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Genotyping of marine viral haemorrhagic septicaemia virus isolated from the Flemish Cap by nucleotide sequence analysis and restriction fragment length polymorphism patterns

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ABSTRACT: A total of 14 viral haemorrhagic septicaemia virus (VHSV) isolates obtained from Greenland halibut *Reinhardtius hippoglossoides* caught at the Flemish Cap, a fishing ground in the North Atlantic Ocean near Newfoundland, were characterised using restriction fragment length polymorphism (RFLP) and nucleotide sequence analysis. RFLP analysis was performed on a 1259 bp fragment of the glycoprotein (G) gene, and a 305 nucleotide region within the nucleoprotein (N) gene was used for sequence analysis. Representative strains of the 4 established genotypes were employed for comparative purposes. Sequencing analysis indicated that the Flemish cap isolates grouped in Genotype 3, which also includes isolates from wild fish caught in the North Sea and coastal waters of the UK and Ireland, isolates derived from outbreaks of VHS in turbot farms in the British Isles, and an isolate from European eel *Anguilla anguilla* caught in northern France. Characterisation using RFLPs resulted in the development of a simple and reliable method of typing VHSV at the genotype level using a 2-step restriction analysis (2-SRA) assay.

KEY WORDS: Viral haemorrhagic septicaemia virus · VHSV · Wild fish · Genotyping · Restriction fragment length polymorphism · RFLP · Sequencing analysis · Phylogenetics

INTRODUCTION

Viral haemorrhagic septicaemia virus (VHSV), the aetiological agent of viral haemorrhagic septicaemia (VHS), is a significant pathogen of farmed rainbow trout *Oncorhynchus mykiss* in Europe. VHS outbreaks have also been reported in other cultured and wild fish species elsewhere (Schlotfeldt et al. 1991, Meier et al. 1994, Ross et al. 1994, Meyers et al. 1999, Isshiki et al. 2001), and in recent years VHS viruses have been isolated from an increasing number of often asymptomatic wild marine fish (Dixon et al. 1997, 2003, Mortensen 1999, Smail 2000, Brudeseth & Evensen 2002, Dopazo et al. 2002, Watanabe et al. 2002, Hedrick et al. 2003).

VHSV belongs to the genus *Novirhabdovirus* of the family *Rhabdoviridae*. It has a genome consisting of approximately 11 200 nucleotides and containing 6 genes (3′ N-P-M-G-NV-L 5′). Initial nucleotide sequence analyses based on different regions of the glycoprotein (G) gene demonstrated the existence of 3 geographically distinct genotypes (Benmansour et al. 1997, Stone et al. 1997, Thiéry et al. 2002). Sequence analysis of the nucleoprotein (N) gene has supported the existence of these 3 established genetic groups (Snow et al. 1999) and, in addition, identified a new genotype (Snow et al. 2004). Further phylogenetic analysis based on the entire G gene of VHSV have confirmed the presence of 4 major genotypes (Einer-Jensen et al. 2004). Following the classification estab-

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lished by these authors, Genotype 1 groups isolates from continental Europe as well as several marine isolates from the Baltic Sea; Genotype 2 includes a number of marine isolates obtained from the Baltic Sea with no clear link to rainbow trout aquaculture; Genotype 3 comprises isolates obtained from fish cultured or caught around the British Isles, as well as a French isolate from European eel *Anguilla anguilla* caught on the Atlantic side of the Loire; Genotype 4 includes VHSV strains isolated from the Pacific coast of North America.

Although there is no doubt about the usefulness and reliability of sequencing analysis, this technology is not practical or affordable for many laboratories. For this reason, other approaches for molecular typing of fish viruses have been developed. Ribonuclease protection assays (RPA) have been employed to characterise genetic differences in closely related RNA viruses, and this technique has been applied to the study of infectious haematopoietic necrosis virus (IHNV; Kurath et al. 1995, Troyer et al. 2000) and VHSV (Snow et al. 1999). Restriction fragment length polymorphism (RFLP) patterns have also been successfully used for genotyping aquabirnaviruses (Heppell et al. 1992, Lee et al. 1996, Biering et al. 1997, Cutrín et al. 2004), and have recently been applied to VHSV by Einer-Jensen et al. (2005), who proposed employing a set of 3 enzymes for typing VHSV isolates.

Here we report the employment of both RFLP and sequence analysis to genotype 15 isolates of VHSV obtained from Greenland halibut caught at the Flemish Cap, a deep fishing ground in the North Atlantic Ocean in international waters near Newfoundland (Dopazo et al. 2002). At least 1 reference strain belonging to each of the genotypes established by Snow et al. (2004) was also included for comparative purposes and for the development of a simplified method of typing VHSV strains by RFLP analysis.

MATERIALS AND METHODS

Cell lines and viruses. The VHSV strains under study were isolated from Greenland halibut *Reinhardtius hippoglossoides* during a 20 d research survey in the Flemish Cap (Newfoundland), a fishing ground with a total area of approximately 30 000 km^2. Fish were collected along different trawling transects at depths between 200 and 500 m. In addition, a number of reference VHSV isolates from different genotypes were used for comparative purposes. All VHSV isolates (Table 1) were grown on epithelioma papillosum cyprini (EPC) and bluegill fry trunk (BF-2) cell monolayers at 15°C using Eagle's minimum essential medium or L-15 supplemented with 100 IU of penicillin ml^{-1}, 100 µg streptomycin ml^{-1} and 10% foetal calf serum. Available DNA sequences of other VHSV viruses listed in Table 1 were used for phylogenetic analysis.

