Vol. 128: 51–62, 2018
https://doi.org/10.3354/dao03215

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published March 22

Virulence marker candidates in N-protein of viral haemorrhagic septicaemia virus (VHSV): virulence variability within VHSV Ib clones

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ABSTRACT: Four major genotypes of viral haemorrhagic septicaemia virus (VHSV), which have been isolated from many marine and freshwater fish species, are known to differ in virulence. While fast and low-cost genotyping systems based on monoclonal antibodies (MAbs) have been developed for typing of VHSV virulence, there is a need for supplementing the knowledge. In particular, 2 field isolates from viral haemorrhagic septicaemia (VHS) outbreaks in sea-reared rainbow trout *Oncorhynchus mykiss* in Sweden, SE-SVA-14 and SE-SVA-1033 (both genotype Ib), have yielded contradictory reactions. In the present study, upon cloning by limited dilution, both isolates appeared to be heterogeneous in terms of reactivity with nucleo (N)-protein-specific MAbs as well their gene sequences. Infection trials in rainbow trout further revealed differences in the virulence of these virus clones derived from the same primary isolate. Based on a comparative analysis of the entire genome of the clones tested, we suggest that the differences in virulence are tentatively linked to substitutions of amino acids (aa) in the N-protein region covered by aa 43–46 and aa position 168, or a combination of the two. The fact that such minor naturally occurring genetic differences affect the virulence implies that even low-virulent VHSV isolates in the marine environment should be considered as a potential threat for the trout farming industry. The described MAbs can represent useful tools for initial risk assessment of disease outbreaks in farmed trout by marine VHSV isolates.

KEY WORDS: Viral haemorrhagic septicaemia virus · VHSV · Virulence · *Oncorhynchus mykiss* · Genotype Ib · Nucleo-protein

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INTRODUCTION

Viral haemorrhagic septicaemia virus (VHSV) is the causative agent of a serious disease, viral haemorrhagic septicaemia (VHS), occurring in wild and farmed fish in the Northern Hemisphere. Until the 1980s, the disease was thought to cause severe mortalities only in farmed rainbow trout *Oncorhynchus mykiss* in Europe. In the last 3 decades, however, VHSV has been isolated from >80 fresh- and seawater fish species in North America, northeast Asia and Europe (Skall et al. 2005). VHSV isolates can be divided into 4 major genotypes and a number of subtypes with distinct geographical distributions (Einer-Jensen et al. 2004). The host range and the virulence appear, at least to some extent, to be linked to the

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genotype. In general, VHSV isolated from marine fish, i.e. genotypes Ib, II and III, range from being non-virulent to causing low virulence to rainbow trout (Skall et al. 2004). The IVa, IVb and IVc isolates of VHSV also show low virulence to rainbow trout (Ito et al. 2004, Emmenegger et al. 2013). The prevalence of VHSV in the sea around northern Europe is periodically very high in herring *Clupea harengus* and sprat *Sprattus sprattus*, and most of the viruses isolated from these fish belong to Ib (Mortensen et al. 1999, EURL 2009, Johansen et al. 2013, Sandlund et al. 2014). None of the VHSV isolates from wild marine fish in this region have been linked to pathology in rainbow trout, but it has been hypothesized that the highly virulent Ia isolates in Europe originally derive from marine finfish isolates. Based on molecular clock analysis, Einer-Jensen et al. (2004) suggested that the virulent Ia isolates from rainbow trout and the marine isolates (Ib, II and III) had split from a common ancestor in the 1950s. This period coincides with the time when the first VHS-like symptoms appeared in rainbow trout farms in Denmark. These findings indicate that VHSV in the marine environment around Europe adapted to rainbow trout in freshwater aquaculture and became highly virulent for this species. The genetic background for increased virulence of VHSV in rainbow trout has been studied by several groups (Betts & Stone 2000, Snow & Cunningham 2000). However, no clear results have pointed to which nucleotide and/or amino acid (aa) substitutions of VHSV are a key for triggering pathogenicity in fish.

Stone et al. (1997) argued that marine isolates of VHSV are a potential threat to the farming industry if provided with the opportunity to adapt under intensive farming conditions. In 1998 and 2000, 2 isolations of VHSV were made from diseased sea-farmed rainbow trout on the Swedish west coast close to Gothenburg. The isolates SE-SVA-14 (Nordblom 1998) and SE-SVA-1033 (Nordblom & Norell 2000) both belong to genotype Ib (Einer-Jensen et al. 2004). Since all other marine fish-derived Ib isolates have no or very low virulence to rainbow trout (Skall et al. 2005), the 2 sea-reared trout-derived isolates have attracted attention in order to assess which part of the viral proteins might be associated with increased virulence to rainbow trout (Campbell et al. 2009). When comparing the full genome sequence of the rainbow trout isolate SE-SVA-1033 with that of a typical Ib isolate from herring, the 4p37 isolate, Mortensen et al. (1999) observed a few nucleotide substitutions in all viral proteins. Single aa substitutions were found in the nucleo (N)-gene (aa 46), glycoprotein (G)-gene (aa 113), non-virion (Nv)-gene (aa 12) and large polymerase (L)-gene (aa 56). However, they were unable to identify which of the aa substitutions played the most important role in VHSV virulence.

VHSV genotype-specific monoclonal antibodies (MAbs) have been produced in order to establish a fast and low-cost genotyping system for VHSV isolates (Ito et al. 2012). In the process, we encountered unpredicted interesting reactions. One MAb, VHS-3.80, normally recognizing Ib, Ic, Id and II isolates, did not react with the SE-SVA-14 isolate nor with a clone from the SE-SVA-1033 isolate designated SE-SVA-1033-3F, although another clone from this SE-SVA-1033 isolate designated as SE-SVA-1033-9C was recognized (Ito et al. 2012). In the present work, we extended these studies by demonstrating heterogeneity of the SE-SVA-14 isolate, and differences in virulence of the individual virus clones derived from both Swedish isolates. By including another N-protein-specific MAb (VHS-4.20) that did not recognize the majority of Ib isolates in our immunoassay, along with full genome sequencing, we were able to determine variations in the N-gene as being important for VHSV virulence to trout.

