

# An RT-PCR-based method for the diagnosis of the sleeping disease virus in experimentally and naturally infected salmonids

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**ABSTRACT:** The sleeping disease (SD) of rainbow trout (*Oncorhynchus mykiss*) is a worldwide disease for which the causative agent, the sleeping disease virus (SDV), has been recently characterized as an atypical alphavirus (Villoing et al. 2000). Up to now, no diagnostic tools were available and thus no epidemiological studies have been undertaken to evaluate the occurrence of this disease on the field. We present in this paper a sensitive and highly specific 1 working day method, which allows the detection of SDV from experimentally and naturally infected fishes. This method, based on a reverse transcriptase/polymerase chain reaction (RT-PCR) assay on total RNA extracted from SDV-infected fish organs, enables the specific DNA amplification of part of the gene encoding the SDV glycoprotein E2, as early as 2 d post-infection (d.p.i.) and as late as 70 d.p.i., at which time clinical signs of infection are no longer apparent. Moreover, we show that this RT-PCR method can be successfully used for the diagnosis of fish infected by a closely related virus, namely salmon pancreas disease virus (SPDV). This report is the first description of a very powerful diagnostic assay which could provide a more accurate replacement for the classical virological, histological and immunochemistry methods.

**KEY WORDS:** Sleeping disease virus · Salmon pancreas disease virus · Alphavirus · Rainbow trout · Salmon · RT-PCR · Diagnosis

## INTRODUCTION

Sleeping disease (SD) syndrome of freshwater farmed rainbow trout has been observed in France for many years (Boucher & Baudin-Laurencin 1994). The most characteristic sign of the disease is the unusual behavior of fish, which stay on their side at the bottom of the tank. Through experimental SD transmission assay, the sequential histology of this disease has been studied (Boucher et al. 1995), and it was revealed that infected fishes successively developed pancreatic, cardiac, and muscular lesions. A viral etiology of SD was suspected (Boucher et al. 1994) and thereafter confirmed by the isolation of a virus, the sleeping disease

virus (SDV) by the inoculation of organ homogenates from diseased fish to cell cultures (Castric et al. 1997). cDNA cloning and nucleotide sequencing of part of the SDV RNA genome has permitted us to assign SDV to the alphavirus, an important genus of the *Togaviridae* (Villoing et al. 2000).

To date, SD is only diagnosed following observation of clinical signs of fishes thought to be infected, and by the implementation of various methods such as virological assay in cell culture or histological studies. A virological assay is time consuming and not always reliable, a major limitation being the fact that the virus must be adapted to grow in cell culture. Histological studies of SDV-infected fish tissues are unable to discriminate between fish infected by SDV from those infected by the infectious pancreatic necrosis virus (IPNV), a birnavirus which induces the same kind of

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lesions in juvenile rainbow trouts. To prevent future SD outbreaks and further spread of this disease in fish farms, an early and reliable viral detection method appears necessary, since to date, there are no effective therapeutics against SD.

The use of a reverse transcription and polymerase chain reaction (RT-PCR) assay for the sensitive detection of RNA viruses has been developed for a number of fish viruses such as IPNV (Lopez-Lastra et al. 1994, Blake et al. 1995, Suzuki et al. 1997), nodaviruses (Nishizawa et al. 1994, Péducasse et al. 1999) and fish rhabdoviruses (Bruchhof et al. 1995, Miller et al. 1998, Guilou et al. 1999). We have investigated the usefulness of an RT-PCR method for the diagnosis of experimentally or naturally SDV-infected rainbow trout and compared the results with those obtained in parallel with virological, histological and immunohistological techniques. We demonstrate in this report the superiority, in terms of sensitivity and specificity, of the RT-PCR method compared to the other methods tested. Furthermore, we have also assessed the usefulness of this method for the early detection of a closely SDV-related virus, namely the salmon pancreas disease virus (SPDV), in experimentally and naturally infected salmon.

## MATERIALS AND METHODS

**Cells and viruses.** Salmonid-derived cell line CHSE-214 (Lannan et al. 1984) was maintained in Eagle's minimum essential medium (MEM, Sigma FRANCE) buffered at pH 7.4 with Tris-HCl, supplemented with 10% fetal bovine serum (FBS, Boehringer-Mannheim, France), 50 I.U. penicillin ml<sup>-1</sup> and 50 mg ml<sup>-1</sup> of strep-

tomicin. Cells were maintained in 75 and 150 cm<sup>2</sup> plastic cell culture flasks at 20°C.

Viruses used for the experimental fish infections were respectively: SDV adapted to grow in CHSE-214 cell culture (strain S49P, 10<sup>6</sup> pfu ml<sup>-1</sup>), a 'wild-type' SDV preparation (an homogenized mixture of kidneys of farmed infected rainbow trouts, 10<sup>6</sup> pfu ml<sup>-1</sup>), SPDV adapted to CHSE-214 cell culture (strain P42P, 4.10<sup>8</sup> pfu ml<sup>-1</sup>). The P42P SPDV strain was originally isolated from kidneys of Atlantic salmon suffering of pancreas disease (kindly provided by Dr McVicar, Marine Laboratory of Aberdeen, UK).