RNA extraction. Confluent monolayers of EPC or BF-2 in 25 cm^2 flasks were inoculated (multiplicity of infection, MOI 0.1–1) with each viral isolate and incubated at 15°C. When early cytopathic effects were visualised, the overlying medium was carefully removed and RNA extraction was performed using Trizol® LS reagent (Gibco, BRL) as described by the manufacturer. The extracted RNA was finally resuspended in 50 µl of nuclease-free water.

Sequencing analysis. Reverse transcription (RT) reactions were performed using 5 µl of purified RNA. cDNA to poly (A)+RNA was prepared by incubation of total RNA with 56 pmol oligo $(dT)_{15}$ primer and 200 U of Moloney Murine Leukemia virus (MMLV) reverse transcriptase (Invitrogen) in a total reaction volume of 20 µl containing 2 mM deoxyribonucleotide triphosphate (dNTP), 0.1 M dithiothreitol (DTT), 5× first strand buffer (Invitrogen) and 20 U RNase Out (Invitrogen).

Each reaction tube was subjected to PCR using a pair of specific primers designed to amplify a 511 nucleotide (nt) region corresponding to bases 166 to 677 of the VHSV N gene referring to the published sequence of the reference strain FR 07/71 (accession no. AJ233396; VNFor 5′-ATGGAAGGAGGAATTCG TGAAGCG 3′, VNRev 5′-GCGGTGAAGTGCTGCA GTTCCC 3′). PCR amplifications were performed in 50 µl volumes under mineral oil, using 28 pmol of each primer, in the presence of 1.5 mM $MgCl_2$, 10 mM dNTP, 2 U BioTaq polymerase (Bioline) and 1× PCR buffer (160 mM $(NH_2)_2SO_4$, 670 mM Tris-HCl [pH 8.8], 0.1% Tween-20). Template material consisted of 5 µl cDNA dilution prepared as described. Amplification was performed during 35 cycles of denaturing (94°C, 1 min), re-annealing (55°C, 1 min) and extension (72°C, 1 min) followed by a single final extension step (72°C, 5 min) on a Techne Genius thermocycler.

Amplified products were verified by electrophoresis on a 1.5% agarose gel and purified using the Wizard® PCR Preps DNA purification system according to the manufacturer's instructions (Promega). Purified products were quantified following electrophoresis on a 1.5% agarose gel alongside standards of known concentration.

Triplicate PCR products were sequenced with the same primers using an Applied Biosystems model 373A automated sequencer with the ABI Prism dye terminator sequencing chemistry according to the manufacturer's instructions (Applied Biosystems).

Sequences were edited using the DNASTAR Lasergene v.6 SeqMan II and EditSeq programs. Nucleotide sequences of 335 nt were aligned by using the DNAS-

Table 1. VHSV isolates and sequences used in this study

VHSV isolate	Genotype	Geographic origin	Host species	Source	Accession no.		
					Gene N	Gene G	Complete sequence
Strains propagated in cell culture							
GH30	3	Flemish Cap	*Reinhardtius hippoglossoides*	Dopazo et al. 2002	AJ849477		
GH32	3	Flemish Cap	*Reinhardtius hippoglossoides*	Dopazo et al. 2002	AJ849478		
GH33	3	Flemish Cap	*Reinhardtius hippoglossoides*	Dopazo et al. 2002	AJ849479		
GH34	3	Flemish Cap	*Reinhardtius hippoglossoides*	Dopazo et al. 2002	AJ849480		
GH35	3	Flemish Cap	*Reinhardtius hippoglossoides*	Dopazo et al. 2002	AJ849481		
GH36	3	Flemish Cap	*Reinhardtius hippoglossoides*	Dopazo et al. 2002	AJ849482		
GH37	3	Flemish Cap	*Reinhardtius hippoglossoides*	Dopazo et al. 2002	AJ849483		
GH40	3	Flemish Cap	*Reinhardtius hippoglossoides*	Dopazo et al. 2002	AJ849484		
GH42	3	Flemish Cap	*Reinhardtius hippoglossoides*	Dopazo et al. 2002	AJ849485		
GH43	3	Flemish Cap	*Reinhardtius hippoglossoides*	Dopazo et al. 2002	AJ849486		
GH45	3	Flemish Cap	*Reinhardtius hippoglossoides*	Dopazo et al. 2002	AJ849487		
GH46	3	Flemish Cap	*Reinhardtius hippoglossoides*	Dopazo et al. 2002	AJ849488		
GH47	3	Flemish Cap	*Reinhardtius hippoglossoides*	Dopazo et al. 2002	AJ849489		
GH48	3	Flemish Cap	*Reinhardtius hippoglossoides*	Dopazo et al. 2002	AJ849490		
FR-07/71	1a	France	*Oncorhynchus mykiss*	Le Berre et al. 1977		AY546616	AJ233396
DK3592B	1a	Denmark	*Oncorhynchus mykiss*	Lorenzen et al. 1993	AF012093	X66134	
DK1p40	1b	Baltic Sea	*Rhinonemus cimbrius*	Mortensen et al. 1999	AJ130919	AY546575	
DK5p31	1b	Kattegat	*Clupea harengus*	Skall et al. unpubl.	AY356674		
DK 1p12	1b	Baltic Sea	*Clupea harengus*	Mortensen et al. 1999	AY356653	AY546574	
DK1p49	2	Baltic Sea	*Clupea harengus*	Mortensen et al. 1999	AY356743		
DK5p551	2	Baltic Sea	*Clupea harengus*	Skall et al. unpubl.	AY356690		
UK 860-94	3	Gigha	*Scophthalmus maximus*	Ross et al. 1994	AJ130915	AY546628	
US-Makah	4	WA, USA	*Oncorhynchus kisutch*	Benmansour et al. 1997	X59241	U28747	
Strains employed only for sequence analysis							
UK H17/5/93	3	North Sea	*Gadus morhua*	Smail 2000	AY356727	AY546630	
DK 4p101	3	North Sea	*Merlangius merlangus*	Mortensen et al. 1999	AJ130918	AY546581	
IR-F13.02.97	3	SW Ireland	*Scophthalmus maximus*	McArdle unpubl.	AJ130916	AY546620	
UK H17/1/95	3	North Sea	*Melanogrammus aeglefinus*	Smail 2000	AY356720		
UK MLA98/6PT10	3	North Sea	*Melanogrammus aeglefinus*	Smail 2000	AY356723		
UK MLA98/6PT11	3	North Sea	*Trisopterus esmarkii*	King et al. 2001	AY356731		
UK DK2p51	3	Skagerrak	*Trisopterus esmarkii*	Mortensen et al. 1999	AJ130917		
UK MLA98/4PT1	3	North Atlantic	*Trisopterus esmarkii*	King et al. 2001	AY356719		
UK MLA98/6WH1	3	North Sea	*Merlangius merlangus*	King et al. 2001	AY356729		
UK MLA98/6PT14	3	North Sea	*Trisopterus esmarkii*	King et al. 2001	AY356730		
UK MLA98/4CO1	3	N. Atlantic	*Gadus morhua*	King et al. 2001	AY356735		
DK-4p51	3	North Sea	*Argentina sphyraena*	Mortensen et al. 1999	AY356736		
UK-MLA98/4PT4	3	N. Atlantic	*Trisopterus esmarkii*	King et al. 2001	AY356739		
UK-MLA98/4PT5	3	N. Atlantic	*Trisopterus esmarkii*	King et al. 2001	AY356740		

