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Identifying potential virulence determinants in viral haemorrhagic septicaemia virus (VHSV) for rainbow trout

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ABSTRACT: We identified viral haemorrhagic septicaemia virus (VHSV) isolates classified within Genotype Ib which are genetically similar (>99.4% glycoprotein amino acid identity) yet, based on their isolation history, were suspected to differ in virulence in juvenile rainbow trout. The virulence of an isolate recovered in 2000 from a viral haemorrhagic septicaemia disease episode in a marine rainbow trout farm in Sweden (SE-SVA-1033) was evaluated in juvenile rainbow trout via intraperitoneal injection and immersion challenge alongside 3 isolates recovered from wild-caught marine fish (DK-4p37, DK-5e59 and UKMLA98/6HE1) suspected of being of low pathogenicity to trout. Mortality data revealed that isolate SE-SVA-1033 caused VHSV-specific mortality in both intraperitoneal and immersion challenges (75.0 and 15.4%, respectively). The remaining Genotype Ib isolates caused significantly lower mortalities using the same experimental infection routes (<35.0 and <2.0%, respectively). Having identified VHSV isolates with clear differences in their pathogenicity, coding and inter-genic non-coding regions of 2 isolates (SE-SVA-1033 and DK-4p37) were determined and compared in order to identify potential markers responsible for the observed differences in virulence. Only 4 predicted amino acid substitutions were identified across the genome sequenced; these occurred in the N (R46G), G (S113G), NV (L12F) and L (S56A) proteins. These findings form the basis for further studies aimed at determining the biological significance of these mutations and suggest that small changes at the molecular level can cause significant changes in the virulence properties of VHSV isolates.

KEY WORDS: Rainbow trout · VHSV · Pathogenicity · Sequencing · Virulence determinants

INTRODUCTION

Viral haemorrhagic septicaemia virus (VHSV) is the aetiological agent of viral haemorrhagic septicaemia (VHS), a disease responsible for substantial losses in rainbow trout *Oncorhynchus mykiss* culture in continental Europe. VHSV is a rhabdovirus comprised of a single-stranded RNA genome encoding nucleo-(N), phospho-(P), matrix-(M), glyco-(G) and RNA-dependent (L) polymerase structural proteins. Classified within the genus *Novirhabdoviridae*, VHSV also encodes a characteristic non-structural (non-virion, NV) protein, situated between the G and L genes, the functional role of which remains uncertain (Alonso et al. 2004, Thoulouze et al. 2004). Four main genotypes of the virus have been identified which are independent of the genomic region used for analysis (Einer-Jensen et al. 2004) and are restricted to 4 broadly distinct geographical locations (Snow et al. 2004).

Until recently, isolates responsible for disease in rainbow trout in Europe have been exclusively restricted to Genotype I (GI). Isolates responsible for VHS disease outbreaks in rainbow trout farms in continental Europe have been subdivided into GIa, while GIb includes mostly isolates recovered from the Baltic Sea (Snow et al. 2004). Evidence suggests that these viruses were introduced into rainbow trout farming from a GIb-like ancestor and that this process may

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have been facilitated by the practice of feeding rainbow trout unpasteurised marine fish products derived from areas where VHSV was enzootic, a practice that was commonplace in the early days of the industry (for a concise review see Skall et al. 2005a). Since GIb isolates are generally of low virulence in rainbow trout, this introduction and spread appears to be associated with an adaptation to causing disease within the environment of rainbow trout culture (Skall et al. 2004). Fish RNA viruses such as VHSV are characterised by having error-prone polymerases and rapid rates of replication. Coupled to selection pressures, these factors undoubtedly contribute to the relatively higher rates of viral evolution observed within aquaculture as compared to those in the natural environment (Einer-Jensen et al. 2004).

