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Development of a monoclonal antibody against viral haemorrhagic septicaemia virus (VHSV) genotype IVa

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ABSTRACT: The viral haemorrhagic septicaemia virus (VHSV) comprises 4 major genotypes and a number of subtypes with, in most cases, distinct geographical distribution. A quick and simple detection method that can discriminate the different genotypes is desirable for a quick and more efficient prevention of the spread of genotypes to new geographical areas. A monoclonal antibody (MAb) against VHSV genotype IVa was produced, with the aim of providing a simple method of discriminating this genotype from the other VHSV genotypes (I, II, III and IVb). Balb/c mice were injected with purified VHSV-JF00Ehil (genotype IVa) from diseased farmed Japanese flounder. Ten hybridoma clones secreting monoclonal antibodies (MAbs) against VHSV were established. One of these, MAb VHS-10, reacted only with genotype IVa in indirect fluorescent antibody technique (IFAT) and ELISA. Using cell cultures that were transfected with each of the viral protein genes, it was shown that the MAb VHS-10 recognizes a nonlinear genotype IVa-specific epitope on the VHSV N-protein.

KEY WORDS: VHSV · Genotyping · Monoclonal antibody · Indirect fluorescent antibody technique · IFAT · ELISA

INTRODUCTION

Viral haemorrhagic septicaemia (VHS) is a serious disease of farmed rainbow trout *Oncorhynchus mykiss* in Europe. In the last 2 decades, the VHS virus (VHSV) has been isolated from wild marine fish in the northern hemisphere along North American, Japanese and northern European coasts. Four genotypes of VHSV with, in most cases, distinct geographical distributions have been described (Snow et al. 2004, Einer-Jensen et al. 2005, Skall et al. 2005).

In Japan, VHSV was first detected in farmed Japanese flounder *Paralichthys olivaceus* in 1996 (Isshiki et al. 2001). Since then, VHSV has been isolated from both farmed and wild flounder (Takano et al. 2000, Nishizawa et al. 2002). Recently, VHSV was isolated from farmed black rockfish *Sebastes inermis* (Isshiki et al. 2003), oblong rockfish *Sebastes oblongus*, Japanese jack mackerel *Trachurus japonicus*, red sea bream *Pagrus major*, and cultured and wild Pacific sandeel *Ammodytes personatus* (Watanabe et al. 2002, Isshiki et al. 2003). All VHSV isolates from Japan were identified as genotype IVa (Ito et al. 2004), except for 1 isolate (KRRV9601) from farmed Japanese flounder from 1996 which belongs to genotype Ib. It was suggested that VHSV genotype Ib was accidentally introduced into Japan by importation of fish or fish products from overseas (Nishizawa et al. 2002, Einer-Jensen et al. 2005). Considering the serious dis-

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ease outbreaks caused by genotype IV VHSV in North America in the last decade, the further geographical spread of this genotype is a serious risk to both the aquaculture industry and wild fish resources.

Once VHSV genotypes are introduced into new areas, they may cause severe outbreaks of VHS in susceptible fish species. According to the OIE (Office International des Epizooties/World Organisation for Animal Health) Aquatic Animal Health Code, even if the same disease agent is present in both the importing and the exporting country, the importing country can demand health certification from the exporting country for imports when the pathogenicity or host range of the strain in the exporting country is significantly higher or larger than in the importing country. In order to prevent the spread or introduction of a new VHSV genotype, issue health certificates, and implement quarantine and disease control programs, a quick and simple detection method to discriminate the different genotypes is desired.

Geno- and subtyping of VHSV isolates are usually undertaken using sequence analyses. In addition, Einer-Jensen et al. (1995) reported that VHSV genotype IV can be discriminated from the other genotypes by RT-PCR using specific primer sets for the amplification of the N gene fragments of the genotype. Access to other tests for VHSV typing, such as a reliable immunoassay that may be more convenient than molecular techniques, would be an improvement. Therefore, a monoclonal antibody (MAb) against a Japanese isolate of VHSV type IVa was produced in order to develop simple immunochemical methods for easy and quick discrimination between genotype IVa and the other genotypes of VHSV.

MATERIALS AND METHODS

Cell line. The fathead minnow (FHM) cell line (Gravell & Malsberger 1965) that was used in this study was maintained in minimum essential medium (MEM; MP Biomedicals) supplemented with 10% fetal bovine serum (FBS; Gibco, Invitrogen) and antibiotics (100 units penicillin ml^{-1} and 100 µg streptomycin ml^{-1}). The cell line and the virus-infected cells were cultivated at 25 and 20°C, respectively.

Virus. VHSV strain JF00Ehi1 from Japanese flounder (Ito et al. 2004) was concentrated and gradient-purified as described by Nishizawa et al. (1991). The purified virus was used for immunization of mice as well as for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Seventy-four VHSV isolates from around the world (Tables 1 & 2) were used to characterize some of the obtained monoclonal antibodies (MAbs). Infectious hematopoietic necrosis virus (IHNV) isolated from cherry salmon *Oncorhynchus masou masou* in Japan (T. Ito unpubl.) and hirame rhabdovirus (HIRRV) isolated from Japanese flounder in Japan (Kimura et al. 1986) were also included as specificity controls.

Immunization of mice. Two 6 wk old Balb/c mice were injected intraperitoneally with 0.1 ml of purified virus suspended in PBS (phosphate-buffered saline) and mixed with an equal volume of adjuvant (Ribi adjuvant system, MPL+TDM emulsion; Funakoshi). The mice were given an intravenous booster injection of purified virus suspended in PBS 6 wk after the first immunization. Three days after the booster, the mice were sacrificed and the spleens removed for fusion.