MATERIALS AND METHODS

Cell lines

The bluegill fry (BF-2) cell line (Wolf et al. 1966) was used for propagation of VHSV genotype Ib, Id, II and III isolates; the epithelioma papulosum cyprini (EPC) cell line (Fijan et al. 1983) for I, Ia, Ic and Ie isolates; and the fathead minnow (FHM) cell line (Gravell & Malsberger 1965) for IVa and IVb isolates. Cell lines were maintained in minimum essential medium (MEM; Mediatech) supplemented with 10% fetal bovine serum (FBS; Equitech-Bio) and antibiotics (100 U penicillin ml^{-1} and 100 mg streptomycin ml^{-1}). Cultivation of the cell lines and the propagation of virus was conducted at 25°C and 15°C, respectively.

Virus

Eighty-one VHSV isolates representing all geno- and subtypes from throughout the world were used to characterize the obtained MAbs (Table S1 in the Supplement at www.int-res.com/articles/suppl/d128p051_supp.pdf). In addition, 18 non-VHSV piscine pathogenic rhabdovirus isolates were used in the assessment of the specificities of the MAbs (Table S2).

Production and characterization of MAb VHS-4.20

The VHSV Ia isolate DK-9695377 (Einer-Jensen et al. 2004, EURL 2009), isolated in 1996 from an outbreak of VHS in a rainbow trout farm in Denmark, was used to immunize BALB/c mice. Virus from BF-2 cell culture supernatant was pelleted by ultracentrifugation (100 000 × *g*). The pellet was washed once in TE buffer (pH 7.5) and resuspended in TE at 2 mg protein ml^{-1}. Immunization was performed as described by Lorenzen et al. (1988).

Cell fusion of spleen cells from an immunized mouse was performed as described by Lorenzen et al. (1988). The immunoglobin (Ig) class of the MAb was determined using a mouse monoclonal isotyping kit (AbD Serotec) according to the manufacturer's instructions.

ELISA for screening of hybridoma cell culture supernatants was performed by sandwich ELISA using a mix of purified rabbit antibodies to VHSV N-, phospho (P-) and G-proteins as catcher followed by virus in the form of Triton X-100-treated cell culture supernatant as described earlier (Ito et al. 2012). Western blotting for determination of protein specificity of MAb VHS-4.20 was done with purified VHSV as antigen as described by Ito et al. (2012). Briefly, the protein bands after sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were transferred from polyacrylamide gels to nitrocellulose membranes in a semi-dry electroblotter. The membrane was reacted with MAb VHS-4.20 diluted at 1:50 with phosphate-buffered saline (PBS) for 30 min at 37°C after blocking with Blok Ace (DS Pharma Biomedical). As a positive control reagent for immunostaining, a mixture of the anti-VHSV MAbs IP1D11 recognizing the viral G-protein, IP5B11 recognizing the N-protein, IP1C6 recognizing the P-protein and IP1C3 recognizing the M-protein (Lorenzen et al. 1988) was used.

In the scoring procedure, priority was given to supernatants able to differentiate between the virulent VHSV genotype Ia and the marine genotypes. The MAb VHS-4.20 was tested against a panel of VHSV isolates (Table S1) and 18 non-VHSV fish rhabdoviruses (Table S2) using the indirect fluorescent antibody technique (IFAT) as follows. The MAbs IP5B11 (Lorenzen et al. 1988), known to react with all VHSV isolates, and VHS-5.18, only reacting with VHSV Ib (Ito et al. 2012), were used as a control.

For characterization of binding specificity, dot-blot analysis was performed: 2 kinds of synthetic oligopeptides mimicking aa positions 155–173, but with a single difference shown in bold on position 168 (GELADTQGVGELQ**H**FTADK or GELADTQGVGELQ**Y**FTADK) in N-protein, were blotted onto a nitrocellulose membrane. The membrane was reacted with MAb VHS-4.20 diluted at 1:50 with PBS for 30 min at 37°C after blocking with Blok Ace. Visualization was performed using horseradish peroxidase (HRP)-conjugated antiserum and 3,3′-diaminobenzidine in PBS, containing 0.06% H_2O_2 after 3× washing by PBS. As a positive and negative control for immunostaining, purified VHSV DK-3592B (Ia) and KRRV9601 (Ib) were used, respectively.

IFAT for MAbs characterization

The binding of the MAbs IP5B11, VHS-5.18, VHS-4.20 and VHS-3.80 to a panel of VHSV isolates and the cross-reaction of MAb VHS-4.20 against 18 non-VHSV fish rhabdoviruses (Table S2) was performed according to the method described by Ito et al. (2010, 2012). Briefly, the binding of MAbs to the large panel of VHSV isolates (Table S1) and non-VHSV fish rhabdoviruses was performed using fixed virus-infected and non-infected EPC cells in black 96-well plates (Corning) for IFAT. The infected cells were fixed with cold acetone for 10 min, then overlaid with MAbs and incubated in a humid chamber for 30 min at 37°C and then rinsed with PBS. Fluorescein isothiocyanate-conjugated goat anti-mouse IgG serum (MP Biomedicals) was applied and the samples were incubated for 30 min at 37°C. After another rinse, the plates were examined with a fluorescence microscope.

Cloning of variants from Swedish rainbow trout virulent isolates

The variant clones from the SE-SVA-14 and SE-SVA-1033 isolates were cloned by repeated limiting dilutions on BF-2 cells. The obtained variant isolates were confirmed by entire nucleotide sequencing and IFAT reaction to MAbs VHS-3.80 and VHS-4.20 as clones.

Sequencing of the variant clones

Each entire viral genome was sequenced as follows. The Swedish VHSV variant clones were concentrated and sucrose gradient-purified as described by Ito et al. (2012). Viral RNA was extracted from each purified variant clone using the TRIzol® LS Reagent (Life Technologies), and submitted to RT-PCR amplification with primer sets (Table S3). Primer se-

quences for gene sequencing were designed according to the KRRV-9601 (Isshiki et al. 2001) (Ib) gene (GenBank accession no. AB672614). The nucleotide sequence of each RT-PCR product except 5′ or 3′ termini was confirmed using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Life Technologies) and ABIPRISM® 3100 Genetic Analyzer (Life Technologies). Each 5′ or 3′ terminus was cloned using the SMARTer RACE cDNA Amplification Kit (Clontech) and primer (5′-CTC GAT GAT GAT GAT GAT CTC-3′) (Schütze et al. 1995) for cloning the 3′ end, respectively. These PCR products were cloned using the TOPO® TA Cloning® Kit for Sequencing (Life Technologies). Plasmids from at least 5 independently derived clones were extracted using QIAprep Spin Miniprep Kit (QIAGEN) and sequenced in both orientations, after which consensus sequences were determined from 5 or more independent clones. The GenBank/EMBL/DDBJ accession numbers for the VHSV isolate sequences SE-SVA-14-3D, SE-SVA-14-5G, SE-SVA-1033-9C and SE-SVA-1033-3F are AB839745–AB839748.