**Fish and fish facilities.** Juvenile rainbow trout (0.5 g) used in this study were the progeny of virus-free genitors bred in our fish facilities. Fish were randomly placed in 50 l (volume of water: 30 l) aerated tanks, with opened circulation of filtered and UV-sterilised freshwater at a controlled temperature of 10 ± 1°C. Commercial food was manually distributed twice a day. Others fish samples used in this study originated from different French fish farms where SD outbreaks were suspected (Table 1).

**Experimental infections.** Experimental infections were conducted as follow: 600 virus-free juvenile rainbow trout were infected by static immersion with S49P SDV strain (final titer: 1 to 2.10<sup>4</sup> pfu ml<sup>-1</sup>) during 2 h at 10°C (±2°C) in an aquarium filled with 3 l of river freshwater. Fish were then transferred to an aquarium filled with 30 l of freshwater. As a control, 600 virus-free juvenile rainbow trout were mock-infected with cell culture medium in the same conditions. Similar experimental infections were also done using 'wild-type' SDV (final titer: 10<sup>4</sup> pfu ml<sup>-1</sup>) and P42P SPDV strain (final titer: 1 to 2.10<sup>4</sup> pfu ml<sup>-1</sup>).

Table 1. Fish samples used in study taken from fish farms where SD outbreaks were suspected. Average freshwater temperature in tanks at sampling time: 14 ± 2°C

Sample reference no.	Sample (no. of fish)	Sampling date	Body weight (g)	Symptoms and observations
9085	Rainbow trout (11)	14 April 1999	150–250	6 fish with SD-like symptoms (less appetite, swam or stayed on their side). 5 fishes were asymptomatic although they came from the same tank
9101	Rainbow trout (7)	19 May 1999	25	Some fish with SD-like symptoms
9106	Rainbow trout (3)	20 May 1999	700	Potential SD-like symptoms; lesions on the flank
9108	Rainbow trout (12, plus 5 fishes fixed in 10% formaldehyde)	26 May 1999	25	7 fish with SD-like symptoms. 5 asymptomatic fish, although they came from the same tank
9109	Rainbow trout (7, plus 5 fishes fixed in 10% formaldehyde)	26 May 1999	25	Fish stayed on their side at the bottom of the tank. Low mortality observed
9115	Rainbow trout (6)	31 May 1999	50	Fish had less appetite and growth stop for 3 wk, but they returned to a healthy state
9127	<i>Salmo salar</i> (4)	9 June 1999	50	Less appetite could be observed, and these fish usually swam at the bottom of the tank

**Sampling and analysing protocol.** For each assay, 30 infected and 6 mock-infected fish were randomly collected at various days post infection (d.p.i.) (from 2 to 41). For wild type SDV strain, fish were also collected at 70 d.p.i. At each sampling time, 10 infected and 2 mock-infected fish were analysed by the RT-PCR-based SDV detection method, 10 infected and 2 mock-infected fish were analysed by virological titering, and the remaining 10 infected and 2 mock-infected fish were analysed by histopathology and immunohistochemistry (IHC).

**Preparation of total RNA from fish.** Total RNA from fish was extracted using the RNA Now™ (Biogentex, USA) procedure. Briefly, fish were crushed in a Dounce homogenizer in a 2 ml tube containing 1 ml of RNA Now lysis buffer. Chloroform (0.2 ml) was added, and the obtained mixture was left on ice for 5 min. It was then centrifuged at 14 000 rpm (20 000 × *g*) for 10 min. The aqueous phase containing RNA was transferred to a new tube, and an equal volume of isopropanol was added. After 1 h at -20°C, the tubes were centrifuged at 14 000 rpm (20 000 × *g*) for 10 min. The RNA pellets were washed with ice-cold 70% ethanol for 5 min, and the pellets were dried 5 min in a concentrator (Speedvac, Savant). Dried RNA pellets were then resuspended in 700 µl DEPC-treated water. When RNA was extracted from individual fish organs (caeca, kidney, brains, heart), the RNA pellets were resuspended in 300 µl.

**Total RNA preparation from SDV or SPDV-infected cells.** SDV was grown in 10 flasks (150 cm<sup>2</sup>) containing RTG-2 cells for 10 d at 10°C. Flasks were placed at -80°C for 3 h, then a pool of scraped cells supernatant was centrifuged at 35 000 rpm (150 000 × *g*) for 90 min. Pellets were resuspended in 500 µl of Eagle-Tris medium and RNA was extracted using the QIAAMP viral RNA extraction kit (Qiagen, France), and the RNA was recovered in 50 µl of DEPC-treated water. The same procedure was used to recover SPDV RNA, except that SPDV was grown on CHSE-214 cells at 14°C.