Cell fusion. Spleen cells from immunized mice were fused with P3-X63-Ag8.653 myeloma cells in 50% polyethylene glycol 1500 (Roche). The cells were resuspended in hypoxanthine-aminopterine-thymidine (HAT)

Table 1. Japanese viral haemorrhagic septicaemia virus (VHSV) isolates that were used for the characterization of monoclonal antibodies (MAbs). All isolates were from farmed diseased fish. Virus genotype classification was based on the method of Einer-Jensen et al. (1995, 2005). NRIA FRA: National Research Institute of Aquaculture, Fisheries Research Agency, Japan

Isolate	Year	Genotype	Host	Source
KRRV9601	1996	Ib	Japanese flounder	Isshiki et al. (2001)
KRRV9822	1998	IVa	Japanese flounder	Nishizawa et al. (2002)
KRRV9906	1999	IVa	Japanese flounder	NRIA FRA (this study)
KRRV0011	2000	IVa	Japanese flounder	NRIA FRA (this study)
ORRV0007	2000	IVa	Japanese flounder	NRIA FRA (this study)
OKRRV0020	2000	IVa	Japanese flounder	NRIA FRA (this study)
YRRV0039	2000	IVa	Japanese flounder	NRIA FRA (this study)
FH0102	2000	IVa	Japanese flounder	NRIA FRA (this study)
JF00Ehi1	2000	IVa	Japanese flounder	Nishizawa et al. (2002), Ito et al. (2004)
KRRV0101	2001	IVa	Japanese flounder	NRIA FRA (this study)
JF01Ehi1	2001	IVa	Japanese flounder	NRIA FRA (this study)
BR01Ehi1	2001	IVa	Black rockfish	NRIA FRA (this study)
JF01Oit1	2001	IVa	Japanese flounder	NRIA FRA (this study)
JF01Kum1	2001	IVa	Japanese flounder	NRIA FRA (this study)
JF01Nag1	2001	IVa	Japanese flounder	NRIA FRA (this study)
KRRV0201	2002	IVa	Japanese flounder	NRIA FRA (this study)
JF02Ehi1	2002	IVa	Japanese flounder	NRIA FRA (this study)
BR02Ehi1	2002	IVa	Black rockfish	NRIA FRA (this study)
JF02Oit1	2002	IVa	Japanese flounder	NRIA FRA (this study)
JF02Wak1	2002	IVa	Japanese flounder	NRIA FRA (this study)
JF02Yam1	2002	IVa	Japanese flounder	NRIA FRA (this study)
JSL02Yam1	2002	IVa	Pacific sandeel	NRIA FRA (this study)
JF02Nag1	2002	IVa	Japanese flounder	NRIA FRA (this study)
JF03Wak1	2003	IVa	Japanese flounder	NRIA FRA (this study)

Table 2. Viral haemorrhagic septicaemia virus (VHSV) isolates from around the world that were used for the characterization of monoclonal antibodies. Virus genotype classification was based on Einer-Jensen et al. (2004, 2005), Snow et al. (2004), Elsayed et al. (2006), Gagné et al. (2007), and Groocock et al. (2007). DTU Vet: National Veterinary Institute, Technical University of Denmark; Affsa: French food safety agency; SVI: State Veterinary Institute in Pulawy, Poland; VRI: Veterinary Research Institute, Brno, Czech Republic; Marine Inst.: Marine Institute, Ireland; USGS: United States Geological Survey; Pac. Biol. Stat.: Pacific Biological Station, Department of Fisheries and Oceans, Canada. Hosts are either from fresh (F), brackish (B) or sea (S) water

