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Influence of storage temperature on infectious hematopoietic necrosis virus detection by cell culture isolation and RT-PCR methods

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ABSTRACT: The detection of infectious hematopoietic necrosis virus (IHNV) in infected rainbow trout *Oncorhynchus mykiss* and in cell culture supernatants stored under different conditions was studied. IHNV-positive fish visceral organ homogenates and cell culture supernatants were incubated at 4 and 25°C. Virus titre was measured by virus isolation on *epithelioma papulosum cyprini* (EPC) cells and the IHNV RNA was detected by RT-PCR and semi-nested RT-PCR. The influence of repeated freezing and thawing on the virus isolation from organ homogenates and from cell culture supernatants was studied as well. It was possible to isolate the virus from IHNV-positive organ material during the 3 d of incubation at 4°C but, only on the first day of incubation at 25°C. Viral RNA could be amplified during the incubation period of 35 d at 4°C but only during 8 d of incubation at 25°C. In IHNV-infected cell culture supernatant stored at 4°C, it was possible to detect virus for 36 and 16 d in supernatant stored at 25°C. Viral RNA could be followed by using molecular methods during the entire experimental period of 123 d. Each cycle of freezing and thawing of samples resulted in a reduction of IHNV titre in the suspension of visceral organs, while the virus titre in cell culture supernatant remained almost the same following 33 freezing-thawing cycles. The present results show that rapid laboratory processing and storage of potentially virus-containing tissue samples as well as the use of different detection methods are very important for efficient IHNV diagnosis.

KEY WORDS: Infectious hematopoietic necrosis virus · IHNV · Diagnosis · Virus isolation · RT-PCR · Storage

INTRODUCTION

Infectious hematopoietic necrosis virus (IHNV) produces systemic infections with high mortality in rainbow trout *Oncorhynchus mykiss* and also in other economically important salmonid species including brown trout *Salmo trutta fario*, Atlantic salmon *Salmo salar* and Pacific salmon *Oncorhynchus* sp. This virus is responsible for increasingly devastating losses of young fish. In hatcheries where the disease breaks out for the first time, total mortality can reach up to 80%.

IHNV was first isolated in western North America and then spread to Japan and Europe. It was first detected in 1987 in Italy (Bovo et al. 1987) and France (Baudin-Laurencin 1987). In the summer of 1997, IHNV was also isolated for the first time from rainbow trout in Slovenia. The results of monitoring ovarian and seminal fluids annually have shown that many fish farms have become infected with IHNV (Jenčič & Hostnik 1998).

IHNV belongs to the new genus *Novirhabdovirus* of the family *Rhabdoviridae* (Walker et al. 2000). Its genome consists of a linear molecule of negative sense, non-segmented, single-stranded RNA, encoding 5 genes for structural proteins and 1 gene for a non-virion protein (Kurath & Leong 1985, Kurath et al. 1985).

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An IHNV infection is often diagnosed on the basis of epidemiological and clinical findings; however, laboratory diagnosis of IHNV can lead to better control methods. The most commonly used test for IHNV detection is the virus isolation test using *epithelioma papulosum cyprini* (EPC) and BF-2 cell lines (Lorenzen et al. 1999). The identification of virus isolates may be performed by the indirect fluorescent or peroxidase antibody test, which detects virus antigen directly in cell cultures using monoclonal anti-IHNV antibodies and anti-mouse conjugate as the next step. Another confirmatory test for IHNV identification is the virus neutralization test on tissue culture cells (Lorenzen et al. 1999). These methods are labor intensive and time-consuming (requiring about 5 to 20 d); samples for the diagnosis must be stored under optimal conditions in order to obtain valid results. The inactivation of IHNV during sampling, transport and manipulation in laboratories is possible and could negatively influence the virus isolation test.

