper III).

Based on these three questions, the differences in viral virulence and the similarities/differences in genomic sequences between different VHSV strains were studied in this thesis. To answer these questions, we first developed a reverse genetics system (rJF-09) as an over-arching method.

To find sequence differences between the VHSV strains, the entire genome of four VHSV strains (DK-3592B, 1p8, NO/650/07, and JF-09) was sequenced in Paper I. We were interested in understanding what rendered virus strains virulent to rainbow trout under experimental conditions. Genome sequencing was carried out with the purpose to identify host-specific virulence residues or motifs (amino acids) for two pathogenic rainbow trout VHSV strains (DK-3592B and NO/650/07). The sequence obtained for these strains will not provide this information per se, but the sequences obtained were compared to VHSV strains (1p8 and JF-09) with low- or non-pathogenic profiles for rainbow trout. The biological characteristics of the 4 strains were studied with regard to virulence profiles using *in vitro* virulence tests whereby the strains were used to infect primary cultures of rainbow trout (RBT) gill epithelial cells (GECs). Previous studies have shown that RBT GECs serve as an excellent proxy of *in vivo* virulence [25,127]. The GEC systems separated the strains into high-virulence and low-virulence strains, with DK-3592B and NO/650/07 being highly virulent and 1p8 and JF-09 having an avirulent profile. These results were also confirmed by *in vivo* challenge in rainbow trout. Aligning the sequences for the 4 isolates, we were able to pin-point hypothetical virulence residues. By reverse genetics using the JF-09 backbone, these positions were mutated in one-by-one and the recombinant JF-09 variants were then used to infect RBT GEC *in vitro* (in Paper I).

In Paper II, focus was on the 3'- and 5'-end UTR regions. The virus genome sequences were aligned and the similarity (or differences) between VHSV strains (DK-3592B, 1p8, NO/650/07, and JF-09) was assessed. The 3'- and 5'-end UTRs were conserved across strains and the 3´ and the 5´-region was found to be complementary. We then studied the importance of specific positions in the 3'-end for viral RNA synthesis for *in vitro* growth characteristics, using a strand-specific quantitative RT-PCR, western blotting, and viral growth kinetics. Various mutations were introduced in the 3´-region and the different mutants were

successively studied with regard to *in vitro* growth characteristics and an *in vivo* pathogenicity using a zebrafish (*Danio rerio*) infection model [128–131].

Temperature sensitivities for the different VHSV strains were studied in Paper III. To explore the importance of the L protein for *in vitro* temperature sensitivity, the L protein encoding regions were interchanged between VHSV genotype IVa and IVb by gene swapping. The growth characteristics at different temperatures were assessed, an approach also used for other NNS RNA viruses [55,114,115,132–135]. This study focused on the importance of the L protein (RNA polymerase) for *in vitro* growth at elevated temperatures.

VIRUSES AND CELLS

The DK-3592B, 1p8, NO/650/07, JF-09, and MI03GL strains were used in this thesis and propagated in *Epithelioma papulosum cyprini* (EPC) cells in L15 cell medium (Invitrogen) containing 10 % fetal bovine serum (FBS; Sigma Aldrich) at 15 °C [25]. The DK-3592B and 1p8 strains were a kind gift of Dr. Niels Jørgen Olesen, Danish Veterinary Institute, Århus, Denmark, and the NO/650/07 strain was kindly provided by Dr. Renate Johansen, Norwegian Veterinary Institute, Oslo, Norway. The JF-09 strain originated from VHSV-infected olive flounder in Jeju, South Korea (2009). The MI03GL strain was isolated in Great Lake, USA (2003) [45]. Viral titration was performed on the EPC cells using the 50% tissue culture infective dose ($TCID_{50}$) method as previously described [136].

In order to use the *in vitro* GEC assay as a proxy of *in vivo* infectivity, primary cultured RBT GECs were isolated from fry of rainbow trout (Fig. 9), using to the following procedure [25,80]:

i. Gills of rainbow trout were dissected and disinfected with antibiotics (400 µg/ml of gentamicin and 200 µg/ml of penicillin-streptomycin B; Invitrogen) and antifungal medicine (250 µg/ml of fungizon; Gibco).
ii. Blood clots in the gill filaments were removed and trypsinized (in vortex for 15 min twice).
iii. Trypsin activation was stopped by the addition of 10 % FBS in phosphate-buffered saline (PBS).
iv. The cells were collected by centrifugation (400 × g for 10 min).

v. The collected cells were seeded to a cell culture flask with L15 (Invitrogen) containing 10 % FBS and 200 µg/ml gentamicin.

vi. The cells were incubated at 20 °C for 24 h to allow for attachment to the bottom of the flask.

vii. Debris and blood cells in the cell culture medium were removed by washing with PBS.

viii. The cell culture medium was exchanged everyday until infection.

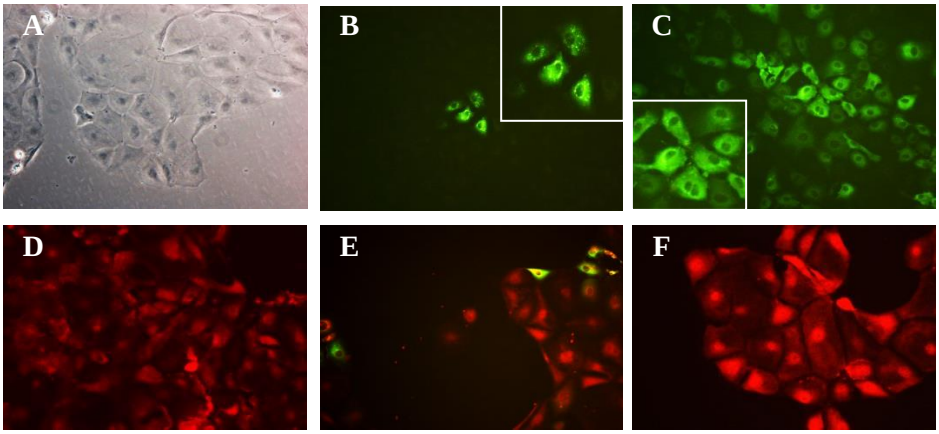
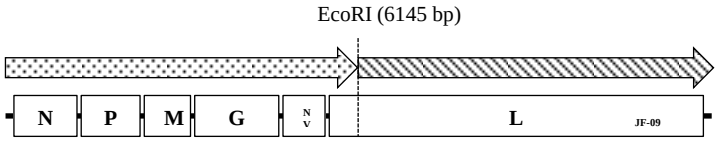


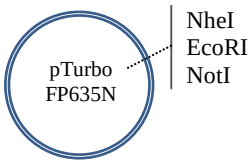
Figure 9. Primary cultured rainbow trout gill epithelial cells. A) Cells are shown by phase contrast imaging. B) Cells infected by a low-virulence VHSV strain (JF-09). C) Cells infected by a high-virulence VHSV strain (DK-3592B). D) Viral-induced Mx protein in cells infected with DK-3592B. E) Combination of viral infection and Mx protein expression in cells by infected with DK-3592B. F) Mx protein expression in the cells by application of salmonid recombinant IFN-alpha from [137]. Viral N protein expression by using MAb IP5B11 as a primary antibody (green fluorescence) and Mx protein expression by using anti-rainbow trout Mx as a primary antibody (red fluorescence) are shown by indirect fluorescent antibody testing.

JF-09 PLASMID AND RECOMBINANT JF-09

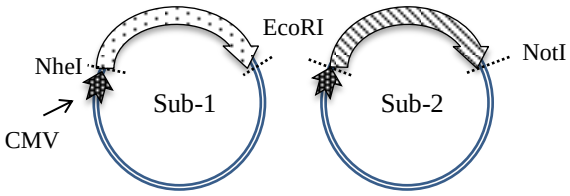
(i) First-strand cDNA is amplified by RT-PCR to produce 6.1 and 5 kb DNA products covering the whole genome.



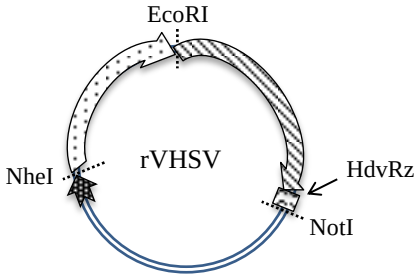
(ii) Parental plasmid, pTurboFP635N, is selected.



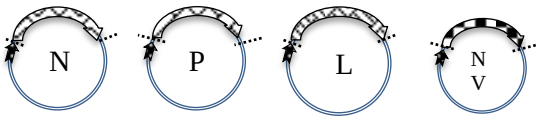
(iii) PCR products (6.1 and 5 kb) are inserted into sub-plasmids.



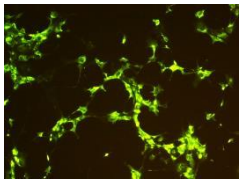
(iv) Sub-plasmids are combined at the *EcoRI* site following enzyme digestion.



(v) Helper plasmids encoding the RNP complex (N, P, and L) and supplementary NV gene are constructed.



(vi) Plasmids are co-transfected into permissive EPC cells.



(vii) Recombinant VHSV (rJF-09) is rescued from the cell culture supernatant and growth of virus was documented by IFAT (as shown).

Figure 10. Schematic layout of the reverse genetics system for production of recombinant VHSV (rJF-09).

In this study, we first developed a recombinant VHSV system (JF-09, genotype IVa) using reverse genetics, based on the following process (Fig. 10):

i. The entire genomic sequencing of JF-09, including 3'- and 5'-end sequences, was completed. On the basis of a two subclones strategy, two viral genomic DNAs were produced by RT-PCR, following ultracentrifugation, to obtain a high-yield viral RNA concentration. The genomic DNAs were long RT-PCR products (6.1 and 5 kb) containing a *NheI* restriction site in front of gene-start sequence, a *NotI* restriction site at the 3'-end tail of the anti-genome sequence, and a common overlapping *EcoRI* restriction site. Precise 3'- and 5'-end sequences are important for the negative strand RNA virus reverse genetics system.
ii. A commercial plasmid (pTurboFP635N; Everogen) containing a standard CMV early promoter site and restrition sites (*NheI, EcoRI,* and *NotI*) was selected for insertion of the viral genome.
iii. The two RT-PCR products (6.1 and 5 kb) were inserted into pTurboFP635N following enzyme digestions at restrition sites the *NheI, EcoRI,* and *NotI* sites, respectively. The common restriction site *EcoRI* was used for both subclones, but only one of the two end restriction sites (*NheI* or *NotI*) was used for either individual subclone: (i) *NheI-EcoRI* and (ii) *EcoRI-NotI*. The CMV promoter sequence was positioned exactly in front of the 5'-end of the anti-genome start sequence.
iv. A full viral genomic plasmid (rJF-09) was constructed following combination of the two subclones by ligation at the *EcoRI* restriction site with the two end sites (*NheI* and *NotI*). The HdvRz sequence was added at the 3'-end of the anti-genome sequence with the purpose to obtain precise cutting, followed by the SV40 early mRNA polyadenylation signal.
v. Helper plasmids, based on pTurboFP635N for RNP (N, P, and L) to initiate RNA synthesis and a supplemental plasmid (NV) to increate viral copy numbers with suppression of the cellular innate immunity, were constructed following the gene RT-PCR amplifications. A Kozak consensus sequence was created in front of the start codon of each ORF.
vi. The full genomic JF-09 plasmid and helper plasmids (N, P, L, and NV) were co-transfected into permissive EPC cells by using chemical-based transfection reagents [74]; $pVHSV_{JF-09}$ (120 ng), pN (70 ng), pP (30 ng), pNV (15 ng), and pL (30 ng) were mixed in 25 µl of Opti-MEM® medium (Invitrogen) and then 1 µl of X-tremeGENE 9 DNA

transfection reagent (Roche) was added. The transfected cells were incubated at 28 °C for 5-8 h for optimal activation of the CMV early promoter, based on the RNA polymerase II system driving the VHSV genes in the plasmids, and the incubation temperature was then reduced to 15 °C for optimal viral RNA synthesis.

vii. Following verification of the virus-induced cytopathic effect (CPE), the recombinant JF-09 strain was harvested from the cell culture medium.

The growth kinetics of the recombinant JF-09 in permissive EPC cells was compared with that of wild-type virus (JF-09 strain, genotype IVa). The full genome sequence of the recombinant JF-09 virus was identical with that of the wild type, except for an amino acid at position 91 in the NV protein (serine (S) to asparagine (N), both polar amino acids).

SEQUENCE COMPARISON

With the aim to analyze genomic differences and similarities, all ORF sequences of the four VHSV strains (DK-3952B, 1p8, NO/650/07, and JF-09) were obtained by RT-PCR, covering the whole coding region. 3'- and 5'-end UTR sequences for the JF-09 strain were obtained by using a SMARTer RACE cDNA Amplification kit (Clontech). The 3'- and 5'-RACEs and RT-PCR products were sequenced by GATC Biotech, Germany. The assembly of contiguous sequences, multiple sequence alignments, and comparative sequence analyses were conducted by using the Vector NTI Advance 11 software (Invitrogen) and the Basic Local Alignment Search Tool (BLAST) in the National Center for Biotechnology Information (NCBI) database. To identify candidate virulence motifs in Paper I where identified by these alignments.

In Vitro PROFILING OF VIRUS ISOLATES

For the *in vitro* growth kinetics of the characterization of VHSV strains, progeny was detected intracellularly by immunofluorescence staining, by quantitative real-time RT-PCR targeting a conserved N protein-coding gene, and viral titration using permissive EPC cells. Cell viability after infection was measured using a CellTiter AQ_{ueous} One Solution reagent (Promega) in 96-well plates [25,80] and the XCelligence system from Roche, giving a real-time-based cell viability assay (Paper I).

With the purpose to mimic in vivo translocation across primary barriers, translocation of VHSV was study in ex vivo systems of gill epithelial cells (GEC) of rainbow trout where GECs were grown on inserted filters (Falcon, 0.4 μm-pore sizes). The establishment of a tigh barrier was measured using standard resistance measurement ($\geq$ 1.0 KΩ×cm^2) (Paper I). Virus particles translocating across the epithelial barrier were collected from the basal medium and inoculated onto EPC cells. Virus growth was detected by indirect fluorescence antibody test (IFAT) [25,80].

A strand-specific quantitative RT PCR assay was established in which the forward primer amplifies negative strand RNA and the reverse primer favors mRNA synthesis. At early time post transfection/infection, this will exceed cRNA levels. We used this setup to detect viral RNA synthesis (positive- or negative strand RNA), in Paper II (Fig. 11) [138,139].

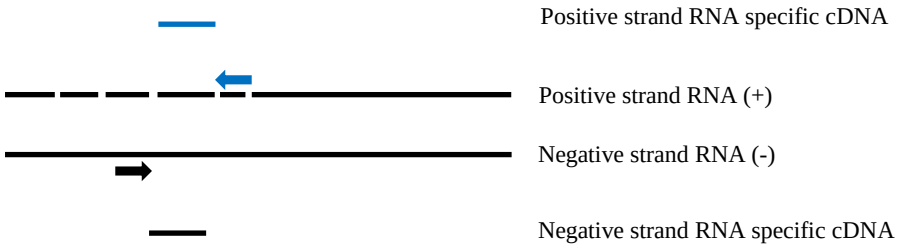


Figure 11. Schematic design of the strand-specific RT-PCR for making strand-specific cDNA. The black arrow is forward primer (+) to detect negative strand RNA (-) and the blue arrow is reverse primer (-) to detect plus strand RNA (+). Strand-specific first strand cDNAs were detected by quantitative real time RT-PCR to measure the strand-specific RNA amounts. Figure prepared by S.-H. Kim.

Western blotting was used to determine production of viral proteins for recombinant VHSV strains using a polyclonal anti-VHSV antibody [73] (Paper II).

MUTAGENESIS OF RECOMBINANT VIRUSES

Three different procedures were used for the manipulation of a parental plasmid (recombinant JF-09), as follows:

i. Site-directed mutagenesis (GENEART® Site-Directed Mutagenesis System; Invitrogen) using gene-specific primers was used for site-directed, single nucleotide mutations in the complete genomic parental plasmid (recombinant JF-09, Papers I and II). The site-directed mutagenesis reaction, with two overlapping primers, methylated the parental plasmid DNA and also amplified the plasmid, including the target nucleotide mutations.

ii. PCR-directed mutagenesis was based on target nucleotide mutation in the gene-specific primers (forward or reverse primer), using a restriction enzyme site. This introduced gene deletions or single nucleotide mutations on a complete genomic plasmid (recombinant JF-09, Paper II).

iii. Restriction site cutting at two identical restriction enzyme sites in the two parental recombinant plasmid backbones (recombinant JF-09 and recombinant MI03GL) was applied (in Paper III) with the purpose to swap the ORF genes between the two VHSV strains.

Following mutagenesis, the reconstructed recombinant VHSVs were rescued from EPC cells transfected with VHSV plasmids and helper plasmids (N, P, L, and NV) by using chemical-based transfection reagents, as previously described [74].

In Vivo ZEBRAFISH INFECTION MODEL

To correlate *in vitro* results with *in vivo* virulence, a zebrafish infection model was used for testing the attenuated virus (A4G-G5A, in Paper II). Wild-type zebrafish (sex ratio, 50:50) were gradually transferred from 28 °C water temperature to 16.5 °C in a closed aquarium (6 L), and fed every day. The water was aerated. The zebrafish infection facility is kept in a closed room with no outlet water being released without documented disinfection and complete killing of the virus. The water temperature is 16.5 ± 1 °C during challenge with VHSV (Fig. 12). Fish were kept at 16.5 ± 1 °C for a minimum of 2 days prior to challenge. One third of the water volume in the tanks was exchanged daily and the water quality was monitored every day for NO2-, NO3-, NH3, pH, KH, and GH. The light regime was 12:12 hours light/darkness. The zebrafish were injected intraperitoneally or intramuscularly with 2 µl of virus solution, in three parallel tanks, using insulin needles fitted with 2.5 µl pipette tips. Non-infected control groups were anesthetized and injected with 2 µl of L15 medium. After

virus challenge, the mortality and health condition of the zebrafish were recorded daily. Humane endpoints were employed and moribund fish were collected, anesthetized and decapitated to reduce further suffering. Fish were counted daily for mortality. Dead fish and euthanized fish were summarized for estimation of cumulative percent mortality (CPM), used to determine virus pathogenicity.