Fish

All fish used in the infection experiments were free of VHSV, infectious hematopoietic necrosis virus (IHNV) and infectious pancreatic necrosis virus (IPNV). The fish were acquired from 2 sources. For the challenge experiment (Expt 1), rainbow trout were bred from a single pair of parental fish in the Tamaki laboratory of the National Research Institute of Aquaculture (NRIA), Fisheries Research Agency (FRA). For Expt 2, outbred rainbow trout fingerlings from multiple parental fish were purchased from a Danish commercial fish farm and moved to the National Veterinary Institute, Technical University of Denmark (DTU Vet) challenge facilities 1 wk before infection.

Infection experiments

In total, 60 rainbow trout (average weight: 10.7 g) were selected for use in Expt 1. Fish were divided into 6 groups, each including 10 individuals per aquarium. The fish were intraperitoneally (IP) injected with 0.1 ml of each virus culture i.e. DK-3592B as positive control, SE-SVA-14-3D, SE-SVA-14-5G, SE-SVA-1033-3F and SE-SVA-1033-9C isolate ($10^{6.1}$–$10^{6.3}$ $TCID_{50}$ $fish^{-1}$). Fish in the negative control group were IP inoculated with 0.1 ml of cell culture supernatant. All fish groups were kept in 60 l tanks at 9.7°C (range: 9.0–11.0°C). The fish were fed commercial diets. Once mortality was observed, moribund fish were collected daily for 31 d. Expt 1 was performed at the Tamaki laboratory of the NRIA in Japan and in accordance with the guidelines for animal experimentation of the NRIA, FRA, Japan.

In Expt 2, approximately 1500 rainbow trout fry (2.0 g on average) were used. At first, fish were divided into 2 groups of approximately 700 fish for IP injection (subdivided into 7 groups) and 800 fish for immersion (subdivided into 8 groups). The IP injected groups were given 0.05 ml of supernatant of each virus culture i.e. SE-SVA-14 wild-type, SE-SVA-14-3D, SE-SVA-14-5G, SE-SVA-1033 wild-type, SE-SVA-1033-3F and SE-SVA-1033-9C isolate ($10^{5.0}$ $TCID_{50}$ $fish^{-1}$). The bath infection groups were immersed for 2 h in $10^{5.0}$ $TCID_{50}$ ml^{-1} of each virus in aerated static water. All fish groups were kept in 8 l tanks at 10.0°C and fed a commercial diet. All groups infected with Ib isolates by IP injection or by immersion were tested in triplicate with at least 30 fish in each tank. Positive and negative control groups were tested in duplicate tanks. Clinically diseased and moribund fish were collected 3× d^{-1} for 28 d. Expt 2 was performed at DTU Vet and carried out in accordance with the recommendations in the current animal welfare regulations and under license 2013-15-2934-00976. The Danish Animal Research Authority approved the applied protocols.

Details of the experimental design and total length and body weight of fish per group in each experiment are shown in Table S4.

Virus re-isolation from experimental fish

All fish that died during Expts 1 and 2 were kept at –80°C until examined virologically on BF-2 cell cultures. In addition, all surviving fish in Expt 1 were sampled for virological examination at the end of the trials. In Expt 1, homogenates of kidney were suspended in approximately 50× volume of MEM and filtered (0.45 µm). Then 100 µl and 10 µl of each filtrate were inoculated onto subconfluent 1 d old BF-2 cells in 24-well dishes respectively and incubated at 15°C. The cells were observed for cytopathic effect (CPE) over a period of 14 d. In Expt 2, kidney, spleen and heart were collected, suspended 1:10 in MEM with 10% FBS, homogenized, and centrifuged at 1000 × *g*. Supernatants were then treated with antibiotics overnight and inoculated onto subconfluent BF-2 cells in 24-well plates. Two wells were for the fi-

nal dilution 1:100, 1 well in 1:1000 and 1 well in 1:10 000 for each sample. The cells were observed for CPE for 7 d and subcultivated onto new cells and observed for another 7 d if no CPE occurred in primary culture. Finally, isolated viruses were identified by ELISA (Olesen & Jørgensen 1991) with modification according to Mortensen et al. (1999). Briefly, plates were coated with a mixture of protein-A purified rabbit anti G-, N- and P-protein of VHSV and used as a first layer. Then the plates were incubated for 1 h at 37°C with the Triton X-100-treated isolated virus after washing with PBS containing 0.05% Tween 20. Subsequently, plates were incubated for 1 h at 37°C with the MAb IP5B11. Detection was performed using HRP-conjugated rabbit anti-mouse immunoglobulins (DAKO, P0260).

Detection of VHSV in fish by RT-PCR

Infected as well as non-infected fish from Expt 1 were tested for the presence of VHSV by RT-PCR. Initially, total RNA was extracted from the samples using TRIzol® Reagent (Life Technologies) following the recommended protocols. Total RNA was dissolved in 100 µl DNase/RNase-free distilled water (Life Technologies) and stored at −85°C until further processing. Then RT-PCR was performed using SuperScript® One-Step RT-PCR System with Platinum® *Taq* (Life Technologies) using the VHSV-specific primers described in the VHS chapter of OIE's manual of diagnostic tests for aquatic animals (OIE 2016). The only modification was that the volume was reduced from 50 to 20 µl.

Statistical analysis

In Expt 1, Fisher's exact test was used to compare survival rates among the infected and the negative control groups. Fisher's exact test was also used to compare survival rates of the groups infected with the sister clone of the same original isolate. The results were judged as statistically significant when $p < 0.05$. In Expt 2, the data was fitted with a Cox proportional hazards model (Therneau & Grambsch 2000) using R (R Development Core Team 2011). Prior to fitting, we evaluated the assumption of proportional hazards (Grambsch & Therneau 1994). Statistical significance ($p < 0.05$) of survival between groups was tested with a robust score test, which takes replicate aquaria into consideration and does not assume independence of observations within the replicates.