**RT-PCR.** cDNA synthesis and DNA amplification were done using primers derived from the published SDV nucleotide sequence (Villoing et al. 2000, accession number AJ238578). Two microliters of extracted RNAs were added to 18 µl of a reverse transcription (RT) mixture containing: 10 mM Tris HCl, pH 8.3, 50 mM KCl, 5 mM MgCl<sub>2</sub>, 1 mM dNTP and 20 pmol of the primer SDV3' (position 2016-2044, 5'-GGATCCATTCAGATGTGGCGTTGCTATGG-3') (Genset, France). RNA was denatured for 5 min at 99°C and 50 units of MuLV reverse transcriptase (Perkin Elmer) were added. The reaction was incubated for 30 min at 42°C; then MuLV was denatured at 95°C for 10 min. To the RT reaction tube, 80 µl of a PCR mixture containing 10 mM Tris HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, and 10 pmol of the forward primer SDV5' (position

1740-1767, 5'-CGTGACCTTCACCAGCGACTCCCA-GACG-3') (Genset, France) were added, and the mixture was heated for 2 min at 94°C and 2.5 units Taq DNA polymerase (Promega) were added (hot-start). PCR was performed with the following conditions: one step at 94°C for 2 min, then 5 cycles at 94°C for 40 s, 57°C for 30 s and 72°C for 30 s, then 35 cycles at 94°C for 40 s, 65°C for 30 s and 72°C for 30 s, then a final extension step at 72°C for 7 min. PCR products (15 µl) were analysed by electrophoresis on a 1.5% agarose gel.

**PCR products sequencing.** PCR products were purified using the high pure PCR product purification kit (Boehringer-Mannheim, France) according to the manufacturer's recommendations. Nucleotide sequencing reactions of RT-PCR products were carried out on a ABI 377A DNA automatic sequencer using the ABI Prism dye deoxy terminator prism kit (Applied Biosystem, Division of Perkin-Elmer, France).

**Virological examination.** Fish or organs were homogenized in a mortar with a pestle and sea sand in 9 volumes of Eagle's solution containing penicillin (200 I.U. ml<sup>-1</sup>), streptomycin (0.2 mg ml<sup>-1</sup>) and kanamycin (0.2 mg ml<sup>-1</sup>). After centrifugation at 2000 × *g* for 15 min at 4°C, the supernatant was passed through a membrane filter (0.45 µm) and was inoculated on CHSE-214 cells. Inoculated cells were incubated at 10°C (SDV) or 14°C (SPDV) for 7 d; then plaques were directly counted when the SDV S49P strain or P42P SPDV were used. For the wild type SDV, a reverse plaque titration assay was performed: serial SDV dilutions in MEM (from 10<sup>2</sup> to 10<sup>12</sup>) were prepared and inoculated in duplicate on CHSE-214 cells in 96-well plates. After a 7 d incubation at 14°C, infected cells were over-infected with the viral hemorrhagic septicemia virus, VHSV, (500 pfu well<sup>-1</sup>). After 3 d, cells were fixed with formalin and stained with violet crystal.

**Histological assessment and immunohistochemistry assays.** Fish were killed and fixed in Bouin's fixative, dehydrated through graded ethanol xylene baths and embedded in paraffin (Gabe 1968). Sections (containing pancreas, heart, muscles and brains) of 5 to 6 µm were first deparaffinised, rehydrated and then stained with haematoxylin-eosin (HE) for histopathological examination. For the immunohistochemistry assays, the slides were first incubated for 5 min in hydrogen peroxide (3% in distilled water) to block the endogenous peroxidase activity. Following washes in PBS, the slides were saturated in PBSA (3% BSA in PBS) and incubated for 1 h with a 1:100 dilution of a rabbit polyclonal anti-E2 SDV antiserum (Villoing et al. 2000). Following 2 washes in PBS-Tween (1% Tween 20 in PBS) and 1 wash in PBS, slides were incubated for 30 min with an anti-rabbit IgG goat antibody conjugated with peroxidase (dilution 1:100). After washes, the peroxidase substrate solution (AEC chromogen,

Sigma, France) was added for 10 min. Following washes in distilled water, samples were counterstained with haematoxylin and mounted in an aqueous mounting medium (Daido Sangyo Co. Ltd., Japan).

## RESULTS

### Assessment of three diagnosis methods on experimentally infected rainbow trout

Three different viral strains were used to infect juvenile rainbow trouts, respectively: a tissue-culture adapted viral strain of SDV (S49P, Castric et al. 1997), a SDV wild type strain, and a tissue-culture adapted viral strain of SPDV (P42P, Castric unpubl.). From 2 to 41 d.p.i., sampled fishes were sacrificed and the detection of virus was determined through (1) virological assay, (2) histological analysis and IHC, and (3) RT-PCR.

