Vol. 74: 151–158, 2007 | DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org | Published February 28

Detection of rainbow trout antibodies against viral haemorrhagic septicaemia virus (VHSV) by neutralisation test is highly dependent on the virus isolate used

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ABSTRACT: Three serological tests, enzyme linked immunosorbent assay (ELISA), 50% plaque neutralisation test (50%PNT) and Western blotting (WB), were used to detect antibodies against viral haemorrhagic septicaemia virus (VHSV) in 50 rainbow trout broodstock from a rainbow trout farm endemically infected with VHS but with no clinical signs of infection. When the sera were examined by 50%PNT using the VHSV reference isolate DK-F1 or the heat attenuated DK-F25 mutant strain, no neutralizing antibodies were found. In contrast, when one of the virus isolates from the farm (homologous virus) was used in the 50%PNT, 90% of the fish were found to be positive. By examining a panel of different VHSV isolates in 50%PNT, it was demonstrated that the virus isolate used as test antigen could significantly affect the sensitivity and titre determination in 50%PNT for detection of rainbow trout antibodies against VHSV. When the sera were examined for the presence of VHSV antibodies by ELISA or WB, 61% were found to be positive. When conducting WB analysis, the viral glycoprotein was the protein most frequently recognized, followed by the viral nucleoprotein.

KEY WORDS: VHSV · Trout antibody · Serology · Plaque neutralisation · ELISA

INTRODUCTION

Viral haemorrhagic septicaemia (VHS) is a serious viral disease affecting a range of fish species (Wolf 1988). The etiological agent, viral haemorrhagic septicaemia virus (VHSV), is included in the genus *Novirhabdovirus* of the *Rhabdoviridae* family (Van Regenmortel et al. 2000). VHS has occurred endemically in the continental part of Europe for decades, and in the past 15 yr many isolations of VHSV have been made from an increasing number of free-living marine and freshwater fish species (Skall et al. 2005), showing that the virus is much more widespread than previously anticipated. VHS has an important economic impact for European rainbow trout farming and is included in the Office International de Epizooties (OIE) list of notifiable diseases (OIE 2004) and in List II of EU Council Directive 91/67/EEC, Annex A (Anon. 1991). Surveillance programs are based on virus isolation in cell cultures and identification by immunological techniques.

Antibodies against VHSV in rainbow trout were first reported in the 1970s (Jørgensen 1971, 1974). Since then, several studies have demonstrated the efficacy of different serological techniques, like immunofluorescence (IF), enzyme linked immunosorbent assay (ELISA), plaque neutralisation test (50%PNT) and Western blotting (WB), for the detection and characterization of rainbow trout antibodies against salmonid rhabdoviruses under experimental and field conditions (Enzmann & Konrad 1990, 1993, Jørgensen et al. 1991, Olesen et al. 1991, Lorenzen et al. 1993, Hattenberger-Baudouy et al. 1995). These studies suggest that serological techniques could be developed and used as epidemiological and diagnostic tools as well as for con-

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trol and surveillance, but more work is needed for standardization and validation in order to include serological tests in surveillance programs (LaPatra 1996) and in international diagnostic manuals such as the OIE Manual of Diagnostic Tests for Aquatic Animals. Surveillances based on serological tests have several advantages compared with virus isolation, especially in cases were water temperature is too high for virus isolation and in endemically infected populations without clinical symptoms of disease. However, the disadvantage of serological test is the slow development of fish antibodies after infection, especially at low water temperature.

In the present study we used 3 different serological tests—ELISA, 50%PNT and WB—to detect antibodies against VHSV in rainbow trout broodstock from a Danish farm endemically infected with VHSV. Different strains of VHSV as antigen source in 50%PNT were tested in order to assess how they could affect the test sensitivity and titre determination.