RESULTS

Development and assessment of reactivity of VHSV MAb VHS-4.20

The cell fusion of spleen cells from mice immunized with the purified VHSV Ia isolate DK-9695377 were screened by ELISA using a small panel of isolates representing most genotypes. The MAb clone VHS-4.20 belonging to the IgG2 group gave a particularly strong reaction in ELISA with most VHSV isolates, except those belonging to the Ib genotype. Interestingly, this behavior was opposite to the earlier-described MAb VHS-5.18, which recognized only Ib isolates (Ito et al. 2012). The 2 MAbs thus appeared to have complementary reactivity. This was confirmed by subsequent IFAT, including a large panel of VHSV isolates representing all VHSV genotypes and the MAb IP5B11 (Lorenzen et al. 1988), known to react with all VHSV isolates, as a positive control. Unexpectedly, MAb VHS-4.20 did, however, also react with 2 Ib isolates: the M. Rhabdo VHSV from wild cod *Gadus morhua* (Vestergård Jørgensen & Olesen 1987) and the rainbow trout virulent Ib isolate SE-SVA-14 from Sweden (Nordblom 1998, our Table 1). Additional testing of MAb VHS-4.20 in IFAT against the 18 non-VHSV piscine rhabdoviruses listed in Ito et al. (2012) confirmed that this MAb was specific for VHSV (Table S5). Western blotting demonstrated that the protein specificity of MAb VHS-4.20 was the viral N-protein (Fig. 1).

The epitope specificity of MAb VHS-4.20 was further assessed by aligning the aa sequence data and the unique binding pattern of the MAb in the large VHSV panel. As MAb VHS-4.20 reacted with all VHSV genotypes but Ib, and unexpectedly the 2 Ib isolates M. Rhabdo and SE-SVA-14, it appeared that the histidine (His) in aa position 168 of the viral N-protein is essential for the epitope (Fig. S1 in the Supplement). In order to confirm this position, dot-blot analysis using synthetic oligopeptides representing the aa in position 155–173 of the recognized, and non-recognized N-proteins respectively was conducted. Only the peptide including His was recognized by MAb VHS-4.20 (Fig. 2), whereas the peptide where the His was substituted by tyrosine (Tyr) was not. When the aa sequence of the SE-SVA-14 isolate was included in the process of epitope mapping of MAb VHS-4.20, 2 genome variants were found in the N-protein. Accordingly, these variants were obtained by repeated limiting dilutions and the variants have been confirmed by the full-length genomes and IFAT as clone (Table 1).

Table 1. Indirect fluorescent antibody technique (IFAT) of viral haemorrhagic septicaemia virus (VHSV) isolates representing all genotypes and subtypes with 4 monoclonal antibodies (MAbs): IP5B11, VHS-5.18, VHS-4.20 and VHS-3.80. '+': positive; '–': negative

Isolate	Geno-type	IFAT			
		IP5B11	VHS-5.18	VHS-4.20	VHS-3.80
DK-F1	I	+	–	+	–
DK-Hededam	I	+	–	+	–
DK-3592B	Ia	+	–	+	–
DK-3971	Ia	+	–	+	–
DK-3946	Ia	+	–	+	–
DK-5151	Ia	+	–	+	–
DK-6137	Ia	+	–	+	–
DK-7974	Ia	+	–	+	–
DK-9695377	Ia	+	–	+	–
DK-200149	Ia	+	–	+	–
DK-200051	Ia	+	–	+	–
FR-07-71	Ia	+	–	+	–
FR-23-75	Ia	+	–	+	–
FR-02-84	Ia	+	–	+	–
CZ-R5	Ia	+	–	+	–
CZ-2077	Ia	+	–	+	–
DK-5927	Ia	+	–	+	–
AU-8/95	Ia	+	–	+	–
CH-F1 262 BFH	Ia	+	–	+	–
PL-202473	Ia	+	–	+	–
M. Rhabdo	Ib	+	+	+	+
1p8	Ib	+	+	–	+
1p40	Ib	+	+	–	+
1p85	Ib	+	+	–	+
1p86	Ib	+	+	–	+
1p93	Ib	+	+	–	+
1p116	Ib	+	+	–	+
1p120	Ib	+	+	–	+
1p121	Ib	+	+	–	+
5p276	Ib	+	+	–	+
SE-SVA-14 wild type	Ib	+	+	+	–
SE-SVA-14-3D	Ib	+	+	+	–
SE-SVA-14-5G	Ib	+	+	–	–
SE-SVA-1033 wild type	Ib	+	+	–	+
SE-SVA-1033-3F	Ib	+	+	–	–
SE-SVA-1033-9C	Ib	+	+	–	+
96-43	Ib	+	+	–	+
4p37	Ib	+	+	–	+
KRRV9601	Ib	+	+	–	+
DK-2835	Ic	+	–	+	+
DK-5123	Ic	+	–	+	+
DK-5131	Ic	+	–	+	+
FiA01a.00	Id	+	–	+	+
FiP02b.00	Id	+	–	+	+
NO-A163-68 EG46	Id	+	–	+	+
GE-1.2	Ie	+	–	+	–
TR206239-1	Ie	+	–	+	–
1p49	II	+	–	+	+
1p52	II	+	–	+	+
1p53	II	+	–	+	+
1p54	II	+	–	+	+
2p51	III	+	–	+	–
4p101	III	+	–	+	–
4p168	III	+	–	+	–
4p51	III	+	–	+	–
UK-H17/5/93	III	+	–	+	–
UK-860/94	III	+	–	+	–
UK-H17/2/95	III	+	–	+	–
F-L59x	III	+	–	+	–
GH30	III	+	–	+	–
IR-F13.02.97	III	+	–	+	–
NO-2007-50-385	III	+	–	+	–
USA-Makah	IVa	+	–	+	–
USA-KHV	IVa	+	–	+	–
USA-Elliot Bay	IVa	+	–	+	–
Minter Creek, WA	IVa	+	–	+	–
Tokul Creek, WA	IVa	+	–	+	–
Port Angels, WA	IVa	+	–	+	–
BC'93	IVa	+	–	+	–
CAN-3624	IVa	+	–	+	–
CAN-99-019	IVa	+	–	+	–
Quatsino, BC	IVa	+	–	+	–
JP-Obama 25	IVa	+	–	+	–
JF00Ehi1	IVa	+	–	+	–
BR01Ehi1	IVa	+	–	+	–
JF01Oit1	IVa	+	–	+	–
JSL02Yam1	IVa	+	–	+	–
PM05Ehi1	IVa	+	–	+	–
MI03GL	IVb	+	–	+	–
Goby 1-5	IVb	+	–	+	–
Lake Ontario, NY	IVb	+	–	+	–
Budd Lake, MI	IVb	+	–	+	–
Skaneateles Lake	IVb	+	–	+	–
CA-NB00-01[a]	IVb (IVc)	+	–	+	–

[a]This isolate was designated as genotype IVb in the original report, but genotype IVc in GenBank

The aa in position 168 of the clone SE-SVA-14-3D that did react with MAb VHS-4.20 was found to be His, while the not-recognized clone SE-SVA-14-5G had Tyr at the same position (Fig. 3). Similarly, only VHSV isolates with His in aa position 168 reacted with MAb VHS-4.20, which was the case for all non-Ib isolates as well as for M. Rhabdo and the SE-SVA-14-3D clone (Fig. 3).