#### Virological analysis

As shown in the Fig. 1, virological assays detected infected fish from 2 to 41 d.p.i., with some variations depending on the viral strain used for the infection trials. The maximum viral titers in fish were obtained at 21 d.p.i. ( $10^{9.3}$  pfu  $g^{-1}$  organ) and the cumulative rate of positive fish was 75%. For the wild type SDV strain, the maximum viral titer was  $10^{6.5}$  pfu  $g^{-1}$  organ at 9 d.p.i., and the cumulative rate of positive fish was 77%. For the SPDV strain, titer reached a maximum of  $10^{8.4}$  pfu  $g^{-1}$  organ at 14 d.p.i., and the cumulative rate of positive fish was 70%.

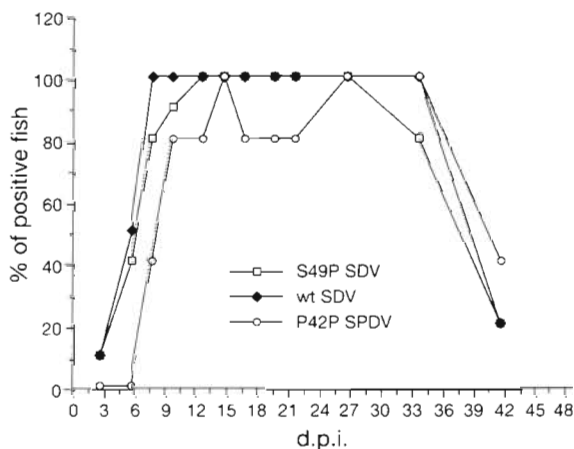


Fig. 1. Detection of SDV and SPDV in experimentally infected fish by a virological assay. Fish samples were taken and analysed at various days post-infection (d.p.i.) by the virological assay as described in 'Materials and methods'. Virus strains used for the infection trials are indicated in the upper right part of the graph. wt: wild type

Virological assay was also used to detect separately virus extracted from fish brain and organs. A typical result obtained with fish infected with the wild type SDV is shown in Fig. 2. From internal organs (body), SDV was recovered in 1 out of 10 fish at 2 d.p.i., and then 50 to 100% of the fish were positive from 5 to 21 d.p.i. From 26 to 41 d.p.i., no more virus was recovered from the body. Interestingly, when the brain (head) was used as starting material, the earliest time of detection of SDV was at 5 d.p.i. in 1 out of 10 fish, then culminated until 31 d.p.i., and was detected up to 41 d.p.i. in 2 fishes. Maximum viral titer was observed in brains at 9 d.p.i. ( $10^{6.5}$  pfu  $g^{-1}$  organ). At 70 d.p.i., no virus was detected any longer using the virological method, either from brains or internal organs (data not shown). The overall detection rate with fish infected by the wild type SDV was 77 and 63% from brains and internal organs, respectively.

When a similar experiment was conducted with SPDV-infected fish, the data were superimposable on those obtained with SDV-infected fish (not shown).

Virological assay was also conducted with 2 fish farm samples: one sample consisted of farmed rainbow trout (sample reference 9085) for which internal organs had been analysed, but no virus found. The other samples analysed were farmed Atlantic salmon (sample reference 9127), and virus was found in the internal organs in only 1 out of the 4 salmon analysed (viral titer  $10^3$  pfu  $g^{-1}$  organ), but the nature of this virus (SDV or SPDV) could not be determined by the virological procedure.

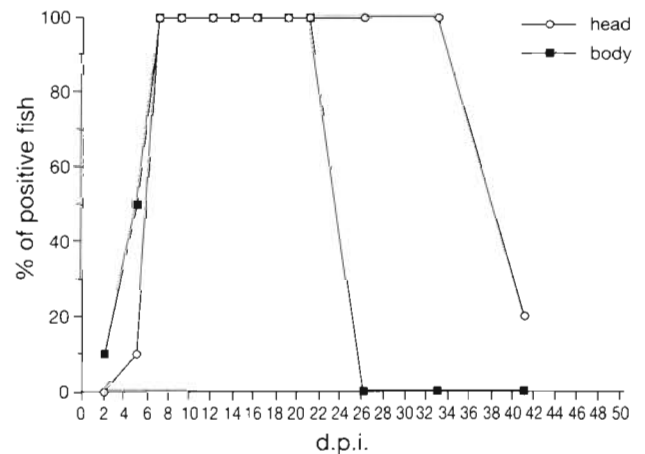


Fig. 2. Virological assay on brain and internal organs from the wild type (wt) SDV-infected rainbow trout. Rainbow trout were infected with wt SDV strain and samples were taken and analysed at various days post-infection (d.p.i.) by the virological assay as described in 'Materials and methods'. Cell culture were inoculated with homogenates of either brains (head) or internal organs (body) as indicated in the upper right part of the graph

### Histopathological and IHC assays

Through histopathological and IHC studies, the earliest time of noticeable changes in infected fish was at 7 d.p.i., when the S49P and the wild type SDV strain as well were used (Fig. 3) and only at 13 d.p.i. for the SPDV. Striking histopathological changes were observed in some fish until 41 d.p.i. for each virus strain. Characteristic SD lesions in the pancreas, which is the primary organ damaged in the histological course of SD, and fish with an IHC-positive pancreas could be seen (Fig. 4) from the earliest stage of infection and afterwards. The IHC method gave a detection rate of 35% with the S49P SDV strain, but was much lower with the wild type SDV and SPDV strains, 3 and 10% respectively. However, IHC-positive fish were not observed any more after 21 d.p.i. with any of the viral strains, reflecting probably the regeneration of the pancreas by the inflammatory cells and the remaining unharmed pancreatic tissue (Murphy et al. 1992). Histological and IHC methods have been carried out on the brains of wild type SDV-infected fish, but no positive fish was detected.