The prevalence of VHSV in wild marine fish appears to be relatively high in the Baltic Sea, particularly around the Danish coast (Skall et al. 2005b). Production of marine farmed rainbow trout in this region has apparently resulted in the more recent introduction and possible adaptation of naturally present marine VHSV viruses in farmed fish. Indeed, outbreaks of VHS have been reported in marine-farmed rainbow trout in Finland, Sweden and, more recently, in Norway (Nordblom 1998, Nordblom & Norell 2000, Einer-Jensen et al. 2004, Raja Halli et al. 2006, Brudeseth et al. 2008). Outbreaks in Finland and Sweden have been attributed to GI isolates and further subdivision of this genotype has been suggested to accommodate an increasing diversity of virus isolates (Einer-Jensen et al. 2004, Snow et al. 2004). Interestingly, virus recovered from the recent Norwegian outbreak has since been characterised as GIII (Brudeseth et al. 2008, Dale et al. 2009), a genotype which includes marine viruses enzootic to the North Sea, East Atlantic, Skagerrak and Kattegat (Skall et al. 2004). This represents the first apparent rainbow trout adaptation of a non-GI VHSV isolate.

The Swedish isolates used in the present study were categorised as GIb isolates (SE-SVA-14 and SE-SVA-1033) based on the deduced nucleotide sequence of the glycoprotein gene (Einer-Jensen et al. 2004). Many genetically similar isolates of low pathogenicity to rainbow trout have been recovered from the Baltic marine environment in recent years. The genetic similarity of these viruses with those responsible for the Swedish outbreaks suggests that adaptation within rainbow trout has happened relatively recently in this instance. This provided a unique opportunity to investigate the underlying genetic determinants of differing virulence in these isolates. The present study thus aimed to characterise differences in pathogenicity between these closely related VHSV isolates belonging to GIb. By sequencing coding and intergenic non-coding regions of the genome between isolates displaying different virulence phenotypes, we exploited a unique opportunity of identifying regions of the viral genome that may influence or determine virulence.

MATERIALS AND METHODS

Fish. Before commencement of the experiment, rainbow trout were tested for a range of pathogens associated with salmonid culture and found to be negative. Fry weighing 4.1 ± 0.3 g (mean ± SE, n = 10) were reared in the Marine Scotland, Marine Laboratory Research Unit in Aultbea, and transported to the Marine Scotland, Marine Laboratory in Aberdeen, where fish were acclimated for 14 d prior to VHSV challenge. Fish were stocked in aerated freshwater tanks (30 l) maintained at 10°C. Prior to initiating the experiment, fish were starved for 24 h, after which they were maintained on a commercial diet.

Selection of candidate VHSV isolates. The selection of isolates was due to their different isolation history and high similarities (>99.4) of the deduced amino acid (aa) sequences of the glycoproteins (GenBank accession nos. AY546623, AY546583, AY546580, AY546631). Information related to the selected isolates is summarised in Table 1.

Cell lines and propagation of virus. Viruses (SE-SVA-1033, DK-5e59, DK-4p37 and UKMLA98/6HE1) were propagated at 15°C on 80% confluent monolayers of a low passage bluegill fry cell line (BF-2, ATCC no. CCL-91) in Eagle's minimum essential media (EMEM; Invitrogen) supplemented with 5% foetal bovine serum (FBS). Once full cytopathic effect (CPE) had been observed, the virus was stored at –80°C and a thawed aliquot titrated on BF-2 cells using the $TCID_{50}$ endpoint method (Reed & Müench 1938, Tafalla et al. 2005).