MATERIALS AND METHODS

Rainbow trout sera. Individual blood samples were obtained by puncture of the caudal vein of 50 rainbow trout *Oncorhynchus mykiss* (Walbaum) broodstock from a fish farm endemically infected with VHSV situated in the south-western part of Denmark. Another 50 samples were taken from a fish farm free of VHSV but infected with infectious pancreatic necrosis virus (IPNV), and used as negative controls. Blood samples were placed at 4°C and allowed to clot overnight. The specimens were centrifuged at 1000 × *g* for 45 min, complement inactivated at 45°C for 30 min (Olesen & Jørgensen 1986), and stored at –80°C until examination.

Virological examination. After blood sampling the fish were killed and organ samples (anterior kidney, spleen and heart) were collected for virological examination. The samples were prepared and processed according to the standard diagnostic procedures outlined by EU Commission Decision 2001/183/EC (Anon. 2001) and inoculated into BF-2 cells in 24-well plates. Plates were incubated at 15°C and observed for cytopathic effect (CPE) for 7 d; a second passage was made if no CPE occurred within the first 7 d. When CPE occurred, virus identification was attempted by ELISA (Olesen & Jørgensen 1991). The neutralisation pattern (serogroups) of the isolated VHS viruses were determined by 50%PNT using a panel of 4 monoclonal antibodies (MAbs) and 1 rabbit antiserum, as described by Olesen et al. (1993).

Viruses and fish cell lines. The following 17 VHSV isolates from freshwater and the marine environment, representing all 3 neutralisation patterns of VHSV (Olesen et al. 1993), were selected and examined: (1) the first Danish VHSV isolate DK-F1 and its heat-adapted mutant strain DK-F25, and the highly pathogenic rainbow trout pathogenic isolate DK-3592B, which all belong to Serogroup I; (2) the Danish isolate DK-5131 and the French isolate F403, both belonging to Serogroup II, (3) 7 Danish isolates (DK-5151, DK-6137 and 5 homologous isolates obtained in the present study from the fish farm DK-201433-10, -15, -40, -42 and -49), 1 French isolate H 11225, and 1 Czech isolate 200317, all 9 of which belong to Serogroup III, and finally (4) 3 isolates from wild marine fish species—2 from the Baltic Sea close to Denmark (DK-1p8 and DK-1p49), and 1 from the USA, Makah. Details and sources of the virus isolates used are given in Table 1.

All viruses except the marine isolates,were propagated in epithelioma papulosum cyprini (EPC) cells

Table 1. Characteristics and origin of virus isolates used in this study. VHSV I, II, III: VHSV with neutralisation pattern Type I, II or III, respectively (Olesen et al. 1993). VET-DTU: National Veterinary Institute, Technical University of Denmark

Virus isolate	Host species	Country/ area	Source
VHSV I			
DK-F1	Rainbow trout	Denmark	Jensen (1965)
DK-F25	Rainbow trout	Denmark	de Kinkelin & Bearzotti (1981)
DK-3592B	Rainbow trout	Denmark	Lorenzen et al. (1990)
VHSV II			
DK-5131	Rainbow trout	Denmark	Olesen et al. (1993)
F-403	Rainbow trout	France	J. Castric, AFSSA, Brest, unpubl. (2002)
VHSV III			
DK-201433 N-10, -15, -40, -42, -49	Rainbow trout	Denmark	This study
DK-5151	Rainbow trout	Denmark	Olesen et al. (1993)
DK-6137	Rainbow trout	Denmark	NET-DTU unpubl. (1991)
F-H11225	Rainbow trout	France	J. Castric, AFSSA, Brest, unpubl. (1998)
CZ 2077-200317	Rainbow trout	Czech Republic	T. Vésely, VRI, Brno, unpubl. (2000)
Marine VHSV			
DK-1p8	Herring	Baltic Sea	Mortensen et al. (1999)
DK-1p49	Herring	Baltic Sea	Mortensen et al. (1999)
RBV-'Makah'	Coho salmon	USA	Brunson et al. (1989)

(Fijan et al. 1983) at 15°C using Eagles MEM supplemented with 10% foetal bovine serum (FBS), Tris buffer and antibiotics in standard concentrations. The marine isolates were propagated in BF-2 cells (Wolf et al. 1966). Supernatants from infected cell cultures showing complete CPE were clarified by centrifugation at 4000 × *g* for 15 min, filtered through 0.45 µm pore filters, divided into aliquots, and frozen at –80°C until used as antigen in serological tests.