TAR Lasergene v.6 MegAlign program. Phylogenetic analysis was performed according to the neighbour-joining (NJ) method as implemented in the software PAUP, version 4.0 beta. The tree was constructed using TreeView 1.5. Bootstrap analysis was performed using 1000 data resamplings. Nucleotide sequences of the Flemish Cap isolates obtained in the present study have been deposited in the GenBank database under accession numbers AJ849477 to AJ849490.

Selection of restriction enzymes for RFLP assays. RFLP analysis was conducted on a fragment of 1259 bp corresponding to a region of the VHSV G gene. Theoretical selection of the endonucleases to be assayed was performed using the software MapDraw from DNAstar (Lasergene) and its enzyme database. From around 250 enzymes initially tested, more than 100 were pre-selected based on the presence of the specific restriction site in any of the reference sequences employed. Further selection was performed considering those giving different fragment profiles among the known genotypes of VHSV (Snow et al. 2004), resulting in 11 endonucleases chosen for evaluation: *Acc*I, *Alu*I, *Bbu*I (or its isozyme *Sph*I), *Bsp*1286I, *Cfo*I (isozyme *Hha*I), *Dde*I, *Eco*RI, *Eco*RV, *Hpa*II (*Msp*I),

*Mbo*II and *Nci*I. The expected number and positions of restriction sites in a PCR product were determined based on published sequences corresponding to the glycoproteins of strain FR07/71 (accession no. AJ233396) for Genotype 1a; DK1p12 (AY546574) and DK1p40 (AY546575) for Genotype 1b; DK1p52 (AY546576) for Genotype 2; UK860/94 (AY546628) for Genotype 3; and US-Makah (U28747) for Genotype 4.

Restriction assay. The primer pair 90103U and 90103L, yielding an amplification fragment of 1259 bp, was employed in this assay. Primer 90103U (5′ TCCCGATTCGACCAGCTCAACTCA 3′) hybridises to positions 3119–3142 and primer 90103L (5′ CCCTCCCACAACCCCCATCC 3′) hybridises to positions 4358–4377.

RT-PCR was performed using the Geneamp Gold RNA PCR reagent kit (Applied Biosystems). Each reaction tube containing purified viral RNA (100–200 ng) and RT mix (5× RT-PCR buffer: 25 mM $MgCl_2$, 10 mM dNTP, RNAse inhibitor [20 U μl^{-1}], 100 mM DTT, 50 µM random hexamers and MultiScribe Reverse Transcriptase [50 U μl^{-1}]) was subjected to 25°C for 10 min and 42°C for 30 min. Both specific primers (0.5 µM each), 10 mM dNTP, 25 mM $MgCl_2$, AmpliTaq Gold DNA polymerase (5 U μl^{-1}) and 5× RT-PCR buffer were then added and the mixture subjected to incubation at 95°C for 10 min, followed by 45 cycles of PCR (denaturation for 60 s at 94°C, annealing for 90 s at 55°C, and amplification at 72°C for 90 s) in a Mastercycler Personal Thermal Cycler (Eppendorf). The polymerisation was concluded with an extension period of 10 min at 72°C.

The RT-PCR products were subjected to electrophoresis in 1.5% agarose gels containing ethidium bromide (0.5 µg ml^{-1}). Specific bands were located under UV light and cut out from the gel, and the DNA was purified using a Gel Extraction Device filtrate vial (Millipore). Purified PCR products were subjected to restriction endonuclease digestion at 37°C for 3 to 4 h in the digestion buffer supplied with the corresponding enzyme.