Histopathological and IHC studies have been also done on 2 fish farm samples (reference nos. 9108 and 9109), but failed to detect any lesions on the fish tested.

### RT-PCR assays

Fig. 5 presents the data obtained for virus detection in experimentally infected fish when a RT-PCR method was used. The earliest time of virus detection in fish

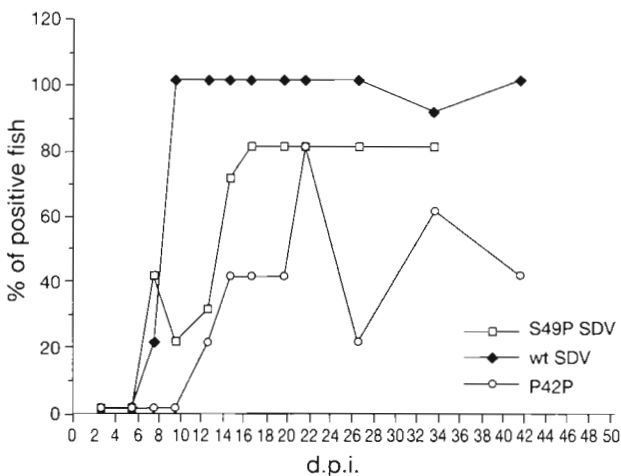


Fig. 3. Analysis of SDV and SPDV-infected fish. Fish samples were taken and analysed at various days post-infection (d.p.i.), by the histopathological and IHC assays as described in 'Materials and methods'. Virus strains used for the infection trials are indicated in the upper right part of the graph. wt: wild type

experimentally infected with the S49P strain of SDV was surprisingly late, 7 d.p.i., when compared to the others methods described above. However, when the wild type SDV strain is used, the virus was detected as soon as 2 d.p.i. on 30% of the infected fish. At longer time post-infections, all the fish were positive independently of the virus strain used, until the last time of sampling (41 d.p.i.). When the wild type SDV strain was used, an additional sampling at 70 d.p.i. was done, and virus was still detected, in contrast to the lack of virus detection when others methods were used (data not shown). Fig. 6 shows a typical result of an RT-PCR assay at 16 and 19 d.p.i. where the expected 284 nucleotides (nt) amplification product is present when RNA was extracted from fish infected with the S49P SDV strain.

RT-PCR-based detection method was also tested on experimentally SPDV-infected rainbow trout. As a preliminary step, RT-PCR was performed with total RNA extracted from SDV or SPDV-infected cells, and the nucleotide sequences of the PCR products were determined. The alignment of the SDV and SPDV PCR products nucleotide sequences emphasized the very high percentage of nucleotide identity between SDV and SPDV in this region (Fig. 7), thus validating the use of the RT-PCR-based method for the detection of SDV and SPDV, as well. RT-PCR carried out on RNA extracted from experimentally SPDV-infected rainbow trout gave positive results from 5 to 41 d.p.i., with an overall positive detection rate of 86% (data not shown).

The RT-PCR-based detection method was also tested on fish samples originating from 7 fish farms suspected of SDV or SPDV infections. RT-PCR was performed on RNA extracted from various fish organs (pylor caeca, kidney, heart and brain) and the more reliable results were obtained with RNA extracted from caeca or kidneys (Fig. 8). All the farmed rainbow trout and Atlantic salmon analysed were positive following RT-PCR assays. Analysis of the nucleotide sequences of the 284 nt long RT-PCR products indicated that all the positive fishes, rainbow trout and Atlantic salmon, had been infected by SDV.

### DISCUSSION

The aim of this study was to determine which technique, among virological, histology/IHC and RT-PCR techniques, would be the most appropriate for routine diagnosis of SDV or SPDV infections. To evaluate the accuracy of each technique, experimental infections were carried out with different viral strains on juvenile fish, given that they are generally more sensitive to infections with SDV or SPDV.