For the confirmation of IHNV infection in fish samples the immunofluorescent antibody test (IFAT) has been extensively used for the detection of viral antigens in cell cultures and fish tissues (reviewed in Sanz & Coll 1992). Other tests employed in routine diagnostics include immuno-capture of viral ribonucleocapsid proteins by ELISA. The ELISA test using polyclonal antibodies, as well as monoclonal antibodies against the G or the N proteins, has been introduced for IHNV diagnosis (Dixon & Hill 1984, Ristow & Arnzen 1989, 1991). Some monoclonal antibodies were found to have lower sensitivity for detecting some IHNV isolates. Monoclonal antibodies specific to the G-protein had cross-reactivity with viral haemorhagic septicaemia virus (VHSV) (Enzmann et al. 1991).

In recent years molecular methods have also been developed and applied to IHNV diagnostics. A reverse transcriptase-polymerase chain reaction (RT-PCR) method which detects specific parts of the IHNV genome has been described (Brushhof et al. 1995, Arakawa et al. 1990, Alonso et al. 1999, Miller et al. 1998, Barlič-Maganja et al. 2002). These methods have been shown to be more sensitive and faster than current procedures.

The aquatic rhabdovirus group, including IHNV, represents viruses which are not stable at room temperature. For example, IHNV in cell culture supernatant was completely inactivated within 8 h at 32°C, and after 7 d at 27°C (Pietsch et al. 1977). It has also been reported that IHNV was inactivated within 140 min at 38°C and within 24 h at 32°C. A reduction of IHNV titre was not observed within 5 h at 22°C in cell culture medium; however, the titre in visceral organ suspension decreased more rapidly (Gosting & Gould 1981). For short-term storage, 4°C was generally the most efficient temperature for preserving infectious virus in ovarian fluids, sera and homogenates of different fish tissues, while for longer periods (up to 1 yr) the infectivity was most efficiently preserved by storage at –20°C (Burke & Mulcahy 1983).

In the present study the effect of different storage conditions on the detection of IHNV by virus isolation and molecular methods was tested. A suspension of IHNV-positive visceral organs as well as IHNV-inoculated cell culture fluid supernatants were stored at 4 and 25°C, respectively, and virus was detected using the cell culture isolation method, RT-PCR and semi-nested RT-PCR. The effects of freezing and thawing of similar samples were also examined.

MATERIALS AND METHODS

Sample preparation. Rainbow trout from 7 to 9 cm in length were collected from a fish farm with a recent history of IHNV. Visceral organs (kidney, spleen, heart) from 10 fish were pooled and used in the present study. Reference strain IHNV 4008, kindly provided by Dr. Bovo (Istituto Zooprofilattico Sperimentale, Padova, Italy), was also included in the experiment as a positive control.

Tissue material was homogenised and diluted 1:10 with cell culture medium (minimal essential medium [MEM], Gibco BRL) containing 10% foetal bovine serum (Svanoclone FBS) and antibiotic Garamycin (Lek d.d.). Specimens were centrifuged at 2500 × *g* for 15 min at 4°C and the obtained supernatant fluids were divided into 2 equal parts (Sample A and Sample B). Sample A was again divided into 2 tubes (Sample A1 and Sample A2). The tube with Sample A1 was stored at 4°C and the tube with Sample A2 was stored at 25°C. Both samples were kept under these conditions for 54 d. Sample B was filter sterilised (Minisart, 0.20 µm) and inoculated on a monolayer of EPC cells. After 5 d of incubation at 15°C, culture medium was collected and centrifuged at 2500 × *g* for 15 min at 4°C. The supernatant fluid was divided into 2 equal parts (Sample B1 and Sample B2). Sample B1 was stored at 4°C and Sample B2 was stored at 25°C. Both samples were kept under these conditions for 123 d.

The persistence of IHNV under different storage conditions was examined by virus isolation, RT-PCR and semi-nested RT-PCR method.

To study the influence of freezing and thawing on the suitability of samples for virus diagnostics, a part of a homogenised IHNV-positive pool of fish visceral organs and the IHNV-infected EPC cell culture fluid were frozen at –70°C. The frozen samples were thawed in cold water. The freezing-thawing procedure was repeated 33 times. After each thawing, the sam-

ples were diluted in MEM (1:10) and centrifuged at 2500 × *g* for 15 min at 4°C. Supernatants were filter sterilised through a 0.20 µm filter and virus titration on EPC cells was performed. In parallel, RT-PCR and semi-nested PCR were done for virus RNA detection in each sample.