DNA fragments from restriction endonuclease digestion were separated by electrophoresis in 2% agarose gels. The gels were stained with ethidium bromide (0.5 mg ml^{-1}) for 30 min and the bands were visualised under UV light. The sizes of the fragments were determined by comparison with molecular weight markers (100 bp DNA ladder; Promega).

Predicting the robustness of the typing system for RFLP assays. The reliability of the typing system was theoretically evaluated (with the software package Lasergene) on all sequences of the G gene available in the GenBank database (70 sequences: 35 for Genotype 1a; 16 for Genotype 1b; 3 for Genotypes 1c, 1d and 2; 9 for Genotype 3; and 1 for Genoptype 4), and the results were expressed as the percentage of strains yielding the expected RFLPs.

RESULTS

Nucleotide sequencing analysis

Sequencing analysis revealed that most of the Greenland halibut (GH) isolates were identical with respect to the 335 nt region of the N gene sequenced. Indeed, a single nucleotide difference was only identified in 1 isolate (GH 34), which thus showed a pairwise nucleotide diversity of 0.3% (99.7% identity, data not shown). The GH isolates were most closely related to the reference strains from Genotype 3, displaying percentage nucleotide identity values of 97.0 ± 0.3% for GH34 and 97.3 ± 0.3% for the remaining GH isolates. The GH isolates were less than 89% identical to Genotypes 1a, 1b, 2 and 4. The highest identity (97.9%) was identified with strains UK-860/97 and IR-F13.02.97, which corresponded to a mismatch of 7 nucleotides. The position of one of those nucleotide substitutions was different depending on the reference strain used for comparison. In terms of amino acid changes, the percentage of identity with the 2 reference strains was increased to 99.1% (only 1 substitution).

As shown in the phylogenetic tree (Fig. 1), the GH isolates were clustered within the Genogroup 3 clade, but due to the overall low diversity within the data set, relationships of the GH isolates to other isolates within Genotype 3 were not resolved.

Evaluation of restriction enzymes against reference strains

Two enzymes (*Hpa*II and *Mbo*II) that yielded non-reproducible results or patterns difficult to interpret were rejected (results not shown). None of the remaining 9 enzymes tested allowed the separation of all genotypes into unique RFLPs, as shown in Table 2. Three enzymes each yielded 3 RFLP profiles for the reference strains *Acc*I, *Alu*I and *Cfo*I, but the first 2 produced results that differed from those theoretically predicted (results not shown). Endonucleases *Bsp*1286I (Fig. 2), *Eco*RV (Fig. 2), *Nci*I (Fig. 2), and *Dde*I all yielded 4 RFLP profiles for the reference strains, but results with *Dde*I were not always reproducible.

Enzymes *Bbu*I and *Eco*RI (Table 2), were poor at differentiating between genotypes, but were considered due to their ability to separate strains of Genotype 3 from other genotypes (*Eco*RI) or to differentiate the GH isolates from any of the described genotypes (*Bbu*I).

Development of a rapid and simple genotyping system for VHSV

Since no single enzyme was identified that could robustly distinguish all genotypes, the enzymes yielding 4 RFLPs (*Nci*I, *Eco*RV and *Bsp*1286I) appeared most suited for use in a 2-step specific system for typing of VHSV isolates. These enzymes alone proved incapable of differentiating Genotypes 2 and 3 (*Nci*I), Genotypes 1a and 1b (*Eco*RV) or 1b and 2 (*Bsp*1286I), respectively. Based on the analysis of robustness (results not shown), *Bsp*1286I was selected as the enzyme of choice for further development of a 2-step discrimination assay. A second enzyme used in this assay to allow subsequent discrimination of Genotypes 1b and 2 was *Nci*I, which was also highly robust (100 % for both genotypes).

Typing of VHSV isolates from the Flemish Cap

Four enzymes (*Bbu*I, *Bsp*1286I, *Dde*I and *Eco*RV) provided a unique RFLP profile for the GH isolates, separating them from all defined genotypes (Table 2).