Isolate	Country	Year	Genotype	Host	Source
F1	Denmark	1962	I	Rainbow trout (F)	Jensen (1965)
Hededam	Denmark	1970	I	Rainbow trout (F)	Vestergård Jørgensen (1974)
I-87	Italy	1969	I[a]	Brown trout (F)	DTU Vet (unpubl.)
DK-687	Denmark	1973	Ia	Rainbow trout (F)	DTU Vet (unpubl.)
DK-978	Denmark	1975	Ia	Rainbow trout (F)	DTU Vet (unpubl.)
DK-3925	Denmark	1987	Ia	Rainbow trout (F)	DTU Vet (unpubl.)
DK-200098	Denmark	2000	Ia	Rainbow trout (F)	Einer-Jensen et al. (2004)
FR-02-84	France	1984	Ia	Rainbow trout (F)	Benmansour et al. (1997)
FR-5874	France	1999	Ia	Rainbow trout (F)	Affsa (unpubl.)
FR-403	France	2000	Ia	Rainbow trout (F)	Affsa (unpubl.)
PL-201234	Poland	2001	Ia	Rainbow trout (F)	DTU Vet (unpubl.)
PL-A	Poland	2002	Ia	Pike fry (F)	SVI (unpubl.)
CZ-7738-R5	Czech Republic	1994	Ia	Rainbow trout (F)	DTU Vet (unpubl.)
CAPM V553	Czech Republic	2000	Ia	Rainbow trout (F)	VRI (unpubl.)
DE-Fi13	Germany	1983	Ia	Rainbow trout (F)	Schütze et al. (1999)
DE-10/90	Germany	1991	Ia	Turbot (S)	Schlotfeldt et al. (1991)
DK-M Rhabdo	Denmark	1979	Ib	Cod (S)	Jensen et al. (1979)
1p8	Baltic Sea	1996	Ib	Herring (S)	Mortensen et al. (1999)
SE-SVA-14	Sweden	1998	Ib	Rainbow trout (S)	Nordblom (1998)
SE-SVA-1033	Sweden	2000	Ib	Rainbow trout (S)	Nordblom & Norell (2000)
DK-2835	Denmark	1982	Ic	Rainbow trout (F)	Einer-Jensen et al. (2004)
DK-960	Denmark	1975	Ic[a]	Rainbow trout (F)	DTU Vet (unpubl.)
DK-992	Denmark	1975	Ic[a]	Rainbow trout (F)	DTU Vet (unpubl.)
AU-13/95 (DK-8077)	Austria	1995	Ic[a]	Rainbow trout (F)	O. Schachner (unpubl.)
FiA01a.00	Finland	2000	Id	Rainbow trout (B)	Raja-Halli et al. (2006)
FiP02b.00	Finland	2000	Id	Rainbow trout (B)	Raja-Halli et al. (2006)
1p52	Denmark	1996	II	Sprat (S)	Mortensen et al. (1999)
4p101	Denmark	1996	III	Whiting (S)	Mortensen et al. (1999)
4p168	Denmark	1996	III	Atlantic herring (S)	Mortensen et al. (1999)
IR-F13.02.97	Ireland	1997	III	Turbot (S)	Marine Inst. Ire (unpubl.)
L59x	France	1987	III	Eel (F)	Castric et al. (1992)
GH30	Flemish Cap	1994	III	Greenland halibut (S)	Dopazo et al. (2002)
Makah	USA	1988	IVa	Coho salmon (F)	Brunson et al. (1989)
KHV	USA	1988	IVa	Chinook salmon (F)	Hopper K (1989)
Elliot Bay herring #5	USA	1993	IVa	Pacific herring (S)	Einer-Jensen et al. (2005)
Minter Creek, WA	USA	2002	IVa	Coho salmon (F)	USGS (unpubl.)
Tokul Creek, WA	USA	2006	IVa	Steelhead (F)	USGS (unpubl.)
Port Angels, WA	USA	2007	IVa	Atlantic salmon (F)	USGS (unpubl.)
BC'93	Canada	1993	IVa	Pacific herring (S)	Meyers & Winton (1995)
CAN-3624	Canada	1995	IVa	Atlantic salmon (S)	Traxler et al. (1995), Pac. Biol. Stat. (unpubl.)
BC-s-99	Canada	1998/99	IVa	Sardine (S)	Hedrick et al. (2003)
Quatsino, BC	Canada	2002	IVa	Sardine (S)	USGS (unpubl.)
J-Obama 25	Japan	1999	IVa	Japanese flounder (S)	Takano et al. (2000)
JF00Ehi1	Japan	2000	IVa	Japanese flounder (S)	Nishizawa et al. (2002)
MI03GL	USA	2003	IVb	Muskellunge (F)	Elsayed et al. (2006)
Goby 1-5	USA	2006	IVb	Round goby (F)	Groocock et al. (2007)
Lake Ontario	USA	2007	IVb	Gizzard shad (F)	USGS (unpubl.)
Budd Lake	USA	2007	IVb	Bluegill (F)	USGS (unpubl.)
Skaneateles Lake	USA	2007	IVb	Smallmouth bass (F)	USGS (unpubl.)
New Brunswick (CA-NB00-001)	Canada	2000	IVb	Mummichog (F)	Oliver (2002), Gagné et al. (2007)

[a]Genotype data was generated based on the Nv gene according to the method of Einer-Jensen et al. (2005) and was provided by K. Einer-Jensen of the National Veterinary Institute, Technical University of Denmark

medium (Dulbecco's modified Eagle's medium [DMEM; MP Biomedicals] supplemented with 10% FBS (fetal bovine serum) and 2% HAT supplement [Gibco, Invitrogen]) and plated in 96-well culture plates. After 12 d of selection in HAT medium, hybridomas producing VHSV-specific antibodies were identified by immunofluorescence assay. Cells showing positive reactions were cloned at least twice by limiting dilution in DMEM supplemented with 10% FBS and 5% (v/v) Briclone (Bio Research). The MAb preparations that were used in subsequent studies were unpurified cell culture supernatants from these hybridomas.

Immunoglobin (Ig) class determination. The Ig class of the MAbs was determined using a mouse monoclonal isotyping kit (AbD Serotec) according to the manufacturer's instructions.

Western blotting. SDS-PAGE was performed according to the method of Laemmli (1970) using 10% (w/v) acrylamide gels under reducing conditions. Western blotting was performed according to the principles described by Lorenzen et al. (1988). Polypeptides that were separated by SDS-PAGE were electroblotted onto a nitrocellulose membrane in a semi-dry electroblotter (Bio-Rad). As a positive control for immunostaining, specific anti-VHSV (JF00Ehi1) rabbit antiserum was used. Visualization was performed using horseradish peroxidase-conjugated antibodies and 3,3′-diaminobenzidine (in PBS) containing 0.06% H_2O_2.

Indirect fluorescent antibody technique (IFAT) for screening of MAbs against VHSV. The binding of MAbs to infected cells was examined using IFAT. VHSV-infected and normal FHM cells on coverslips (11 × 40 mm) were fixed with cold acetone for 10 min. The coverslips were stored at –20°C until used. The coverslips were then overlaid with MAbs and incubated in a humid chamber at 37°C for 30 min and then rinsed with PBS. Fluorescein isothiocyanate-conjugated goat anti-mouse IgG serum (MP Biomedicals) was applied and the samples were again incubated at 37°C for 30 min. After another rinse, the coverslips were mounted in PBS-buffered glycerol, and examined with a fluorescence microscope.

IFAT for characterization of MAbs against VHSV. IFAT characterization of selected MAbs for their binding to the VHSV isolates (Table 2) was performed using VHSV-infected and normal epithelioma papillosum cyprini (EPC) cells (Fijan et al. 1983) in 96-well plates (Corning) after fixation with a mixture of acetone and ethanol (8:2) for 10 min. The subsequent procedure was the same as described previously. MAb IP5B11 (Lorenzen et al. 1988) was used as a positive control.