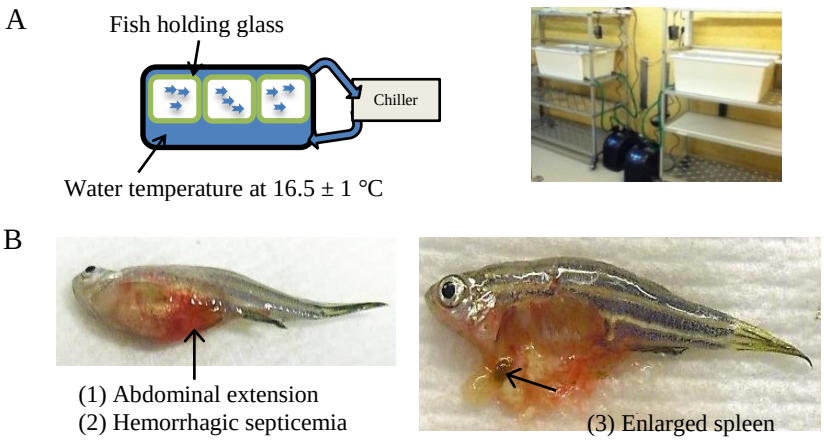


Figure 12. A) Viral hemorrhagic septicemia virus (VHSV) infection at 16.5 ± 1 °C. B) Three typical gross signs of VHS in zebrafish. Figure and photos by S.-H. Kim.

RESULTS AND GENERAL DISCUSSION

HOST-SPECIFIC VIRULENCE IN RAINBOW TROUT

The *in vitro* primary cultured RBT GEC system was previously used our laboratories (at NMBU) as a proxy to predict *in vivo* virulence in rainbow trout [25]. The experiments reported herein have further substantiated previous findings and show that virulence in rainbow trout of VHS virus strains from all genotypes can be assessed by their ability to infect and cause CPE in GECs. Further, translocation across GEC is similarly a proxy of virulence, as has also been shown before [25]. Such a system has several advantages over *in vivo* testing: (i) it is faster, simpler and much cheaper; (ii) it will allow for screening of a large number of virus variants within a short time; (iii) it is a good replacement for *in vivo* testing and therefore contributes to reducing the number of animal used for experimental purposes. Further it can be used for functional testing of neutralizing antibodies and even anti-viral therapies, an approach explored to a very little extent in aquaculture.

The main purpose of Paper I was to identify amino acid positions correlating with virus virulence and GEC was used to predict virulence changes, *i.e.* using a gain-of-virulence approach. Hypothetical virulence positions were identified by comparing the genome sequence of VHS virus strains defined by their high or low virulence to rainbow trout. Further, strains of high and low virulence profiles *in vitro* were or had been tested by *in vivo* challenge in rainbow trout. Single amino acid residues were mutated, one-by-one, using a low-virulent backbone (JF-09 strain, genotype IVa) and adding amino acids from a high-virulent profile or imprint, using a reverse genetics system. By using the GEC model it was possible to rule out most of the identified positions that differed between the high and low virulence strains as a marker of virulence. Of the 8 different positions tested, a single amino acid mutation, I1012F, of the L protein, which is a multifunctional component of the catalytic RdRp complex, resulted in gain-of-virulence. This amino acid is located in the conserved region (CR) IV and this mutant virus increased its replication rate close to 5.6-fold compared with wild type JF-09. It also induced cytopathic effects in GEC monolayers, whereas the remaining six recombinant JF-09 variants did not change the pathogenicity profile *in vitro* relative to the wild-type strain (JF-09). The mutant, I1012F, was compared to a high-virulent strain (DK-3592B), which showed 50-fold higher replication level than wild type JF-09, *i.e.* 9-fold higher than the mutated variant. From this we concluded that the L protein plays a role in infectivity of GECs

and the I1012F variant has an intermediate virulence profile *in vitro*. The underlying mechanisms are not understood but the L protein in NNS RNA viruses has six conserved regions (CRs I-VI) [52]. Following alignment with other NNS viruses, the 1012 position is located at the C-terminus of CR IV (Table 3).

Table 3. Alignment of the 1012 point (VHSV) in CR IV in the L with NNS RNA viruses.

VHSV (JF-09)	1009	AYQ**I**RNITDQKESEMFVTKFATAKTM…KRSQAKEIYTIWRT	1067
VHSV (1p8)	1009	AYQ**I**RNITDQKESEMFVTKFATAKTM…KRSQAKEIYTIWRT	1067
VHSV (DK-3592B)	1009	AYQ**F**RNITDQKESEMFVTKFATAKTM…KRSQAKEIYTIWRT	1067
VHSV (NO/650/07)	1009	AYQ**F**RNITDQKESEMFVTKFATAKTM…KRSQAKEIYTIWRT	1067
IHNV (Q82685)	1011	NYM**I**RSITDEKESEMFVTKFASARTM…MKSQQKETYTIWRT	1069
SHRV (Q9QJT4)	1008	AHL**I**RDITDEKESEMFVTKFATARTM…KKSRAKETYTIWRS	1066
TPMV (Q9JFN3)	1130	ASE**I**VDNSITGARESLAGLLDTTKGL…YDYNQFRQFNRLMQ	1190
SVCV (Q91DS4)	1003	LSE**F**RSSTFVGVTDSLIGLFQNSKTI…SELSSLEHLGSYRK	1061
ISFV (Q5K2K3)	1004	LSE**F**KSGTFMGVASSVVSLFQNSRTI…SEVNSLEHLCKYKG	1062
CHAV (P13179)	1004	LSE**F**KAGTFMGVASSIVSLFQNSRTI…SELTSLEHLSNYTD	1062
BDV (Q8JMN0)	925	MSA**L**FALSNVAYGLSIIDLFQKSSTV…VALESVRYKESIIQ	976
RABV (Q8B6J5)	1028	LSE**L**FSSSFLGIPESIIGLIQNSRTI…SEIHGINRMTQTPQ	1086
SENDZ(P06447)	1060	AHE**I**LGNSLTGVREAIAGMLDTTKSL…YDLLQYETLTRTLR	1120

*SHRV=Snakehead rhabdovirus, TPMV=Tupaia paramyxovirus, SVCV=Spring viraemia of carp virus, ISFV=Isfahan virus, CHAV=Chandipura virus, BDV=Borna disease virus, RABV= Rabies virus, and SENDZ= Sendai virus.

The function of CR IV still remains unclear [50,140,141]. Studies in Sendai virus and parainfluenza-3 virus have shown that single point mutations in this region rendered the virus temperature sensitive [132,133]. Further, a single point mutation at the 1321 position of the L protein of human respiratory syncytial virus (hRSV) also rendered the virus avirulent [134] and deletion of codon 1313 changed hRSV to an attenuated and temperature-sensitive vaccine candidate [135]. The CR-IV region of the polymerase likely plays a role in protein structure and possibly interacts with the incoming RNA strand (template strand) but very little is actually understood as regards the underlying mechanisms. Our studies on the impact of the mutation (of the L) on transcription or replication are not studied in detail for VHSV apart from the observed increased replication level. A single mutation in CR IV (L992F) in Sendai virus resulted in improved replication [132]. Further we have not examined combinations of mutations, *i.e.* position 1012, combined with any of the other residues found different between high and low virulent strains. Like for IPN virus [142], it is not unlikely that combined mutations play a role. Thus, there are numerous additional studies to be carried out

to better understand the mechanisms involved. Concluding, this is the first study to clearly demonstrate *in vitro* that the virulence of VHSV can be linked to a specific amino acid residue (I1012F) in CR IV of the L protein. Yet another implication of these findings is that the L protein plays a role in defining the host specific virulence of VHSV strains. This remains a speculation but it certainly warrants further studies along these lines. Particularly since some VHS virus variants (genotypes) infect a multitude of species while others have a relatively narrow species range.

While the involvement of the polymerase in VHS virus virulence has been studied to a very little extent, several studies have addressed the possible involvement of the G protein in this regard. This is also the reason we included a mutation in the G-protein but could not find this had any impact on virus virulence. Our findings concur with what has been reported recently in which IHN virus harboring the G or NV proteins from high- or low-virulent VHSV strains without seeing any difference between the two profiles when tested in rainbow trout using *in vivo* challenge methods [68].

SPECIFICITY OF VIRAL RNA SYNTHESIS

Yet another aspect of VHS virus replication and virulence that has not been studied in any detail is the untranslated 3' and 5' region of the virus genome. It is well known that these regions play an important role for establishing a successful reverse genetics systems but the importance of these regions in virus virulence has remained largely unexplored. Working with reverse genetics and aligning virus sequences we noted the similarity of the conserved 3'- (3'-CAUAGUAUUUU) and 5'- (UAAAAGAUAUG-5') end sequences in terms of complementarity. There are previous studies addressing this in other NNS RNA viruses, like vesicular stomatitis virus (VSV; prototype rhabdovirus) where it was suggested that the 3´ region plays an important role for initiation of transcription and replication [143]. Further the indications are that there are there is one entry sequence for transcription (at the start point of the N protein) and another for initiation of replication, at the start point of 3' UTR [104–106]. There are no previous studies on the function of the UTR related to transcription or replication of novirhabdoviruses and this was a good motivation for initiating these studies. Studies on VSV virus gave some guidance and our first approach was to design a PCR method whereby we could differentiate between transcription bias versus replication bias

during the virus replication cycle. Our approach was simple, we designed a strand-specific quantitative RT-PCR in which forward primers are used to transcribe cDNA from negative strand RNA (for determining the replication rate) and reverse primers are used to transcribe cDNA from positive strand RNA, mRNA and cRNA [138,139]. Our challenge was that the single-sided quantitative RT-PCR (ssqRT-PCR) would not differentiate between mRNA and cRNA, and since it is anticipated that the CMV promoter will produce cRNA from the anti-genome (of the plasmid), which might account for a portion of the measured RNA(+) measured by ssqRT-PCR. To overcome this we performed a transfection where the pL plasmid was omitted (as a control) and we were then able to measure the relative contribution from the CMV-driven part. This was shown to be very low, less than 1%, of the total cRNA. Thus, we were now able to differentiate between transcription and replication during early and late infection stages.

Next, we spent a lot of time deciding which nucleotide positions we should mutate. Through various approaches we ended up with modeling the 2-D image (tentative) of the complementary 3´and 5´ends, giving us a panhandle-like structure (see Fig. 13).

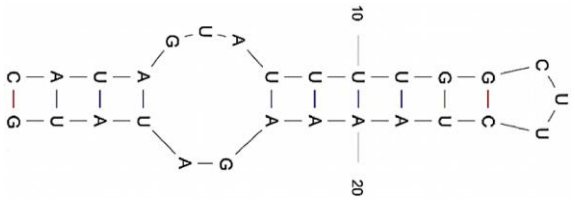


Figure 13. Tentative panhandle-like structure with an open inner ring structure. The "upper" sequence corresponds to the 3´end while the lower is the 5´.

Despite a panhandle structure has never been documented for the genus *Novirhabdovirus* (or NNS RNA viruses) we decided to target what appeared as "opening" and "closing" nucleotides of the inner ring structure. Positions 4 (A) and 5 (G) (counting from the 3´ end) were permutated and position 7 (A) was exchanged with a C and position 8 (U) with an A. We also included a single mutation in position 8, U8C. These mutations were vigorously tested with regard to transcription and replication capacity, and for *in vitro* and *in vivo* virulence. What we found from these studies was that an A4G-G5A mutation resulted in lower transcription efficiency and corresponding lower protein levels were found in transfected or infected cells. ssqRT-PCR studies showed that A4G-G5A mutant had a

significant decrease in total positive-strand synthesis of 51% at 2 days after transfection when compared to a non-mutated control, a recombinant wild type virus. There was however no difference indicating lower level of virus negative-strand synthesis. By western blot viral proteins G and NV proteins were low at 2 days post transfection (Fig. 14), distinct at 5 days post transfection for the wild-type and mutants U8C and A7C-U8A, while for A4G-G5A G protein was hardly seen. For rescued virus the same pattern was obtained (Fig. 15).

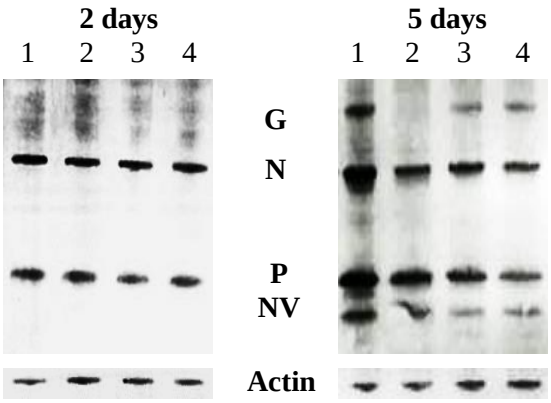


Figure 14. Western blot of transfected cells at 2 and 5 days post transfection. Lane 1:wt, lane 2:A4G-G5A, lane 3:A7C-U8A, and lane 4:U8C. Note lane 2 at 5 day, the G protein of A4G-G5A is hardly discernible.

When infecting permissive cell lines with rescued virus, A4G-G5A showed delayed expression of N and P protein compared to WT-JF-09 (Fig. 15).

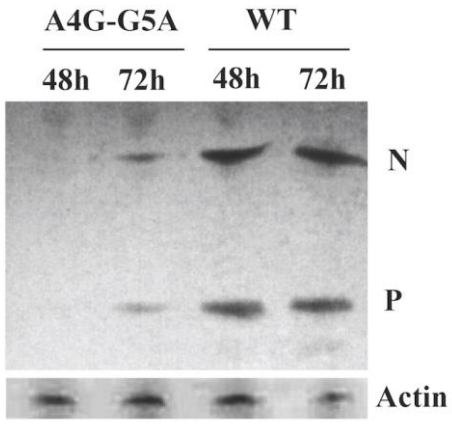


Figure 15. Western blot of permissive cells with rescued virus showing weak N and P proteins expression at 72h post infection for A4G-G5A versus WT (wild-type).

Comparing viral titers, the A4G-G5A mutant took an additional 24 hours for this variant to reach a viral titer comparable to WT-VHSV.

In vivo studies in zebrafish, the A4G-G5A variant exhibited reduced pathogenicity with accumulated mortality at half (50 % approximately) compared to wild-type virus. In conclusion, we showed that a single point mutation in the 3'-end promoter sequence reduced viral RNA synthesis, reduced the growth of the virus *in vitro* with slower formation of viral progeny, having an impact on viral virulence *in vitro* and *in vivo*. Collectively, these studies show that the primary sequence is important for production of viral virulence. It is possibly that the mutations affect recognition by the polymerase complex of the viral RNA and thus delay synthesis of mRNA, production of viral proteins and ultimately genome replication.

Additional studies should be pursued to address various combinations of mutations in the 3´ region, and also possible interactions with the N protein during initiation of transcription. These phenomena are largely unexplored in novirhabdoviruses. Furthermore, the A4G-G5A can possibly be useful for vaccination in aquaculture operation using the strain as a live, attenuated vaccine.

TEMPERATURE SENSITIVITY

The water temperature significantly affects VHS virus virulence in fish (in all species affected by the virus) and is possibly linked to virus replication being less efficient at temperatures away from optimum, that being an increase or decrease of temperature, plus for poikilothermic hosts immune response have a temperature optimum [108,110,111,144]. In general VHS virus infections are more frequently seen at lower water temperatures but different VHS virus strains have different optima. To assess the function of the L protein under different temperature conditions (Paper III), we first evaluated the sensitivity to increased temperatures for strains DK-3592B, 1p8, NO/650/07, MI03GL, and JF-09 (Fig. 16) grown in EPC cells.

In a previous study, the structural G protein of novirhabdoviruses, which is a determinant for viral neutralization and attachment to cell membranes, was found not to be the only protein involved in defining temperature sensitivity, and the suggestion was that the polymerase complex plays an even more important role [62,119]. Previous studies of other NNS RNA virus species also indicated that the polymerase (L) protein is important for *in vitro* temperature sensitivity [55,114,115,132–135].

The results in Paper III showed that the JF-09 strain (genotype IVa) was replicating at 23 °C, whereas other strains tested, DK3592B, 1p8, NO/650/07 and M103GL, did not. With the purpose to examine the role of the L protein, we exchanged the L-gene between genotype IVa and IVb (JF-09: IVa; [145] and MI03GL: IVb [45]) keeping the rest of the genome unchanged. The purpose being to study gain- and/or loss-of-function related to temperature sensitivity. The results showed that the temperature sensitivities of recombinant VHSVs strains harboring heterologous L proteins were different from the parental strains at high temperatures (21.5 ºC and 23 ºC), grown in EPC cells. However there was no significant difference at a low temperature (15 ºC). These findings indicate that the VHSV L protein plays a role in determining the temperature optimum and changes render the virus strains sensitivity to high temperatures, here 21.5 ºC and 23 ºC. These findings concur what has been found in other NNS RNA viruses [55,114,115,132–135].

This is the first report on novirhabdoviruses showing that differences in temperature-sensitivity among VHSV strains is linked to the L protein, and that both gain- or loss-of-function can be observed within genotype IV.

At present climate change is likely to impact modern aquaculture in many ways and geographic distribution of viruses (strains or species) will likely be influenced by environmental alterations. Here we showed that an Asian VHSV strain (JF-09, genotype IVa) has a different property than other VHSV strains under increased temperature (23 ºC). Furthermore, the JF-09 strain also showed improved *in vitro* growth at higher temperature in a non-permissive cell line (GEC). These findings warrant further studies including studies of gene switch between different genotypes than those tested here.