Reactivity of MAb VHS-3.80

The MAb VHS-3.80 was described to recognize the motif ExDGKV in aa position 43–48 of the N-protein found in Ib, Ic, Id and II isolates, but not in the SE-SVA-14 isolate nor in the clones from this isolate or in a clone from the SE-SVA-1033 isolate designated SE-SVA-1033-3F (Ito et al. 2012). These 3 clones had the

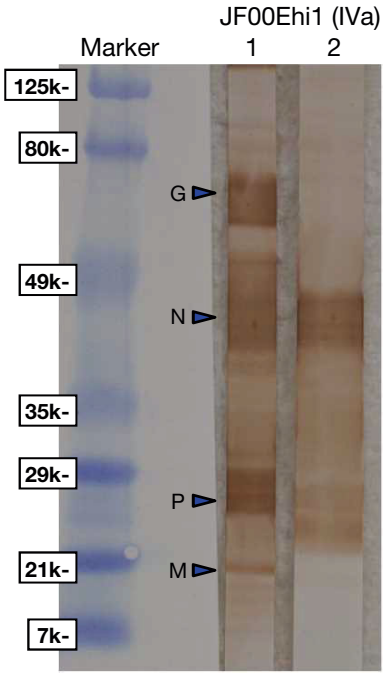

Fig. 1. Identification of protein specificity of established monoclonal antibody (MAb) VHS-4.20. Immunoblotting with MAb and with a mixture of MAb anti-G (IP1D11), MAb anti-N (IP5B11), MAb anti-P (IP1C6) and MAb anti-M (IP1C3) as positive controls (Lorenzen et al. 1988). The purified JF00Ehi1 (genotype IVa, Ito et al. 2012) of viral haemorrhagic septicaemia virus (VHSV) isolates was used as antigens: Strip 1: JF00Ehi1 with mixture of positive-control MAbs; Strip 2: JF00Ehi1 with MAb VHS-4.20

motif KEDGKV or EEDRKV and therefore did not react with MAb VHS-3.80 (Fig. 3)

Comparative analysis of aa among Swedish clones

The full-length genomes of 2 clones of each of the 2 SE-SVA isolates, SVA-14-3D, SVA-14-5G, SVA-1033-3F and SVA-1033-9C, were sequenced in order to assess their degree of similarity. The sequencing demonstrated that the 2 SE-SVA-14 clones were very similar, with only 3 aa substitutions among the 3440 aa, and substitutions were only observed in the N- and L-genes (Table 2). The 2 SE-SVA-1033 clones were more different, with 11 aa substitutions, with most substitutions in the L-gene (5 aa), followed by the N-gene (2 aa) and the G-gene (3 aa) (Table 2).

Infection experiments with 4 VHSV Ib clones in rainbow trout

Infection experiments were conducted in Japan (Expt 1) and in Denmark (Expt 2) and the obtained results are summarized in Table 3. In Expt 1, the survival rates of the rainbow trout IP injected with the VHSV clonal isolates SE-SVA-14-3D (90%), SE-SVA-14-5G (80%) and SE-SVA-1033-9C (90%) were in the same range as the negative control (100%), while the group infected with clone SE-SVA-1033-3F (10%) was significantly lower ($p < 0.01$) and in the range of DK-3592B (20%) as the positive control. VHSV was isolated from all dead fish and from some of the surviving fish in the infected groups. VHSV was not detected from the negative control group.

In Expt 2, survival rates of rainbow trout IP infected with SE-SVA-14-3D and SE-SVA-1033-9C were 69 ± 5.4% and 60 ± 8.7%, while the negative control behaved as expected, with a survival rate of 97 ± 3.6%. The immersion challenge included a positive control group (DK-3592B) that showed a survival rate of 37 ± 12.7%. A summary of the statistical R analysis of the survival analysis between the Ib VHSV clones SE-SVA-14 and SE-SVA-1033 and the corresponding original isolates used in Expt 2 are shown in Fig. 4 and Table 4. The survival proportion in the group injected with SE-SVA-14 wild type was significantly lower than in the groups injected with SE-SVA-14-3D and SE-SVA-14-5G, and the survival of the SE-SVA-14-3D group was also significantly lower than the SE-SVA-14-5G group. Survival proportion in the group injected with SE-SVA-1033 wild-type was significantly lower than in the group injected with SE-SVA-1033-9C. In addition, the survival proportion of SE-SVA-1033-3F group was also significantly lower than in the SE-SVA-1033-9C group but not significantly different from the wild type. Although no significant differences were observed between the survival proportion in the group infected by immersion with SE-SVA-14 wild type and SE-SVA-14-3D, the

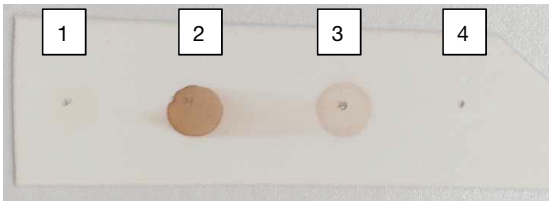

Fig. 2. Epitope mapping of monoclonal antibody (MAb) VHS-4.20 using synthetic oligopeptides by dot-blot analysis