ELISA. The reactivity of selected MAbs against a panel of VHSV (Table 2) was evaluated using double-sandwich ELISA as described by Olesen & Jørgensen (1991), except that a mixture of protein-A purified rabbit anti G-, N- and P-protein of VHSV was used as a first layer. As a positive control, MAb IP5B11 against VHSV N-protein (Lorenzen et al. 1988) was used. This MAb has been proven to react with all known VHSV isolates (tested against >1000 isolates, N. J. Olesen pers. obs.).

Dot-blot analysis. The mixture of purified virus (JF00Ehi1) and SDS sample buffer (Bio-Rad) (1:1) without 2-mercaptoethanol was blotted onto a nitrocellulose membrane. This membrane was immunostained after blocking with 2% bovine serum albumin in PBS. The procedure for visualization was the same as that in Western blotting. The purified virus was used as a positive control.

Transfection. EPC cells were transfected at 25°C using FuGENE (Roche). Transfection was done with a purified plasmid containing pTARGET expression vectors (Promega) into which the entire amplified N, P, M or G gene was inserted according to the manufacturer's instructions, and tested using IFAT. The sequence of the entire genome of VHSV isolate JF00Ehi1 was registered with DDBJ/EMBL/Genbank by the authors as accession no. AB490792.

Alignments of amino acids of N-protein. Amino acid sequences of N-protein were obtained from DDBJ/EMBL/Genbank. The amino acid sequences in positions 1 to 50 were aligned for this study.

RESULTS

Production and screening of hybridomas

Twelve days after fusion, ~70% of the 600 seeded wells contained hybridomas. In the initial IFAT, ~6% of the hybridomas reacted with VHSV-infected FHM cells and showed negative reactions with uninfected cells. Subcultivation and repeated screening using IFAT yielded 10 positive hybridoma clones, which were named VHS-1 to 10.

Ig class determination

MAb VHS-10 belonged to the IgG2a subclass with a kappa light chain. The other 9 MAbs were determined to belong to the IgG1 subclass with a kappa light chain.

Reactivity of MAbs against fish rhabdoviruses using IFAT

Established MAbs were tested against fish novirhabdoviruses (VHSV, IHNV and HIRRV) using IFAT to determine their specificity to VHSV. All 10 MAbs specifically reacted with VHSV.

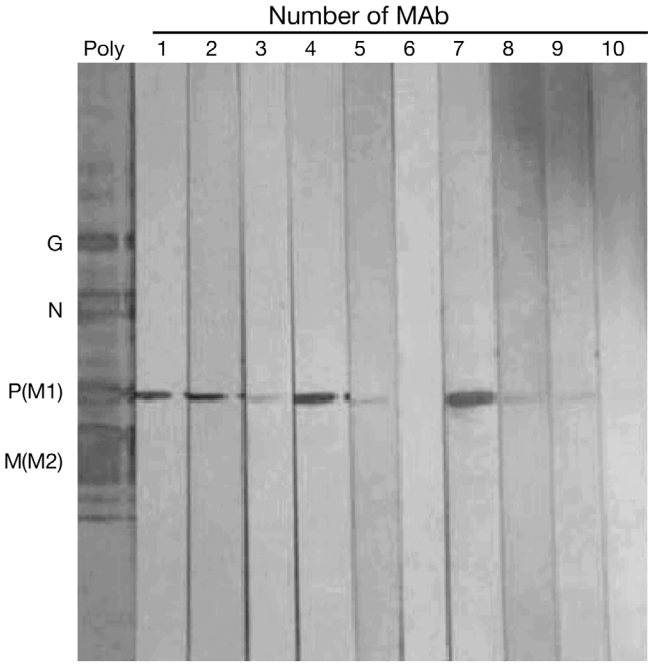

Fig. 1. Western blotting with monoclonal antibodies (MAbs) and rabbit serum against viral haemorrhagic septicaemia virus (VHSV) to analyze the antigen recognized by each MAb. The loaded antigens were purified VHSV (JF00Ehi1) virions. G: glycoprotein, N: nucleoprotein, P(M1): phosphoprotein, M(M2): matrix protein

Western blotting

Western blotting results are shown in Fig. 1. Eight of the 10 MAbs recognized P-protein; however, VHS-6 and VHS-10 did not react with any virus proteins after Western blotting.

Characterization of MAbs

Four (VHS-2, -4, -5 and -10) of the 10 MAbs that were selected based on their staining intensity in IFAT against the JF00Ehi1 isolate were again tested using IFAT against the Japanese VHSV isolates given in Table 1. Three of the 4 MAbs reacted with all 24 Japanese isolates, but VHS-10 did not react with the KRRV9601 isolate that belonged to genotype Ib (Table 3). MAb VHS-10 was then further characterized against the worldwide panel of VHSV given in Table 2 using IFAT and ELISA. MAb IP5B11, which was used as a positive control, reacted with all 51 isolates including all genotypes (I, Ia–d, II, III, IVa–b), while MAb VHS-10 only reacted with genotype IVa of VHSV in both IFAT and ELISA (Table 4). In IFAT, the reactivity of MAb VHS-10 against 5 selected VHSV isolates from various genotypes in infected FHM cells is shown in Fig. 2.