Experimental infection to assess mortality induced by different isolates of VHSV. To enable an assessment of mortality rates, 24 fish were stocked into each of 20 tanks. Fish in duplicate tanks were intraperitoneally (i.p.) infected with isolates SE-SVA-1033 (Tanks 1 and 2), DK-5e59 (Tanks 3 and 4), DK-4p37 (Tanks 5 and 6), UK MLA98/6HE1 (Tanks 7 and 8) or control media (Tanks 9 and 10) in an inoculum volume of 100 µl, representing a dose of 1.07×10^6 $TCID_{50}$ $fish^{-1}$. Prior to the injection protocol, trout were anaesthetised by immersion in a bath containing methane tricaine sulphonate (MS-222; concentration = 0.1 mg ml^{-1}) for 2 to 3 min, before being injected with either treatment. Fish in duplicate tanks were also exposed to isolates SE-SVA-1033 (Tanks 11 and 12), DK-5e59 (Tanks 13 and 14), DK-4p37 (Tanks 15 and 16), UK-MLA98/6HE1 (Tanks 17 and 18) or control media

Table 1. Viral haemorrhagic septicaemia virus (VHSV) isolates used in the present study. Isolates were chosen due to their genetic similarities and suspected differences in their virulence against rainbow trout based on their isolation history. Source indicates original report

VHSV isolate	Genotype	Year isolated	Region of isolation	Host species	Isolation history	Accession no.	Source
DK-4p37	GIb	1997	North Sea	Blue whiting *Micromesistius poutassou*	Healthy marine fish[a]	AY546580	Snow et al. (1999)
DK-5e59	GIb	1998	Kattegat	Dab *Limanda limanda*	Healthy marine fish	AY546583	Einer-Jensen et al. (2004)
SE-SVA-1033	GIb	2000	Kattegat	Rainbow trout *Oncorhynchus mykiss*	VHSV outbreak[b]	AY546623	Nordblom & Norell (2000)
UKMLA98/6HE1	GIb	1998	North Sea	Herring *Clupea harengus*	Healthy marine fish	AY546631	King et al. (2001)

[a]Found to be of low pathogenicity to rainbow trout during experimental immersion challenge of trout (Skall et al. 2004)
[b]Found to be highly pathogenic to rainbow trout during experimental immersion challenge of trout (H. Skall unpubl. data)

(Tanks 19 and 20) by immersion using an infectious dose of 1×10^4 $TCID_{50}$ ml^{-1} for a period of 5 h before reestablishing a freshwater normal flow rate (50 l h^{-1}). Tanks were monitored twice daily for fish showing signs of ill health.

RNA extraction. Viral RNA was extracted using the Qiagen QIAamp Viral RNA kit following the recommended protocol. Viral RNA was eluted with 60 µl Buffer AVE and left to sit for 1 min. A final centrifuge at 8000 × *g* for 1 min was performed and eluate transferred to a fresh 0.8 ml tube and stored at –20°C.

Reverse transcription (RT). First-strand cDNA was synthesised from viral RNA using the ThermoScript™ RT *Taq* polymerase following the manufacturer's (Invitrogen) recommended protocol. Briefly, a 0.8 ml tube containing 1 µl random hexamers, 9 µl RNA and 1 µl PCR-grade dH_2O (Sigma) was incubated at 65°C for 5 min before being immediately transferred to ice. After a brief centrifugation, the following reagents were added: 4 µl 5′ cDNA synthesis buffer, 1 µl 0.1 M dithiothreitol (DTT), 1 µl dH_2O and 1 µl ThermoScript™ RT (15 U μl^{-1}). Tubes were then incubated at room temperature (25°C) for 10 min prior to mixing thoroughly using a bench-top vortex, and then incubated at 50°C for 1 h. The RT reaction was heated to 85°C for 5 min to terminate the reaction and immediately transferred and stored at –20°C.