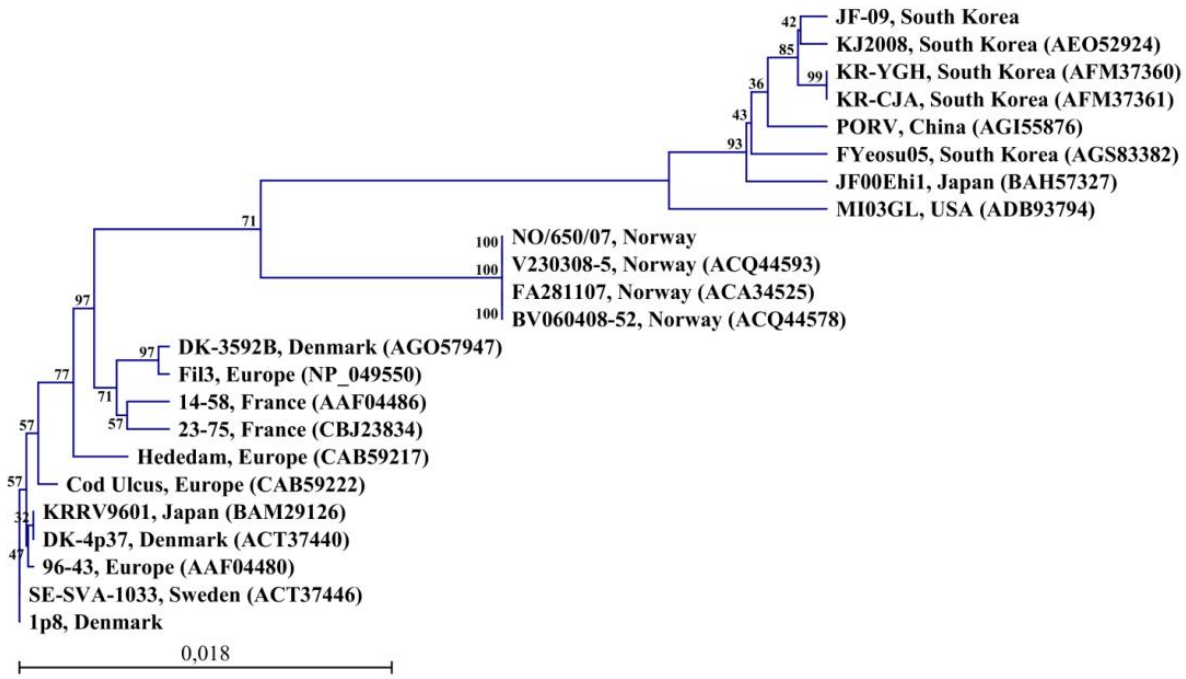


Figure 16. The phylogenetic tree based on the RNA polymerase (L) protein, showing the genetic distance among viral hemorrhagic septicemia viruses. Generated by using the CLC Main Workbench 6, set at root 1p8.

CONCLUSIONS

VHS in rainbow trout was last diagnosed in Norway in 2007 and since then there are no reports of VHS in Norway. Screening programs aimed to define the prevalence of VHSV infection in marine (wild) fish in Norwegian coastal areas were last performed between 2009 and 2011 and showed that VHS virus is present in wild fish populations [146] and may serve as a source of transfer to farmed fish species. Further, VHS is an important disease in rainbow trout aquaculture in continental Europe and is an important disease in farmed marine fish species in many Asian countries, including South Korea. Further to this, disease outbreaks in wild fish populations in the Great Lakes in North America have resulted in huge die-offs likely with ecological impacts.

The work carried out in this thesis concludes that the RNA polymerase (L) protein plays an important role for VHSV virulence and temperature sensitivity. In detail, the findings reported here show that a specific sequence in 3'-end UTR is required for optimal viral replication and formation of infectious progeny *in vitro* and *in vivo*. There are few studies prior to those published as part of this thesis, focusing on the importance of the L protein for VHSV virulence.

Summary points

i. A single point codon (I1012F) in the VHSV L protein CR IV is a determinant for in vitro virulence to GEC cells and possibly plays a role for cross-species infectivity (Paper I).
ii. A specific sequence in 3'-UTR is required for virulence of VHSV (Paper II).
iii. The VHSV L protein is a determinant of *in vitro* temperature sensitivity at elevated temperatures. Swapping the L proteins results in gain- and/or loss-of-function VHSV genotype IV strains (Paper III).

FUTURE PERSPECTIVES

This work also creates new questions and set direction for future research:

i. The I1012F-variant should be studied for *in vivo* virulence in rainbow trout and this combination should be combined with those identified as being different for high and low virulent strains.
ii. The I1012F-variant should be tested for its virulence in olive flounder *in vivo*.
iii. A F1012I-variant (loss-of-virulence study) should be applied to virulent strains (DK-3592B or NO/650/07) and studied *in vitro* and *in vivo* of rainbow trout.
iv. The 3'-end UTR-variant (A4G-G5A) should be used in vaccine trials with olive flounder to evaluate their suitability as live vaccine candidates. And combinations of mutations for these positions should be studied in more detail.

Furthermore, there are also obvious limitations when it comes to explaining several details of the VHSV virulence. To strengthen the present concepts, the following future studies should be conducted:

i. Addressing the functions of CR IV in the L protein.
ii. Addressing the importance of (long-range) RNA-RNA interaction and importantly, if a panhandle structure is actually formed in VHSV.
iii. Pinpointing single amino acid residues important for temperature sensitivity.

The following should be applied to under aquaculture conditions to prevent VHS in farmed fish:

i. The 3'-end UTR variant should be tested as a live vaccine with the purpose to acquire protective immunity against VHSV.
ii. With awareness of the temperature sensitivity, the water temperature of in-land fish farming systems will be controlled to reduce VHSV virulence.

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A Single Amino Acid Mutation (I1012F) of the RNA Polymerase of Marine Viral Hemorrhagic Septicemia Virus Changes *In Vitro* Virulence to Rainbow Trout Gill Epithelial Cells

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ABSTRACT

Viral hemorrhagic septicemia virus (VHSV) is separated into four different genotypes (I to IV) with different sublineages (K. Einer-Jensen, P. Ahrens, R. Forsberg, and N. Lorenzen, J. Gen. Virol. 85:1167–1179, 2004; K. Einer-Jensen, J. Winton, and N. Lorenzen, Vet. Microbiol. 106:167–178, 2005). European marine VHSV strains (of genotypes I to III) are, in general, nonpathogenic or have very low pathogenicity to rainbow trout after a waterborne challenge, and here we also show that genotype IVa is nonpathogenic to trout. Despite several attempts, it has not been possible to link genomic variation to *in vivo* virulence. *In vitro* virulence to gill epithelial cells (GECs) has been used as a proxy for *in vivo* virulence, and here we extend these studies further with the purpose of identifying residues associated with *in vitro* virulence. Genotype Ia (DK-3592B) and III (NO/650/07) isolates, which are pathogenic to rainbow trout (O. B. Dale, I. Orpetveit, T. M. Lyngstad, S. Kahns, H. F. Skall, N. J. Olesen, and B. H. Dannevig, Dis. Aquat. Organ. 85:93–103, 2009), were compared to two marine strains that are nonpathogenic to trout, genotypes Ib (strain 1p8 [H. F. Mortensen, O. E. Heuer, N. Lorenzen, L. Otte, and N. J. Olesen, Virus Res. 63:95–106, 1999]) and IVa (JF-09). DK-3592 and NO/650/07 were pathogenic to GECs, while marine strains 1p8 and JF-09 were nonpathogenic to GECs. Eight conserved amino acid substitutions contrasting high- and low-virulence strains were identified, and reverse genetics was used in a gain-of-virulence approach based on the JF-09 backbone. Mutations were introduced into the G, NV, and L genes, and seven different virus clones were obtained. For the first time, we show that a single amino acid mutation in conserved region IV of the L protein, I1012F, rendered the virus able to replicate and induce a cytopathic effect in trout GECs. The other six mutated variants remained nonpathogenic.

IMPORTANCE

This is the first study to clearly link *in vitro* virulence of viral hemorrhagic septicemia virus (VHSV) with an amino acid residue in the L protein, a site located in conserved region IV of the L protein. *In vitro* virulence is documented by induction of cytopathic effects and viability studies of gill epithelial cells, and the observed cellular responses to infection are associated with increased viral replication levels. There are no previous studies addressing the importance of the L protein or the RNA-dependent RNA polymerase for virus virulence *in vitro* or *in vivo*. Therefore, the findings reported here should broaden the search for pathogenicity traits in novirhabdoviruses, and there is a possibility that the polymerase participates in defining the host species virulence of various VHSV strains.

Viral hemorrhagic septicemia virus (VHSV) is an enveloped, nonsegmented negative-sense (NNS), single-stranded RNA virus that belongs to the *Novirhabdovirus* genus of the family *Rhabdoviridae* (1). The virus genome contains the nucleocapsid (N) protein, polymerase-associated phosphoprotein (P), matrix (M) protein, surface glycoprotein (G), and viral polymerase (L) genes and a nonstructural (NV) protein gene (1). Until the late 1980s, VHSV was thought to cause only a disease of farmed rainbow trout (*Oncorhynchus mykiss*) in Europe, but with the detection of VHSV in returning Chinook (2) and Coho (3) salmon in the United States, later defined as genotype IVa, its detection in Atlantic cod (*Gadus morhua*) (4), and later the identification of a VHSV (genotypes I to III) in a vast number of marine fish species in Europe (5), the understanding of the virus's origin, its species range, and the dynamics of the interchange between wild and farmed populations changed. Investigations into the source of the trout-pathogenic European genotype Ia isolate showed that it likely emerged from a marine source some 60 years earlier (6). Today, VHSV is separated into four different genotypes (I to IV) with different sublineages (6, 7), and the different genotypes are separated geographically. All European marine VHSV strains have been shown to be nonpathogenic or have very low pathogenicity to rainbow trout after a waterborne challenge (5, 6, 8). The marine source of genotype I (6–11) and the later emergence of a trout-virulent genotype III isolate of marine origin in Norway (12) show that VHSV is capable of crossing host species barriers. Obviously, this creates a continuous concern to aquaculture operations, but it has not been possible to link virulence traits to specific sequence motifs of any of the expressed virus proteins. One approach has

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TABLE 1 Primers used for complete genomic sequencing of VHSV strains

Primer	Sequence[a]
VHSV-f1-Forward	GTATCATAAAATATGATGAGTTATG
VHSV-f1-Reverse	CCCTCCAAGGAGTCCACTGC
VHSV-f2-Forward	CTGATGAGGCAGGTGTCGGA
VHSV-f2-Reverse	ACTGAGCCCTTGCAGAGTAC
VHSV-f3-Forward	GAACGGGAAGAAGACCGACA
VHSV-f3-Reverse	GGGTCAGTCCTTTGGGTCTA
VHSV-f4-Forward	ATGTGGGGGGAAAAGGCCAC
VHSV-f4-Reverse	GACAGTTTCTTCGCTCCCCC
VHSV-f5-Forward	GGACACATGATCACAGGGTG
VHSV-f5-Reverse	CAAGCACATGGAGGTAGGGAA
VHSV-f6-Forward	TGTCCTTCGCGAGATGATCG
VHSV-f6-Reverse	CTGGCAGATGGCTGTGAGGA
VHSV-f7-Forward	AGTTCGAGTTCAAGGAAGGG
VHSV-f7-Reverse	GCGATGCTCTCTCGGAGAAC
VHSV-f8-Forward	GCTGACGAAATCTACGACAC
VHSV-f8-Reverse	GATTCCTTGCAACCGGACCT
VHSV-f9-Forward	CAATCTCAGAATGCGTGAAC
VHSV-f9-Reverse	GAGTTCCATGTAGGCTTTTG
VHSV-f10-Forward	ATCATGGGAGGAAGAGAAGG
VHSV-f10-Reverse	GAGATTGCTTTGGCGAGTGT
VHSV-f11-Forward	AGACATCTCATACCAAGCAC
VHSV-f11-Reverse	GGATGATGTGACGTGCAGCT
VHSV-f12-Forward	GACCCCGACAATTTGCTCAG
VHSV-f12-Reverse	GTATAGAAAATAATACATACCACACC

[a] The GenBank accession no. is AB179621.

TABLE 2 Primers used for plasmid construction

Application and primer	Sequence[a]
pVHSV construction	
VHS-segment1-Forward-NheI	AATGCTAGCGTATCATAAAAGATGATGAG TTATGTTAC
VHS-segment1-Reverse-EcoRI	TATCGGATGTAGAATTCCTCCCTAATCTTG
VHS-segment2-Forward-EcoRI	CAAGATTAGGGAGAATTCTACATCCGATA
VHS-segment2-Reverse-NotI	TTAGCGGCCGCGTATAGAAAATGATACAT ACCACAACC
VHS-HdvRz-Reverse-NotI	TTAGCGGCCGC**CTCCCTTAGCCATCCGAG TGGACGTGCGTCCTCCTTCGGATGCCC AGGTCGGACCGCGAGGAGGTGGAGAT GCCATGCCGACCC**GTATAGAAAATGAT ACATACCAC
Helper plasmid construction	
N-Forward-NheI	GCTAGC*GCCACCATG*GAAGGGGGAATCC
N-Reverse-NotI	ATGCGGCCGCTTAATCAGAGTCCCCTGG
P-Forward-NheI	GCTAGC*GCCACCATG*ACTGATATTGAGA
P-Reverse-NotI	ATGCGGCCGCCTACTCCAACTTGTCCAAC
L-Forward-NheI	GCTAGC*GCCACCATG*GAGATGTTCGAGC
L-Reverse-NotI	ATGCGGCCGCTCATTGCTCTCCAAATGG
NV-Forward-NheI	GCTAGC*GCCACCATG*ACGACCCAGTCGG
NV-Reverse-NotI	ATGCGGCCGCTCATGGGGGAGATTCGGAG

[a] Restriction enzyme sites are underlined, the HdvRz sequence is in boldface, and the Kozak consensus sequence is in italics.

been to identify pathogenicity markers of these new variants through genome sequencing (10, 13), but so far it has not been possible to tie such variation or specific amino acid motifs of the viral proteins to virulence (14). We have shown that the ability of defined VHSV strains to infect and translocate across primary cultures of rainbow trout gill epithelial cells (GECs) correlate with *in vivo* virulence by using a bath challenge of trout fry (15). Isolates not able to infect (flat) cultures of GECs and/or translocate across polarized GECs also did not cause death postchallenge. In this study, we obtained the same results and found that one genotype Ia isolate (6) and a genotype III (NO/650/07) variant (12), both pathogenic to trout (by *in vivo* challenge), are able to infect and translocate across GECs. In contrast, two marine strains nonpathogenic to trout, genotype Ib strain 1p8 (16) and genotype IVa strain JF-09, lack the ability to infect, replicate in, and translocate across GECs cultures *in vitro*. Thus, we found that the *in vitro* GEC infection model is a good proxy of *in vivo* virulence to trout. All four isolates were sequenced and aligned, and we selected eight conserved amino acid substitutions contrasting high- and low-virulence strains. Reverse genetics was used and the JF-09 backbone was point mutated at the selected residues to obtain seven different mutant JF-09 virus clones (all able to replicate in epithelioma papulosum cyprini [EPC] cells). Through this gain-of-virulence approach, we show for the first time that the replacement of a single amino acid of the L protein, isoleucine (I) at position 1012, which is in conserved region IV (CRIV), with phenylalanine (F) renders mutant JF-09 able infect, replicate in, and induce a cytopathic effect (CPE) in trout GECs. The other mutated variants of JF-09 were nonpathogenic, like wild-type (WT) JF-09.

MATERIALS AND METHODS

Cells and viruses. VHSV isolates designated DK-3592B (genotype Ia), 1p8 (genotype Ib), NO/650/07 (genotype III), and JF-09 (genotype IVa) were used in this study and propagated in EPC cells (ATCC CRL-2872) in L15 cell medium (Invitrogen) containing 10% fetal bovine serum (FBS; Sigma-Aldrich) at 15°C (15). Strains DK-3592B and 1p8 were a kind gift of Niels Jørgen Olesen, Danish Veterinary Institute, Århus, Denmark, and NO/650/07 was kindly provided by Renate Johansen, Norwegian Veterinary Institute, Oslo, Norway. Isolate JF-09 originated from VHSV-infected olive flounder (*Paralichthys olivaceus*) in South Korea. All strains were plaque purified and propagated in EPC cells in L15 cell medium (Invitrogen) containing 10% FBS (Sigma-Aldrich) at 15°C. Titration was performed on EPC cells by the 50% tissue culture infective dose ($TCID_{50}$) method as described previously (17). In addition, we used primary cultured rainbow trout GECs isolated from fry of rainbow trout (18, 19) as modified by Brudeseth et al. (15).

Complete genomic sequencing of strain JF-09. Gene-specific primers (GSPs) for sequencing were designed to produce overlapping fragments (Table 1). First-strand cDNA synthesis was carried out with the Transcriptor First Strand cDNA synthesis kit (Roche). Reverse transcription (RT)-PCR was done with a thermal cycler with denaturation at 98°C for 10 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min. In order to identify the 3′-terminal region of the genomic RNA, a poly(A) tail was added to the 3′ end by using the poly(A) polymerase enzyme with the mMESSAGE mMACHINE T7 Ultra kit (Ambion), and RNA was purified with the RNeasy Plus minikit (Qiagen). cDNA synthesis was conducted with a SMARTer rapid amplification of 5′ cDNA ends (RACE) cDNA Amplification kit (Clontech) with a VHSV-f1-Reverse GSP (Table 1). The 5′-terminal region of genomic RNA was identified by RACE with a JF09 5′ end (5′-CCTCGGTGCCCTGCTCCACCTGGAGTCC-3′) GSP and a SMARTer RACE cDNA amplification kit (Clontech). The amplified cDNAs of 3′ and 5′ ends were cloned with a TOPO TA Cloning kit (Invitrogen). The 3′ and 5′ RACE and RT-PCR products were sequenced by GATC Biotech, Germany. The assembly of contiguous sequences, multiple-sequence alignments, and comparative sequence analyses were conducted with Vector NTI Advance 11 software (Invitrogen) and BLAST searches of the NCBI database.