ELISA. The ELISA used to detect rainbow trout antibody against VHSV was performed according to the general principles of indirect ELISA described by Voller et al. (1979) and followed the ELISA technique previously described (Jørgensen et al. 1991, Olesen et al. 1991). The ELISA test used was an antigen-capture ELISA, making each fish serum its own negative control by incubating the sera on wells with and without virus, respectively, and by only taking differences in optical density into account. This is necessary due to the sticky nature of fish antibodies, which have a tendency to bind non-specifically to the plates (Olesen et al. 1991). Briefly, microtitre plates (Nunc) were first coated with protein-A purified rabbit anti-virus immunoglobulin (Ig) diluted in carbonate buffer. After washing the plates with PBS containing 0.05% Tween-20 (PBS-T), the VHSV suspension diluted to 1:10 in PBS-T with 1% bovine serum albumin (PBS-T-BSA) was added and incubated for 1 h at room temperature. After washing with PBS-T and blocking for 2 h at room temperature with PBS-T-BSA, the plates were incubated overnight at 4°C with serial 2-fold dilutions (1:40 to 1:10240) of trout serum in PBS-T-BSA. Positive- and negative-control trout sera were included on each plate. Following another washing step with PBS-T, the plates were incubated for 1 h at room temperature with MAb 4C10 against rainbow trout IgM (Thuvander et al. 1990), followed by another washing cycle and incubation for 1 h at room temperature with horseradish-peroxidase (HRP)-conjugated rabbit antiserum against mouse Ig (Dako A/S). After a final washing step, the bound enzyme was visualized by addition of H_2O_2-orthophenyline diamine substrate (Sigma-Aldrich), and the reaction was stopped after 15 min at room temperature by addition of 1 M H_2SO_4. Absorbance was measured spectrophotometrically in a Titertek Multiscan reader at a wavelength of 492 nm (A_{492}). The ELISA titre of a trout serum was defined as a reciprocal value of the highest serum dilution giving a difference in absorbance of 0.5 or more between wells with and without virus, respectively.

50%PNT. Prior to neutralisation, the virus isolates were plaque-titrated in microplates with EPC cells using overlay medium containing 1% methylcellulose in order to determine the dilution to be used in the test. The 50%PNT with complement addition was performed in 96-well microtitre plates as previously described (Olesen & Jørgensen 1986) but with some modifications. Briefly, 0.05 ml (per well) of serial 2-fold dilutions of trout serum in dilution medium (Eagles NEM with Tris buffer and 5% FBS), beginning with a dilution ratio of 1:10, were mixed in round bottom, 96-well microplates (Nunc) with an equal volume of trout complement diluted to 1:30. After incubation for 30 min at 15°C on a rocker platform, 0.1 ml of virus dilution adjusted to 8×10^3 PFU ml^{-1} was added to each well and the plate was incubated overnight at 15°C on a rocker platform. Each serum-complement-virus mixture was then adsorbed to 2 replicate wells (0.01 ml per well) with monolayer EPC cells in 96-well microplates (Life Technologies) for 1 h at 15°C. The cell cultures were then overlaid with 1% methyl cellulose medium and incubated for 5 d at 15°C. Finally, the cells were fixed in 10% P-buffered formaldehyde (0.15 ml per well, 3 h at room temperature), followed by 10 washes with tap water and staining with 0.5% crystal violet (0.05 ml per well, 30 min at room temperature). After 10 more washes, the microplates were air-dried and the plaques counted by placing the plates on a light table. The 50%PNT titre was calculated as the reciprocal value of the highest trout serum dilution causing a 50% reduction of the average number of plaques in control cultures inoculated with normal trout serum, complement and virus.