PCR and product purification. Triplicate PCR reactions were conducted on each sample to produce a consensus nucleotide sequence per isolate, using primers for each gene designed based on GIb published sequences available in GenBank. The sequencing strategy consisted of amplifying each of the genes individually along with the intergenic regions, with an approximate 100 bp overlap between products to enable successful alignment of generated sequences. In the case of the L gene (approximately 6 kb), the gene was divided into 5 regions for amplification purposes (4 products of approximately 1.5 kb, and a 637 bp product at the 5′-end). Briefly, 1 µl of product was added to the PCR master mix which consisted of 17.55 µl dH_2O, 2.5 µl NH_4 10× PCR buffer, 0.75 µl $MgCl_2$ 50 mM, 0.5 µl dNTPs 10 mM, 0.2 µl *Taq* polymerase and 0.5 µl each forward and reverse primers (28 pmol). The PCR conditions were an initial denaturation cycle of 94°C for 2 min, then 35 cycles of 94°C for 1 min, 50 to 55°C for 1 min and 72°C for 1 min 30 s, and a single elongation cycle at 72°C for 10 min before holding at 4°C. PCR products were run on tris-borate-EDTA (TBE) ethidium bromide impregnated gels at 100 V for 50 min, and products visualized using UV light. Products were excised and purified using a Qiagen MinElute kit following the manufacturer's recommended protocol. The product was eluted by adding 20 µl of molecular grade dH_2O (Sigma) and allowing to

stand for 1 min before being centrifuged at 8000 × *g* for 1 min. Eluate was transferred to a clean 1.5 ml tube and stored at –20°C. DNA quantity was established using a NanoDrop ND-1000 spectrophotometer.

Sequencing of the amplicons. Products were sequenced using a Beckman Coulter CEQ™ 8800 DNA Analysis System following the manufacturer's recommended methods for sequencing and subsequent precipitations. Additional sequencing of the G gene from virus recovered directly from infected kidney tissue of dead fish was conducted to confirm that the consensus sequence determined from the challenge isolates reflected that which was actually responsible for the observed mortality (data not shown).

Assembling and comparing isolate sequences. Sequence runs were visually checked and verified using Sequencher™ (version 4.5) and assembled based on the consensus nucleotide sequences generated. Primer sequences were eliminated from the analysis. Comparative analysis of isolates was performed using the Clustal W programme available from the European Molecular Biology Laboratory European Bioinformatics Institute (EMBL-EBI) (www.ebi.ac.uk/ clustalw/). Nucleotide sequences were translated into aa sequences using the Transcription and Translation Tool available from Attotron Biosensor Corporation (www.attotron.com/cybertory/analysis/trans.htm). Observed point mutations at the aa level were determined for remaining isolates (DK-5e59 and UKMLA98/6HE1) following the protocol detailed above.

RESULTS

Mortality study

Mean cumulative mortalities resulting from the challenges are shown in Figs. 1 & 2. Rainbow trout i.p. infected with the SE-SVA-1033 isolate incurred a cumulative mortality of 75% (Fig. 1), with the majority of fish lost between 6 and 10 d post-virus exposure. Cumulative mortality attributable to this isolate was similar to the typical mortality course for pathogenic freshwater VHSV isolates (Meyers & Winton 1995, Skall et al. 2004). The remaining isolates DK-5e59, DK-4p37 and UKMLA98/6HE1 resulted in cumulative mortalities of 34.6, 23.1 and 19.2%, respectively. The time-course of mortality in this group of isolates was similar to that observed in fry challenged with SE-SVA-1033; however, cumulative mortality was considerably reduced. No mortality was recorded in the negative control immersion group, thus validating the infection strategy. Chi-squared analysis of remaining treatment groups revealed there was a statistically significant difference in the mortality rates incurred between treatments (χ^2 = 42.10, df = 3, $p < 0.001$). A further test only incorporating mortality rates from trout challenged with low virulent isolates revealed no significant difference between treatments (χ^2 = 3.45, df = 2, $p > 0.05$). Trout challenged with the SE-SVA-1033 isolate by immersion incurred a cumulative mortality of 15.4%, whereas the only other recorded mortality in the immersion challenge was a single fish loss (1.9%) from one of the UKMLA98/6HE1 tested tanks (Fig. 2).