Table 3. Results of indirect fluorescent antibody technique (IFAT) tests of selected monoclonal antibodies (MAbs) for various viral haemorrhagic septicaemia virus (VHSV) isolates from Japan

Isolate (genotype)	MAb			
	VHS-2	VHS-4	VHS-5	VHS-10
KRRV9601(Ib)	+	+	+	–
KRRV9822 (IVa)	+	+	+	+
KRRV9906 (IVa)	+	+	+	+
KRRV0011 (IVa)	+	+	+	+
ORRV0007 (IVa)	+	+	+	+
OKRRV0020 (IVa)	+	+	+	+
YRRV0039 (IVa)	+	+	+	+
FH0102 (IVa)	+	+	+	+
JF00Ehi1 (IVa)	+	+	+	+
KRRV0101 (IVa)	+	+	+	+
JF01Ehi1 (IVa)	+	+	+	+
BR01Ehi1 (IVa)	+	+	+	+
JF01Oit1 (IVa)	+	+	+	+
JF01Kum1 (IVa)	+	+	+	+
JF01Nag1 (IVa)	+	+	+	+
KRRV0201 (IVa)	+	+	+	+
JF02Ehi1 (IVa)	+	+	+	+
BR02Ehi1 (IVa)	+	+	+	+
JF02Oit1 (IVa)	+	+	+	+
JF02Wak1 (IVa)	+	+	+	+
JF02Yam1 (IVa)	+	+	+	+
JSL02Yam1 (IVa)	+	+	+	+
JF02Nag1 (IVa)	+	+	+	+
JF03Wak1 (IVa)	+	+	+	+

Determination of the antigen recognized by MAb VHS-10 using transfected cell cultures

Initially, dot-blot analysis was carried out to investigate the antigen that is recognized by VHS-10. VHS-10 reacted strongly with the purified virus but not with SDS-treated virus under nonreducing conditions (data not shown), indicating recognition of a nonlinear epitope. Therefore, EPC cells were transfected to express the viral proteins N, P, M and G of the JF00Ehi1 isolate. In IFAT, the reactivity of the MAb VHS-10 against EPC cells that were transfected with pTARGET-vhs N, P, M and G is shown in Fig. 3. VHS-10 reacted only with EPC cells that were transfected with pTARGET-vhs N. It was thus concluded that MAb VHS-10 recognized a genotype IVa-specific epitope of the viral N-protein.

Alignments of amino acids of VHSV N-protein

Alignments of the amino acid (AA) sequences from positions 1 to 50 of the N-protein of several VHSV isolates are shown in Fig. 4. When these sequences from the N-protein of genotypes I, Ia, Ib, Ie, II, III, IVa and IVb were compared, unique substitutions were ob-

Table 4. Results of indirect fluorescent antibody technique (IFAT) and ELISA tests of selected monoclonal antibodies (MAbs) for various viral haemorrhagic septicaemia virus (VHSV) isolates from around the world. For ELISA results: (+++) >2.0 of A_{492}; (++) >1.2 of A_{492}; (+) >0.8 of A_{492}; (–) <0.8 of A_{492}

Genotype	Isolate	IFAT		ELISA	
		IP5B11	VHS-10	IP5B11	VHS-10
I	F1	+	–	+++	–
I	Hededam	+	–	+++	–
I	I-87	+	–	+++	–
Ia	DK-687	+	–	+++	–
Ia	DK-978	+	–	+++	–
Ia	DK-3925	+	–	+++	–
Ia	DK-200098	+	–	+++	–
Ia	FR-02-84	+	–	+++	–
Ia	FR-5874	+	–	+++	–
Ia	FR-403	+	–	+++	–
Ia	PL-201234	+	–	+++	–
Ia	PL-A	+	–	+++	–
Ia	CZ-7738-R5	+	–	+++	–
Ia	CAPM V553	+	–	+++	–
Ia	DE-Fi 13	+	–	+++	–
Ia	DE-10/90	+	–	+++	–
Ib	DK-M Rhabdo	+	–	+++	–
Ib	1p8	+	–	+++	–
Ib	SE-SVA-14	+	–	+++	–
Ib	SE-SVA-1033	+	–	+++	–
Ic	DK-2835	+	–	+++	–
Ic	DK-960	+	–	+++	–
Ic	DK-992	+	–	+++	–
Ic	AU-13/95	+	–	+++	–
Id	FiA01a.00	+	–	+++	–
Id	FiP02b.00	+	–	+++	–
II	1p52	+	–	+++	–
III	4p101	+	–	+++	–
III	4p168	+	–	+++	–
III	IR-F13.02.97	+	–	+++	–
III	F-L59x	+	–	+++	–
III	GH30	+	–	+++	–
IVa	Makah	+	+	+++	+++
IVa	KHV	+	+	+++	+++
IVa	Elliot Bay herring #5	+	+	+++	+++
IVa	Minter Creek, WA	+	+	+++	+++
IVa	Tokul Creek, WA	+	+	+++	+++
IVa	Port Angels, WA	+	+	+++	+++
IVa	BC'93	+	+	+++	+++
IVa	CAN-3624	+	+	+++	+++
IVa	BC-s-99	+	+	+++	+++
IVa	Quatsino, BC	+	+	+++	+++
IVa	J-Obama 25	+	+	+++	+++
IVa	JF00Ehi1	+	+	+++	+++
IVb	MI03GL	+	–	+++	–
IVb	Goby 1-5	+	–	+++	–
IVb	Lake Ontario	+	–	+++	–
IVb	Budd Lake	+	–	+++	–
IVb	Skaneateles Lake	+	–	+++	–
IVb	New Brunswick (CA-NB00-001)	+	–	+++	–

served at AA positions 38 (substitution of valine [V] → alanine [A]), 40 (glycine [G] or glutamic acid [E] → proline [P]) and 43 (aspartic acid [D] or glutamic acid [E] or alanine [A]→ threonine [T]) in genotype IVa.

DISCUSSION

It was previously demonstrated that VHSV isolates are serologically homogeneous and that only 1 serogroup with a number of subtypes can be identified when a panel of poly- and monoclonal antibodies are used for serotyping (Olesen et al. 1993). In contrast, subsequent studies showed that VHSV isolates can be clearly discriminated into 4 major genotypes and a number of subtypes (Ia–e, II, III, and IVa–b; Snow et al. 2004, Einer-Jensen et al. 2005, Gagné et al. 2007). These genotypes appear to have geographically distinct distributions, with genotype I, II and III being found in Europe and genotype IV being found in North America and Asia.