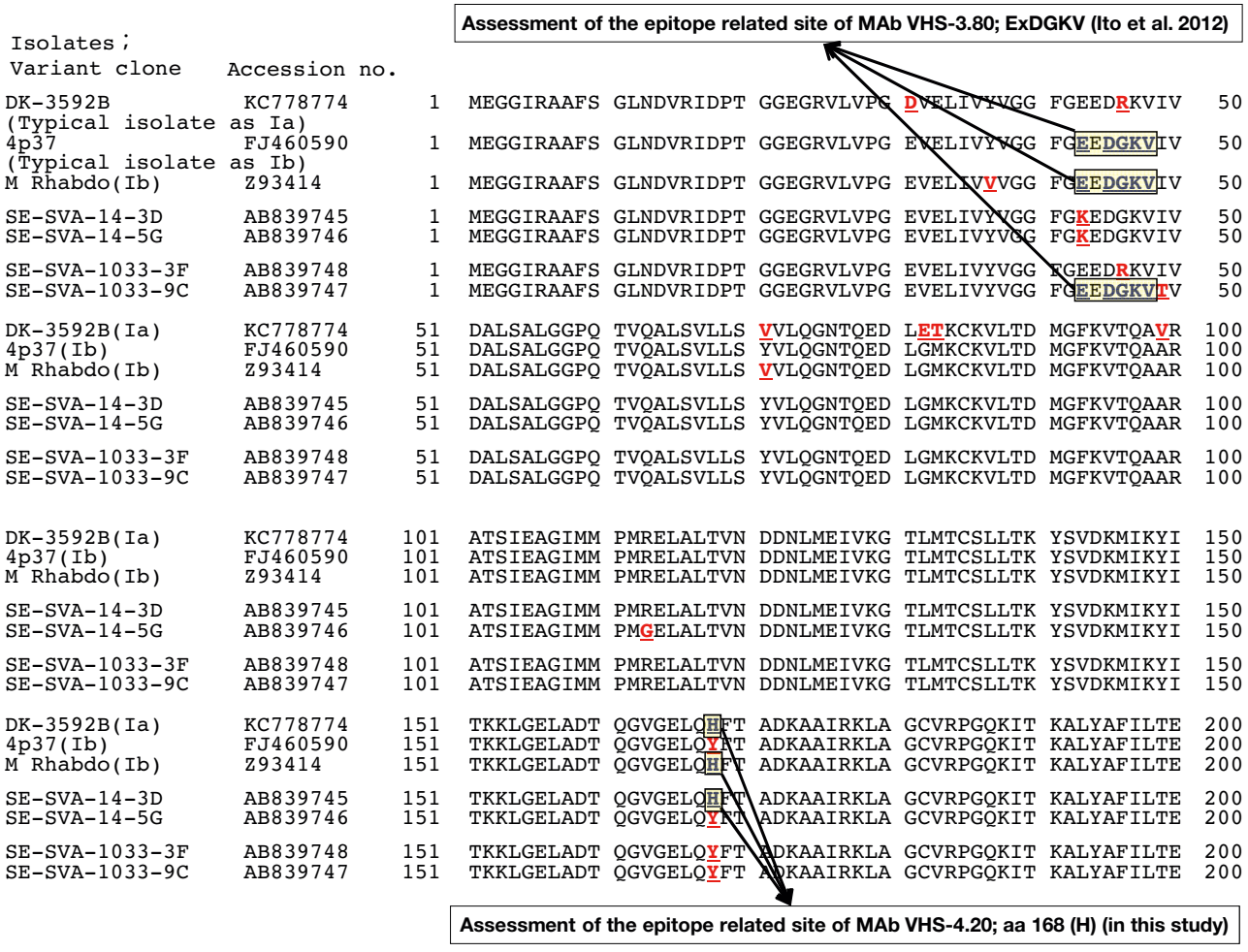

Fig. 3. Comparison of partial alignment of the amino acids of the N-proteins among 4 variant clones obtained from rainbow trout virulent Swedish viral haemorrhagic septicaemia virus (VHSV) Ib isolates SE-SVA-14 and SE-SVA-1033 with the typical Ib isolate 4p37 and M. Rhabdo, and the typical Ia isolate DK-3592B. Amino acid substitutions are marked in red

Table 2. Comparative analysis of amino acid (aa) substitution among the Swedish VHSV Ib clones: SE-SVA-14-3D vs. SE-SVA-14-5G, and SE-SVA-1033-3F vs. SE-SVA-1033-9C. '–': no substitutions

Protein	Product size (aa)	Substitution (n)	Identity (%)	Substituted (aa)
SE-SVA-14-3D vs. SE-SVA-14-5G				
N	404	2	99.5	R113G, H168Y
P	222	0	100	–
M	201	0	100	–
G	507	0	100	–
Nv	122	0	100	–
L	1984	1	99.9	I474V
All encoding regions	3440	3	99.9	–
SE-SVA-1033-3F vs. SE-SVA-1033-9C				
N	404	2	99.5	R46G, I49T
P	222	0	100	–
M	201	0	100	–
G	507	3	99.4	N230H, M292T, R371S
Nv	122	1	99.2	L12F
L	1984	5	99.7	S56A, P112L, L182P, E849H, N1317K
All encoding regions	3440	11	99.7	–

Table 3. Obtained survival rates of rainbow trout in the viral haemorrhagic septicaemia virus (VHSV) infection trials (Expts 1 and 2) and detection or re-isolation of VHSV in the challenged fish (dead and surviving; no. positive/no. tested). Fish were tested as individual fish for VHSV by cell culture followed by either ELISA or by RT-PCR of kidney material for confirmation. DTU Vet: National Veterinary Institute, Technical University of Denmark; NRIA: National Research Institute of Aquaculture; IP: intraperitoneal; NT: not tested. SD was determined across the 3 replicate aquariums in Expt 2

Expt (Location)	Infection route	Group	Survival rate (%) (mean ± SD)	Dead fish			Surviving fish	
				Cell cultivation	Cell cultivation + ELISA	RT-PCR	Cell cultivation	RT-PCR
1 (NRIA)	IP injection	DK-3592B	20[a]	8/8	NT	8/8	1/2	0/2
		SE-SVA-14-3D	90	1/1	NT	1/1	0/9	0/9
		SE-SVA-14-5G	80	2/2	NT	2/2	0/8	0/8
		SE-SVA-1033-3F	10[a,b]	9/9	NT	9/9	0/1	0/1
		SE-SVA-1033-9C	90	1/1	NT	1/1	1/9	1/9
		Negative control	100	NT	NT	NT	NT	0/10
2 (DTU Vet)	IP injection	SE-SVA-14 wild-type	16 ± 1.7	NT	77/81	NT	NT	NT
		SE-SVA-14-3D	69 ± 5.4[c,d]	NT	34/35	NT	NT	NT
		SE-SVA-14-5G	88 ± 1.9[d]	NT	9/10	NT	NT	NT
		SE-SVA-1033 wild type	39 ± 16.7	NT	66/67	NT	NT	NT
		SE-SVA-1033-3F	60 ± 8.7[c,d]	NT	35/42	NT	NT	NT
		SE-SVA-1033-9C	81 ± 1.2[d]	NT	16/20	NT	NT	NT
		Negative control	97 ± 3.6	NT	0/3	NT	NT	NT
	Immersion	DK-3592B	37 ± 12.7	NT	50/50	NT	NT	NT
		SE-SVA-14 wild type	92 ± 1.7	NT	8/8	NT	NT	NT
		SE-SVA-14-3D	96 ± 4.2	NT	3/4	NT	NT	NT
		SE-SVA-14-5G	98 ± 3.2[d]	NT	0/2	NT	NT	NT
		SE-SVA-1033 wild type	95 ± 4.0	NT	4/5	NT	NT	NT
		SE-SVA-1033-3F	87 ± 12.9	NT	7/15	NT	NT	NT
		SE-SVA-1033-9C	96 ± 2.3	NT	1/4	NT	NT	NT
		Negative control	94 ± 4.4	NT	0/6	NT	NT	NT