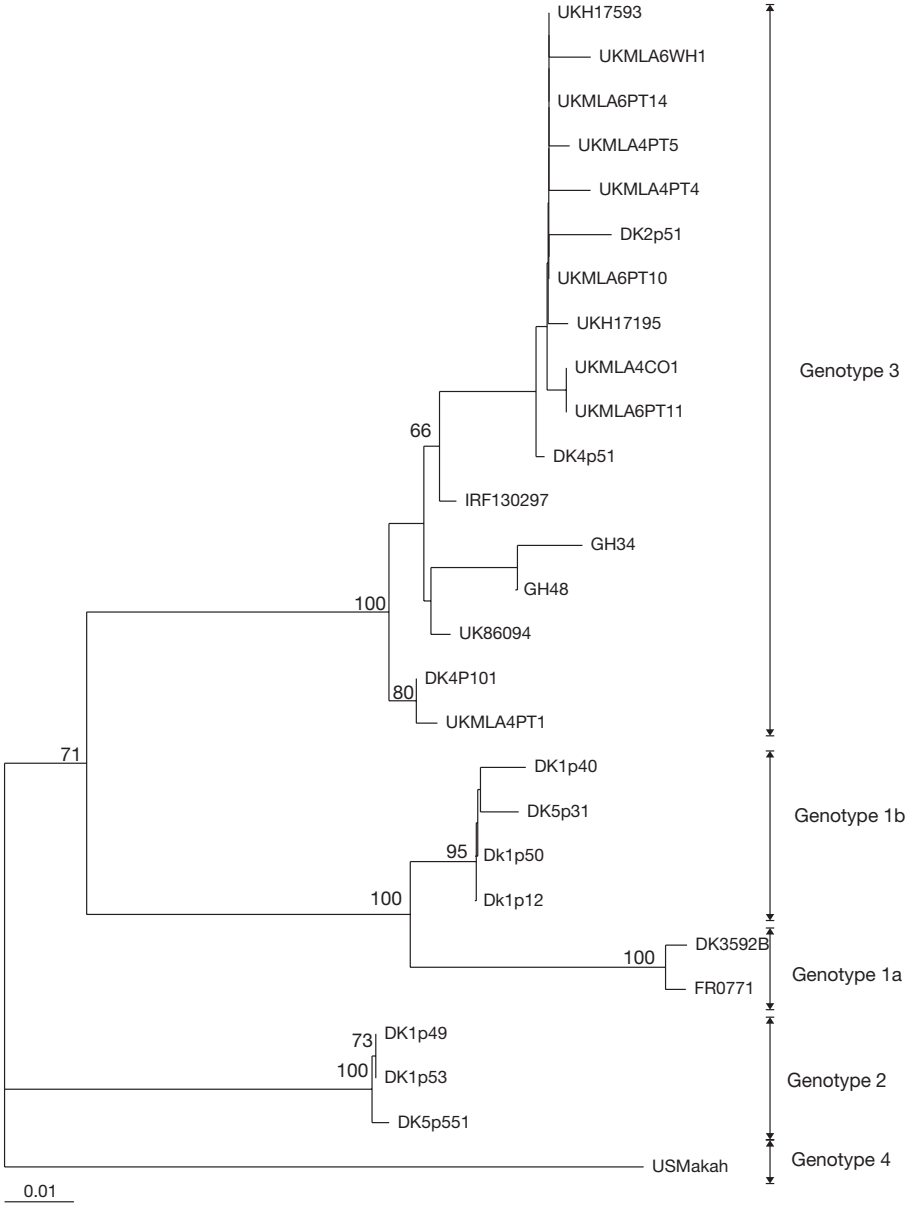

Fig 1. Phylogenetic relationships among GH isolates and reference strains from different genotypes of VHSV (for further details see Table 1). Phylogeny was inferred by using the neighbour-joining method included in PAUP version 4.0 beta. Bootstrap values are indicated as percentages of 1000 resamplings. Only values ≥60 % are presented

Table 2. Distinctive restriction fragment length polymorphism patterns (RFLPs) obtained with endonucleases applied to Greenland halibut (GH) isolates and different genotypes of viral haemorrhagic septicaemia virus (VHSV). gt: genotype

Endonucleases	RFLP patterns				
	1	2	3	4	5
*Acc*I	1a	1b, 2, 3, GH	4		
*Alu*I	1[a], 2, GH	3	4		
*Bbu*I (*Sph*I)	All genotypes	GH			
*Bsp*1286I	1a	1b, 2	3	4	GH
*Cfo*I (*Hha*I)	1a, 2, GH	1b, 3	4		
*Dde*I	1[a]	2	3	4	GH
*Eco*RI	3	Remaining gt			
*Eco*RV	1[a]	2	3	4	GH
*Nci*I	1a	1b	2, 3, GH	4	

[a]Genogroup 1, including Genotypes 1a and 1b

Two additional enzymes (*Alu*I and *Cfo*I) separated the GH isolates from the remaining Genotype 3 strains (Table 2). *Bbu*I was the only enzyme generating one unique RFLP for the GH isolates and a second for all other known genotypes.

DISCUSSION

The distinction of 4 main genotypes within VHSV, which correlate with geographical areas of isolation, has been clearly demonstrated on the basis of phylogenetic studies using both N and G genes (Einer-Jensen et al. 2004, Snow et al. 2004).

To date, Genotype 3 included marine isolates from the North Sea/coastal waters of the British Isles together with 2 isolates recovered from outbreaks of VHS in Irish and Scottish turbot farms (Snow et al. 1999, 2004, Einer-Jensen et al. 2004) as well as 1 isolate originating from eel caught in northern France (Thiéry et al. 2002, Einer-Jensen et al. 2004). Results obtained in the present study based on sequencing of a 335 nt region of the N gene indicated that the Flemish Cap isolates must also be grouped in Genotype 3, extending the geographic area in which this genotype is found to the eastern rim of the North Atlantic Ocean. Although the Flemish Cap is certainly far away from the European waters where isolates belonging to this genotype have been obtained, the spreading of the virus could be explained if migratory patterns of Greenland halibut are taken into account. This species is widely distributed in the North Atlantic and can cover extreme distances during migration, from Norway and Iceland to the Grand Banks of Newfoundland (Vis et al. 1997).

Due to the sampling procedure, the low variability observed among the GH isolates (at least regarding this sequence) can be extrapolated to the whole population of Greenland halibut and suggests that the strains observed must share a common and relatively recent origin. On the other hand, inclusion of GH isolates in Genotype 3 will increase the variability within this genotype. In fact, the number of differences between any of the 14 Flemish cap isolates and the other members of Genotype 3 previously sequenced ranged from 7 to 10 nt (for the 335 nt sequenced fragment). Snow et al. (2004) reported a mean of 4 nucleotide differences among 30 strains of this genotype, only 3 of which showed similar differences to those observed in the present report.