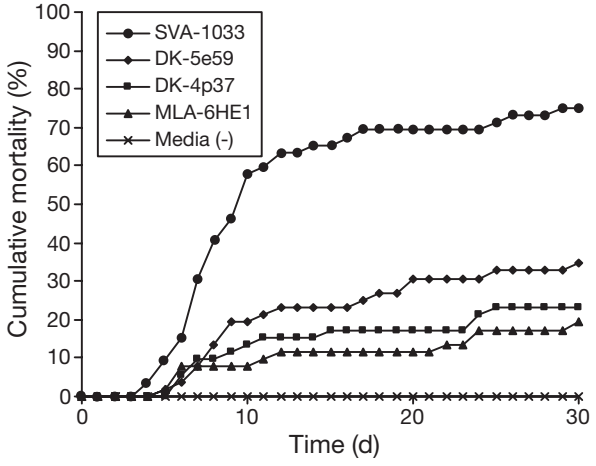

Fig. 1. *Oncorhynchus mykiss*. Mean cumulative mortality (%) versus time in rainbow trout exposed to viral haemorrhagic septicaemia virus isolates SE-SVA-1033, DK-5e59, DK-4p37 or UKMLA98/6HE1 via intraperitoneal injection of 50 µl at a dose of 1.07×10^7 $TCID_{50}$ ml^{-1}. Media was administered to a negative control group and in all cases tanks were duplicated. The length of the trial was 30 d

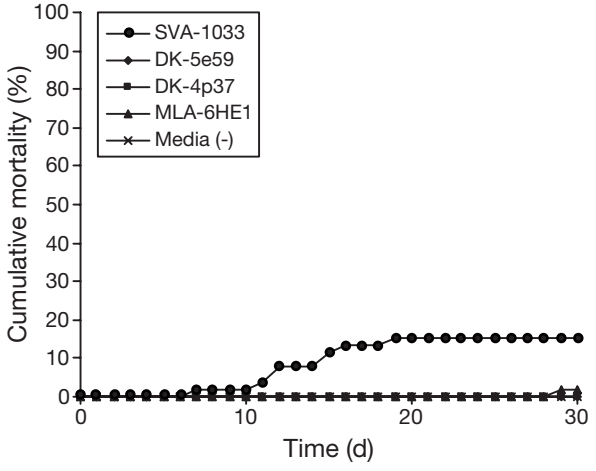

Fig. 2. *Oncorhynchus mykiss*. Mean cumulative mortality (%) versus time in rainbow trout exposed to viral haemorrhagic septicaemia virus isolates SE-SVA-1033, DK-5e59, DK-4p37 or UKMLA98/6HE1 via immersion challenge with a dose of 10^4 $TCID_{50}$ ml^{-1} for 5 h. Media was administered to a negative control group and in all cases tanks were duplicated. The length of the trial was 30 d

Comparative analysis of sequencing data

The complete coding regions for VHSV isolates SE-SVA-1033 and DK-4p37 were determined and submitted to GenBank (accession nos. FJ460591 and FJ460590, respectively). Generated and aligned sequences revealed the anticipated 6 open reading frames (ORFs), encoding 6 proteins (Table 2). Excluding the RNA 3′ lead terminus and 5′ terminal trailer regions, a total sequence of 10 845 nucleotides (nt) was obtained. Within the coding region, comparative analysis between sequences revealed a high genetic similarity, with only 16 nt substitutions between isolates (Table 2). Further comparative analysis of the deduced aa sequence data within the coding regions revealed 4 aa substitutions, a divergence of 0.1% between isolates. There were single aa substitutions recorded within each of the N-(Arg46Gly), G-(Ser113Gly), NV-(Leu12Phe) and L-(Ser56Ala) proteins. In addition, data revealed the genetic identity of the P and M proteins at the aa level. Within the 5 intergenic non-coding regions (approximately 4.7% of the genome sequenced), ranging in lengths of between 74 and 127 nt, there was a single nucleotide substitution (0.2%) situated 100 nt downstream of the P gene between the isolates sequenced. Comparison of the SE-SVA-1033 and DK-4p37 isolate sequences with the complete genomic sequence of another GIb isolate (UK-96-43) revealed similarity (at the aa level) between isolates as follows: N (99.8, 100%), P (99.5%), M (100%), G (99.2, 99.0%), NV (99.2, 100%) and L (99.9, 99.95%), respectively.