Host range and pathogenicity appear, to some extent, to be linked to genotype (Skall et al. 2004); all rainbow trout pathogenic isolates from continental Europe thus belong to genotype Ia, while genotype Ib isolates from the North and Baltic Seas are primarily from wild marine fish and are non- or lowly virulent to rainbow trout. Within genotype IV, major differences in host range and geographical distribution are also observed. It is thus important to know the actual distribution of existing isolates and to prevent the spread of the different genotypes and subtypes of VHSV. With the development of MAb VHS-10, quick and reliable immunochemical methods can be easily implemented to discriminate the IVa from the other genotypes.

MAb IP5B11 (Lorenzen et al. 1988) reacts with all known VHSV isolates. This MAb was produced from mice that were immunized with VHSV strain F1 (genotype I) (Jensen 1965) and reacts with a linear epitope on the viral N-protein. Virus neutralizing MAbs are directed towards the viral G-protein. A

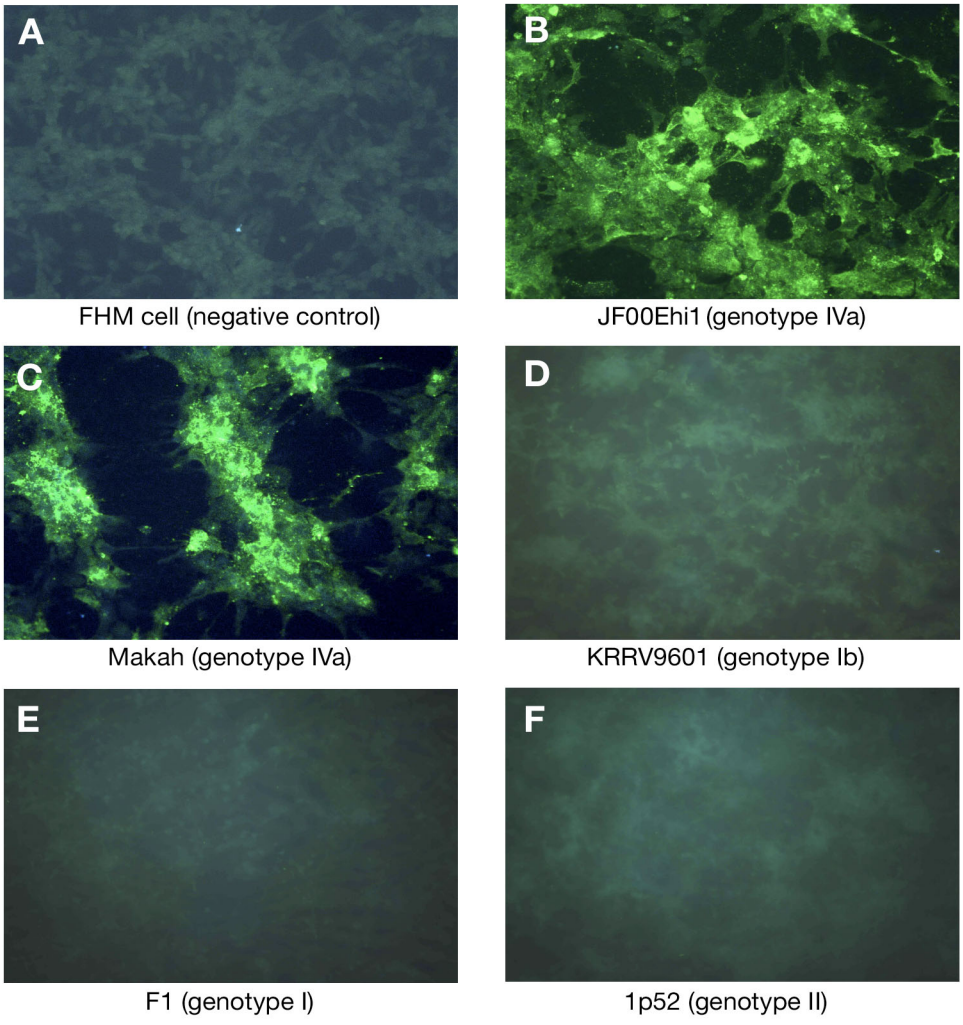

Fig. 2. Reactivity of MAb VHS-10 to virus-infected cells as assessed using indirect fluorescent antibody technique (IFAT). (A) Uninfected fathead minnow (FHM) cells, (B) JF00Ehi1-infected FHM cells, (C) Makah-infected FHM cells, (D) KRRV9601-infected FHM cells, (E) F1-infected FHM cells, (F) 1p52-infected FHM cells

panel of such virus neutralizing MAbs that was produced in VHSV F1 immunized mice (Lorenzen et al. 1990) was able to discriminate VHSV isolates into 3 serologically distinct subgroups (Olesen et al. 1993).

