Detection of infectious pancreatic necrosis (IPN)-virus in experimentally infected Atlantic salmon parr by RT-PCR and cell culture isolation.

T. Taksdal¹, B.H. Dannevig¹, E. Rimstad²

¹ National Veterinary Institute, PO Box 8156 Dep., N-0033 Oslo, Norway. ² Norwegian School of Veterinary Science, PO Box 8146 Dep., N-0033 Oslo, Norway

Abstract
Fifty samples from clinically healthy Atlantic salmon parr were examined nine months after IPNV infection. The present RT-PCR protocol detected a higher number of IPNV positive samples than the standardised cell culture method. Cortisol treatment of the fish did not have any impact on the number of IPNV positive fish detected.

It is well established that salmonids may become persistently infected with infectious pancreatic necrosis virus (IPNV), either after an outbreak of IPN or through sub-clinical infection (Reno et al. 1978, Swanson & Gillespie 1979, Ahne & Thomsen 1986, Bootland et al. 1991, McAllister et al.1993). Persistently infected fish may be a potential source of IPNV. However, earlier studies have revealed that the infection may remain undetected, even when samples have been examined in cell culture (Agius et al. 1982, Johansen et al. 1997, Taksdal et al.1998). Methods that can detect IPNV in covertly infected fish may thus be beneficial in the surveillance and prevention of spread of the infection.

Reverse transcription - polymerase chain reaction (RT-PCR) has been found to be a very sensitive tool for the detection of RNA viruses, and regarded as promising for IPNV detection (Rimstad et al. 1990, Lopez-Lastra et al. 1994, Wang et al. 1997). However, to our knowledge, no protocols for RT-PCR have been documented to be more sensitive than cell culture isolation for the detection of IPNV from fish tissues.

Earlier findings have shown that stress may enhance virus replication in persistently IPNV-infected fish (Frantsi & Savan 1971, Roberts & McKnight 1976, McAllister et al. 1994, Taksdal et al. 1998). Therefore, to potentially induce detectable levels of IPNV, Atlantic salmon parr that were survivors of an IPN outbreak were injected with cortisol (Pickering and Duston 1983, Wendelaar Bonga 1997). Here we report the results of IPNV examination of samples from a group of fish collected three weeks after intra-peritoneal injection of a depot of cortisol and from a group of untreated fish. The samples were examined by both an improved RT-PCR protocol and a standardised cell culture method currently used at the National Veterinary Institute (NVI) in Norway.
Fish and sampling

Atlantic salmon fry were bath-challenged with IPNV, serotype Sp (similar to strain N1) just after the onset of first feeding, at approximately 500 degrees x days after hatching as previously described (Taksdal et al. 1997). Accumulated mortality 60 days post infection ranged from 20 to 60% in different tanks. The survivors were gathered into a single tank and then kept for 7 months at a water temperature of 10.8 - 11.5°C until sampling. The mean weight of the fish at sampling was 15g. Kidney samples from 50 fish were examined. Twenty-five of these had been injected intraperitoneally with 2.5 mg cortisol in 0.1 ml cocoa butter (Pickering and Duston 1983) three weeks prior to sampling. Each kidney sample was divided into two parts. One half, to be used for virus isolation in cell culture, was transferred to transport medium (Eagle’s minimal essential medium (EMEM), pH 7.6, supplemented with 10% newborn bovine serum, Tris-buffer and antibiotics) and then kept on ice for a maximum of 24 h. The other half, to be used for RT-PCR, was frozen at -70°C.