Plasmid constructions. The complete genomic plasmid was constructed by assembling two overlapping subclones in modified pTurboFP635-N vectors (Evrogen) with GSPs (Table 2). In the final construct, the trailer end of antigenome cDNA was fused with hepatitis delta virus ribozyme (HdvRz) (20), followed by the simian virus 40 early mRNA polyadenylation signal. For construction of helper plasmids, RT-PCR was used to amplify the open reading frames (ORFs) of the N, P, L, and NV

TABLE 3 Primers used for point mutations

Primer	Sequence[a]	Protein
T288A-Forward	GAAGTGTGTCAAC**G**CCGACGTTCAGATGA	G
T288A-Reverse	TCATCTGAACGTCGG**C**GTTGACACACTTC	
E57D-Forward	TCTAGAGTCTCAGA**T**GATCTAAGGACCTG	NV
E57D-Reverse	CAGGTCCTTAGATC**A**TCTGAGACTCTAGA	
VSE55-57ILD-Forward	ACAACTCTTTCTAGA**A**TCT**T**AGA**T**GATCTAAGGACCTGC	NV
VSE55-57ILD-Reverse	GCAGGTCCTTAGATC**A**TCT**A**AGA**T**TCTAGAAAGAGTTGT	
G80R-Forward	GATAAGTCTTCTAGAG**A**GAACTCATTACATACT	NV
G80R-Reverse	AGTATGTAATGAGTTC**T**CTCTAGAAGACTTATC	
G149E-Forward	GATGTCAAGGGAAAAG**A**AAAAAGAAAGTT	L
G149E-Reverse	AACTTTCTTTTTT**T**CTTTTCCCTTGACATC	
E298K-Forward	CCAGGTCGTAT**A**AACGGATGATGAAAACT	L
E298K-Reverse	AGTTTTCATCATCCGTTT**T**ATACGACCTGG	
I1012F-Forward	AATCGCCTACCAA**T**TTCGAAACATCACAG	L
I1012F-Reverse	CTGTGATGTTTCGAA**A**TTGGTAGGCGATT	

[a] Point-mutated nucleotides are in boldface.

genes with GSPs (Table 2). A Kozak consensus sequence was created in front of the start codon of each ORF. The amplified genes were cloned into modified pTurboFP635-N vectors and were under the control of the cytomegalovirus promoter. The recombinant JF-09 (rJF-09) isolate was rescued from EPC cells transfected with VHSV plasmids and helpers with FuGENE HD transfection reagent (Roche) as described previously (21).

***In vitro* replication of VHSV in rainbow trout GECs.** For immunofluorescence staining (IFAT), GECs (1×10^6) were seeded into 24-well plates and cultured for 24 h at 20°C (15). The cells were infected with VHSV isolates (multiplicity of infection [MOI] of 10) and further incubated at 15°C. Sampling was performed at the time points indicated (see below) by fixing the cells in a 4% paraformaldehyde solution for 20 min. VHSV was identified by IFAT with monoclonal antibody (MAb) IP5B11 (anti-N protein) (15) as the primary antibody. Anti-rainbow trout Mx antibody was used to detect Mx protein in infected cells (22). Stained cells were washed and examined with an inverted Olympus IX81 fluorescence microscope.

Virus replication in GECs (1×10^5) was assessed by quantitative real-time RT-PCR. Every 24 h, 100 μl cell culture supernatant was sampled for RNA extraction with a viral RNA minikit (Qiagen) and cDNAs were synthesized with a Transcriptor First Strand cDNA synthesis kit (Roche). Quantitative RT-PCR was carried out with a LightCycler 480 SYBR green I master mix and the LightCycler 480 system (Roche) with a pair of GSPs (0.5 μM), VHSV 23-N-Forward (5′-CGCCATCATGATGAGTCGGATG CTG-3′) and VHSV 23-N-Reverse (5′-CTTCTCTGTCACCTTGATCCC CTCC-3′), targeting a conserved N protein gene in four VHSV isolates. The specificity of the PCR products was confirmed by melting curve analysis and subsequent agarose gel electrophoresis.

For viability analysis, GECs (6.4×10^4) were seeded into 96-well plates and cultured for 24 h at 20°C. Three different virus preparations (MOIs of 0.15, 1.5, and 15) of each of the four VHSV isolates were added to the cell cultures. Cell viability was measured with a CellTiter AQ_{ueous} One solution reagent (Promega) (15).

Transepithelial passage through GECs. An *in vitro* system was set up to determine the ability of VHSV to translocate across the barrier of GECs (15, 24). GECs (2.5×10^5) were seeded onto insert filters (Falcon; 0.4-μm pore size). Infection (MOI of 0.4) of cells was initiated when the transepithelial electrical resistance (TER) exceeded 1.0×10^3 Ω/cm^2 (15). Transepithelial passage of virus to the bottom well was detected by inoculation onto EPC cells, assessment of a CPE, and detection by IFAT (15).

***In vivo* virulence of WT JF-09 in rainbow trout.** Strains DK-3592B, 1p8, and NO/650/07 had all been tested for *in vivo* virulence in rainbow trout and shown to be pathogenic (DK-3592B [15] and NO/650/07 [12]) or nonpathogenic (1p8 [15]). The *in vivo* pathogenicity of WT JF-09 to rainbow trout and olive flounder was tested by using artificial infection. Olive flounder (20 ± 2 g) were divided into three groups kept in tanks containing 20 liters of UV-disinfected seawater at 11 ± 0.5°C in a controlled indoor seawater facility. The fish were acclimatized to their respective rearing conditions for 7 days prior to infection. Ten fish per group were injected intramuscularly (i.m.) with 100 μl of a viral suspension ($10^{6.5}$ or $10^{5.5}$ $TCID_{50}$ per fish), and six fish were given phosphate-buffered saline (PBS) as a control. The rainbow trout infection trial was carried out in triplicate tanks by immersion and intraperitoneal (i.p.) injection (average weight, 2.0 g). Two tanks were infected by immersion with DK-3592B as a positive control, and two tanks (per administration) of fish treated by virus-free cell medium immersion and injected to simulate stress conditions served as noninfected negative controls. Immersion infection was carried out for 2 h with 8 liters of softened tap water with a viral suspension added. The challenge dose of WT JF-09 was $10^{4.5}$ $TCID_{50}$/ml of water, and that of DK-3592B was $10^{3.7}$ $TCID_{50}$/ml of water. WT JF-09 was also injected i.p. at $10^{5.5}$ $TCID_{50}$ per fish under anesthesia (benzocaine, 10 mg/liter). Approximately 35 fish were placed into each tank. During the infection trials, fish were kept in tanks containing 8 liters of soft, fresh, unchlorinated water (around 3 German degrees [dH]) maintained at 10.5 ± 1.1°C. Fish that died during the experiment were sampled for virus examination. Deaths were recorded daily.

Sequencing of ORFs and amino acid comparison analysis. ORF sequences were obtained by first-strand cDNA synthesis with the Transcriptor First Strand cDNA synthesis kit (Roche) with extracted total RNAs according to the manufacturer's instructions. Consensus GSP sets (Table 1) were used for complete genomic sequences (by GATC Biotech) from cDNAs of four VHSV isolates (DK-3952B, 1p8, NO/650/07, and JF-09). The assembly of contiguous sequences, multiple-sequence alignments, and comparative sequence analyses were conducted with Vector NTI Advance 11 software (Invitrogen).

Mutated variants of JF-09. Site-directed mutagenesis (GENEART site-directed mutagenesis system; Invitrogen) with GSPs (Table 3) was performed, and seven different variants were generated, $T288_GA$, $VSE55\text{-}57_{NV}ILD$, $E57_{NV}D$, $G80_{NV}R$, $G149_LE$, $E298_LK$, and $I1012_LF$ (see Fig. 4B). Mutated plasmids $pVHSV_{JF\text{-}09}$ (120 ng), pN (70 ng), pP (30 ng), pNV (15 ng), and pL (30 ng) were mixed into 25 μl Opti-MEM medium (Invitrogen), and 1 μl of X-tremeGENE 9 DNA transfection reagent (Roche) was added to rescue mutated virus variants. The mixtures were incubated for 10 min at room temperature and added to subconfluent layers of EPC cells (1×10^5) on a 24-well plate. The cells were incubated at 28°C for 5 h and then shifted to 15°C. The cell culture supernatants (passage 0) were clarified by centrifugation, 10-fold diluted, and inoculated onto a subconflu-

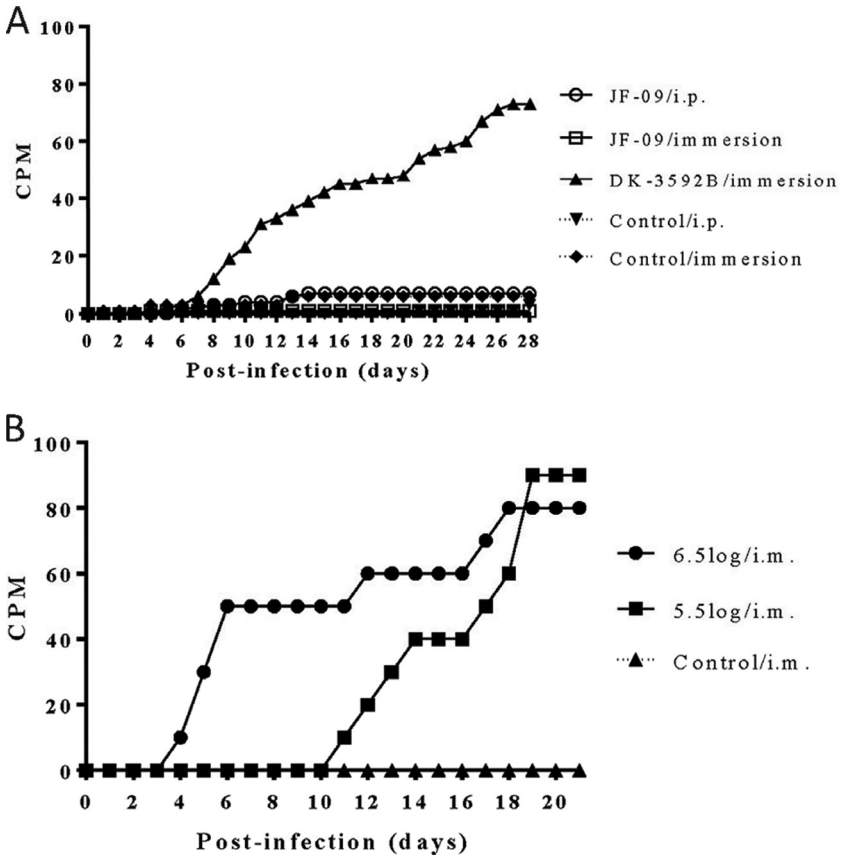

FIG 1 (A) *In vivo* virulence of WT JF-09 in rainbow trout. Challenges were performed by i.p. injection and by immersion (for details, see Materials and Methods). The CPM was 7% in the injected groups, which is not different from that in the uninfected group following an immersion challenge. DK-3592B was used as a positive control and gave a CPM of 73%. (B) Challenge of olive flounder with WT JF-09. Fish were injected i.m. with the WT JF-09 strain at the indicated titers per fish, and controls were injected with PBS. Onset of death occurred earlier in the high-challenge group. In both groups, the CPM was >80%.

ent layer of EPC cells. The rescued viruses were confirmed by IFAT in EPC cells.

***In vitro* virulence of mutated (I1012F) JF-09.** To evaluate the increase in virulence, mutated JF-09 was added (MOI of 1, 5, or 10) to GECs (2×10^5) on 24-well plates and incubated at 15°C for 48 h. VHSV protein expression was identified with a standard IFAT with a polyclonal antibody (23) or MAb IP5B11 (15) as the primary antibody. Virus quantities in sampled supernatants (100 μl) were estimated by quantitative real-time RT-PCR (QuantiFast SYBR Green RT-PCR; Qiagen) with a pair of GSPs [VHSV(4)-N-Forward and VHSV(4)-N-Reverse]. The mutated variants of JF-09 were compared to DK-3592B and WT JF-09. To assess cell viability, GECs (1×10^4) were seeded into 96-well plates and the viruses (I1012F, DK-3592B, and JF-09) were added (MOI of 1 or 5) to the cells at 15°C. Cell viability was measured with CellTiter AQ_{ueous} One solution reagent (Promega) (15).

Statistical analysis. A *t* test was used to calculate differences when relevant, and a *P* value of <0.05 was considered statistically significant.

RESULTS

Strain JF-09 is nonpathogenic to rainbow trout and highly pathogenic to olive flounder in an *in vivo* challenge. An experimental challenge of rainbow trout with WT JF-09 gave a cumulative percent mortality (CPM) of 7% by i.p. injection ($10^{5.5}$ $TCID_{50}$ per fish) and a CPM of 1% by immersion challenge ($10^{4.5}$ $TCID_{50}$/ml of water) (Fig. 1A), while DK-3592B gave a CPM of 73% ($10^{3.7}$ $TCID_{50}$/ml of water, immersion infection; positive control). In contrast, we confirmed the virulence of WT JF-09 by an *in vivo* challenge of olive flounder, which gave a CPM of ≥80% (Fig. 1B).

Replication and induction of a CPE in primary trout cells can be used to differentiate between low- and high-virulence strains. DK-3592B and NO/650/07 infected, replicated, and caused a CPE in GECs, a pattern typical of high-virulence strains (Fig. 2A to C). 1p8 and JF-09 infected GECs (Fig. 2A) but did not replicate (Fig. 2B) and did not cause a CPE or loss of viability in GECs (Fig. 2C) infected at an MOI of ≤10. Progeny of 1p8 and JF-09 were detected by IFAT in GECs (Fig. 2A), but at the same time, we found induction of Mx protein expression at 60 h postinfection (Fig. 2A), likely limiting the replication of the virus. We were also interested in assessing the impact of the MOIs (of all four strains) used to infect GECs, and MOIs of 15, 1.5, and 0.15 were tested and cell viability was monitored over a period of 96 h. At high MOIs (≥1.5), DK-3592B and NO/650/07 induced a CPE earlier (Fig. 2C), while for JF-09 and 1p8, there was no difference between MOIs of 0.15 and 1.5,which also did not differ from uninfected controls. At an MOI of 15, there was reduced viability, significantly lower than that of controls, from 3 days postinfection with JF-09 and 1p8 onward (Fig. 2C). This shows that when given as a large infectious dose (single-cycle infection), low-virulence strains replicated and induced a CPE in GECs but the reduction in

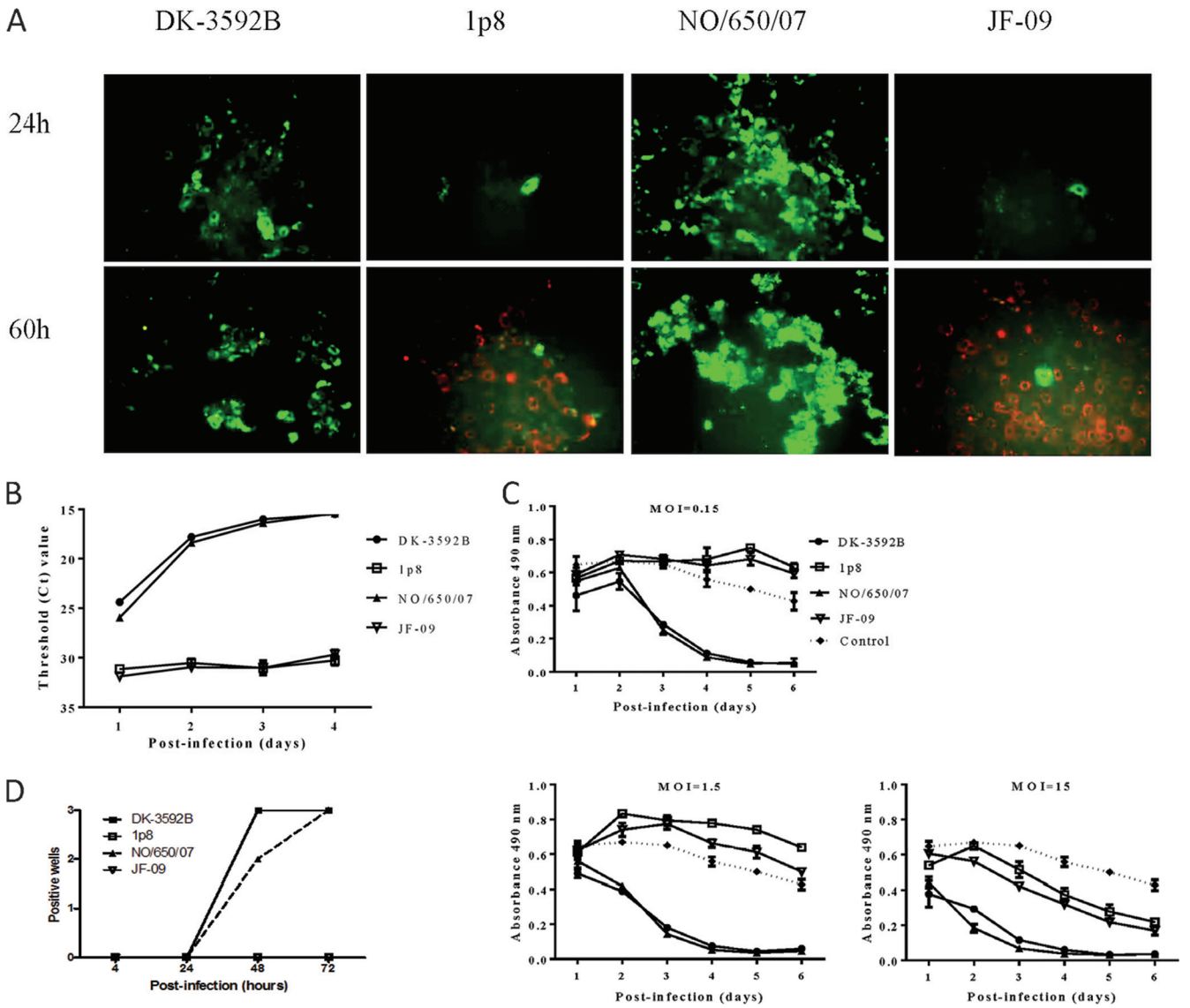

FIG 2 *In vitro* replication and translocation of strains DK-3592B, 1p8, NO/650/07, and WT JF-09 in polarized GECs. (A) GECs were infected at an MOI of 10. VHSV N protein expression (green fluorescence) and Mx protein expression (red fluorescence) in GECs were detected by IFAT at 24 and 60 h postinfection. Note the high number of Mx-positive cells and few virus-positive cells for JF-09 and 1p8. No Mx staining was observed for DK-3592B and NO/650/07. (B) Viral replication in GECs was quantified by real-time RT-PCR targeting the VHSV N gene up to 4 days postinfection (MOI of 10) (average ± the standard error of the mean [SEM]; $n = 3$). The cycle threshold in a real-time PCR was determined. (C) Viabilities of infected GECs at three different MOIs (0.15, 1.5, and 15) compared with that of the uninfected control. Results are presented as the mean absorbance at 490 nm ± SEM ($n = 3$). Labeling is identical for all MOIs. (D) The translocation of viruses through GECs is presented as the number of positive wells in three parallel experiments at different time points postinfection (MOI of 0.4).

viability was significantly less ($P < 0.01$) than that caused by DK-3592B and NV/650/07 (Fig. 2C).