Identified point mutations across a range of isolates

Screening of all isolates in the present study for the presence of the single aa substitutions observed between isolates DK-4p37 and SE-SVA-1033 indicated that only mutations within the N and L proteins could be correlated with the high virulent phenotype (Table 3).

DISCUSSION

The present study sought to characterise the virulence of several closely related GIb isolates in rainbow trout, which were expected to differ in virulence, in order to identify candidate virulence determinants in rainbow trout. The expected virulent isolate was selected based on its recovery from a VHS disease episode in rainbow trout in 2000 and earlier infectivity trials. The remaining isolates were obtained from healthy wild marine fish in the locality; these isolates are generally known to be of low virulence to rainbow trout, but are thought to have the potential for introduction and adaption to the host species within marine rainbow trout farms (see Einer-Jensen et al. 2004, Skall et al. 2004).

Results of the infection trial confirmed the relatively high virulence of isolate SE-SVA-1033 irrespective of infection protocol. Immersion mortality rates herein were similar to unpublished mortality data reported by Skall et al. (2004), whereby an immersion challenge of isolate SE-SVA-1033 was performed on rainbow trout fingerling at a dose of 2×10^4 $TCID_{50}$ ml^{-1} resulting in 20.0%

Table 2. Comparative analysis of (a) nucleotide (nt) and (b) amino acid (aa) substitution rates between 2 viral haemorrhagic septicaemia virus (VHSV) isolates (SE-SVA-1033 and DK-4p37) with different virulence properties

(a) Nucleotide substitution SE-SVA-1033 vs. DK-4p37

Gene 3′ - 5′	Product size (nt)	Substitution (n)	Identity (%)	Starting position	End position
N	1215	4	99.7	1	1215
P	669	2	99.7	1314	1982
M	606	0	100	2100	2705
G	1524	1	99.9	2794	4317
NV	369	2	99.5	4392	4760
L	5958	7	99.9	4888	10845
Combined	10341	16	99.8	–	–

(b) Amino acid substitution SE-SVA-1033 vs. DK-4p37

Gene 3′ - 5′	Product size (aa)	Substitution (n)	Identity (%)	Substituted (aa)	Substitution position (aa)
N	404	1	99.8	Gly–Arg	45
P	222	0	100	–	–
M	201	0	100	–	–
G	507	1	99.8	Gly–Ser	113
NV	122	1	99.2	Phe–Leu	12
L	1985	1	99.95	Ala–Ser	56
Combined	3446	4	99.9	–	–

Table 3. Amino acid (aa) substitutions recorded between 4 GIb isolates across the N, G, NV genes and partial sequence of the L gene (67 to 486 nucleotides). Isolates were chosen based on their isolation history

Gene	DK-4p37	DK-5e59	SE-SVA-1033	UKMLA98/6HE1	Residue (aa) position
N	Gly	Gly	Arg	Gly	46
G	Gly	Ser	Ser	Ser	113
NV	Phe	Leu	Leu	Phe	12
L	Ala	Ala	Ser	Ala	56

cumulated mortality. Fish succumbing to VHSV infection displayed the characteristic pathological symptoms of *Rhabdoviridae* infection (Essbauer & Ahne 2001). While mortalities were recorded from fish inoculated with the remaining isolates via i.p. injection, significantly reduced mortality rates indicated these isolates were less virulent. The immersion challenge results supported this interpretation, as do results obtained in previous challenges using VHSV isolates recovered from wild-caught marine fish (Dixon et al. 1997, Skall et al. 2004, Brudeseth et al. 2008). Having tested the virulence of all 4 isolates in rainbow trout, the SE-SVA-1033 isolate and the isolate having the most similar G gene sequence, DK-4p37, were selected for comparative sequence analysis of their entire coding regions. Unlike the Swedish strain obtained from a VHS disease episode in 2000, isolate DK-4p37 was recovered from asymptomatic blue whiting *Micromesistius poutassou* taken from the North Sea in 1997 (Snow et al. 1999, King et al. 2001, Einer-Jensen et al. 2004, Skall et al. 2004).