Virus isolation and immunofluorescent detection. Sample B was inoculated onto confluent EPC cells in 96-well microplates. Ten-fold dilutions from 10^0 to 10^{-7} were prepared in mock plates (the 180 µl cell culture medium and 20 µl sample) and inoculated, using 50 µl per well. After adsorption for 30 min at 15°C, 50 µl of cell culture medium supplemented with 10% FBS were added and plates were incubated at 15°C. After 6 d of incubation, the cell culture medium was removed and cells were fixed with 85% acetone at –20°C. The detection of IHNV by an IFAT was carried out with monoclonal antibodies (BioX) following the protocol recommended by the supplier. Briefly, monoclonal antibodies were diluted 1:40 in PBS-T (0.01 M phosphate buffered saline plus 0.05% [v/v] Tween 20) and added on air-dried cell monolayer in 96-well microplates. After 1 h incubation period at 37°C, the cells were rinsed with PBS-T and rabbit anti-mouse FITC-conjugated antibodies (Dako) were added in each well. After 1 h incubation at 37°C microplates were rinsed and cell monolayer was examined by UV light microscopy (Zeiss, Axiovert 25).

Virus titration. Titration of IHNV in virus-positive material and in cell culture supernatant was performed in triplicate by end-point dilution on EPC cell cultures in 96-well microplates. The IHNV titre was determined for Samples A1, A2, B1 and B2 stored under different conditions at specific time intervals. The distribution of samples for virus titration in 96-well microplates was performed as described above. The plates were incubated at 15°C and examined microscopically for cytopathic effect 6 d post inoculation. The virus titre was than calculated as $TCID_{50}$ ml^{-1}.

RT-PCR and semi-nested RT-PCR. All samples stored under different conditions were tested by RT-PCR. When results of the RT-PCR method were negative, semi-nested RT-PCR was performed.

Total RNA was extracted from tested specimens by TRIzol LS reagent (Gibco BRL). Briefly, 250 µl of homogenised tissue or cell culture supernatants were mixed with 750 µl TRIzol LS reagent. The extraction procedure followed the manufacturer's instructions and the extracted RNA was dissolved in RNase-free water.

Reverse transcription and PCR amplification of the gene fragment encoding the G-protein was carried out using single step RT-PCR and oligonucleotide primers as described previously (Barlič-Maganja et al. 2002). Additional semi-nested amplification of the obtained products was performed including the same reverse primer as in RT-PCR and an internal forward primer. As a negative control, non-infected cells as well as pools of IHNV-negative fish samples were used. The products of both reactions were analysed by 1.8% agarose gel electrophoresis.

RESULTS

Sample A1 stored at 4°C and Sample A2 stored at 25°C had the initial infectivity $10^{4.8}$ $TCID_{50}$ ml^{-1}. Virus isolation from Sample A2 was negative after incubating the sample at 25°C for more than 1 d. The virus titre in Sample A1 decreased during the first 3 d and was not detectable on Day 4 by cell culture inoculation (Table 1).

Table 1. *Oncorhynchus mykiss.* IHNV detection in the fish visceral organ homogenates (Sample A) stored at 4°C (Sample A1) and 25°C (Sample A2) by virus isolation, RT-PCR and semi-nested RT-PCR. +: positive result; –: negative result (no evidence of virus); ND: not done