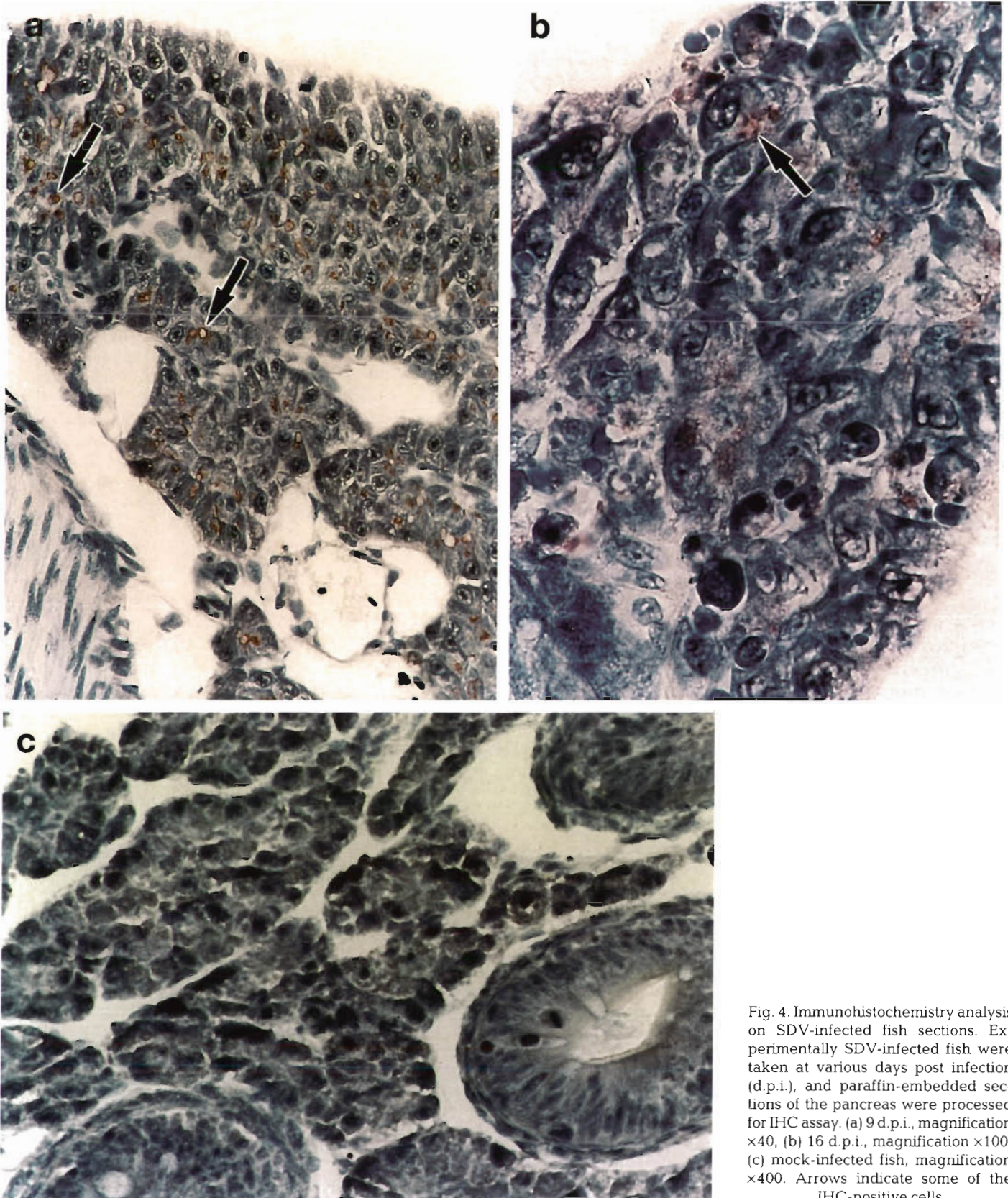


Fig. 4. Immunohistochemistry analysis on SDV-infected fish sections. Experimentally SDV-infected fish were taken at various days post infection (d.p.i.), and paraffin-embedded sections of the pancreas were processed for IHC assay. (a) 9 d.p.i., magnification  $\times 40$ , (b) 16 d.p.i., magnification  $\times 100$ , (c) mock-infected fish, magnification  $\times 400$ . Arrows indicate some of the IHC-positive cells

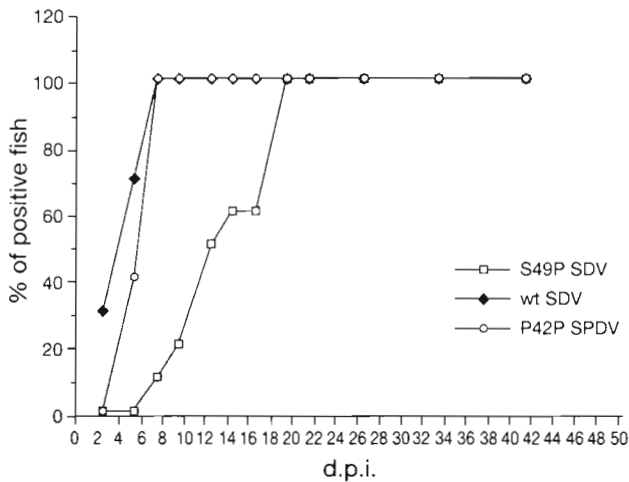


Fig. 5. Detection of SDV and SPDV in experimentally infected fish by a RT-PCR assay. Fish samples were taken and analysed at various days post-infection (d.p.i.) by the RT-PCR assay as described in 'Materials and methods'. Virus strains used for the infection trials are indicated in the upper right part of the graph. wt: wild type