Aligned sequences revealed the RNA genome sequenced comprised 10 845 nt, of which 10 338 nt (95.35%) encoded 6 genes (3′-N, P, M, G, NV, L-5′), as previously reported by Betts & Stone (2000). Collectively across coding regions, comparative analysis revealed only 16 nt substitutions, leading to the substitution of 4 aa between predicted proteins. This indicated a divergence between isolates of just 0.1%, potentially revealing areas of the genome where virulence determinants might be located. This finding substantiates the notion proposed by Betts & Stone (2000) that only a few aa substitutions are necessary to increase the pathogenicity of the virus. Intriguingly, there was complete identity of the deduced aa sequence of the P and M genes between isolates, with only 2 (0.3%) silent nt substitutions identified in the P gene, suggesting that the observed differences in virulence may not be attributable to these genes.

Previous studies have successfully identified virulence determinants in another rhabdovirus, rabies virus (RV), whereby a single aa point mutation at residue 333 within the G protein was sufficient to have shown a dramatic impact on virulence (see Shimizu et al. 2007). Takayama-Ito et al. (2006) also identified an additional 3 residues in the G protein at positions 242, 255 and 268 responsible for virulence in mice. The close proximity of these residues led to speculation as to their importance in the stability of an epitope within the G protein crucial to RV virulence in mice. While this evidence reflects the importance of the G gene in effecting virulence, it also emphasizes the notion that only a few aa substitutions are necessary to determine virulence amongst rhabdoviruses for a prescribed host. Sequencing data previously available for the G protein of both isolates of VHSV used here identified 2 nt substitutions leading to a single aa substitution (Ser113Gly position; 0.2%); this finding was further verified in the present study, both from infected cells and harvested tissue. The G protein is responsible for host cellular entry of the virus by means of receptor-mediated endocytosis, is the target molecule for neutralizing antibodies (Boudinot et al. 2004), and has already been demonstrated to play a decisive role in determining the virulence of VHSV (Béarzotti et al. 1995, Stone et al. 1997). The position of this mutation proved interesting. Mas et al. (2002) had already described a VHSV enhancer peptide spanning the region between residues 99 and 113 of the G gene and, intriguingly, the mutation detected between isolates tested in the present study fell within this region, occurring at the latter end of this domain. However, analysis of published sequences revealed the DK-4p37 G gene was genetically identical to another rainbow trout virulent Swedish isolate, SE-SVA-14 (accession no. AY546622), which had also been shown to be highly virulent. Indeed, an initial pilot study testing the SE-SVA-14 and SE-SVA-1033 isolates via i.p. or immersion at a higher dose (9.67×10^6 $TCID_{50}$ $fish^{-1}$, 1×10^5 $TCID_{50}$ ml^{-1} 5 h, respectively) revealed high mortalities (100 and 91.7% i.p. and 58.3 and 58.3% immersion) in rainbow trout fry (approximately 1.7 g) taken from the same stock (S. Campbell unpubl. data). These data suggest that the mutation Gly-113-Ser is not a virulence determinant. The fact that this mutation is not conserved across the remaining isolates sequenced in the present study (DK-5e59 and UKMLA98/6HE1) further supports this conclusion.