The Flemish Cap isolates showed the highest relatedness to 2 of the 15 strains employed as reference for Genotype 3: UK 860/94 and IR-F13.02.97 isolates (99.1% amino acid similarity). This high level of homology with 2 strains that were involved in epizootic outbreaks in farmed turbot (Ross et al. 1994) emphasises the risk that 'wild marine' VHSV strains could represent for the turbot farming industry, as has been previously suggested by other authors (Snow & Smail 1999, King et al. 2001, Snow et al. 2004). In addition, it brings into question the management of VHSV in the European Union, based on the maintenance of approved zones. In fact, the well documented existence of VHSV pathogenic or potentially pathogenic for cultured turbot in wild marine environments is at odds with the existence of approved zones, at least as it refers to marine aquaculture.

On the basis of the presumed higher evolutionary pressures exerted on the G gene, and with the hope of finding a higher diversity among the GH isolates, the G gene was chosen for the RFLP analysis. Nevertheless, no alteration of the results of typing was expected since the same genotyping results have been reported using N and G genes (Einer-Jensen et al. 2004, Snow et al. 1999, 2004)

All enzymes used in this study were chosen on the basis of distinctive predicted restriction sites using a selection of the published sequences corresponding to the different genotypes. No enzyme was found to allow detection of differences among the GH isolates, but 1 enzyme, *Bbu*I (isozyme *Sph*I), was observed to completely differentiate these isolates from all known genotypes. Therefore, we are now employing this enzyme to type VHSV isolates from the Flemish Cap.

Although we were not able to find a single enzyme that allowed identification of all individual genotypes, we propose a new method of typing new VHSV isolates that can be as simple as a 2-step restriction analy-

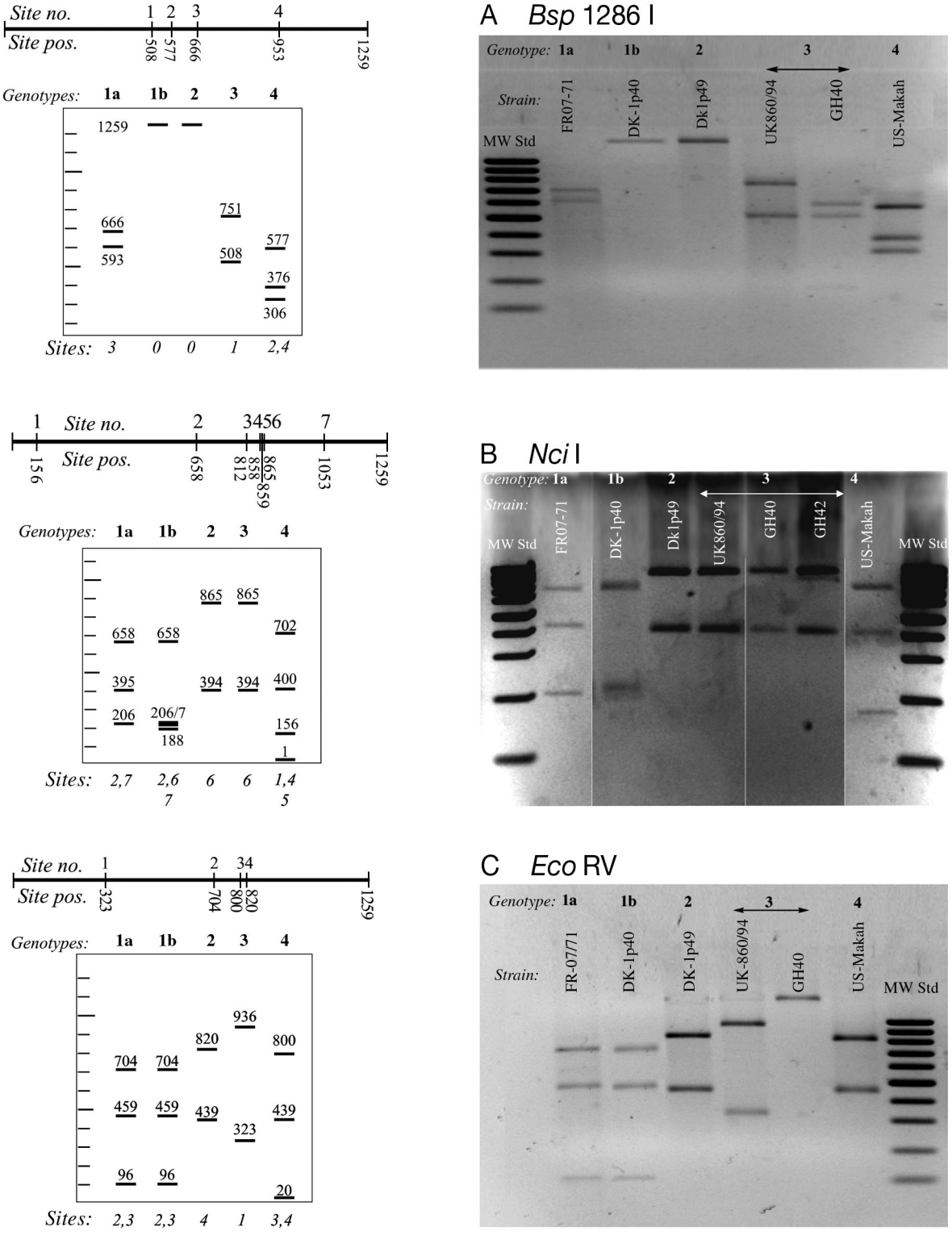

Fig. 2. Expected restriction sites and obtained restriction patterns for 3 endonucleases assayed. A: endonuclease *Bsp*1286I; B: *Nci*I; C: *Eco*RV. The scaled line shows the site number and site position (nucleotide number) for the sequence of the strain FR 07/71 (accession no. AJ233396). The square figures show the expected profiles and fragment sizes (bp) for reference strains of each genotype. Genotype 1a: reference strain FR 07/71 (AJ233396); 1b: DK1p12 (AY546574); 2: DK1p52 (AY5646576); 3: UK860/94 (AY546628); 4: US-Makah (U28747)