RT-PCR

Samples were diluted 1:10 in PBS (RNase free), and RNA was extracted using TRIzol® (Gibco BRL) according to the manufacturer’s instructions. Purified RNA was then quantified by measuring the optical density at 260 and 280nm. In the following RT step, 2µg RNA was used. Ready-To-Go™ RT-PCR beads (Amersham Pharmacia) were used according to the manufacturer’s instructions, with some modifications. Briefly, the tubes were kept on ice, H₂O (45µl minus the volume of RNA sample) was added, and they were incubated for 5 min. Then 2µl of random hexamers (0.1µg/µl) and RNA sample were added. The tubes were then incubated at 42°C for 120 min, after which they were put on ice and 1.5µl of each of the primers (15pmol/ml) DIAIPNF (5’ ATC TGC GGT GTA GAC ATC AAA G –3’ and DIAIPNR (5’- TGC AGT TCC TCG TCC ATC CC–3’) were added. The primers target the positions 2132-2153 and 2355-2336 on the NS/VP3 region of segment A of IPNV serotype Sp. The cycling conditions (Touchdown thermal cycler, Hybaid, Teddington, UK) were 94°C for 5 min., followed by 40 cycles at 95°C for 30 s., 55°C for 15 s., 72°C for 30 s. and finally 72°C for 7 min. The PCR products were visualized by agarose gel electrophoresis.

Three controls were included for each RT-PCR run: one positive tissue sample collected from Atlantic salmon during an IPN outbreak, one negative tissue sample and one negative control where water was included instead of the RNA sample.

The amplified DNA fragments from one fish and from the positive control were cloned using TOPO TA cloning kit (Invitrogen, Leek) and sequenced using an automated DNA-sequencer (ABI Prism 377, Perkin Elmer Applied Biosystems). Alignment of the nucleotide sequences to published IPNV sequences was performed using the Vector NTI program (InforMax).

Cell culture, virus isolation and identification

BF-2 cells (ATTC CCL 91) were used for virus isolation as described by Taksdal et al. 1997, with minor modifications. Briefly, cells were grown to 70-80% confluency in 24-well tissue culture plates at 20 °C in EMEM supple-
mented with 10% foetal bovine serum, Tris-buffer and antibiotics. The samples were homogenised (10%, w/v) in transport medium. After centrifugation, the supernatants were added to the cell cultures in final dilutions of 1 and 0.1% (w/v), one well per dilution. The remaining supernatants were frozen at -70°C. The cell culture medium was passaged to new cell cultures after one week of incubation at 15°C. One week thereafter, medium from the cultures exhibiting cytopathic effect were further examined by a neutralization test using rabbit antiserum against IPNV. For re-examination, the frozen supernatants from tissue homogenates were thawed and added to both BF-2 and CHSE-214 cells. The CHSE-214 cells were grown in Leibovitz’s L-15 medium with 5% FBS and antibiotics.

**Laboratory findings**

The samples collected from non-injected fish were all negative by virus isolation in BF-2 cell cultures. However, one sample was positive in CHSE-214 cells. By RT-PCR, eight (32%) of the 25 samples, including the CHSE-214 positive sample, gave positive amplifications of the expected DNA fragment size, 224bp. (Table 1). Regarding the samples collected from cortisol-injected fish, one sample was positive by virus isolation in BF-2 cells. At re-examination of the frozen supernatants all tissue homogenates were negative in BF-2 cells, including the sample that gave positive result at first examination (Table 1). However, in CHSE-214 cells, IPNV was isolated from one other sample. The number of RT-PCR-positive samples was identical to that found in the non-injected fish i.e. 8 (32%) of 25. The RT-PCR positive samples included the CHSE-214-positive sample, but not the BF-2-positive sample. The nucleotide sequences of the 224 bp DNA fragments from one of the samples, and the positive control, both showed 99% identity to IPNV serotype Sp.

The intention of the present study was to establish a detection method for persistent IPNV infection in Atlantic salmon. It was assumed that cortisol treatment would induce a stress response in the fish and thus activate and/or enhance IPNV replication, which subsequently should increase the possibility to detect the infection. A similar test for the detection of carriers of *Aeromonas salmonicida* has been established (McCarthy 1977, Hiney et al. 1994). However, in our study, no difference in IPNV prevalence was observed between cortisol-injected and non-injected fish. This indicates either that cortisol injection as performed in the present study did not induce immune suppression in the fish, or that any immune suppression induced failed to enhance virus replication. To further develop methods to increase IPNV replication for subsequent detection purposes, the mechanisms suppressing virus replication in persistently infected fish should be elucidated.