Numerous nt substitutions were recorded in the N and NV genes (4 and 2, respectively), leading to single aa substitutions within each gene (0.3 and 0.8%,

respectively). The function nucleoproteins play in virulence within rhabdoviruses is far from being understood (Shimizu et al. 2007). Structurally associated and tightly bound to viral genomic RNA (Leong et al. 1983, Said et al. 1998), the N protein facilitates encapsulation of the viral RNA genome (Zhang et al. 2008). Positioning of the substitution Arg46Gly revealed it was not within the central portion of the nucleocapsid protein which has been reported to be particularly important in binding the RNA genome (Said et al. 1998, Betts & Stone 2000). This point mutation was unique to isolate SE-SVA-1033 across the range of isolates tested. Because the N protein has functional roles in the transcription and replication of the virus (see Zhang et al. 2008), the mutation could contribute to the virulence of isolate SE-SVA-1033, although this requires further investigation.

Further downstream, a single substitution (Phe12-Leu) was the only aa substituted in the NV protein between isolates. In addition to SE-SVA-1033, isolate DK-5e59 was the only remaining isolate to encode a Phe at this point mutation. Due to the relatively small size of the viral genome (approximately 11 000 nt), Alonso et al. (2004) postulated the NV gene (369 nt) must have an important role in viral replication, although it was not essential for pathogenesis in snakehead rhabdovirus (SHRV). However, Thoulouze et al. (2004) showed the gene to be essential for pathogenicity of another salmonid rhabdovirus, infectious haematopoietic necrosis virus (IHNV), whereby removal of the NV gene significantly reduced the virulence of the virus for rainbow trout, as also demonstrated by Brémont (2005). In addition to running an *in vivo* study characterising the pathogenicity of IHNV, an *in vitro* study demonstrated the necessity of the NV gene for efficient replication of the virus in cell culture (Thoulouze et al. 2004). The biological significance of the substitution within this gene in determining virulence cannot be fully elucidated until the functional role of the gene is established.

Located further downstream of the NV gene, consisting of 5958 nt (1985 aa), the L gene had 7 nt substitutions between the isolates, but these resulted in only a single aa substitution (Ser56Ala; 0.1%). This point mutation was unique to isolate SE-SVA-1033 amongst those characterised. Primarily associated with the copying of genomic RNA during viral transcription and replication, the L protein is highly conserved, reflecting the functional constraints on the protein (Betts & Stone 2000). Whether the mutations observed play a decisive role in determination of virulence is unclear, although one could postulate that any such role might be expected to be related to replication efficiency of the virus.

During the undertaking of the present study, the assumption had been that virulent determinants would more likely be located within the coding regions of respective genes. While the present study did sequence the intergenic regions, sequencing of the terminal regions (167 and 151 nt, respectively) as described by Schütze et al. (1999) was not undertaken. However, it would be impossible to suggest that virulence determinants could not be located within these regions, and this remains a potential area of further research.

Despite numerous nt substitutions across the entire coding region (n = 16), most did not change the deduced aa sequences, as previously discussed by Betts & Stone (2000). Sequences generated in the present study could be combined with those sequences generated from further studies of genetically similar isolates to identify candidate genes or genetic changes that may prove beneficial in later *in vitro* studies, e.g. reverse genetics systems that allow for the manipulation of RNA viruses (Wu & Rupprecht 2008). These techniques provide researchers with an opportunity to decipher the potential influences a particular sequence will have on the overall phenotype. Subsequent transfection trials on specific cell lines using plasmids carrying specific genes of interest could allow researchers to establish the biological function and significance of respective genes with regard to virulence. Successful identification of these virulence determinants could lead to the development of improved control strategies and routine diagnostic tools to differentiate between isolates with different virulence properties.

In conclusion, comparative sequence analysis confirms the close genetic relationship (99.9%) between a VHSV isolate recovered from a VHS disease episode (SE-SVA-1033) with that of a naturally occurring VHSV isolate in a marine species (DK-4p37). Sequencing revealed only 4 aa substitutions between isolates, which may, in isolation or in synergy, represent determinants of virulence for isolate SE-SVA-1033 in rainbow trout. Although the present study is unable to conclusively identify which aa substitutions are responsible for influencing VHSV virulence, it provides a sound basis for further studies aimed at understanding the biological significance of such mutations in determining the virulence of VHSV.

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