In the 50%PNT with the marine VHSV isolates, serum-complement-virus mixture was adsorbed onto BF-2 cells instead of EPC cells for 1 h at 15°C, overlaid with 1% methyl cellulose medium and incubated for only 24 h at 15°C. Then, the infected cells were visualized by immunostaining using MAb IP5B11 against the VHSV N protein as the first antibody layer, HRP-conjugated as the second antibody layer and 3-amino-9-ethylcarbazole as the substrate (Lorenzen et al. 1988, 1990).

Western blotting (WB). A WB analysis was performed in order to identify individual virus proteins recognized by the trout sera. Purification of viral proteins, SDS-PAGE and transfer methods have been previously described (Lorenzen et al. 1988, 1993). The WB experiments were carried out using VHSV DK-F1 and without 2-mercaptoethanol in the sample buffer (non-reduced conditions). Trout sera were diluted to 1:50 in PBS-T-BSA and incubated overnight at 4°C with strips of nitrocellulose membrane with viral proteins. After incubation, the strips were washed 3 times for 5 min with PBS-T. Then, the strips were incubated for 1 h at room temperature with MAb 4C10 against rainbow trout IgM, followed by 3 washes with PBS-T and incubation for 1 h at room temperature with HRP-conjugated rabbit anti mouse Ig. After a final washing step, the bands reacting with the sera were visualized by

Table 2. Detection of VHSV and antibodies against VHSV by ELISA, 50%PNT and WB in 50 rainbow trout from an endemically VHSV-infected farm; ns: no sample. G: viral glycoprotein, N: viral nucleoprotein, M1 and M2: viral matrix protein 1 and 2

	ELISA[a]		50%PNT			WB[b]				Virus
Virus isolate:	DK-F25	DK-201433-40	DK-F25	DK-F1	DK-201433-40	DK-F1				isolation
Serum no.	Titre	Titre	Titre	Titre	Titre	G	N	M_1	M_2	
1	640	640	0	0	2560	(+)	(+)	(+)	–	–
2	320	160	0	0	2560	++	(+)	(+)	–	–
3	320	640	0	0	10240	+	(+)	–	–	–
4	40	160	0	0	320	–	–	–	–	–
5	160	40	0	0	5120	+++	(+)	–	–	–
6	2560	640	0	0	2560	(+)	(+)	(+)	(+)	–
7	0	0	0	0	1280	(+)	(+)	–	–	–
8	0	0	0	0	1280	(+)	(+)	–	–	–
9	320	160	0	0	320	(+)	–	(+)	–	–
10	0	0	0	0	0	–	–	–	–	+
11	640	40	0	0	1280	–	–	–	–	–
12	0	0	0	0	160	–	–	–	–	+
13	40	0	0	0	1280	(+)	(+)	–	–	–
14	0	0	0	0	640	–	–	–	–	+
15	160	0	0	0	10240	(+)	(+)	–	–	+
16	0	0	0	0	640	(+)	–	–	–	–
17	320	160	0	0	160	+++	(+)	–	–	–
18	40	0	0	0	80	–	–	–	–	–
19	1280	640	0	0	2560	+	(+)	+	–	–
20	0	0	0	0	2560	++	(+)	–	–	–
21	1280	640	0	0	10240	+++	–	–	–	–
22	160	0	0	0	640	–	–	–	–	+
23	2560	160	0	0	640	+	–	–	–	–
24	0	0	0	0	2560	(+)	(+)	–	–	–
25	0	0	0	0	320	–	–	–	–	+
26	>10240	>2560	0	0	10240	(+)	–	–	–	–
27	640	40	0	0	2560	+	–	–	–	–
28	40	0	0	0	320	–	–	–	–	–
29	0	0	0	0	160	–	–	–	–	–
30	160	160	0	0	10240	+	–	–	–	–
31	0	0	0	0	5120	(+)	–	–	–	–
32	160	40	0	0	1280	–	–	–	–	–
33	80	0	0	0	2560	++	–	–	–	–
34	640	640	0	0	10240	+	–	–	–	–
35	160	160	0	0	1280	–	–	–	–	–
36	160	0	0	0	5120	–	–	–	–	–
37	0	0	0	0	2560	–	–	–	–	–
38	0	0	0	0	320	–	–	–	–	–
39	0	0	0	0	40	–	–	–	–	+
40	ns	ns	ns	ns	ns	ns	ns	ns	ns	+
41	40	0	0	0	5120	+	–	–	(+)	–
42	40	0	0	0	2560	–	–	–	(+)	+
43	320	160	0	0	2560	–	–	–	–	–
44	0	0	0	0	5120	++	–	–	(+)	–
45	0	0	0	0	2560	–	–	–	–	–
46	0	0	0	0	40	(+)	(+)	–	–	+
47	0	0	0	0	640	+++	–	–	–	–
48	40	40	0	0	5120	+	–	–	–	–
49	0	0	0	0	0	–	–	–	–	+
50	40	0	0	0	2560	+	–	–	–	–
No. of positive[c]	30	20	0	0	44	29	14	5	4	11
	61%	41%	0%	0%	90%	30 (61%)				22%