Nonvirulent and low-virulence strains of VHSV replicate less and are unable to translocate across polarized GECs (mimicking primary gill barriers) (15). We therefore included a functional assay in which GECs were grown on filters and polarized and the different VHSV isolates were inoculated onto the apical side, i.e., on top of the insert filters (15). DK-3592B and NO/650/07 were detected in the bottom samples, indicating translocation across the epithelium at 48 and 72 h postinfection. Detection was by culture on EPC cells confirmed by IFAT. In contrast, no 1p8 or JF-09 virus was detected in bottom wells over the observation period (Fig. 2D), showing that these strains did not translocate across GECs *in vitro*. We also observed the extent to which the tight junctions of the cells were maintained; this was confirmed by measuring the TER (10^3 Ω/cm^2) in parallel following DK-3592B infection, which was 3.74×10^3 Ω/cm^2 at 72 h postinfection (well above the 1.0×10^3 Ω/cm^2 threshold).

Virulent rJF-09 was rescued from EPC cells. rJF-09 was rescued from transfected EPC cells, and the growth pattern and final virus titer were compared to those of WT JF-09 and found to be identical (Fig. 3A). The full sequence of the rescued recombinant virus was also obtained, and the sequences were identical, except for position 91 in the NV protein (S91N, both polar amino acids).

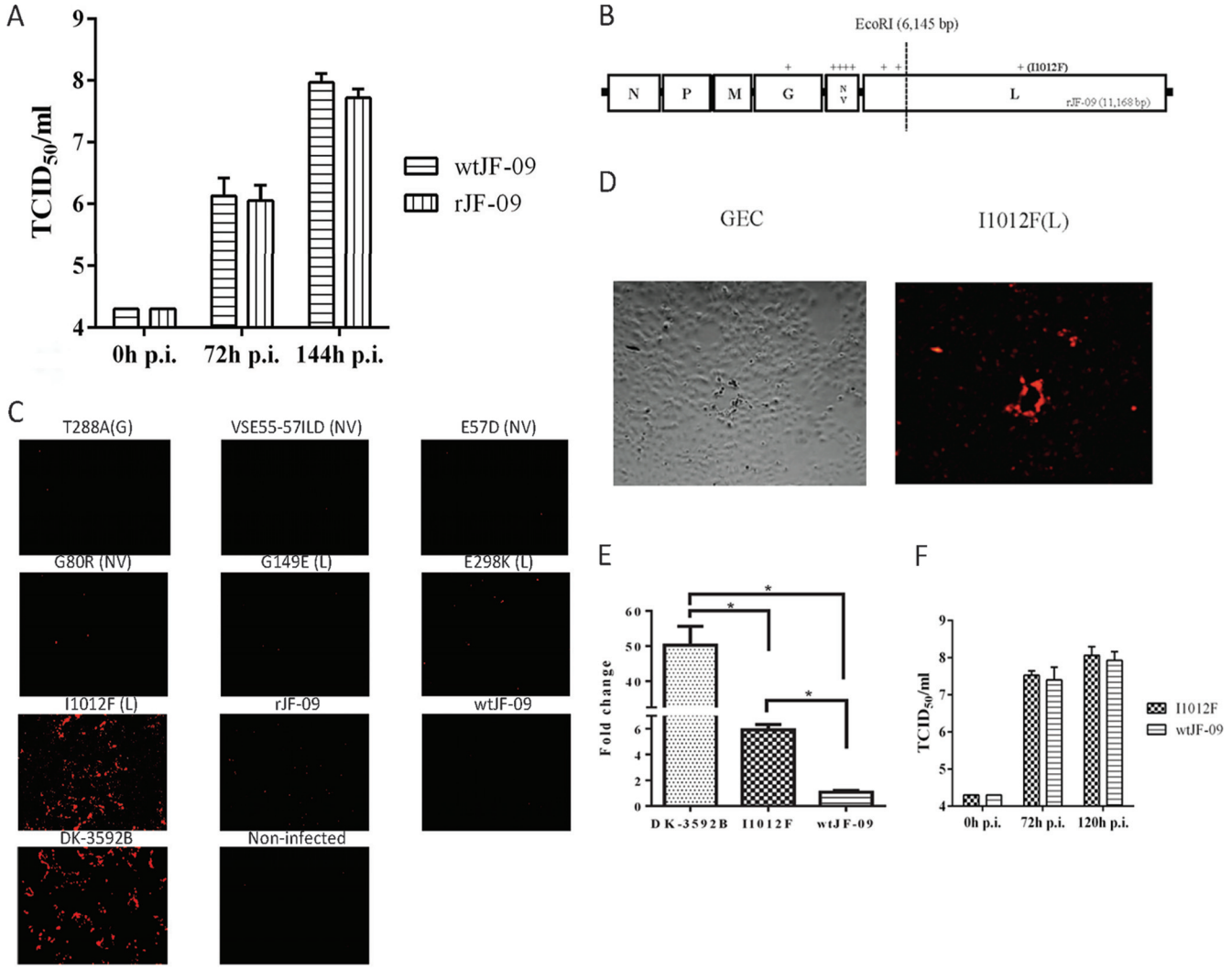

FIG 3 Infectivity of mutated variants of JF-09 in rainbow trout GECs. (A) Growth kinetics of rJF-09 and WT JF-09 in (permissive) EPC cells over 144 h of incubation (MOI of 0.1) (mean ± SEM; $n = 3$). rJF-09 and WT JF-09 show similar growth patterns. (B) Point mutations were introduced into the rJF-09 backbone at eight positions (+), as indicated. (C) Viral protein expression (red fluorescence) in GECs infected with the different mutated clones, shown by IFAT at 24 h postinfection (p.i.) (MOI of 10). A noninfected control and a positive control, DK-3592B, were included, and in addition, we also included WT JF-09 and rJF-09 as controls. DK-3592B infected the GECs (as expected), and the only other positive well is that containing the I1012F mutant. (D) Infection with the I1012F mutant at an MOI of 1 was also included. Shown are a phase-contrast micrograph (left) and viral protein expression (red fluorescence; right) at 24 h postinfection. (E) Replication of the DK-3592B, I1012F, and WT JF-09 viruses was assessed by real-time RT-PCR (N GSPs) at 48 h postinfection (MOI of 5). The I1012F mutant shows a replication level 5.6 times as high as that of WT JF-09, while that of DK-3592B is 50 times as high (mean ± SEM; $n = 3$). (F) WT JF-09 and I1012F mutant clone replication in permissive EPC cells. The replication kinetics and final titers of the two variants are identical (mean; $n = 3$).

This had no impact on the growth pattern in permissive EPC cells (Fig. 3A).

I1012F mutant JF-09 replicates and produces progeny in trout GECs. We then aligned the sequences of the four strains included in this study and found 13 positions that differed between high- and low-virulence strains (Table 4). We selected eight positions (Fig. 3B) in the G (one residue), NV (four residues), and L (three residues) proteins and generated mutated clones with one amino acid mutation per clone in the rJF-09 backbone. For the NV ORF, we also introduced a three-amino-acid en-block mutation, VSE to ILD (positions 55 to 57). The candidate proteins (G, NV, and L) were chosen because the G protein is a membrane protein involved in virus attachment and entry (25) and the NV protein has been shown to have an impact on antiapoptosis functions and virulence (23). There are very few, if any, studies addressing to what extent the polymerase plays a role in the virulence of novirhabdoviruses, but it is well known that a single amino acid mutation in PB2 (polymerase B2 protein) of human influenza virus renders the virus able to replicate in chicken cells (26). The various mutants (Table 4) were harvested from transfected EPC cells, all of the mutated variants grew in EPC cells and induced a CPE, and the harvested supernatants were used to infect GECs. We found that the I1012F mutant JF-09 virus infected GECs at MOIs ranging from 1 to 10 (Fig. 3C and D). None of the other mutated variants of rJF-09 were able to infect or replicate in GECs (Fig. 3C). Furthermore, we assessed the replication of the I1012F mutant JF-09 virus by real-time RT-PCR and found a close-to-6-fold increase in virus replication over that of WT JF-09, while the replication level

TABLE 4 Amino acid positions conserved across the entire genomes of high (DK-3592B and NO/650/07)- and low (1p8 and JF-09)-virulence strains[a]

Virulence group and strain	N (404)		P (222)	M (201)	G (507)	NV (122)			L (1984)		
	5	401	39	2	288	55-57	57	80	149	298	1012
Low virulence											
1p8	I	G	P	A	**T**	**ILN**	**N**	**K**	**G**	**E**	**I**
JF-09	I	G	S	A	**T**	**VSE**	**E**	**G**	**G**	**E**	**I**
High virulence											
DK-3592B	L	E	T	T	**A**	**ILD**	**D**	**R**	**E**	**K**	**F**
NO/650/07	L	E	T	T	**A**	**ILD**	**D**	**R**	**E**	**K**	**F**

[a] Viral proteins and the total numbers of amino acids (in parentheses) are shown at the top. The mutations in the backbones of the JF-09 G, NV, and L proteins are in boldface. The mutations are T288A (G), VSE55-57ILD (NV), E57D (NV), G80R (NV), G149E (L), E298K (L), and I1012F (L), corresponding to the residues indicated.

of the DK-3592B strain again was 50-fold higher (Fig. 3E). We also compared the replication of WT JF-09 with that of the I1012F mutant isolate in a permissive cell line (EPC), and the titers obtained were similar and the growth curves of the two strains overlapped (Fig. 3F). The entire genome of strain I1012F was also sequenced after culture in EPC cells and GECs, and the nucleotide sequences of the viruses obtained from the two cell lines were identical to that of the original clone, rJF-09, showing that no other compensatory mutations had occurred.

The I1012F mutant strain has an intermediate-virulence phenotype in trout GECs. As a next step, we measured the cell viability (cellular integrity) of infected GECs with a standard 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay and found a significant drop in cell viability by 48 h and beyond (72 and 96 h postinfection; Fig. 4A), where the I1012F mutant strain appeared as an intermediate-virulence variant between DK-3592B and WT JF-09. WT JF-09 was not different from the noninfected control. We also included GECs from different-size rainbow trout to account for the possible effect of the size of the donor fish (Fig. 4B) and found no effect. Further, rJF-09 and WT JF-09 had an amino acid difference in NV (S91N) and this had no impact on *in vitro* viru-

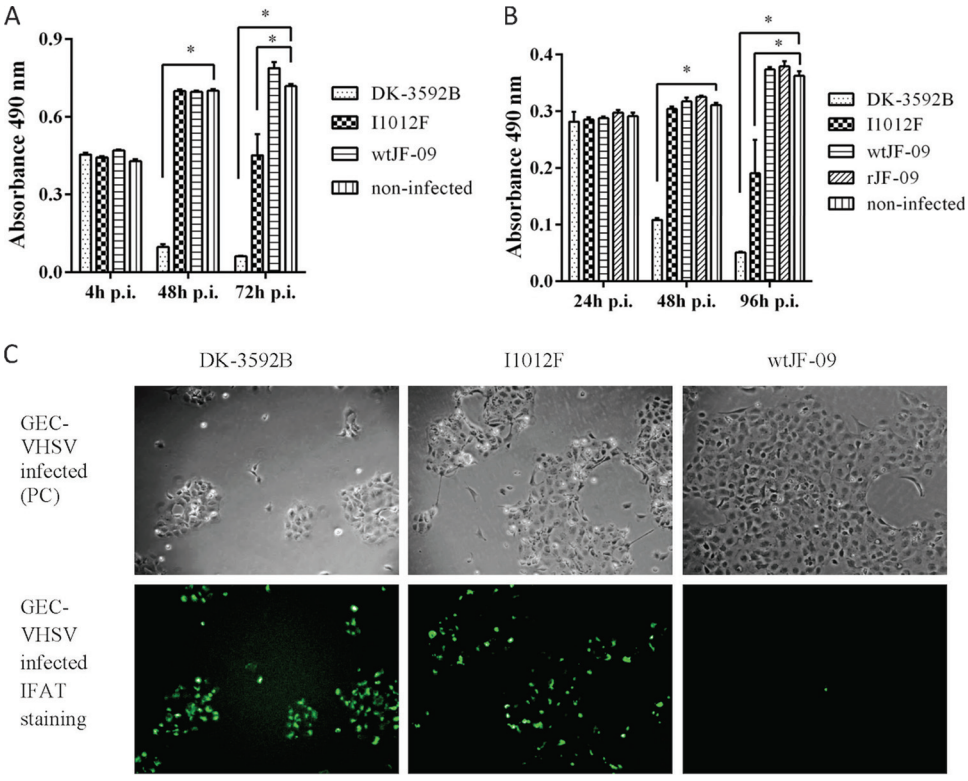

FIG 4 Infectivity of the I1012F clone in rainbow trout GECs. (A) GECs (25 g) from rainbow trout were infected (MOI of 5) with the WT JF-09, I1012F, and DK-3592B (positive control) viruses and monitored for 72 h postinfection (p.i.). The *in vitro* virulence of the I1012F mutant is intermediate between those of the two other strains (MOI of 1). Asterisks indicate significant differences ($P < 0.05$) from WT JF-09. (B) GECs (150 g) from rainbow trout were infected (MOI of 5) with the WT JF-09, rJF-09, I1012F, and DK-3592B (positive control) viruses and monitored for 72 h postinfection. Also here, the *in vitro* virulence of the I1012F mutant is intermediate between those of the two other strains (MOI of 1). Asterisks indicate significant differences ($P < 0.05$) from WT JF-09. (C) Phase-contrast (PC) images (top) and viral N protein expression (green fluorescence images, bottom) shown by IFAT at 48 h postinfection (MOI of 5).

TABLE 5 Alignment of VHSV (including position 1012) with the L proteins of NNS viruses maps the residue to CRIV

Virus[a]	Accession no.	Sequence[b]
VHSV isolates:		
JF-09		(1009) AYQIRNITDQKESEMFVTKFATAKTM (1035)
DK-3592B		(1009) AYQFRNITDQKESEMFVTKFATAKTM (1035)
IHNV	Q82685	(1011) NYMIRSITDEKESEMFVTKFASARTM (1037)
SHRV	Q9QJT4	(1008) AHLIRDITDEKESEMFVTKFATARTM (1034)
TPMV	Q9JFN3	(1130) ASEIVDNSITGARESLAGLLDTTKGL (1156)
SVCV	Q91DS4	(1003) LSEFRSSTFVGVTDSLIGLFQNSKTI (1029)
ISFV	Q5K2K3	(1004) LSEFKSGTFMGVASSVVSLFQNSRTI (1030)
CHAV	P13179	(1004) LSEFKAGTFMGVASSIVSLFQNSRTI (1030)
BDV	Q8JMN0	(925) MSALFALSNVAYGLSIIDLFQKSSTV (951)
RABV	Q8B6J5	(1028) LSELFSSSFLGIPESIIGLIQNSRTI (1054)
SENDZ	P06447	(1060) AHEILGNSLTGVREAIAGMLDTTKSL (1086)

[a] Abbreviations: SHRV, snakehead rhabdovirus; TPMV, Tupaia paramyxovirus; SVCV, spring viremia of carp virus; ISFV, Isfahan virus; CHAV, Chandipura virus; BDV, Borna disease virus; RABV, rabies virus; SENDZ, Sendai virus.
[b] The start and end positions are shown in parentheses at the left and right, respectively.

lence to GECs; both were avirulent (Fig. 4B). A CPE was observed, and virus protein (N) was detected in infected GECs at 48 h postinfection (Fig. 4C). On the basis of these studies, we conclude that the I1012F mutant gains the abilities to infect trout GECs, produce viral progeny, and induce a CPE in infected cells. Finally, the entire genome of the I1012F mutant strain was also sequenced after passage in GEC cultures and the nucleotide sequence obtained was identical to that of the original I1012F mutant clone. We also aligned JF-09 with other NNS strains, with a particular focus on the CR. Position 1012 is located in the C-terminal part of CRIV, toward CRV (Table 5).

DISCUSSION

The main finding of this study is that a single amino acid mutation (I1012F) of the viral polymerase renders an otherwise nonpathogenic marine isolate of VHSV able to infect, produce live progeny, and induce a CPE in primary cultures of trout gill cells. Exchange of amino acids of the G protein, the NV protein, or two other residues of the polymerase did not change the pathogenicity profile of the mutated virus strains.

There are no previous publications addressing the importance of the polymerase for *in vitro* virulence of VHSV strains. WT JF-09 and 1p8 are both able to infect GECs but do not replicate and do not induce a CPE in cells infected at an MOI of ≤10 (Fig. 2A to C). Mutation of residue 1012 (I1012F) of the L protein enhanced the replication capacity of the polymerase, while two other point mutations had no such effect. The viral polymerase (L) protein is involved in many aspects of viral replication and transcription as a large RNA-dependent RNA polymerase (RdRp) complex component of VHSV, and here we also have an indication that the RdRp also plays a role in host specificity, with similarities to the viral heterotrimeric polymerase complex in influenza A virus (27). The L protein of NNS RNA viruses has six CRs (CRI to CRVI) (28). Following an alignment with other NNS viruses, some of which have relatively high levels of identity in the CRIV region, we found that position 1012 is located in the C-terminal region of CRIV, toward CRV. The RdRp activity maps to the CRIII region, which is also involved in polyadenylation, while CRV is involved in capping activities (29–31). However, the function(s) of CRIV remains unclear, apart from the fact that it is a key structural component of the ring domain of the polymerase (30, 31). It has been shown that point mutations (in the CRIV region) of Sendai virus and parainfluenza 3 virus render these viruses temperature sensitive (32, 33). Further, mutation of codon 1321 of the L-encoding gene of human respiratory syncytial virus (hRSV) rendered the virus avirulent (34). Additionally, deletion of codon 1313 also yielded an attenuated and temperature-sensitive vaccine candidate of hRSV (35). We show here that a single point mutation (I1012F) in CRIV of VHSV increases virulence in nonpermissive cells. We have not explored if the mutation has an impact on transcription and/or replication, but a single mutation in CRIV (L992F) of Sendai virus resulted in better replication than transcription (33). CRIV constitutes a part of the ring domain that creates a cage structure that is likely involved in the spatial coordination of template entry and product exit (28). Mutations in this region of the L protein of NNS RNA viruses could thus potentially influence the functionality of viral transcription or replication, but additional studies are obviously needed to elucidate the mechanisms involved. Finally, I1012F mutant rJF-09 does not replicate to levels comparable to those of DK-3592B, but 5.6-fold higher replication levels than WT JF-09 at 48 h postinfection resulted in loss of GEC viability over 72 h of incubation and production of viral progeny. Thus, the I1012F variant has an intermediate virulence between those of highly virulent trout isolates and nonvirulent marine strains of VHSV.