It was not possible to determine the protein specificity of the MAb VHS-10 as no reaction was observed during Western blotting, whether under reduced or nonreduced conditions. In ELISA, no binding of VHS-10 was observed if the virus suspension was mixed with 0.1% SDS before incubation (data not shown). It was therefore anticipated that MAb VHS-10 could only recognize the 3-dimensional conformation of a viral protein. In IFAT, VHS-10 reacted strongly with EPC cells that were transfected with the N gene of the homologous viral isolate (JF00Ehi1), whereas no staining was observed in cells that were transfected with the P, M or G genes of the same virus isolate, indicating recognition of a conformational epitope on the N-protein. Comparison of the genotype IVa deduced AA with the AA of genotypes I, II, III and IVb showed differences at the positions between AA 38 and 44 in the viral N-protein (Fig. 4.). In particular, the substitution at AA 40 (glycine [G] or glutamic acid [E] → proline [P]) is interesting as P and G are well known as secondary structure breakers (Colloc'h & Cohen 1991). According to an analysis of the break point of the secondary structure in proteins using SOSUIbreaker software (http://bp.nuap.nagoya-u.ac.jp/sosui/sosuibreaker/sousuibreaker_submit.html), G does not seem to be a breaker while P is (data not shown). Therefore, this substitution might especially be related to the specificity of MAb VHS-10. It is thus most likely that VHS-10 only reacts with an epitope where the AA sequence AGPFGTD in positions 38 to

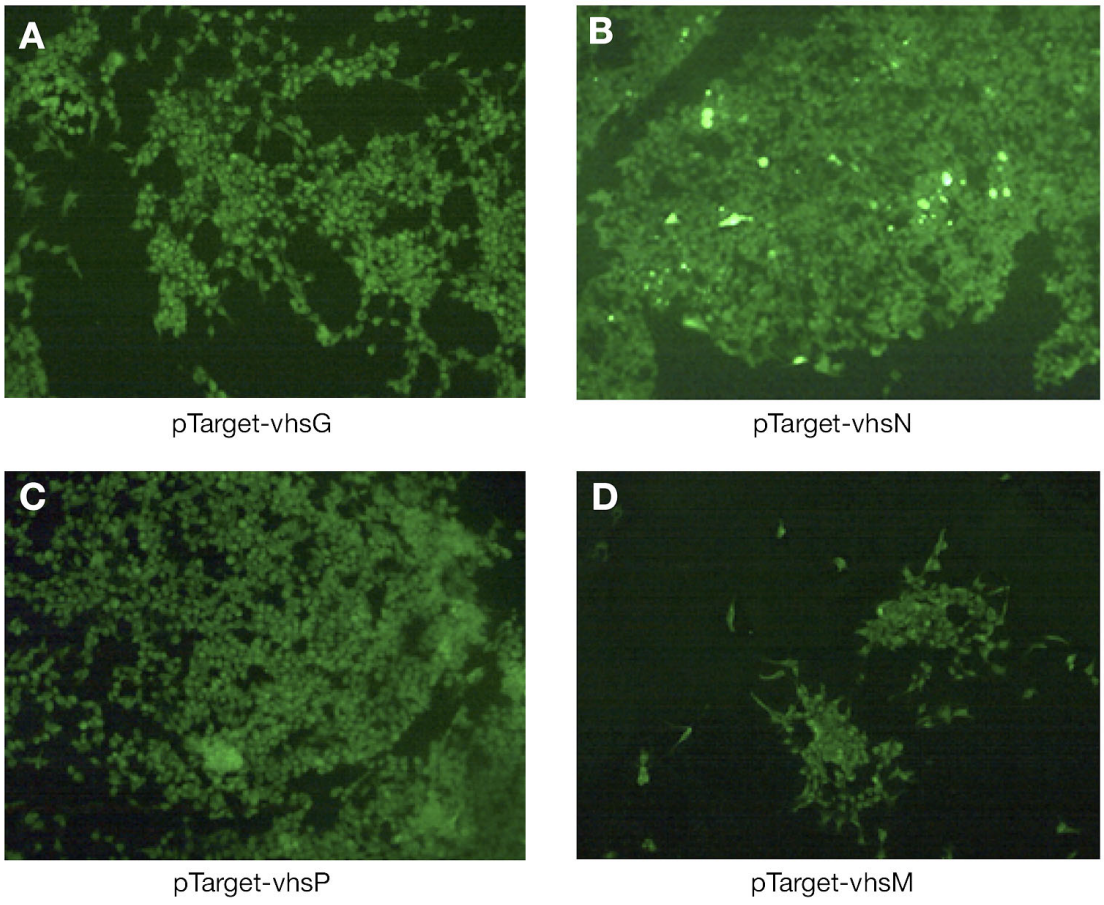

Fig. 3. Reactivity of MAb VHS-10 to epithelioma papillosum cyprini (EPC) cells that were transfected with the N, P, M, or G gene of viral haemorrhagic septicaemia virus (VHSV) strain JF00Ehi1 as assessed by indirect fluorescent antibody technique (IFAT). (A) G gene transfected EPC cells, (B) N gene transfected EPC cells, (C) P gene transfected EPC cells, (D) M gene transfected EPC cells

44 of the N-protein is included. By examining these AA positions in detail, one can observe that genotype III has a specific sequence with substitutions at positions AA 42 (glycine [G] → serine [S]) to 43 (aspartic acid [D] or glutamic acid [E] or threonine [T] → alanine [A]). In genotype I and Ia, AA 46 is substituted with arginine (R) or lysine (K) from glycine (G). From these findings, it can be deduced that the amino acids in positions AA 38 to 46 of the N-protein form a variable region.

The VHSV genotype IVb recently emerged in the Great Lakes region and was isolated from >25 different fish species during serious die-offs with high mortality rates. Since the infectivity of these isolates is very significant in many fish species (Elsayed et al. 2006, Groocock et al. 2007, Lumsden et al. 2007), there are concerns that spread to other areas may also result in significant disease outbreaks involving several fish species. In this study, VHS-10 did not react with 6 isolates of genotype IVb from different fish species in USA and Canada; it is therefore anticipated that only IVa of genotype IV reacts with MAb VHS-10.