[a]In ELISA and 50%PNT: titre 0 is equivalent to titres <40
[b]In WB: (+) = very weak; + = weak; ++ = moderate; +++ = strong reaction
[c]Sera with titres of ≥40 in ELISA and ≥160 in 50%PNT

3-amino-9-ethylcarbazole in sodium acetate buffer (0.1 M, pH 5.5). The reaction was stopped after 30 min at room temperature by washing the strips with distilled water.

RESULTS

Clinical and virological examination

None of the 50 fish collected from the endemically VHSV-infected farm showed any clinical signs of VHS. However, 11 fish (22%) were shown by cell cultivation to be positive for VHSV (Table 2). The neutralisation patterns of these VHSV isolates were examined and revealed that all belong to Serogroup III (data not shown). None of the 50 fish collected from the VHSV-free farm showed any clinical signs of diseases; IPNV was isolated from 2 fish.

Detection of antibodies

When sera from the VHSV-infected farm were examined by 50%PNT using VHSV DK-F1 or the heat attenuated DK-F25 mutant, no neutralizing antibodies were found. In contrast, when one of the virus isolates from the farm (DK-201433-40 from Trout No. 40) was used, 90% (44 from 49) of the fish had neutralizing antibody titres of 160 or more and were thus considered positive. The titres ranged from 40 to 10240 and most of the sera had titres around 2560 (median titre 2560) (Table 2).

When the sera were examined by ELISA using the VHSV DK-F25 as antigen, 30 (61%) were positive, with titres ranging from 40 to 2560 except one that was higher than 10240 (Table 2). The median titre was 160. Seven of the sera had a titre of 40; however, in 4 of sera, the A_{492} value was very close to the cut-off value. In the group of positive sera, most (16 from 30) had titres ranging from 160 to 640. When the homologous VHSV isolate DK-201433-40 was used in the ELISA test, only 20 of the 49 sera (41%) were found positive, with titres ranging from 40 to 2560 (median titre 160) (Table 2). Most of the sera that were negative with the DK-201433-40 isolate but positive with DK-F25 had low titres (40) with the latter.

When WB was performed, positive bands were visualised in 30 of the sera (61%) but different reaction patterns were observed. Viral glycoprotein (G) was the protein most frequently recognized (in 29 of the 30 sera with positive reactions), followed by the nucleoprotein (N) (14 of 30 sera). The membrane proteins (M_1, M_2) were recognized by only 5 and 4 sera, respectively. Of the 49 sera analysed, 19 (39%) did not react with any viral proteins (Table 2). WB only tested the reaction against one virus isolate (DK-F1).

In order to examine the specificity of the serological techniques, 50 broodstock fish sera collected in an approved VHS-free farm were examined (this farm had been officially tested and assessed to be free of VHS for more than 30 yr). The farm is, however, infected with IPN. All sera from this farm were negative for antibodies against VHSV, except one that had a weak positive reaction in ELISA at a titre of 40. When these sera were examined for the presence of antibodies against IPNV by ELISA (plates coated with protein-A purified rabbit anti-IPNV and a virus suspension of the reference strain Sp (Spjarup) of IPNV diluted to 1:5), 13 of them (26%) were positive with titres ranging from 80 to 10240 (data not shown).