Time (d)	Virus isolation ($TCID_{50}$ ml^{-1})		RT-PCR		Semi-nested RT-PCR	
	Sample A1 (4°C)	Sample A2 (25°C)	Sample A1 (4°C)	Sample A2 (25°C)	Sample A1 (4°C)	Sample A2 (25°C)
0	$10^{4.8}$	$10^{4.8}$	+	+	+	+
1	$10^{3.9}$	–	ND	+	ND	ND
2	$10^{2.1}$	–	+	ND	ND	ND
3	$10^{0.6}$	–	ND	+	ND	ND
4	–	ND	+	+	ND	+
6	–	ND	ND	–	ND	+
8	–	ND	ND	–	+	+
13	ND	ND	+	ND	ND	–
20	ND	ND	+	ND	+	ND
22	ND	ND	+	ND	+	ND
35	ND	ND	–	–	+	ND
54	ND	ND	–	–	–	–

Table 2. *Oncorhynchus mykiss*. IHNV detection in the cell culture supernatants stored at 4°C (Sample B1) and 25°C (Sample B2) by virus isolation and RT-PCR test. +: positive result; –: negative result (no evidence of virus); ND: not done

Time (d)	Virus isolation ($TCID_{50}$ ml^{-1})		RT-PCR	
	Sample B1 (4°C)	Sample B2 (25°C)	Sample B1 (4°C)	Sample B2 (25°C)
0	$10^{5.2}$	$10^{5.2}$	+	+
5	$10^{5.1}$	$10^{3.6}$	ND	ND
13	$10^{5.1}$	$10^{2.1}$	ND	ND
14	$10^{4.8}$	$10^{1.5}$	ND	ND
16	$10^{4.8}$	$10^{0.4}$	+	+
18	$10^{4.7}$	–	ND	ND
30	$10^{1.2}$	–	ND	ND
34	$10^{0.4}$	–	ND	ND
36	$10^{0.2}$	ND	+	+
37	–	ND	ND	ND
49	–	ND	+	+
57	–	ND	+	+
73	ND	ND	+	+
91	ND	ND	+	+
123	ND	ND	+	+

The viral RNA in Sample A1, stored at 4°C, could be detected by RT-PCR during the experimental period of 22 d and by semi-nested RT-PCR during a period of 35 d. IHNV RNA in Sample A2, stored at 25°C, was detected by RT-PCR on Day 4 and by semi-nested RT-PCR on Day 8 but not thereafter (Table 1).

With IHNV-infected cell culture medium (Sample B1) stored at 4°C the virus titre decreased slowly (Table 2). The IHNV could be detected on cell culture through Day 36. The reduction of virus titre was more drastic in Sample B2. Virus isolation from Sample B2 stored at 25°C was possible only through Day 16. Viral RNA could be detected by RT-PCR throughout the entire experimental period of 123 d in both samples (Table 2).

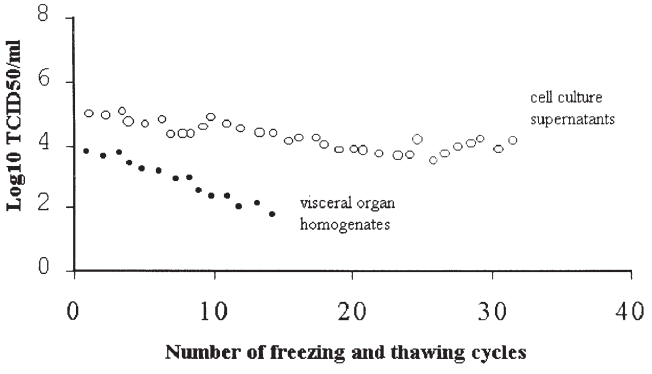

Fig. 1. IHNV in *Oncorhynchus mykiss*. IHNV titre in the cell culture supernatants and in the visceral organ homogenates measured after each freezing-thawing cycle

The suspension of fish visceral organs and the supernatant fluid from IHNV infected cell culture that were frozen at –70°C were thawed 33 times and virus titre on cell culture was determined. The initial virus titre in the suspension of visceral organs and in the supernatant of cell culture was $10^{4.8}$ and $10^{5.2}$ $TCID_{50}$ ml^{-1}, respectively. The freezing-thawing procedure had minimal influence on the virus titre in the supernatant of infected cell culture fluid during the whole experiment (Fig. 1). The virus titre in the suspension of visceral organs decreased and, due to the cytotoxic effect, IHNV could not be isolated after 13 freezing-thawing cycles. The molecular methods allowed the detection of viral RNA throughout the experiment (data not shown).