The importance of the G protein in VHSV virulence has been subjected to a lot of research, motivated partly by the fact that the G protein is involved in virus attachment and entry (25). However, it has not been possible to link particular residues or motifs of the G protein to virulence. In a recent study, it was shown that when the entire G protein of a nonvirulent marine VHSV isolates was used to create a $VHSV_G/IHNV_{NPMLNv}$ hybrid (infectious hematopoietic necrosis virus [IHNV] backbone), this hybrid virus attained full virulence to rainbow trout (14). We also could not identify any residue of the G protein that explains the differences in virulence between high- and low-virulence strains. All together, these findings strongly indicate that the G protein plays lesser of a role in the virulence of VHSV than has been discussed earlier.

We found that infected GECs show strong upregulation of Mx protein expression. At an MOI of <10, we did not see induction of a CPE and the virus could not be rescued from cell cultures. In contrast, when a high infectious dose level was used (MOI of 15), a CPE was observed but it was less extensive than that caused by the high-virulence strains. At such a high MOI, more than 99.95% of the cells will be infected with more than one virus particle (Poisson distribution), while at an MOI of 5, this fraction drops to 95.96%, and at an MOI of 0.15, only 13.93% of the cells (total) will be infected. This shows that low-pathogenicity strains rely on a high number of cells being infected with more than one virus particle for successful infection to take place. The fact that low-virulence strains induce Mx protein expression (and also interferon I; not shown) is in contrast to what has been found in previous *in vivo* studies (36), where low-virulence strains were not found to induce interferon and Mx responses in infected fish. These observations were obtained after an *in vivo* challenge of rainbow trout, and the authors also discussed the possibility that the nonvirulent strains did not pass across primary barriers (skin, gills, gut) and thus the lack of innate immune responses reflects a lack of infection and a lack of virus replication (36).

Findings herein corroborate and extend results from a previous study showing that high-virulence strains of VHSV translo-

cate across GECs, in contrast to their low-virulence counterparts. Translocation occurs without disruption of cellular tight junctions, documented by TER measurements in wells infected with DK-3592B, showing that epithelial tight junction was still intact and higher than $1.0\times 10^3\ \Omega/cm^2$ (15, 19, 24) during the experimental period. Interestingly, the abilities to replicate and induce cytopathic responses in nonpolarized cultures of GECs correlate with translocation abilities. These findings are in contrast to what has been seen in a permanent rainbow trout GEC line (RTgill-W1), where the VHSV IVa and IVb strains, both of which are nonpathogenic to rainbow trout, were able to replicate, cause a CPE, and produce infectious progeny (37), and no conclusion could be drawn with regard to pathogenicity on the basis of *in vitro* replication abilities, further substantiating the suitability of the GEC model.

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Specific nucleotides at the 3′-terminal promoter of viral hemorrhagic septicemia virus are important for virulence in vitro and in vivo

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ABSTRACT

Viral hemorrhagic septicemia virus (VHSV), a member of the *Novirhabdovirus* genus, contains an 11-nucleotide conserved sequence at the terminal 3′- and 5′-untranslated regions (UTRs) that are complementary. To study the importance of nucleotides in the 3′-UTR of VHSV for replication of novirhabdoviruses, we performed site-directed mutagenesis of selected residues at the 3′-terminus and generated mutant viruses using a reverse genetics approach. Assessment of growth kinetics and in vitro real-time cytopathogenicity studies showed that the order of two nucleotides (A4G5) of the 3′-terminus of VHSV directly affects growth kinetics in vitro. The mutant A4G-G5A virus has reduced total positive-strand RNA synthesis efficiency (51% of wild-type) at 48 h post-transfection and 70 h delay in causing complete cytopathic effect in susceptible fish cells, as compared to the WT-VHSV. Furthermore, when the A4G-G5A virus was used to challenge zebrafish, it exhibited reduced pathogenicity (54% lower end-point mortality) compared to the WT-VHSV. From these studies, we infer that specific residues in the 3′-UTR of VHSV have a promoter function and are essential to modulate the virulence in cells and pathogenicity in fish.

Introduction

Viral hemorrhagic septicemia virus (VHSV) of the genus *Novirhabdovirus* and the family *Rhabdoviridae* belongs to the order *Mononegavirales* (King et al., 2012). It has a linear non-segmented negative strand (NNS) RNA genome of 11 kb which contains the nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), RNA polymerase (L), and nonstructural NV gene (Schutze et al., 1999). The VHSV genome contains an 11 nucleotide-long, conserved sequences at the 3′- and 5′-termini that are unusual for high A/U content and complementary (Whelan et al., 2010). The terminal conserved and complementary sequences at the 3′- and 5′-termini are also found in other members of the order *Mononegavirales* (family *Rhabdoviridae*, *Filoviridae*, and *Paramyxoviridae*) (King et al., 2012). Based on vesicular stomatitis virus (VSV, the genus *Vesiculovirus* in the family *Rhabdoviridae*), it has been shown that the initiation of transcription and replication likely occurs at two different sites in the 3′-region of the virus (Whelan and Wertz, 2002; Chuang and Perrault, 1997;Qanungo et al., 2004). Furthermore, two polymerase complexes, one containing L and P and the other replicase complex containing N, P and L were proposed to control transcription and replication in VSV (Qanungo et al., 2004) but it is still unclear how the polymerase complexes are initiated at the 3′-terminus versus the N gene start (Galloway and Wertz, 2009). It is believed that the intracellular concentration of N that encapsidate the nascent leader RNA is a regulator for RNA-dependent-RNA polymerase (RdRp) activity switching from transcription to replication (Blumberg and Kolakofsky, 1981; Blumberg et al., 1983;Whelan et al., 2010).

In the family *Paramyxoviridae* and *Rhabdoviridae* (lyssavirus, rabies virus, and vesiculovirus), the terminal promoter regions have been shown to be multifunctional and not only are required for control of transcription and replication but also for encapsidation and assembly of newly synthesized RNAs (Whelan et al., 2010). However, no such information exists for the novirhabdoviruses and the underlying mechanisms governing the switch from transcription to replication mode have never been examined for the novirhabdoviruses. Our notion was that the conserved primary sequence which has A/U-rich content at the 3′- untranslated region (UTR) terminus may function as a potential promoter that could impact the transcription or/and replication of the virus (Grinnell and Wagner, 1984). We found that the 3′-UTR terminus nucleotides are 3′-C1AUA4G5UA7U8UUU11 and while C1AUA4 and U8UUU11 are complementary with the 5′-terminus, G5UA7 is not. The in silico secondary RNA structure is similar to the panhandle secondary RNA structure of influenza virus (*Orthomyxoviridae*) (Baudin et al., 1994) but there are no documentation in the literature that a panhandle structure is formed in novirhabdoviruses. We used this information as a guide and mutated the positions defined at the end and beginning of complimentarity (position 4 and 8 from the 3′ end) and beginning of non-complimentarity (positions 5 and 7), with the purpose to understand the importance of the 3′-terminal primary sequence for virus transcription and

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replication using site-directed mutagenesis and reverse genetics. The resulting mutant VHSVs were characterized by strand-specific quantitative RT-PCR (Matzinger et al., 2013;Purcell et al., 2006), growth kinetics, in vitro real-time cytopathogenicity, and challenge studies in zebrafish to assess the role of 3′- terminal specific sequence (A4G5) in replication and pathogenicity of the virus.

Results

Reduced RNA synthesis process rate of A4G-G5A corresponds with lower protein synthesis

To compare the viral protein expression level of VHSV mutants to that of rWT-VHSV (1st passage), the virus-specific N protein was detected in *Epithelioma papulosum cyprini* (EPC) cells by the indirect fluorescent antibody test (IFAT) (Fig. 1). The A4G-G5A variant exhibited reduced infectivity as evidenced by the reduced fluorescence (if any), whereas the other variants (A7C-U8A and U8C) yielded strong fluorescence which was comparable to rWT-VHSV (Fig. 1). The rescued viral titer of A4G-G5A variant (from supernatant) was 3 logs less than other variants and WT-VHSV control at 5 days post-transfection (not shown). To clarify the reason of the low infectivity of the A4G-G5A, we examined the efficiencies of RNA synthesis processes at 2 days post-transfection by using a strand-specific quantitative RT-PCR (ssqRT-PCR) to separately quantify the positive strand RNAs (mRNA and cRNA) and negative-strand RNA (viral RNA) at early time-points post-transfection. We anticipated that the reverse primer (−) would favor mRNA (+) and cRNA (+) levels while the forward primer (+) will favor negative-strand RNA expressions. We found that A4G-G5A variant had significant decrease of total positive-strand (51%) at 2 days post-transfection compared to a non-mutated control (rWT), while there was no difference indicating lower level of virus negative-strand (Fig. 2a). ssq RT-PCR will not differentiate between mRNA and cRNA and the CMV promoter will produce cRNA from the antigenome plasmid which may account for a portion of the measured RNA (+) by ssqRT-PCR. The relative contribution was shown to be minimal (< 1%) of the total cRNA using a control without pL helper plasmid (Fig. 2b). These findings are in favor of a reduction in cRNA synthesis in the A4G-G5A variant. By western blot viral proteins generated from mRNA (G and NV) are low at 2 days post-transfection (Fig. 3b) while present at 5 days post-transfection, expect for A4G-G5A where G protein is hardly discernible (Fig. 3b). Post-infection using rescued A4G-G5A showed delayed expression of N and P protein compared to WT-VHSV (Fig. 3a). Concurrently it took an additional 24 h for this variant to reach a viral titer comparable to WT-VHSV (Fig. 4).

3′-terminal mutations impact growth kinetics that corresponds with cytopathic effects of recombinant VHSV strains in vitro

The rescued recombinant VHSV strains (2nd passage) were then used to study infection kinetics in EPC (MOI = 0.1). A4G-G5A had a slower growth pattern (in conformity with what was shown for the viral protein expression; Fig. 3). We observed that A4G-G5A virus grew even slower with no increase in titer by 2 days post-infection, whereas the final titer was comparable to the WT virus (Fig. 4). These studies indicate that the A4G-G5A virus has delayed growth during early stages of infection.

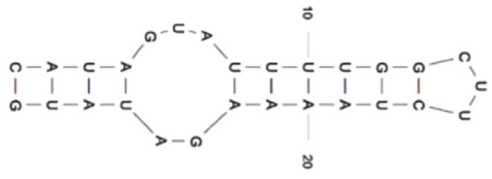
To study the cell viability post-infection in real-time, we used the XCELLigence (Roche) system, which shows that U8C virus induced a drop in cell viability 4 h earlier than the WT-VHSV (Fig. 5a). For A7C-U8A virus, the loss of cell viability was delayed by 30 h and for A4G-G5A virus, the delay was 70 h, when compared to WT-VHSV (Fig. 5a).

Table 2
List of primers used for the point mutagenesis and quantitative RT-PCR.

Point mutation at 3′-terminal promoter[a]		Gene bank
A4G-G5A-Forward	gtcagatccgctagcgta CT ataaaagatgatgagttatgttac	
A4G-G5A-Reverse	gtaacataactcatcatcttttat AG tacgctagcggatctgac	
A7C-U8A-Forward	gtcagatccgctagcgtatca GT aaagatgatgagttatgttac	
A7C-U8A-Reverse	gtaacataactcatcatcttt AC tgatacgctagcggatctgac	
U8C-Forward	gtcagatccgctagcgtatcat G aaagatgatgagttatgttac	
U8C-Reverse	gtaacataactcatcatcttt C atgatacgctagcggatctgac	
3′-UTR-Forward	cattgacgcaaatgggcggta	
3′-RACE-Reverse	atccacgatcaccttcccatcatctg	
Strand-specific quantitative RT PCR		
VHSV-G-Forward	ggacacatgatcacagggtg	
VHSV-G-Reverse	gacagtttcttcgctccccc	
Carp-40s-Forward	ccgtgggtgacatcgttaca	AB012087
Carp-40s-Reverse	tcaggacattgaacctcactgtct	
Virus replication analysis (in zebrafish)		
VHSV-N-Forward	cgccatcatgatgagtcggatgctg	
VHSV-N-Reverse	cttctctgtcaccttgatcccctcc	
Zf-β-actin-Forward	atggatgaggaaatcgctg	AF025305
Zf-β-actin-Reverse	atgccaaccatcactccctg	

[a] Target nucleotides are in capital letter and red color.

Table 1
List of terminal RNA sequences of VHSVs and an in silico secondary RNA structure.

	RNA sequence[a]		Gene bank
Fil3 (genotype Ia)	3′-CAUAGUAUUUUCUA	UAAUAAAAGAUAUG_-5′	Y18263.1
23–27 (genotype Ia)	3′-CAUAGUAUUUUCUA	UAAUAAAAGAUAUG_-5′	FN665788.1
MI03GL (genotype IVb)	3′-CAUAGUAUUUUAUA	UAAUAAAAGAUAUGU-5′	GQ385941.1
JF00Ehi1 (genotype IVa)	3′-CAUAGUAUUUUCUA	UAGUAAAAGAUAUG_-5′	AB490792.1
KRRV9822 (genotype IVa)	3′-CAUAGUAUUUUAUA	UAAUAAAAGAUAUG_-5′	AB179621.1
JF-09 (genotype IVa)	3′-CAUAGUAUUUUCUA	UAGUAAAAGAUAUG_-5′	
Conserved sequence	3′-CAUAGUAUUUU_UA	UA_UAAAAGAUAUG_-5′	
Complementary nucleotides		G^5UA^7	
	3′-CAUA[4]	U^8UUU	
	5′-GUAU	A AAA	
		A G	
in silico secondary RNA structure ($\Delta G = -8.43$)[b]			

[a] Different nucleotides among the VHSV RNA sequences are in italics and red color.
[b] The in silico secondary RNA structure was created by 'Mfold' web server.

Relative rate of virus replication of A4G-G5A virus was drastically lower (5%), compared to WT-VHSV at 72 h post-infection (Fig. 5b). Significant difference in cytopathic effect (CPE) was detected at 72 h post-infection between EPC cells infected with A4G-G5A and WT-VHSV (MOI=1) (Fig. 6).

All variants (A4G-G5A, A7C-U8A, and U8C) were sequenced by RACE with a GSP, 3′-RACE-Reverse (Table. 2), for the 3′-terminal nucleotides followed by a previously described method (Kim et al., 2014) with the purpose to ensure that the introduced mutation was still present following four passages in EPC cells. No mutation or reversion to wild-type was observed.

Pathogenicity studies in fish

To study if the observed attenuation would also impact in vivo virus virulence, we infected zebrafish with these viruses. Since it has been shown that VHSV is pathogenic to zebrafish after lowering of the water temperatures, we compared virulence of WT-VHSV and the A4G-G5A variant. Fish injected i.m. with 4×10^3 $TCID_{50}$ per fish (WT-VHSV) showed close to 90% cumulative percent mortality (CPM) over a period of 15 days post-challenge in zebrafish (Fig. 7a). When fish were injected i.p., the CPM was 83.3%. When the injection dose was lowered to 4×10^2 $TCID_{50}$ per fish (WT-VHSV) by i.m. and i.p., both gave 72.2% CPM. Injection of 4×10^3 and 4×10^2 $TCID_{50}$ per fish of A4G-G5A, i.p. resulted in 37.0% and 18.5% CPM, respectively (Fig. 7a), lowered by more than 50% compared to the WT-VHSV. There was also a delay in onset of mortality by almost 7 days (Fig. 7a). When the dose was lowered to 4×10 $TCID_{50}$ per fish for the A4G-G5A strain, no mortality was seen over a period 15 days post-challenge. All dead fish had signs of typical VHSV infection, hemorrhagic septicemia, ascites, and exophthalmia. VHSV was detected and confirmed from randomly sampled dead fish by cell culture and RT-PCR. A4G-G5A was detected at early time post-infection by RT-PCR only in fish infected with high-doses (Fig. 7b).