Depending on the virus strain used in this study, data obtained were variable in terms of efficiency of virus detection in infected fish. For example, the virological procedure was more efficient with the cell culture-adapted S49P SDV strain, enabling very early SDV

detection, as soon as 2 d.p.i. However, for the wild type SDV strain, which is probably more closely related to the viral strains present in the field, the best results were obtained by the RT-PCR method, which provided the earliest time of detection, and, in most cases enabled the latest detection of the virus up to 70 d.p.i. (data not shown), in contrast to the 2 other methods which did not detect virus at that time. The presence in infected fish of circulating viral neutralizing antibodies, which induce a drastic loss of viral infectivity in cell culture, may explain the absence of positive detection by the virological or histological and IHC methods at 70 d.p.i. In addition, it has previously been observed in SPDV-infected Atlantic salmon that kidney and plasma become non-infectious when peak pathology occurs (Houghton et al. 1995). Thus, a similar phenomenon may also occur following SDV infections that would explain the sudden decrease in the positive fish detection rate after 33 d.p.i., and also the decrease in viral titers (data not shown). That observation, and the finding that no virus can be detected in fish infected for more than 1 mo, emphasize the disadvantage of the virological method as a tool for routine diagnosis. Similarly, at 70 d.p.i., no detection of wild type SDV-infected fish was observed either by the histological or IHC methods, which would seem to imply that it may not be possible to use these methods on a routine basis. Moreover, it is noteworthy that a very low detection

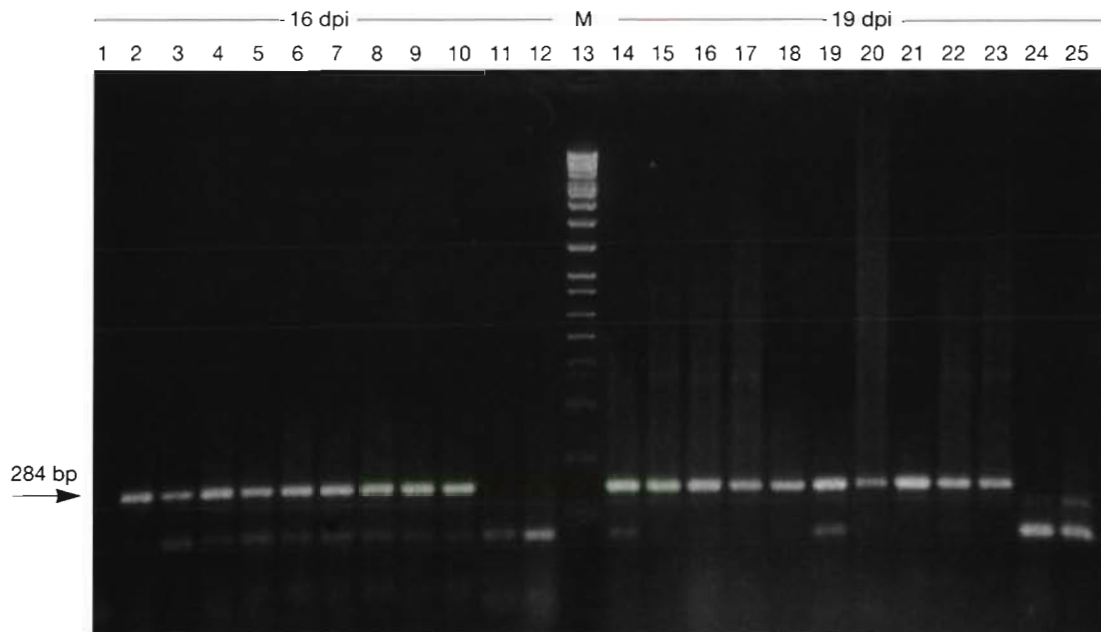


Fig. 6. Detection of SDV by RT-PCR in wild type (wt) SDV-infected fish. Total RNA was extracted from wt SDV-infected fish at 16 or 19 d.p.i. RT-PCR assay was conducted as described in 'Materials and methods'. PCR products were analysed on a 1% agarose gel and visualized by ethidium bromid staining. Arrow indicates the expected 284 nt PCR product. Lanes 1 to 10 and 14 to 23: infected fish samples at 16 and 19 d.p.i. respectively. Lanes 11 & 12 and 24 & 25: mock-infected fish samples at 16 and 19 d.p.i. respectively. Lane 13, M: DNA ladder (Raoul marker, Appligene)

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1                               50
SDV CCGTCACCTT CACCAGCGAC TCCAGACGTT TTACGTGCGA GGAGCCGGTC
SPDV .....T.....T.....T.....A....

51                               100
SDV CTAACGGCCG CCAGCATCAC CCAGGGCAAG CCGCACCTCA GATCGTCAAT
SPDV .....T.....T.....T.....A.....G....