In recent years, genotype characteristics have been used to evaluate viral properties and molecular epidemiology of VHSV from both freshwater and marine fishes. Although genotyping is usually based on sequencing of the viral genomes, a simple method for genotyping such as IFAT or ELISA is desirable. There are so far no reports on MAbs that discriminate the different genotypes of VHSV. The study described here demonstrates the possibility of producing MAbs that recognize a specific genotype or subtype of VHSV. The establishment of monoclonal antibodies that are specific to the various genotypes and subtypes of VHSV has, therefore, been initiated in a collaborative study involving the National Research Institute of Aquaculture (Japan) as the Japanese national reference laboratory for fish diseases and the OIE reference laboratory for VHS in Denmark, with the goal of producing a panel of MAbs that would enable the quick and easy typing of VHSV isolates.

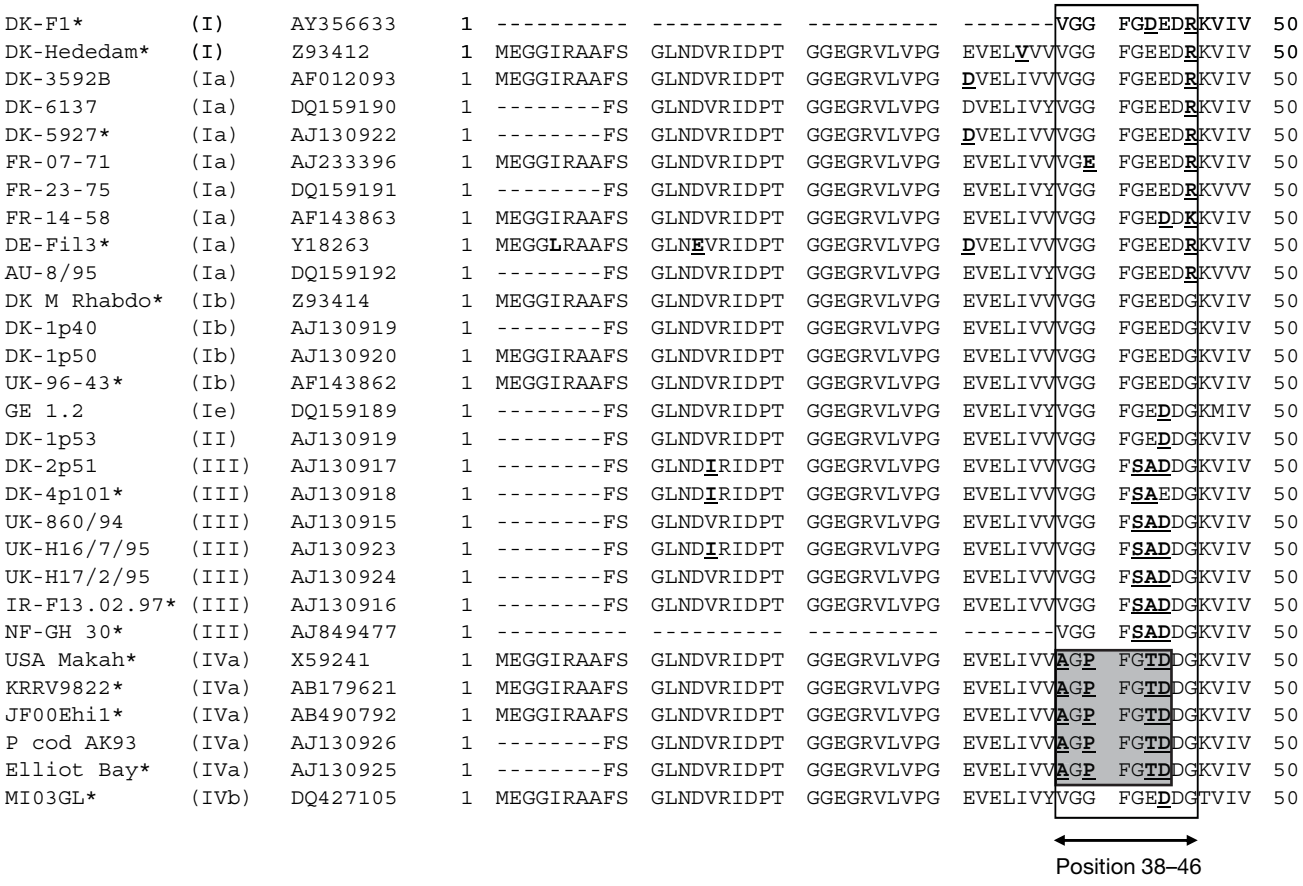

Fig. 4. Partial nucleoprotein gene (N) amino acid alignment of viral haemorrhagic septicaemia virus (VHSV) isolates. Amino acids that are shaded grey (AA 38–44 of the full N gene) and those that are marked with a rectangle (AA 38–46) are referred to in the 'Discussion'. (*) Virus isolates that were used for indirect fluorescent antibody technique (IFAT) and ELISA in this study as listed in Table 3

Acknowledgements. The Japanese VHSV isolates were collected through cooperation among the members of the study group of VHSV infection in Japan that consists of some local governments, Hiroshima University and National Research Institute of Aquaculture. The KRRV9601 isolate was provided by Isshiki, Mie University; the HIRRV isolate by Oseko, National Research Institute of Aquaculture; and the non-Japanese isolates from the virus library of the OIE reference laboratory for VHS, Århus, Denmark. We thank our colleagues in Europe, North America and Japan for providing the viruses in our panel, especially J. R. Winton (Western Fisheries Research Center, Seattle) and G. H. Groocock (Department of Microbiology and Immunology, College of Veterinary Medicine, Cornell University, New York) for providing the American genotype IVa and IVb isolates. We also thank all our colleagues at the National Research Institute of Aquaculture (Japan) and the National Veterinary Institute (Denmark) for their scientific and technical contribution to this study. Part of this work was undertaken while the first author was visiting the National Veterinary Institute, Technical University of Denmark as long-term researcher with an overseas fellowship from the Fisheries Research Agency. This work was mainly supported by a grant-in-aid from the Ministry of Agriculture, Forestry and Fisheries of Japan.

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