Discussion

Here we show that the primary sequence at 3′-terminus of VHSV is important for production of virus progeny in cell culture and pathogenicity in zebrafish. It has been shown that VSV, a prototype of the family *Rhabdoviridae*, has conserved complementary sequence of 3′- and 5′-termini which have been studied to understand RNA synthesis processes (Whelan et al., 2010). A single entry model predicts that a single polymerase entry site is located at the extreme 3′-terminus to make mRNA or copy viral RNA genome (Emerson, 1982). Later studies have shown that there are different sites initiating transcription and replication in the 3′-UTR of VSV (Whelan and Wertz, 2002;Chuang and Perrault, 1997; Qanungo et al., 2004). In NNS RNA viruses, the gene order is conserved with N, P, and M genes near the 3′-terminus and L gene at the 5′-terminus. The 3′-terminus promoter sequence seems a major determinant for control of gene expression in the viral life cycle (Whelan et al., 2010). The 3′- and 5′-termini of NNS RNA viruses contain specific sequences required for encapsidation of the RNA strand, binding of the RdRp, leader synthesis, transcription, replication, and assembly/budding of viral particles (Whelan et al., 2010). For VSV, positions 47–50 of the 3′-UTR leader-N gene junction were found essential for transcription and positions 15–50 at the terminus were dispensable for replication (Whelan and Wertz, 1999). Another study indicated that the signals for both transcription and replication for VSV were contained and overlapped within positions 1–24 at the 3′-terminus and positions 25–47 were needed for optimal transcription (Li and Pattnaik, 1999). There are no previous studies addressing the involvement of the terminal UTRs of novirhabdoviruses. Here we aimed at elucidating the importance of some positions of the primary sequence of the 3′-terminus of VHSV for virus propogation in vitro and in vivo pathogenicity using an in silico approach (Table 1). To differentiate between the positive-strand RNA (mRNA or cRNA) and negative-strand RNA (vRNA) formation, we used a previously published the ssq RT-PCR method (Matzinger et al., 2013;Purcell et al., 2006). The A4G-G5A in 3′-terminus had a significant negative effect (51% of rWT) on total positive-strand RNA synthesis (mRNA or cRNA) at 2 days post-transfection (Fig. 2). However, we cannot clearly differentiate between mRNA (transcription) and cRNA (replication) synthesis by ssqRT-PCR although the western blot studies are indicative of lower protein synthesis in the attenuated variant (A4G-G5A). We would need to follow up using an RNA assay (like Northern blotting) to differentiate between transcription and replication. Furthermore, A4G-G5A showed delayed expression of N and P protein post-infection compared to WT-VHSV (Fig. 3) and it took 5 days for this variant to reach a viral titer ($10^{7.55}$ $TCID_{50}$/ml) comparable to WT-VHSV ($10^{7.88}$ $TCID_{50}$/ml) which plateaued at 4 days post-infection, likely due to running out of available cells to infect (Fig. 4). Concurrent with this observation, the A4G-G5A variant also induced full CPE 70 h later than WT-VHSV (Fig. 5a).

As mentioned earlier, previous studies are indicative of transcription and replication being initiated at different sites for VSV, transcription initiated at the start of the N-gene (Whelan and Wertz, 2002; Chuang and Perrault, 1997;Qanungo et al., 2004). Moreover, there is an alternative possibility that RdRp always enters at the 3′-terminus (scanning) but the transcription is initiated at the start of the N-gene regardless of the 3′-terminus specific sequence (Whelan et al., 2010). The template-dependence of NNS RNA viruses for the RNA synthesis initiation is found to vary. Initiation of VSV is dependent on the first two nucleotides at 3′-terminus while for respiratory syncytial virus (RSV, *Paramyxoviridae*) initiation is independent of the first two nucleotides on the promoter terminus (Morin et al., 2012;Noton and Fearns, 2011). The transcription of RSV polymerase could be initiated at the 3rd nucleotide of the 3′-terminal promoter unlike that of VSV (Tremaglio et al., 2013) and the RSV polymerase–promoter interactions display sophisticated mechanisms for the viral promoter activity (Noton et al., 2012;Tremaglio et al., 2013).

Our findings would be suggestive of the initiation of replication is affected by the primary sequence near the 3′-terminus for VHSV and positions 4 and 5 play a key role like that of VSV (Whelan and Wertz, 2002;Chuang and Perrault, 1997;Qanungo et al., 2004;Whelan and Wertz, 2002) and these mutation were found genetically stable. While it is still not fully understood how the 3′-terminal sequences regulate transcription or/and replication efficiency for NNS RNA viruses in general, we are just beginning to understand how the 3′-terminus regulates these mechanisms for VHSV. This study showed that the primary sequence is an important promoter but it is not sufficient to document the importance of any secondary RNA structure (like double strand RNA promoter). The RNA synthesis profile should be examined to distinguish between effects of primary sequence versus secondary structure.

The introduced mutations at the 3′-terminus of VHSV affect formation of virus progeny in vitro and this is also corroborated by in vivo studies. We used the zebrafish model to study the impact of mutations on pathogenicity. It has been shown previously that VHSV is pathogenic to adult zebrafish (Encinas et al., 2010; Novoa et al., 2006) when the water temperature is lowered to 18 °C and below. The attenuation for the A4G-G5A strain is consistent over two virus doses, 4×10^2–4×10^3 $TCID_{50}$ per fish, and for the two doses tested there is a marked drop in end-point mortality compared to wild type virus (Fig. 7). Injection of 4×10 $TCID_{50}$ per fish of the A4G-G5A variant does not result in mortality (Fig. 7).

In conclusion this is the first study to show the importance of some positions (A4G5) of the primary sequence at the 3′-terminus as a potential promoter that governs progeny formation in VHSV, the genus *Novirhabdovirus*, in vitro. Similarly it is important for in vivo virulence in zebrafish (Fig. 7).

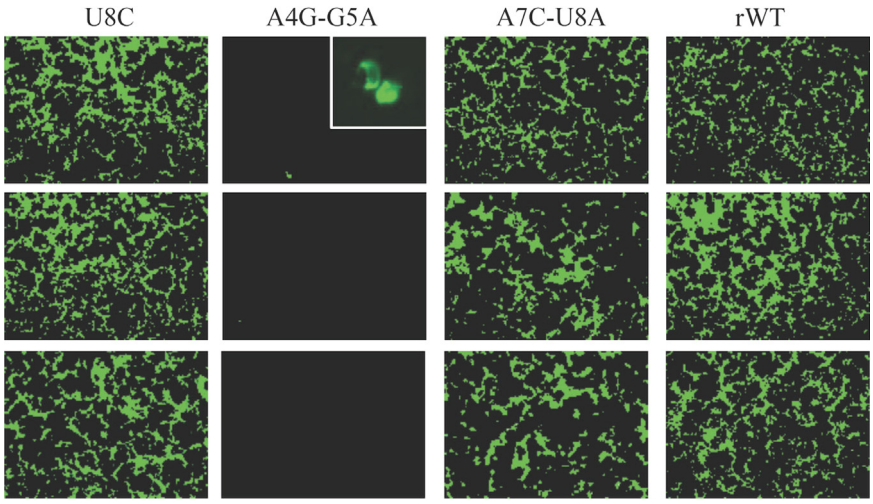


Fig. 1. Viral N protein expression of rescued recombinant VHSVs (1st passage; green fluorescence) in EPC cells (3 parallels) were shown by IFAT at 2 days post-infection. For the A4G-G5A variant, only a few cells showed positive fluorescence (shown in insert). A non-mutated recombinant VHSV (rWT) was used as a control.

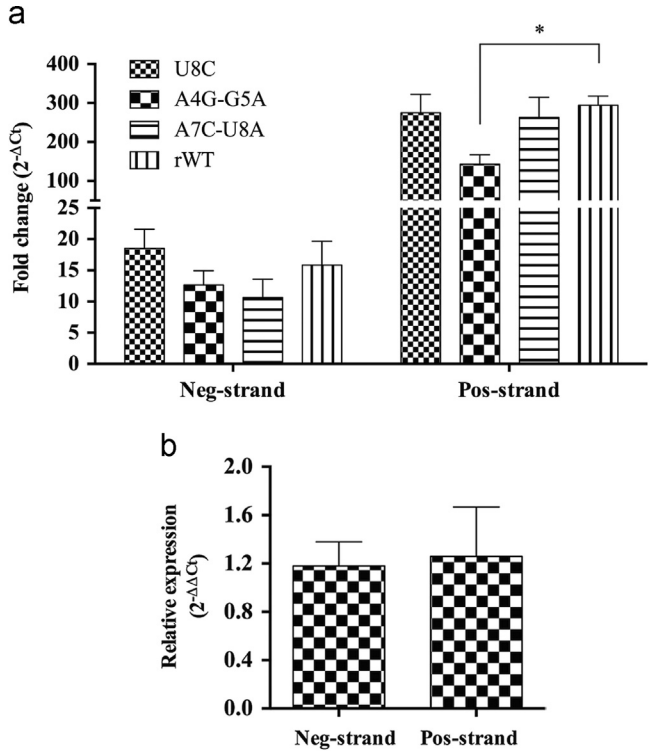


Fig. 2. Relative RNA quantification using strand-specific quantitative RT-PCR. a) Relative quantification of RNA using VHSV-G-Forward/Reverse of different mutants relative to a non-mutated control plasmid (rWT) at 2 days post-transfection. Negative- and positive-strand RNA quantification of mutated plasmids (A4G-G5A, A7C-U8A, and U8C). The results are expressed as mean ± SEM; $n=3$. An asterisk (*) denotes significant reduction ($P<0.05$) compared to a control reference (rWT). b) Relative quantification of A4G-G5A excluding pL (plasmid encoding the polymerase) showing background cRNA generation from plasmid (0.8% of pos-strand for A4G-G5A in a).

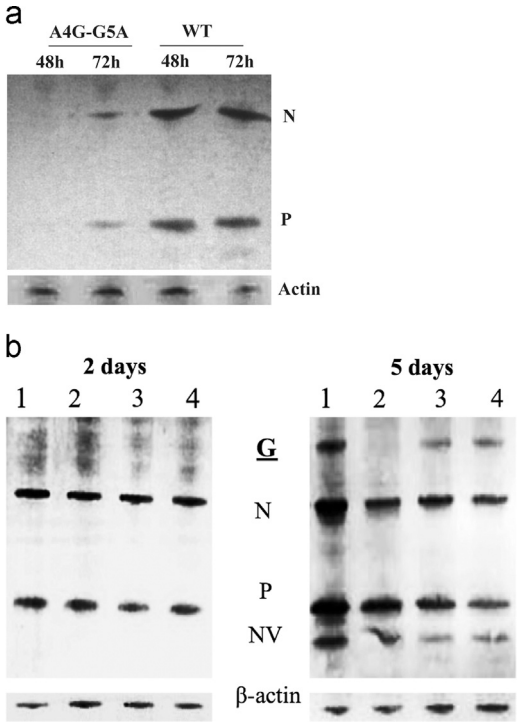


Fig. 3. Kinetics of viral protein expression of strain A4G-G5A. a) Viral specific proteins (Nucleocapsid protein, 42 kDa and Phosphoprotein, 26 kDa) of strain A4G-G5A and WT-VHSV in cell lysates were detected by western blotting post-infection of EPC cells (MOI=1), 48 and 72 h post-infection. b) Expression of VHSV proteins post-transfection by western blot using polyclonal antibody at 2 and 5 days post-transfection (lane 1: U8C, lane 2: A4G-G5A, lane 3: A7C-U8A, and lane 4: WT-VHSV).

Materials and methods

Virus and cells

VHSV (JF-09) was isolated from VHSV-infected olive flounder (*Plathychtis japonicus*) (juvenile) in a fish farm located in South Korea (June 2009) (Kim et al., 2014). The virus was propagated in *Epithelioma papulosum cyprini* (EPC) cells in L15 cell medium (Invitrogen) containing 10% FBS (Sigma-Aldrich) at 15 °C.

Site-directed mutagenesis

VHSV has conserved complementary RNA sequence in 3′-end (3′-CAUAGUAUUUU) and 5′-end (UAAAAGAUAUG-5′) (Table 1). By "introducing" a RNA hairpin loop, 3′-GGCUUC-5′, between conserved 3′- and 5′- terminal sequences, it was possible to predict an in silico secondary RNA structure by 'Mfold' web server (Table 1) (Zuker, 2003;Cheong et al., 1996, 1990;Varaniet al., 1991). Using the in silico secondary RNA structure as a guide, point-mutations were introduced mutations in positions A4G5 and A7U8 by site-directed mutagenesis (GENEART®, Invitrogen), referred to as A4G-G5A, A7C-U8A, and U8C. A previously constructed plasmid of

complete genome (rJF-09) (Kim et al., 2014) was used for this purpose. The point-mutated sequences were confirmed by sequencing (GATC Biotech) with GSPs (3′-UTR-Forward, Table 2).

Transfection and characterization of virus infection by IFAT

VHSV plasmids (250 ng), pN (60 ng), pP (50 ng), pL (50 ng), and pNV (30 ng) were mixed in 25 μl Opti-MEM® medium (Invitrogen) and 1.5 μl of FuGENE HD transfection reagent (Roche) was added. The mixtures were incubated for 10 min at room temperature and added to sub-confluent layers of EPC cells (1×10^5) in a 24-well plate. The cells were incubated at 28 °C for 5 h and then transferred to 15 °C and incubated further for desired time points or till cytopathic effects (CPE) was evident. The cell culture supernatants were collected at 4 days post-transfection, clarified by centrifugation, 10-fold diluted and inoculated onto EPC cells (2×10^5) in a 24-well plate. The cells were incubated at 15 °C for 48 h and fixed with of 4% paraformaldehyde for 20 min. The rescued viral N proteins were identified by using the standard indirect fluorescent antibody test (IFAT), using MAb IP5B11 as the primary antibody and Alexa 488 anti-mouse IgG (Molecular Probes, Invitrogen) as secondary antibody. The stained cells were washed and examined under an Olympus IX81 fluorescence microscope.

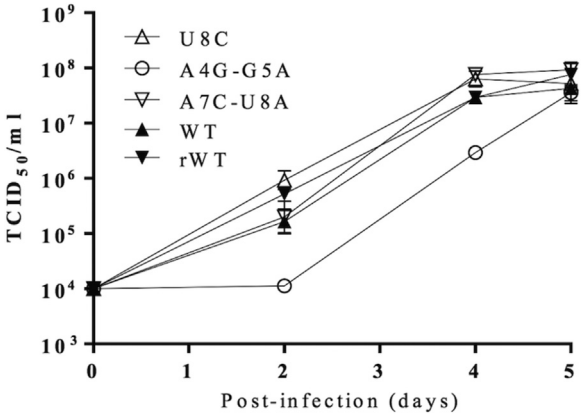


Fig. 4. In vitro viral growth pattern following infection of EPC cells (MOI=0.1). A non-mutated recombinant VHSV (rWT) and wild-type VHSV (WT) were used as a control. The titer values are presented as mean ± SEM from three parallels.

Strand-specific quantitative RT-PCR

Strand-specific quantitative RT-PCR (ssqRT-PCR) assay based on a published method (Matzinger et al., 2013;Troutt et al., 1992; Purcell et al., 2006) was modified for our study. VHSV plasmids (250 ng), pN (60 ng), pP (50 ng), pL (50 ng), and pNV (30 ng) were mixed in 25 μl Opti-MEM® medium (Invitrogen) and 1.5 μl of FuGENE HD transfection reagent (Roche) was added. The mixtures were incubated for 10 min at room temperature and added on sub-confluent layers of EPC cells (1×10^5) in a 24-well plate. The cells were incubated at 28 °C for 5 h and shifted to 15 °C. At 2 days post-transfection, the cells were harvested and total RNA were extracted from the cells by using a RNeasy® Plus Mini kit (Qiagen). To eliminate background plasmid DNA contamination prior to reverse transcription, the samples were DNase treated twice (TURBO DNA-free™ kit, Ambion). 11 μl of DNase treated total RNA was used as a template with a single GSP (0.5 μM), VHSV-G-Forward or VHSV-G-Reverse targeting G protein gene, in a final volume of 20 μl to create strand-specific cDNA templates (Transcriptor First Strand cDNA Synthesis kit, Roche). Quantitative RT-PCR was carried out by using LightCycler 480 SYBR green I master mix and LightCycler 480

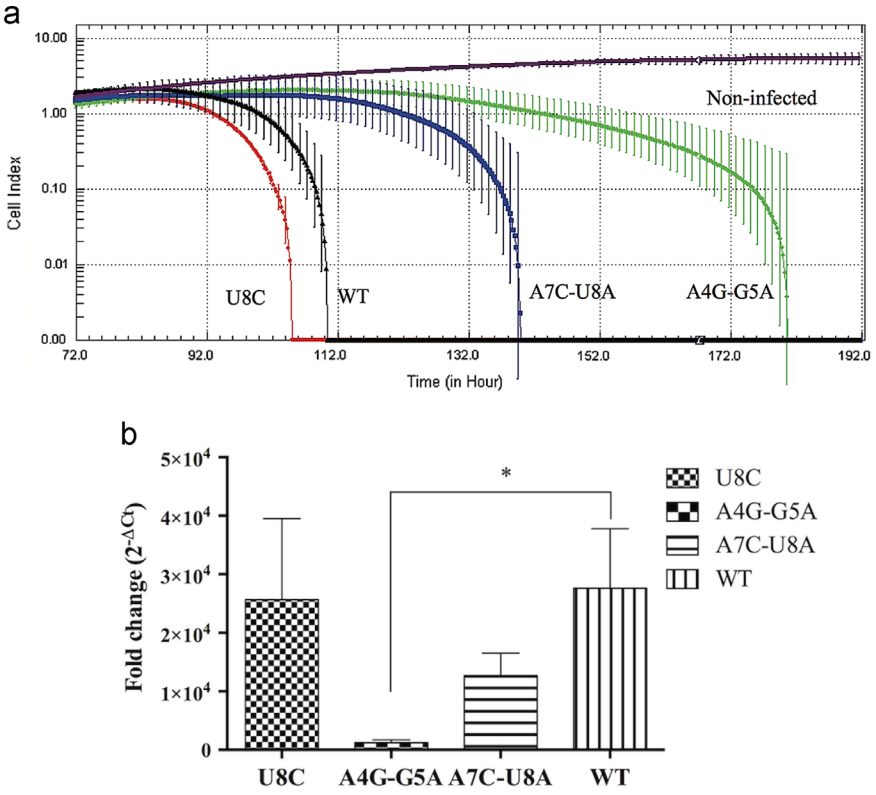


Fig. 5. Real-time cytopathic effects (CPE) of EPC cells after infection with recombinant VHSV strains (MOI=0.01). a) CPE was monitored in real-time by use of xCELLigence (see *Material and methods*). The results are expressed as mean ± SD ($n=3$). The first time point shown is 72 h post-infection since this was the earliest occurrence of reduced cell index (CI). b) Viral quantification in infected EPC cells (MOI=0.01) at 72 h post-infection by quantitative RT-PCR. The results are expressed as mean ± SEM ($n=3$). Wild-type VHSV (WT) was used as a reference. An asterisk (*) denotes significant reduction ($P<0.05$) compared to WT.