sis (2-SRA) assay applied to the 1259 cDNA fragment corresponding to the G gene (Table 3). In the first step, *Bsp*1286I specifically identifies isolates to genotype level unless they belong to Genotype 1b or 2. In such a case, the application of the second enzyme, *Nci*I, to the original 1259 bp cDNA fragment allows specific characterisation of the isolate into either Genotype 1b or 2. Although these enzymes were selected for their demonstrated robustness, any combination of 2 of the enzymes *Bsp*1286I, *Nci*I and *Eco*RV could be used. Since RFLPs rely on the presence of exact sequences reflecting the recognition site of each enzyme, it is important to recognise that a single change in this sequence could lead to different restriction patterns and different typing results. For this reason, we recommend routinely performing the analysis simultaneously using both enzymes to minimise the risk of potential misclassification of isolates.

Regarding the Flemish Cap isolates, it was interesting to observe that most of the enzymes did not place them within Genotype 3, although many provided a characteristic profile different to the RFLPs corresponding to any other strain. These results corroborate the data obtained by sequencing, which indicate the existence of a higher diversity within Genotype 3 than that detected until present. This finding does not question the reliability of the RFLP system, since it is possible to admit the existence of 2 different profiles within Genotype 3 when certain enzymes are used, profiles which are characteristic and distinguishable from other genotypes.

Although other methods that provide rapid information of sequence heterogeneity, such as RPAs, have been used to group VHSV isolates (Snow et al. 1999), the RFLP assay is the first approach that provides a direct correlation with sequence genotyping, allowing a considerable saving of time and economic resources. At the time of completion and submission of the present article, a similar study was published by Einer-Jensen (2005). In their report, the authors performed an analysis on a large number of VHSV isolates and available sequences of the G gene and proposed the use of 3 enzymes to differentiate the VHSV genotypes. Although their report is undoubtedly novel and useful, we believe that the method we propose here simplifies the typing of VHSV strains, since in most cases, a single enzyme is enough to differentiate genotypes; only if a certain RFLP is obtained does it become necessary to employ a second enzyme to separate specific genotypes.

In summary, the results obtained in this study indicate that VHSV strains isolated from GH caught at the Flemish Cap must be grouped into Genotype 3, as has been demonstrated on the basis of sequencing analysis. The inclusion of GH strains increases the variability previously recognised within Genotype 3 (Snow et al. 2004), differences that are corroborated by the results obtained by RFLP assays. Moreover, the use of RFLPs provides a simpler and faster alternative to direct sequence analysis, even distinguishing between 2 main subgroups established within Genotype 1 (1a and 1b). Further studies using strains belonging to sublineages 1c and 1d recently identified by Einer-Jensen et al. (2004) will be necessary to determine if the restriction enzymes chosen in this study will also be able to correctly type these isolates.

Table 3. Typing of VHSV strains by the 2-step restriction assay (2-SRA)

Step 1			Step 2		
Enzyme 1	RFLP	Genotype	Enzyme 2	RFLP	Genotype
*Bsp*1286I	1	1a			
	2	1b, 2	*Nci*I	1	1b[a]
				2	2[a]
	3	3			
	4	4			
	5	GH			

[a]2 sites, yielding distinctive fragment profiles

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LITERATURE CITED

Benmansour A, Basurco B, Monnier AF, Vende P, Winton JR, de Kinkelin, P (1997) Sequence variation of the glycoprotein gene identifies three distinct lineages within field isolates of viral haemorrhagic septicaemia virus, a fish rhabdovirus. J Gen Virol 78:2837–2846

Biering E, Melby HP, Mortensen SH (1997) Sero- and genotyping of some marine aquatic birnavirus isolates from Norway. Dis Aquat Org 28:169–174

Brudeseth BE, Evensen Ø (2002) Occurrence of viral haemorrhagic septicaemia virus (VHSV) in wild marine fish species in the coastal regions of Norway. Dis Aquat Org 52: 21–28

Cutrín JM, Barja JL, Nicholson BL, Bandín I, Blake S, Dopazo CP (2004) Restriction fragment length polymorphisms and sequence analysis: an approach for genotyping infectious pancreatic necrosis virus reference strains and other aquabirnaviruses isolated from northwestern Spain. Appl Environ Microbiol 70(2):1059–1067

Dixon PF, Feist S, Kehoe E, Parry L, Stone DM, Way K (1997) Isolation of viral haemorrhagic septicaemia virus from Atlantic herring *Clupea harengus* from the English channel. Dis Aquat Org 30:81–89

Dixon PF, Avery S, Chambers E, Feist S and 7 others (2003) Four years of monitoring for viral haemorrhagic septi-

caemia virus in marine waters around the United Kingdom. Dis Aquat Org 54:175–186

Dopazo CP, Bandín I, López-Vázquez C, Lamas J, Noya M, Barja JL (2002) Isolation of viral hemorrhagic septicemia virus from Greenland halibut *Reinhardtius hippoglossoides* caught at the Flemish Cap. Dis Aquat Org 50: 171–179

Einer-Jensen K, Ahrens P, Forsberg R, Lorenzen N (2004) Evolution of the fish rhabdovirus viral haemorrhagic septicaemia virus. J Gen Virol 85:1167–1179