101                              150
SDV GTTGCCAGC GGAGGCAAAG AGGTGAAAGC GAGGATTCCA TTCCCGTTCC
SPDV .....T.....G.....A.....A.....C.....G.....

151                              200
SDV CGCCAGAGAC TGCGACTTGC AGAGTGAGCA TCGCCCACT GCCATCGATT
SPDV ....G..A..C..A.....TG..A.....G.....C

201                              250
SDV ACCTATGAGG AAAGCGATGT TCTGCTGGCC GGCCTGCGA AATACCCCGT
SPDV .....C.....C.....A.....T.....C.....T..

251                              284
SDV GCTGCTAACT ACACGGAACC TTGGTTTCCA TAGC
SPDV .....C.....T.....

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Fig. 7. Comparison of part of the SDV and SPDV E2 gene sequence. The nucleotide sequences of the 284 nt PCR products generated by RT-PCR from total RNA extracted from SDV (upper) and (lower) SPDV-infected cells were compared. Dots indicate identical nucleotides

rate was observed in fish infected with the wild type SDV and the SPDV strain, 3 and 10% respectively, when the IHC method was used. One probable explanation for this is that the anti-SDV E2 polyclonal antiserum used for immunological detection was raised by immunizing a rabbit with part of the SDV E2 glycoprotein expressed in *E. coli*, and thus this antiserum may not be appropriate to recognize the native SDV (or SPDV) E2 glycoprotein. In the future, the availability of antiserum directed against other SDV structural proteins, such as the capsid protein, may help to increase the detection rate of infected fish by the IHC method.

The ability to detect virus in symptomatic or asymptomatic fish is a crucial criterion for the choice of a

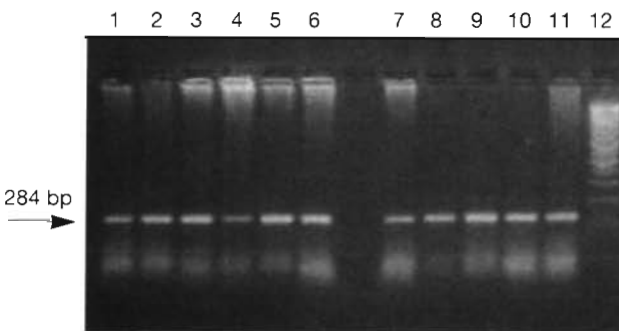


Fig. 8. Detection of SDV by RT-PCR on total RNA from naturally infected fish. RNA was extracted from pyloric caeca from adult rainbow trout originating from a fish farm (sample ref. 9085). RT-PCR assay was conducted as described in 'Materials and methods'. PCR products were analysed on a 1% agarose gel and visualized by ethidium bromid staining. Arrow indicates the expected 284 nt PCR product. Lanes 1 to 6: fish showing SD-like symptoms. Lanes 7 to 11, asymptomatic fish. Lane 12: DNA ladder (Raoul marker, Appligene)

reliable routine diagnostic method. In this respect, the RT-PCR is certainly the most appropriate method, since the late detection of virus in experimentally or naturally infected rainbow trout was only successful using the RT-PCR method.

The neurotropism of the mamalian alphaviruses has been well documented (for a review see Strauss & Strauss 1994, Griffin 1998). Interestingly, in fish infected by the wild type SDV strain, when the virological method was used to detect the virus in either the fish body or the brain, SDV was recovered from the brain at a longer time post-infection than in the body (26 to 41 d.p.i., Fig. 2), and furthermore until 70 d.p.i. using the RT-PCR method (data not shown). Whether or not these results reflect a possible neurotropism of SDV associated with viral persistence remains to be determined.

SPDV isolated from Atlantic salmon (Nelson et al. 1995) induces in fish lesions very similar to those induced by SDV. The immune cross-protection between SPDV and SDV in infected rainbow trout has been demonstrated (Boucher et al. 1996), suggesting a very close relationship between SDV and SPDV. This has been recently confirmed by nucleotide sequencing of their respective sub-genomic RNAs (Weston et al. 1999, Villoing et al. 2000). The results obtained in the present study from the SPDV infection trials show that the RT-PCR method is also suitable for the diagnosis of SPDV and SDV as well.

The superiority of the RT-PCR method, compared to the other methods, for the routine diagnosis of SDV or SPDV in the field has been demonstrated since positive results (presence of SDV) were obtained in all the fish farm samples tested, whereas the virological and histological/IHC methods failed to detect the virus. Moreover, lesions induced by a birnavirus, IPNV, and SDV/SPDV are also very similar, and both virus (IPNV and SDV/SPDV) seem able to co-infect fish (unpubl. result). Thus, in the future, a further step to optimize the RT-PCR-based diagnosis method will be to define multiplex IPNV/SDV RT-PCR conditions which will provide an appropriate and not yet available differential tool for diagnosing pancreatic diseases in salmonids (McLoughlin 1997), similar to those which have been it has been successfully designed for other viruses (Wirz et al. 1993).

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