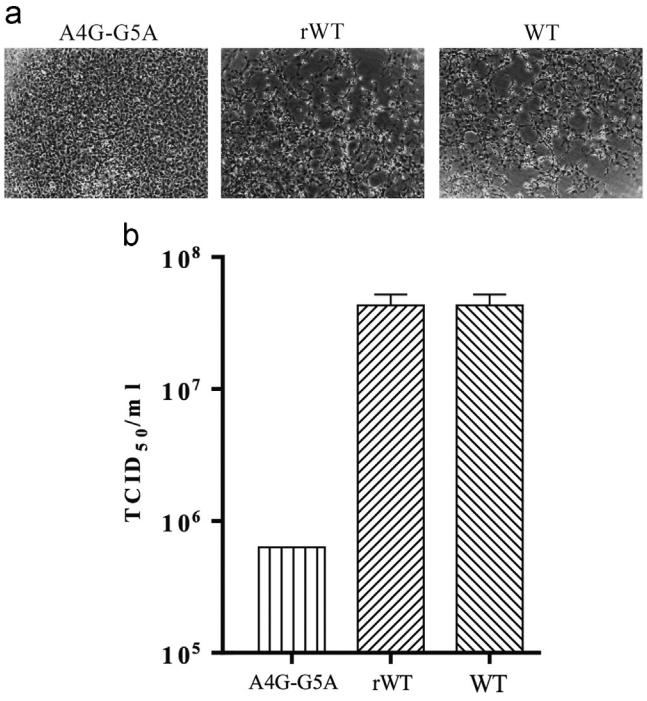


Fig. 6. a) Cytopathic effects (CPE) in EPC cells 72 h post-infection (MOI=1). b) The viral titer 72 h post-infection (mean ± SEM; n=3 parallels). A non-mutated recombinant VHSV (rWT) and wild-type VHSV (WT) were used as controls.

system (Roche). 2 µl of the strand-specific cDNA templates were used with a pair of GSPs (0.5 µM), VHSV-G-Forward and VHSV-G-Reverse, in a final volume of 20 µl. The mixtures were first incubated at 95 °C for 10 min, followed by 40 amplification cycles: 10 s at 95 °C, 20 s at 60 °C, and 8 s at 72 °C. Random hexamer primers (Transcriptor First Strand cDNA Synthesis kit, Roche) were used for cDNA templates for gene expression of carp 40 s, which is a reference gene to normalize the data (Joerink et al., 2006). The GSPs used for strand-specific quantitative RT-PCR are listed in Table 2. To compare the impact of pCMV driven cRNA synthesis from the plasmid relative to viral cRNA (polymerase), the A4G-G5A and rWT plasmids were transfected into the EPC cells without the pL helper plasmid followed by ssq RT-PCR.

Western blotting

EPC cells infected by A4G-G5A (2nd passage) and WT-VHSV (MOI=1) as described above and cells were lysed by using CelLytic M reagent (Sigma), at 48 and 72 h post-infection. Proteins were separated by SDS-PAGE and blotted onto a polyvinylidene difluoride (PVDF) membrane. The VHSV proteins were detected by western blotting using polyclonal antibody against VHSV (Ammayappan and Vakharia, 2011).

Growth kinetics

Growth kinetics was assessed by the $TCID_{50}$ method and the VHSV variants (2nd passage, MOI=0.1) were used to infect EPC cells (1×10^5) in 24-well plates in three parallels at 15 °C. After virus infection, the supernatants were collected at designated time points, clarified by centrifugation and titrated on EPC cells by the 50% tissue culture infective dose ($TCID_{50}$) method (Kärber, 1931).

For monitoring of cytopathic effects in real-time, VHSV variants (MOI=0.01) were used to infect EPC cells (1×10^4) in E-Plates (xCELLigence, Roche) in three parallels at 15 °C. The xCELLigence system showed a parameter termed 'Cell Index' which is derived as a relative change in measured electrical impedance and a correlate of cell status (integrity).

To measure viral replication in the cells by quantitative real-time RT-PCR, EPC cells (1×10^5) in a 24-well plate were infected by VHSVs (MOI=0.01) for the parallel sampling and collection was at 72 h post-infection, a starting point of CPE, at 15 °C. For the viral replication quantification, total RNAs were extracted from infected cells in a 24-well plate by using a RNeasy® Plus Mini kit (Qiagen). Quantitative real-time RT-PCR was carried out by using Quanti-Fast® SYBR® Green RT-PCR (Qiagen) and LightCycler 480 system (Roche). Total RNAs (10 ng) were used as templates with a pair of GSPs (1 µM), VHSV-G, in a final volume of 25 µl. The mixtures were subject to reverse transcription at 50 °C for 10 min, first incubated at 95 °C for 5 min and followed by 40 amplification cycles: 10 s at 95 °C, 30 s at 60 °C. Carp 40 s, which is a reference gene, was used to normalize the data (Joerink et al., 2006). The GSPs used for virus replication are listed in Table 2.

Pathogenicity studies in zebrafish

Wild-type zebrafish (*Danio rerio*) of 0.5–0.8 g (female:male=1:1) were obtained from the zebrafish experimental facility at the Norwegian University of Life Sciences. The fish were gradually transferred to lower water temperatures (1 °C per day) and kept to acclimate for 2 days at 16.5 ± 1 °C before virus infection. The water and feed were provided from the zebrafish facility. For each experiment, zebrafish were moved to closed aquaria (6 L) at 16.5 ± 1 °C and maintained at these conditions by water exchange (2 L) every day. The water quality was monitored by daily measurement of ammonia. The fish were

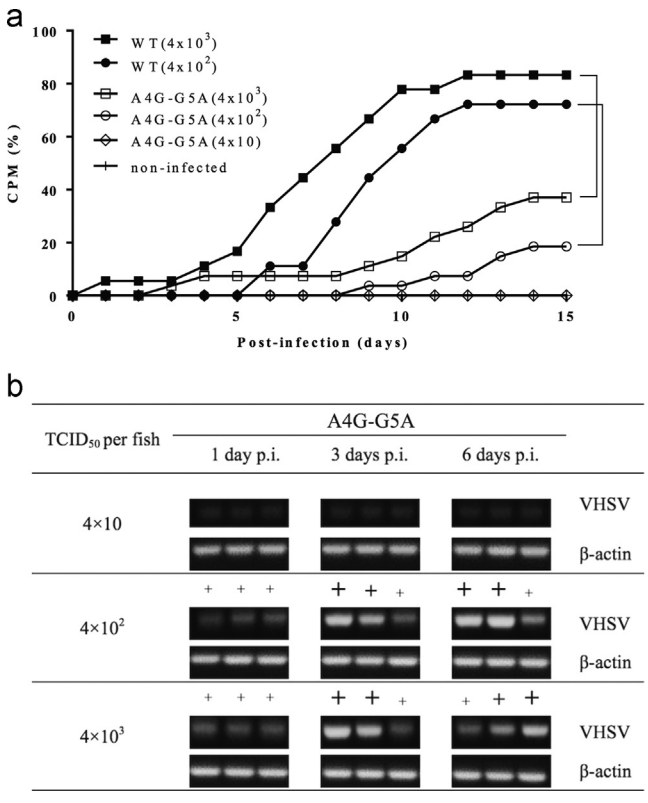


Fig. 7. a) In vivo viral pathogenicity in zebrafish. Cumulative percentage mortality (CPM) 15 days post-infection. Zebrafish were infected by intraperitoneal (i.p.) injection with WT-VHSV (WT), and A4G-G5A at three different doses; 4×10, 4×10^2 and 4×10^3 $TCID_{50}$ per fish. A non-infected control group was injected with 2 µl of L15 medium i.p.. Comparable infection groups are indicated by ']'. b) PCR amplicons (VHSV N gene) in A4G-G5A infected fish at 1, 3, and 6 days post-infection. Infection doses ($TCID_{50}$ per fish) are shown. Strength of bands is indicated semi-quantitatively.

anesthetized with benzocaine and injected intraperitoneally (i.p.) or intramuscularly (i.m.) at two infection doses for WT-VHSV (4×10^2 $TCID_{50}$ per fish or 4×10^3 $TCID_{50}$ per fish; 18 fish per group per dose) in three parallels using an injection volume of 2 μl and the fish injected i.p. at three infection doses for A4G-G5A variant (4×10 $TCID_{50}$, 4×10^2 $TCID_{50}$ or 4×10^3 $TCID_{50}$; all doses per fish; 36 fish per group per dose) in three parallels using an injection volume of 2 μl. Control groups were anesthetized and injected with 2 μl of L15 medium. Randomly selected healthy fish without gross pathology were decapitated and the whole organs were collected for virus detection by RT-PCR at early time post-infection. The selected fish (9 fish per group) were excluded from estimation of cumulative mortality. The GSPs used for virus replication are listed in Table 2.

Data analysis

The comparative delta delta Ct ($2^{-\Delta\Delta Ct}$) method was used to calculate relative difference of the RNA synthesis in this study (Livak and Schmittgen, 2001). Specificity of the PCR products was ensured by checking the melting temperature and profile of each melting curve. A *t* test was used to calculate differences with *P*-value < 0.05 considered as significantly different.

Acknowledgment

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Short communication

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

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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

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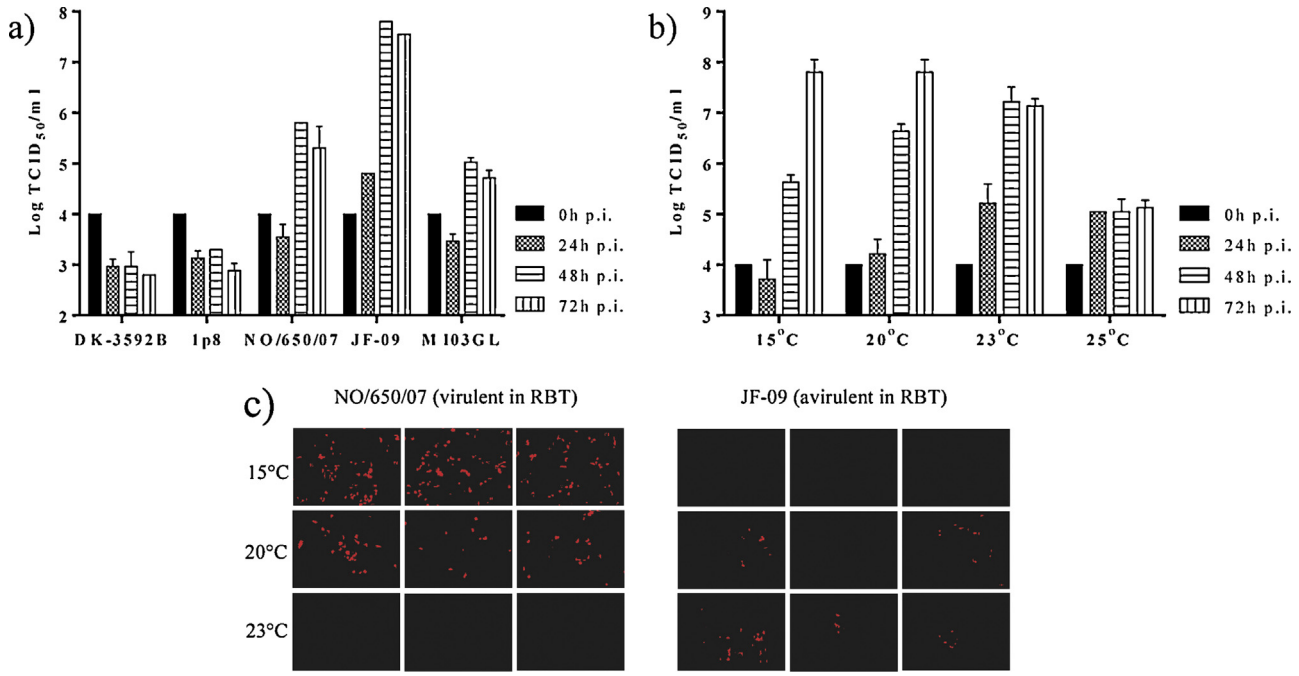

Fig. 1. ***In vitro*** temperature sensitivity of VHSV strains. (a) Different temperature sensitivities for five VHSV strains tested in permissive cells (EPC) at 23 °C. Virus titers are given ($TCID_{50}$/ml) over a 72 h incubation period (MOI = 1). (b) Temperature sensitivity of JF-09 in permissive 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

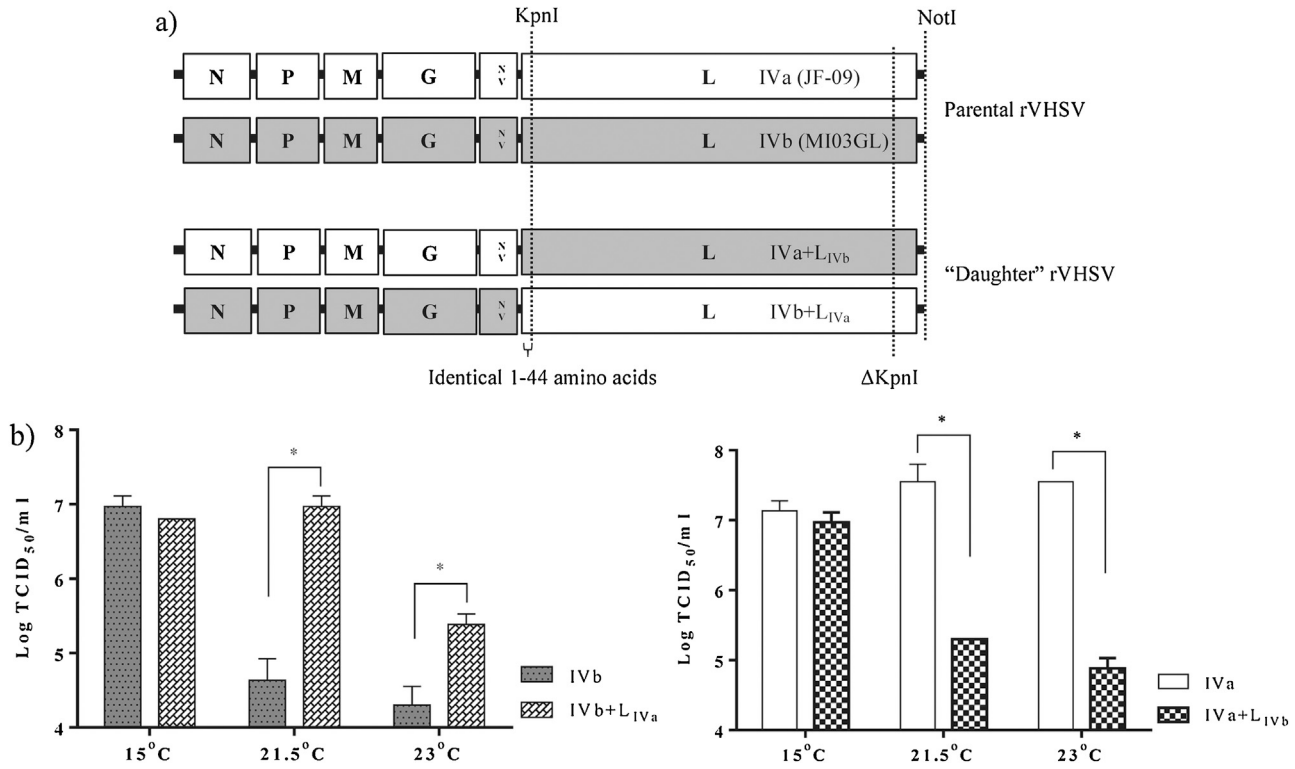

Fig. 2. ***In vitro*** growth of recombinant VHSVs genotype IV a and b strains harboring heterologous L protein. (a) A construction map of recombinant VHSVs harboring heterologous L protein ("daughter" rVHSV). (b) Temperature sensitive of the rVHSVs in permissive cells (EPC) was shown by viral titration ($TCID_{50}$/ml) at 72 h post-infection (MOI = 0.1; 15 °C, 21.5 °C, and 23 °C) for the parental and hybrid strains. The viral titers in supernatants are presented as a mean ± SD from three parallels. *T*-test was used to calculate differences where relevant and a *p*-value < 0.05 was considered statistically significant (*).

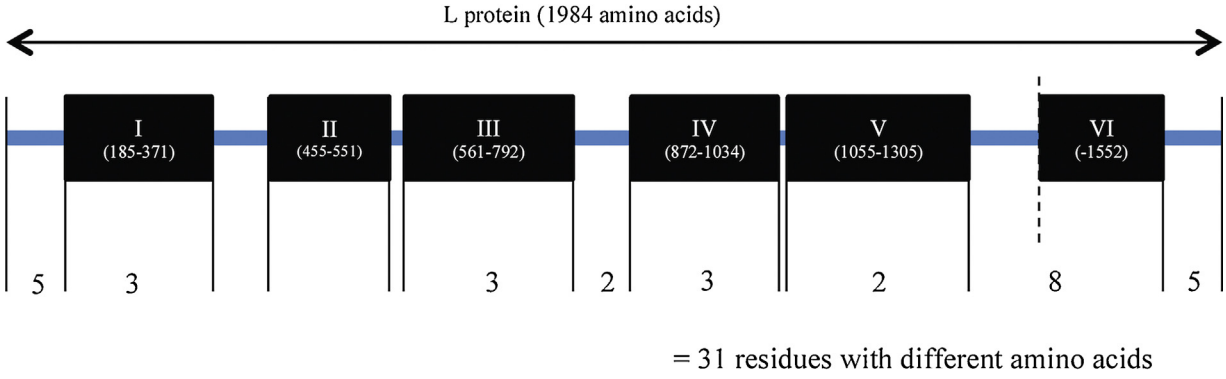

Fig. 3. A schematic linear map of VHSV L protein (1984 amino acids) with the six conserved regions (CR I-VI). 31 residues, which are different between JF-09 (IVa) and MI03GL (IVb), were shown by numbers. An unclear border of N-terminus of CR VI was indicated with the dotted line. The gaps are the areas in-between defined motifs. The positions of CRs were analyzed with the Conserved Domains in NCBI database (pfam00946, pfam14318, and pfam14314), based on previous NNS RNA virus studies (Poch et al., 1990; Ogino and Banerjee, 2011).

this should be addressed in future studies. Finally, the findings reported here underlines the importance of the L protein for virulence of VHSV strains and conforms with a recent study showing that a point mutation of the CR IV region is important for virus virulence *in vitro* (Kim et al., 2014).

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