DISCUSSION

Our laboratory performs virus diagnosis on fish samples coming from different fish farms. Only fresh fish samples are known to be suitable for valid IHNV diagnosis by means of the virus isolation test (Blancou & Håstein 2000). Specimens, especially from free-ranging fish, are sometimes submitted for diagnosis in a decomposed state due to the delay in recovery, storage or dispatch. Using the virus isolation test, only positive results from such samples are valid. When the samples are not transported correctly, or they are stored too long at room temperature, we are unable to isolate IHNV on cell culture. Thermal inactivation and a decrease in IHNV titre within 1 h at 27°C has already been reported (Gosting & Gould 1981).

The objective of our study was to evaluate the usefulness of RT-PCR followed by semi-nested RT-PCR for the detection of IHNV in fish samples stored under different conditions. The efficiency and sensitivity of molecular detection methods were compared with the cell culture isolation method from clinical samples and from supernatants of infected cell culture.

In previous studies of IHNV detection by molecular methods, conditions were optimised and the methods were suggested as a rapid alternative method to the virus isolation method (Barli-Maganja et al. 2002). For virus detection in visceral organs of IHNV-infected fish, the virus isolation test, RT-PCR and semi-nested RT-PCR methods were compared. Molecular methods were found to be as sensitive as virus isolation on cell culture. The virus from freshly obtained fish samples (pooled visceral organs) stored at 4°C could be detected for a period of 3 d, while the same samples stored at 25°C were positive in the virus isolation test for only 24 h (Table 1). It has already been reported that the retention of viral infectivity in different fish samples is greatly dependent upon the storage temperature and the length of storage time (Burke & Mulcahy 1983). In visceral

organs, like spleen and kidney, the initial infectivity dropped after 4 h at room temperature and the virus could not be isolated from samples stored at room temperature for 2 d. After storage at 4°C for 3 d the virus titre in spleen and kidney considerably decreased and the virus could not be isolated from kidney after 1 wk or from spleen after 4 wk. Bacteria and fungi-free IHNV-infected cell culture supernatants stored at 4°C were positive in the virus isolation test for 36 and 16 d when stored at 25°C (Table 2). The IHNV titre decreased rapidly in samples stored at 25°C but slowly in samples stored at 4°C. This observation may indicate that other factors such as neutralising antibodies or cellular enzymes can inactivate virus in tissue samples.

To detect IHNV in fish samples stored under different conditions for longer periods of time, molecular methods were confirmed to be more effective and sensitive. Viral RNA could be detected in visceral organ samples stored at 25°C for 8 d and in samples stored at 4°C for 35 d (Table 1). The antigen-antibody complexes that might produce lower virus titre or negative results in virus isolation tests do not influence the detection of viral RNA by RT-PCR. It was also indicated that sample contamination with fungi and bacteria had less influence on the RT-PCR results. To avoid false negative RT-PCR results due to the presence of inhibitors, additional internal controls for the amplification of RNA should be included in the test (Smith et al. 2000).

The effect of intracellular enzymes, released during the freezing and thawing procedures, on the IHNV infectivity was examined. After 33 freezing-thawing cycles, maintenance of IHNV titre in cell culture suspension, but reduction of virus titre in fish organ suspension, was observed (Fig. 1).

Rapid and sensitive diagnostic methods are critical for timely and correct diagnosis. The storage conditions of samples are very important for effective accurate diagnostic methods. The virus isolation method for IHNV detection is reliable only when fresh fish samples stored at 4°C are used for the diagnosis. When it is not possible to inoculate cell culture within 48 h after the collection of the tissue material, homogenised filter sterilised tissue supernatants have to be prepared and stored in frozen conditions. For samples for which storage conditions are unknown, examination using molecular methods is recommended. RT-PCR has been shown here to provide better sensitivity and reliability, as compared to the virus isolation test, when used to detect IHNV in decomposed tissue material.

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