Einer-Jensen K, Winton J, Lorenzen N (2005) Genotyping of the fish rhabdovirus, viral haemorrhagic septicaemia virus, by restriction fragment length polymorphism. Vet Microbiol 106:167–178

Hedrick RP, Batts WN, Yun S, Traxler GS, Kaufman J, Winton JR (2003) Host and geographic range extensions of the North American strain of viral hemorrhagic septicemia virus. Dis Aquat Org 55:211–220

Heppell J, Berthiaume L, Tarrab E, Lecomte J, Arella M (1992) Evidence of genomic variations between infectious pancreatic necrosis virus strains determined by restriction fragment profiles. J Gen Virol 73:2863–2870

Isshiki T, Nishizawa T, Kobayashi T, Nagano T, Miyazaki T (2001) An outbreak of VHSV (viral hemorrhagic septicemia virus) infection in farmed Japanese flounder *Paralichtys olivaceus* in Japan. Dis Aquat Org 47:87–99

King JA, Snow M, Skall HF, Raynard RS (2001) Experimental susceptibility of Atlantic salmon *Salmo salar* and turbot *Scophthalmus maximus* to European freshwater and marine isolates of viral haemorrhagic septicaemia virus. Dis Aquat Org 47:25–31

Kurath G, Highman KH, Björklund HV (1995) The NV genes of fish rhabdoviruses: development of RNAses protection assays for rapid assessment of genetic variation. Vet Res 26(5–6):477–485

Lee MK, Blake SL, Singer JT, Nicholson BL (1996) Genomic variation of aquatic birnaviruses analyzed with restriction fragment length polymorphisms. Appl Environ Microbiol 62(7):2513–2520

Le Berre M, de Kinkelin P, Metzger A (1977) Identification serologique des rhabdovirus des salmonides. Bull Off Int Epizoot 87:391–393

Lorenzen N, Olesen NJ, Jørgensen PEV (1993) Antibody response to VHS virus proteins in rainbow trout. Fish Shellfish Immunol 3:461–473

Meier W, Schmitt M, Wahli T (1994) Viral hemorrhagic septicemia (VHS) of nonsalmonids. Annu Rev Fish Dis 4: 359–373.

Meyers TR, Short S, Lipson K (1999) Isolation of the North American strain of viral hemorrhagic septicemia (VHS) associated with epizootic mortality in two new host species of Alaskan marine fish. Dis Aquat Org 38:81–86.

Mortensen HF, Heurer OE, Lorenzen N, Otte L, Olesen NJ (1999). Isolation of viral haemorrhagic septicaemia virus (VHSV) from wild marine fish species in the Baltic Sea, Kattegat, Skagerrak and the North Sea. Virus Res 63: 95–106

Ross K, McCarthy U, Huntly PJ, Wood BP, Stuart D, Rough EI, Smail DA, Bruno DW (1994) An outbreak of viral haemorrhagic septicaemia (VHS) in turbot (*Scophthalmus maximus*) in Scotland. Bull Eur Assoc Fish Pathol 14:213–214

Schlotfeldt HJ, Ahne W, Vestergard-Jorgensens PE, Glende W (1991) Occurrence of viral haemorrhagic septicaemia in turbot (*Scophthalmus maximus*) – a natural outbreak. Bull Eur Assoc Fish Pathol 11:105–107

Smail DA (2000). Isolation and identification of Viral Haemorrhagic Septicaemia (VHS) viruses from cod *Gadus morhua* with the ulcus syndrome and from haddock *Melanogrammus aeglefinus* having skin haemorrhages in the North Sea. Dis Aquat Org 41:231–235

Snow M, Smail, DA (1999). Experimental susceptibility of turbot *Scophthalmus maximus* to viral haemorrhagic septicaemia virus isolated from cultivated turbot. Dis Aquat Org 38:163–168

Snow M, Cunningham CO, Melvin WT, Kurath G (1999) Analysis of the nucleoprotein gene identifies distinct lineages of viral haemorrhagic septicaemia virus within the European marine environment. Virus Res 63:35–44

Snow M, Bain N, Black J, Taupin W, Cunningham CO, King JA, Skall HF, Raynard RS (2004) Genetic population structure of marine viral haemorrhagic septicaemia virus (VHSV). Dis Aquat Org 61:11–21

Stone DM, Way K, Dixon PF (1997) Nucleotide sequence of the glycoprotein gene of viral haemorrhagic septicaemia (VHS) viruses from different geographical areas: a link between VHS in farmed fish species and viruses isolated from North Sea cod (*Gadus morhua* L.) J Gen Virol 78: 1319–1326

Thiéry R, de Boisséson C, Jeffroy J, Castric J, de Kinkelin P, Benmansour A (2002) Phylogenetic analysis of viral haemorrhagic septicaemia virus (VHSV) isolates from France (1971–1999) Dis Aquat Org 52:29–37.

Troyer RM, LaPatra SE, Kurath G (2000) Genetic analyses reveal unusually high diversity of infectious haematopoietic necrosis virus in rainbow trout aquaculture. J Gen Virol 81:2823–2832

Vis ML, Carr SM, Bowering WR, Davidson WS (1997) Greenland halibut (*Reinhardtius hippoglossoides*) in the North Atlantic are genetically homogeneous. Can J Fish Aquat Sci 54(8):1813–1821

Watanabe L, Pakingking R, Iida H, Nishizawa T, Iida Y, Arimoto M, Muroga K (2002) Isolation of aquabirnavirus and viral hemorrhagic septicemia virus (VHSV) from wild marine fishes. Fish Pathol 37(4):189–191

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