[a]Survival rate significantly different from negative control ($p < 0.01$, Fisher's exact test); [b]Survival rate significantly different from sister clone of same original isolate ($p < 0.05$, Fisher's exact test); [c]Survival proportion significantly different from sister clone of same original isolate ($p < 0.05$, survival analysis by R); [d]Survival proportion significantly different from original isolate ($p < 0.05$, survival analysis by R)

Table 4. Detailed results of statistical analysis between the Ib VHSV clones SE-SVA-14 and SE-SVA-1033 in Expt 2 using survival analysis of rainbow trout. IP: intraperitoneal. Significant differences in survival ($p < 0.05$) marked in **bold**

Infection route and compared groups	p-value (robust score test)
IP injection	
SE-SVA-14 wild type vs. SE-SVA-1033 wild type	**0.027**
SE-SVA-14 wild type vs. SE-SVA-14-3D	**0.021**
SE-SVA-14 wild type vs. SE-SVA-14-5G	**0.022**
SE-SVA-14-3D vs. SE-SVA-14-5G	**0.029**
SE-SVA-1033 wild type vs. SE-SVA-1033-3F	0.085
SE-SVA-1033 wild type vs. SE-SVA-1033-9C	**0.033**
SE-SVA-1033-3F vs. SE-SVA-1033-9C	**0.028**
Immersion	
SE-SVA-14 wild type vs. SE-SVA-1033 wild type	0.186
SE-SVA-14 wild type vs. SE-SVA-14-3D	0.125
SE-SVA-14 wild type vs. SE-SVA-14-5G	**0.045**
SE-SVA-14-3D vs. SE-SVA-14-5G	0.485
SE-SVA-1033 wild type vs. SE-SVA-1033-3F	0.240
SE-SVA-1033 wild type vs. SE-SVA-1033-9C	0.611
SE-SVA-1033-3F vs. SE-SVA-1033-9C	0.188

survival proportion in the group immersed with SE-SVA-14 wild type was significantly lower than in the groups immersed with SE-SVA-14-5G. VHSV was re-isolated from tissues of almost all dead fish in the inoculated groups. Failed detection of VHSV in a few of the dead fish might be due to either a low non-specific mortality, as also observed in the negative control groups mock-inoculated by injection or immersion (3 and 6%, respectively), or poor quality of the sampled dead individuals.

DISCUSSION

Previous studies on mammal rhabdoviruses have successfully identified virulence determinants of rabies virus, whereby a few point mutations of aa within the G-protein was sufficient to

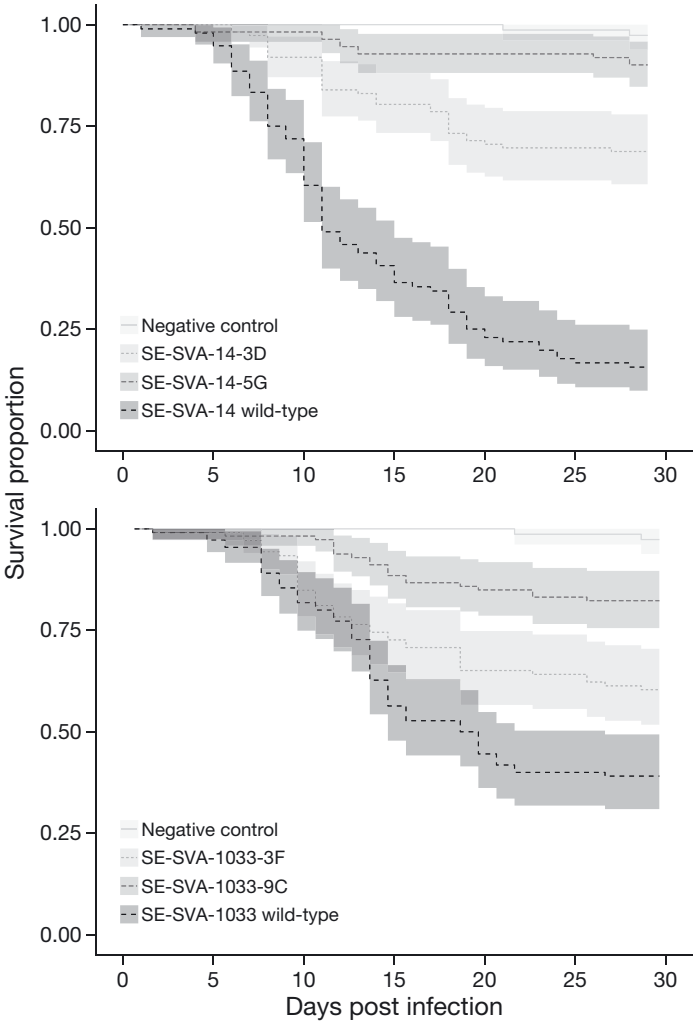

Fig. 4. Survival proportions of rainbow trout infected by the viral haemorrhagic septicaemia virus (VHSV) Ib clones obtained from rainbow trout virulent Ib VHSV isolates studied by intraperitoneal injection. (a) SE-SVA-14 wild-type isolate and the variant clones. (b) SE-SVA-1033 wild-type isolate and the variant clones. Gray shading shows 95% confidence intervals