Effect of virus isolates used as antigen in 50%PNT

When a panel of 13 different VHSV isolates was used in 50%PNT for detection of neutralising antibodies in 5 selected trout sera, significant differences were observed among the virus isolates included (Table 3). The highest titres were observed by using the homologous VHSV DK-201433-40 in the 50%PNT. Trout Serum No. 8 recognised this isolate very specifically, with no cross-neutralisation to any of the other virus isolates. The only VHSV isolates that were not neutralised by any of the sera were the DK-F1 and DK-F25

Table 3. 50%PNT titres using different VHSV isolates and 5 sera from a rainbow trout farm endemically infected with VHSV. VHSV I, II, III: VHSV with neutralisation pattern Type I, II or III, respectively (Olesen et al. 1993); titre 0 is equivalent to titres <40

Virus isolate	Serum no. 6	8	21	26	47
VHSV I					
DK-F1	0	0	0	0	0
DK-F25	0	0	0	0	0
DK-3592B	1280	0	2560	640	320
VHSV II					
DK-5131	640	0	640	40	0
F-403	2560	0	2560	80	2560
VHSV III					
DK-201433–40	1280	640	5120	5120	1280
DK-5151	320	0	160	640	640
DK-6137	160	0	320	320	320
F-H11225	1280	0	80	640	0
CZ 2077–200317	2560	0	5120	160	1280
Marine VHSV					
DK-1P8	1280	0	320	160	0
DK-1P49	320	0	1280	0	0
RBV-'Makah'	0	0	0	0	0

Table 4. 50%PNT titres using 5 homologous VHSV isolates and 16 sera from a rainbow trout farm endemically infected with VHSV

Serum no.	VHSV isolate DK-201433-10	DK-201433-15	DK-201433-40	DK-201433-42	DK-201433-49
8	320	nd	640	nd	640
10	40	0	0	40	0
12	40	40	160	80	40
14	160	160	640	320	160
15	>5120	>5120	>5120	>5120	>5120
18	320	80	80	80	640
21	2560	2560	>5120	>5120	5120
22	40	nd	640	nd	80
25	80	nd	320	nd	40
26	>5120	>5120	>5120	>5120	>5120
39	0	nd	40	nd	0
42	1280	1280	2560	>5120	1280
44	1280	nd	5120	nd	2560
46	40	nd	40	nd	40
47	nd	2560	640	>5120	nd
49	0	0	0	0	0

reference strains, and the marine isolate RBV-'Makah' from the USA.

When 5 of the 11 VHSV isolates from the infected farm were used in 50%PNT against sera from 16 fish from the same farm, no significant differences among the viruses were observed (Table 4).

DISCUSSION

All standardised and approved procedures for the diagnosis and surveillance of VHS are based on virus isolation in cell cultures inoculated with homogenized organ material from fish, followed by identification by serological or molecular methods. However, the detection of virus-specific antibodies in fish sera would have several advantages when compared with the traditional virus isolation and identification methods. While virus can usually only be isolated for a short period during the initial phase of the infection, humoral antibody response can be detected up to 1 yr after infection (Enzmann & Konrad 1993). Moreover, sampling of test material can be done without sacrificing the fish, and fish sera are more stable for sending and storage than are virus isolates. The reasons why serological tests were not implemented for fish health surveillance a long time ago are that fish antibodies are more unstable and more likely to give false positive results than antibodies from mammals and birds; the time from infection to a detectable antibody response is very long (4 to 6 wk, depending on water temperature); and, finally, that the development of the antibody response in fish is more variable than in mammals (Lorenzen & LaPatra 1999).