Nevertheless, when examining these samples by RT-PCR, 32% of the fish in both untreated and cortisol-treated groups were positive. The present RT-PCR protocol is, to our knowledge, the first to be reported as being more sensitive than virus isolation in cell culture for the detection of IPNV. Blake et al. (1995) reported a RT-PCR protocol with similar sensitivity to virus isolation in the detection of aquatic birnaviruses when examining pooled spleen and liver samples from yearling brook trout (*Salvelinus fontinalis*) survivors of natural IPN infections. The RT-PCR-positive samples contained between $10^{3.0}$ and $10^{5.5}$ TCID$_{50}$ ml$^{-1}$ of
IPNV. Yoshinaka et al. (1998) found a detection limit of $10^4 \text{TCID}_{50} \text{ml}^{-1}$ in a multiplex RT-PCR for simultaneous detection of both IHNV and IPNV.

Other studies quantify the sensitivity by the lowest amount of purified IPNV RNA necessary for a positive RT-PCR result. Lopez-Lastra et al. (1994) detected 1 pg of purified IPNV RNA while Wang et al. (1997) detected IPNV by single-tube RT-PCR with an estimated sensitivity of 15 fg of purified IPNV RNA, which should correspond to approximately 200 virus particles.

The use of nested PCR will in most cases further improve the sensitivity, as shown by Suzuki et al. who detected just 1 fg of virus RNA (Suzuki et al. 1997), their PCR being more sensitive than virus isolation in a cell culture. Using the same PCR method, Kitamura et al. (2000) reported that positive samples from pearl oysters had virus titres between $10^{2.7}$ and $10^{3.1} \text{TCID}_{50} \text{ml}^{-1}$. Moya et al. (2000) stated that nested PCR is the most sensitive method for the detection of IPNV. However, no parallel examinations of virus isolations in cell culture were carried out to support this statement.

In the present RT-PCR protocol, an unusual long incubation time for reverse transcription as compared to other protocols was used (2 h versus normally 30 min). Initial studies indicated that this improved the sensitivity. The reasons for this can only be speculated, but most RT-PCR protocols are based on amplification from ssRNA templates and not dsRNA like the IPNV genome. Heating of the samples prior to RT (60-100°C for 5-10 min is usually used) to split double-strands and to denaturate secondary RNA structures, was found to lower the sensitivity of the present RT-PCR, and thus omitted. Overall, these findings indicate the importance of optimising each step in the RT-PCR protocol for improved performance.

The capability of cell cultures to support virus replication varies and is dependent on several factors regarding the cells and growth conditions, as well as possible host-produced interfering humoral responses. The present protocol using BF-2 cells has been optimised and standardised and is used in routine IPNV diagnostics at the NVI in Norway. In the repeated testing of kidney samples, two IPNV-positive samples were detected using CHSE-214 cells while none were detected using BF-

2 cells. Both the number of samples tested, and the difference found between the two cell lines, were too low to evaluate which cell line was best for the detection of IPNV. The present protocol was applied in an inter-laboratory comparison of the susceptibility of selected cell lines to fish pathogenic viruses in which eleven European national reference laboratories participated. One conclusion from that study was that both BF-2 and CHSE-214 were recommended for isolation of IPNV (Lorenzen et al. 1999). However, increasing the cell culture area from approximately 2 cm² as in the present study to 25 cm² (e.g. replacing the 24-wells plates with flasks), as well as increasing the number of passages, would probably increase the sensitivity of the cell culture isolation accordingly.

It can be concluded that the present RT-PCR was more sensitive than the standardised cell culture isolation for the detection of IPNV. Cortisol injection of covertly infected parr did not have any impact on the number of IPNV positive fish detected.

The authors thank Hilde Welde, Inger Austrheim, Inger Böckerman and Sigrun Hannevik for skilful technical assistance. This study was partly supported by the Norwegian Research Council (NFR).

References