have a dramatic impact on virulence (Takayama-Ito et al. 2006). The authors of that article suggested that only a few aa substitutions are necessary to determine virulence among rhabdoviruses for a prescribed host. With regard to VHSV virulence determinants for rainbow trout *Oncorhynchus mykiss*, several studies have focused on comparative studies of the G-protein in high- and non-virulent isolates (Béarzotti et al. 1995, Betts & Stone 2000, Snow & Cunningham 2000). From the results of comparing the virulent isolates DK-Hededam (I) and FR-14-58 (Ia) with 2 non-virulent Ib isolates, VHSV 96-43 and cod ulcus (synonym of M. Rhabdo), Betts & Stone (2000) suggested that only a limited number of genetic changes in various viral proteins may be involved in virulence determination. Snow & Cunningham (2000) reported that the virulence in rainbow trout of the VHSV genotype III strain UK-860/94, which was isolated from an outbreak in farmed turbot, increased after 5 *in vivo* passages in rainbow trout. However, the viral G-gene sequences were examined for all 5 passages and were 100% identical, indicating that the observed change in virulence was not a result of G-protein mutations. Using recombinant viruses, Einer-Jensen et al. (2014) explored how the interchange of the G- and Nv-protein from VHSV strains with significant differences in virulence was not found to be associated with variability of the G-protein nor with the Nv-protein. Furthermore, Kim et al. (2014) reported that the *in vitro* virulence for gill epithelial cells increases by the substitution Ile1012Phe of the L-protein between VHSV Ia, Ib, III and IVa isolates. Finally, Ito et al. (2016) suggested that substitutions of the aa in positions 118–123 of the N-protein in VHSV III isolates are candidates for virulence of VHSV III in rainbow trout. In the present study, based on the results from the *in vivo* testing of clones obtained from the Ib isolates SE-SVA-14 and SE-SVA-1033, and based on a comparative analysis of the encoding regions of the generated clonal genomes, we suggest that the substitutions of aa in the N-protein regions aa 43–46 and aa 168, or a combination of the 2, could be such virulence determinants to rainbow trout of VHSV Ib isolates. Judging from previous reports and the present study, it seems like the virulence determinants of VHSV to rainbow trout varies according to the genotype and probably also according to host species.

In Expt 1, the survival rate of the rainbow trout group infected with the SE-SVA-1033-3F clone was significantly lower than in the group infected with the SE-SVA-1033-9C clone. In Expt 2, the survival proportion in the SE-SVA-14-3D group was significantly lower than in the SE-SVA-14-5G group, and significantly lower in the SE-SVA-1033-3F group than in SE-SVA-1033-9C. These results suggest that virulence of clones SE-SVA-1033-3F and SE-SVA-14-3D are higher than the other clones of the same original isolate. In Expt 2, the wild types of SE-SVA-14 and SE-SVA-1033 showed high virulence compared with each variant clone. The reason might be that the wild strains contain more highly virulent clones. The original viruses were isolated from pooled material sampled from >1 fish. Upon injection, the SE-SVA-14 wild type showed significantly lower survival than both established clones, and SE-SVA-14-3D was more virulent than SE-SVA-14-5G.

Unfortunately, the significant difference in the mortality rate between SVA-14-5G and SVA-14-3D was not confirmed by Expt 1. The differences in the results between the experiments in Denmark and Japan might have been caused by the number of fish used for the experiments. The aa substitution in position 113 (arginine (Arg) → glycine (Gly)) of SE-SVA-14-5G of the N-protein was observed. However, when compared with other VHSV isolates which included all genotypes, the Gly in aa position 113 of the N-protein is exceptional (data not shown). Consequently, it was suggested that this aa substitution was not related to the virulence of VHSV. Expt 1 was purposely carried out with fish that had been bred from a single parent pair. The purpose was to increase the accuracy of experiments using the same fish strain. On the other hand, Expt 2 was conducted with fish bred from multiple parent fish to avoid the bias of the genetic background.

When the deduced aa sequences of the coding regions of all 4 SE-SVA VHSV clones were compared with the aa sequences of the low-virulent Ib isolate 4p37 (GenBank accession no. FJ460590) and the highly virulent Ia isolate DK-3592B (accession no. KC778774), variation was found in 64 aa positions across the 6 encoded viral proteins (Table S6). Among these, at least, the 2 more virulent SE-SVA clones, SE-SVA-14-3D and SE-SVA-1033-3F, only shared aa with DK-3592B in aa position Arg46 and His168 of the N-protein, respectively. Furthermore, the aa in position 43–48 (EEDRKV) of SE-SVA-1033-3F in the N-protein were identical with the aa sequence of DK-3592B (Fig. 3). Therefore, these regions may be related to the virulence to rainbow trout of the VHSV Ib isolates. The M. Rhabdo isolate with the same reaction pattern as SE-SVA-14-3D towards MAb VHS-4.20 and with a substitution at position aa Tyr168His compared to the consensus protein has been reported as non-virulent to rainbow trout (Skall et al. 2004). The aa sequence of this virus in the positions aa 43–48 (EEDGKV) is identical to the aa sequence in 4p37, which is known to be a low-virulent Ib isolate (Campbell et al. 2009; our Fig. 3). Therefore it is suggested that the aa substitution Glu43Lys of the N-protein is responsible for influencing the virulence of the SE-SVA-14-3D in rainbow trout (Fig. 3).

In addition, since variability in these domains affects recognition by the MAbs VHS-3.80 and VHS-4.20, these MAbs can be useful for initial evaluation of risk for disease outbreak in sea-reared rainbow trout.

Further analysis by reverse genetics is needed to fully confirm the importance of the described variability of the N-protein for virulence of VHSV isolates to rainbow trout. However, it may be speculated that the occurrence of the described variability in the more virulent sub-isolate clones reflects a quasi-species effect allowing VHSV Ib isolates to adapt to rainbow trout. Among other VHSV genotypes linked to wild marine fish, a rainbow trout virulent isolate of genotype III (Dale et al. 2009) was isolated in Norway from diseased rainbow trout, similar to the case of Ib in Sweden. Thus, marine isolates of VHSV may be a potential threat to the fish-farming industry if provided with the opportunity to adapt to rainbow trout under intensive farming conditions.

Acknowledgements. We thank our colleagues at the National Research Institute of Aquaculture, Japan Fisheries Research and Education Agency and the National Veterinary Institute, Technical University of Denmark for technical support and useful advice. This work was mainly supported by a grant for international corroborated study from the Japan Fisheries Research and Education Agency and through funding from the European Commission for the European Union Reference Laboratory for Fish Diseases. Part of this work was supported by European Commission grants FAIR-4064 and FP7 311993 TargetFish.

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Editorial responsibility: Stephen Feist, Weymouth, UK

Submitted: January 23, 2017; Accepted: January 30, 2018
Proofs received from author(s): March 17, 2018