The serological techniques most commonly used for the detection of fish antibodies against VHSV are 50%PNT, IF and ELISA (Olesen et al. 1991, Jørgensen et al. 1991, Ahne & Jørgensen 1993). Other techniques such as WB (Lorenzen et al. 1993) or counter-current immunoelectrophoresis (Enzmann & Konrad 1990, 1993) are less commonly used. In previous studies (Olesen et al.1991, Jørgensen et al. 1991), we compared 3 serological techniques for the detection of antibodies against VHSV and infectious hematopoietic necrosis virus (IHNV). Sera collected from rainbow trout that had been naturally or artificially infected with VHSV or IHNV were tested by ELISA, IF and 50%PNT. The most sensitive method for the detection of anti-IHNV antibodies was IF, while 50%PNT and ELISA were less sensitive. In the case of antibodies against VHSV, test sensitivity decreased in the following order: ELISA, IF and 50%PNT. However, a considerable number of sera contained cross-reacting antibodies, which could be considered as false-positive reactions, were detected by ELISA and IF but not by 50%PNT. The lower sensitivity of the 50%PNT was believed to be due to the fact that this method detects only neutralising antibodies that react with the viral glycoprotein (Olesen et al. 1991), whereas the other techniques detect antibodies directed against all the viral structural proteins. 50%PNT was thus considered the least sensitive but the most specific of the tests. It was concluded that ELISA was the most suitable method for screening large numbers of sera due to its high sensitivity and reproducibility, and relatively low cost in terms of time and materials. IF and 50%PNT could be used to confirm the results, but are not recommended for large scale screening because they are time-consuming and require more technical expertise. In addition, 50%PNT is hampered by the need for fresh or properly stored trout serum that is free of neutralizing antibodies as a complement source.

In the present study, ELISA was far more sensitive than 50%PNT when using VHSV DK-F25. However, when 50%PNT was performed using one of the virus isolates from the farm, VHSV DK-201433-40 90% of the sera were found positive, whereas only 61% were found positive by ELISA when using this same isolate.

When 50%PNT was used to examine selected sera against a broad panel of different VHSV isolates, significant differences in neutralisation titres were observed. Only one serogroup of VHSV exists when using poly- and monoclonal antibodies for typing (Olesen et al. 1993). We anticipated at that time that mammalian antibodies would be more exact and specific for uniform serological typing of the viruses than fish antibodies, due to fewer possibilities for diversification of

antibody responses in lower vertebrates, as stressed by Du Pasquier (1982). It was therefore surprising to observe that a change of VHSV isolate in a 50%PNT test could change the number of positive trout sera from 0 to 90%, and furthermore to observe the significant differences in titres of 5 selected rainbow trout sera against different VHSV isolates. This could reflect a much more refined and specific humoral antibody response in fish than previously recorded. However, when examining different virus isolates from the same farm, all titres obtained were similar, reflecting the high homology of VHS viruses isolated from the same site and VHS outbreak (Einer-Jensen et al. 2004).

The specificity of the tests used was high and only one low-titre false-positive reaction was observed in ELISA using the DK-F25 strain on 50 negative control sera from a farm free of VHS. No false positives were observed in 50%PNT or ELISA with the homologous VHSV.

In the present work, VHSV could be isolated from 11 broodfish with no clinical signs of diseases. Serum antibodies might bind to VHSV *in vivo* and give false-negative virus isolations, but the fact that some fish were positive for both VHSV and anti-VHSV antibodies, with both low and high titres, might indicate that the antibody blocking of viruses does not play in important role in virus isolation. The existence of fish with both VHSV in the tissue and significant levels of anti-VHSV antibodies (e.g. Trout No. 15 and 42) indicates that humoral immunity is not sufficient for a complete neutralisation of virus in infected fish.

An end-point serum neutralisation test, which is easier to perform and more suitable for processing large numbers of serum samples, was developed by Hattenberger-Baudouy et al. (1995). In a large-scale serological survey conducted in France, which involved 229 farms and in which more than 14000 trout sera were analysed, the relationship between the serum-neutralizing antibody of the fish and the presence of VHSV and/or IHNV in a trout farm population was confirmed by this method. Thus, this technique was recommended for epidemiological studies and could be useful in the implementation of fish surveillance programmes. However, it is obvious from our results that the VHSV isolate used in neutralisation tests plays a very important role in the outcome of the tests. The VHSV DK-F25 belonging to neutralisation pattern Type I had been used in 50%PNT at our laboratory since 1984, because trout sera at that time were shown to give higher titres with that isolate than with several other VHSV isolates tested (Olesen & Jørgensen 1986). However, in this study, the homologous neutralisation pattern Type III isolate was much more efficient in detecting antibodies against VHSV than DK-F25 or DK-F1 in 50%PNT. Therefore, if a standardised neutralisation test is to be recommended in future surveillance of VHS, it is necessary to first be aware of which virus isolates are to be used.

With regard to 50%PNT, the reaction is dependent on the ability of fish antibodies to recognise a few neutralising epitopes on the viral glycoprotein (Lorenzen & LaPatra 1999), while a broad range of viral antigens are putative binding sites for fish antibodies in ELISA (and IF) tests. It was therefore anticipated that only a minor effect would be observed if different VHSV isolates were used in ELISA. Use of the homologous VHSV in ELISA instead of the traditional DK-F25 strain did not increase the number of positives; on the contrary, the number of positive sera decreased by 20%. This might be due to fewer antigens presenting epitopes in the preparation of the detergent-treated homologous virus suspension compared with the DK-F25 virus preparations, or to the simple fact that different amounts of virus were used in the 2 ELISAs.

Previous studies have demonstrated that conventional WB assay is inadequate for detecting an antibody response to natural VHSV infection. This is possibly due to the negative, denaturing effect of mercaptoethanol on protein conformation, which eliminates the tertiary structure and allows the antibodies to attach only to linear epitopes; in contrast to this, rainbow trout tend to produce antibodies that only recognize discontinuous epitopes (Lorenzen et al. 1993). Lorenzen et al. (1993) reported that WB assays revealed that rainbow trout are able to produce antibodies that recognize all viral proteins of VHSV, but this usually only occurred after repeated immunization of fish with whole virus particles. In this study, we found that 61% of the sera recognized at least one of the VHSV proteins, the same percentage that was found to be positive in ELISA using the DK-F25 strain. One interesting finding is that 29 sera reacted with the glycoprotein of the VHSV DK-F1 strain used in WB, whereas none of the sera showed neutralizing antibodies when assessed by 50%PNT using the same VHSV isolate. This indicates that the sera recognised epitopes on the viral glycoprotein that were not neutralising this particular virus. The neutralisation of VHSV is specifically related to antibodies binding to the glycoprotein (Lorenzen & LaPatra 1999).

To conclude, in terms of laboratory equipment, ELISA is more suitable to use in generalised laboratories than are neutralisation tests. ELISA is usually very sensitive, rapid, independent of complement and cost-effective for analysing high numbers of sera. However, the variable specificity of the ELISA test makes the neutralisation test the technique of choice for the screening of non-infected populations where false-positive reactions could have damaging consequences. False-positive sera usually have a low titre, so the criteria for defining a positive sample must be consid-

ered carefully. The end-point neutralisation test may be easier for most laboratories to use than 50%PNT. In any case, the choice of virus strain is extremely important for the outcome of neutralisation tests, and should reflect the most recent and common type of virus in the area.

Acknowledgements. We thank the technicians at DFVF for their help, especially M. Eliassen and N. Nicolaisen. We also acknowledge our colleagues at DFVF for their contribution to this study. J.M.F.G. was the recipient of a postdoctoral research fellowship from 'Secretaría de Estado de Educación y Universidades, Ministerio de Educación, Cultura y Deporte' of Spain, and was co-financed by the European Social Program.

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Editorial responsibility: Jo-Ann Leong, Kaneohe, Hawaii, USA

Submitted: June 14, 2005; Accepted: November 8, 2006
Proofs received from author(s): February 7, 2007