Bull. Eur. Ass. Fish Pathol., 28(4) 2008, 148

First isolation and identification of Sleeping Disease Virus (SDV) in Germany

S.M. Bergmann[1*], D. Fichtner[1], R. Riebe[1] and J. Castric[2]

[1]Friedrich-Loeffler-Institut (FLI), Institute of Infectology, Federal Research Institute for Animal Health, Südufer 10, 17493 Greifswald-Insel Riems, Germany, [2]AFSSA Brest, BP 70, 29280 Plouzané, France.

Abstract

Sleeping disease (*SD*) is widespread in Northern Europe. The SD virus (*SDV*) affects fresh water reared rainbow trout (*Oncorhynchus mykiss*) and induces the typical listless or "sleepy" behaviour, where fish tend to aggregate at the lower parts of the pond. In Germany, SDV was isolated from diseased rainbow trout fingerlings in RTG-2 and CHSE-214 fish cell lines at 15°C. Genome fragments of the German SDV isolates G1 and G 2 and the reference viruses S49P (French SDV) and P42P (Scottish SPDV) were first identified by reverse transcription polymerase chain reaction (RT-PCR) as a 303 bp instead of a 284 bp fragment and also by indirect immunofluorescence assay (iIFA) after transfection of SDV RNA by electroporation to avoid contamination with *Infectious Pancreas Necrosis Virus* (IPNV) which is wide spread in German rainbow trout farms.

Introduction

A syndrome in salmonids named *Sleeping Disease* (SD) has been observed in farmed rainbow trout (*Oncorhynchus mykiss*) in France, especially in Brittany, for several years (Boucher and Baudin Laurencin, 1994, Boucher et al., 1994). More recently SD has also been observed in Italy (Bovo pers. comm.) and the UK (Branson, 2002, Graham et al., 2003). Affected trout display unusual behaviour including resting on their side on the bottom of the pond. The viral nature of this disease agent was recognised by Boucher and Baudin Laurencin (1994) and SD virus (*SDV*) was characterised as an *Alphavirus* within the family *Togaviridae* (Villoing et al., 2000). SDV is closely related to SPDV, the virus responsible for *Salmon Pancreas Disease* (SPD) (Weston et al., 2002, Desvignes et al., 2002), a disease of sea-reared salmon (*Salmo salar*). SPD was first described by Munro et al. (1984) in Scotland and produces pathological changes in the pancreas, heart and skeletal muscles of affected salmon (Nelson et al., 1995). The disease has also been reported in Ireland (Murphy et al., 1992), Norway (Poppe et al., 1989), USA (Kent and Elston, 1987), France and Spain (Raynard et al., 1992). Both viruses, SDV and SPDV are classified as atypical a*lphaviruses* (Weston et al., 1999; Villoing et al., 2000; Weston et al., 2002) with similarity of 91.1 % over their complete genome. When compared to the non-structural proteins of the other, typical a*lphaviruses*, the identity is 41.7 to 43.6 % only (Villoing et al., 2000). SDV and SPDV exhibit 95 % amino acid identity over their non-structural proteins and induce cross-protection in fish (Boucher and Baudin Laurencin, 1996). This paper describes the first isolation and identification of SDV in Germany.

* Corresponding author's E-mail: sven.bergmann@fli.bund.de

Materials and methods

Cells and viruses

Chinook salmon embryo (CHSE 214) cell line no. 341 (*Collection of Cell Lines in Veterinary Medicine* (*CCLV*), Insel Riems, Germany) and rainbow trout gonad (RTG-2) cell line no. 88 (CCLV) were used for isolation and further propagation of the viruses.

CHSE 214 was propagated in Eagle's MEM containing Hanks BSS and Eagle's MEM with Earles BSS 1:1 and RTG-2 was cultured in Eagle's MEM with Hanks BSS buffered with 850 mg $NaHCO_3$/l, including 10% foetal calf serum without any antibiotics. CCLV lines 173 (Epithelioma Papillosum Cyprinii, EPC), 290 (Bluegill fry, BF-2) and 57 (Fathead Minnow, FHM) were grown under the same conditions as RTG-2.

Cells were cultivated at 26°C in 12.5 cm^2 or 25 cm^2 cell culture flasks (Falcon, Nunc). After 24 hours, when a complete monolayer had formed, cells were infected by reference virus or pooled organ extracts directly into the medium and incubated for five to seven days at 15°C, then frozen at –20°C. For this study, reference virus strains S49P (French SDV isolate), P42P (Scottish SPDV isolate) and isolates G1 and G2 were propagated in CHSE 214 and RTG-2 at 15°C (Table 1).

Preparation of the tissue samples

Frozen organs (a pool of 1 g tissue from heart, kidney, spleen and muscle) from a single rainbow trout (Table 2) were homogenised with a pestle, mortar and sterile sand in 10 ml of cell culture medium (dilution 1:10) that contained 0.5 ml of Baytril 10% suspension (Bayer, Germany), incubated over night at 4°C and clarified by centrifugation (2000 g, 20 min, 4°C). Resulting supernatants were diluted 1:10 and 1:100 (w/v) with culture medium (EMEM + HBSS supplemented with 10 % foetal bovine serum) and inoculated directly onto CHSE-214 and RTG-2 cell monolayers in 12.5 cm^2 or 25 cm^2 cell culture flasks (Falcon, Germany ; Nunc, Denmark). After 7 to 10 days incubation at 15°C monolayers showing no cytopathic effect (cpe) were sub-cultured onto fresh cells and incubated for a further 7 to 10 days. Virus isolates were harvested, identified and stored at –20°C.

RNA extraction and purification of the viruses by electroporation

Total RNA from organ pools (a pool of approximately 100 mg tissue of heart, spleen, kidney, muscle) of rainbow trout suspected of infection with SDV was extracted by Trizol®reagent (Invitrogen, Germany) according to manufacturer's instructions with some modifications. Frozen organ pools were

Virus isolate	Origin	Reference
SDV S49P	France, rainbow trout	Castric et al., 1997
SPDV P42P	Scotland, salmon	Lopez-Doriga et al., 2001
SDV G 1 (DF 11/03)	Germany, rainbow trout	This paper
SDV G 2 (DF 18/03)	Germany, rainbow trout	This paper

Table 1. Virus isolates.

homogenised using a hand mixer (Roth, Germany) and mixed with 0.5 ml cell growth medium containing 0.5 µl of 10 % Baytril (Bayer, Germany). After four-hour incubation time at 4°C, the solution was centrifuged at 4000 rpm for 20 min at 4°C (Hettich, Germany). The pellet was dissolved in 200 µl of the cell free supernatant and 1 ml Trizol®Reagent was added. The preparation was gently shaken for 5 min in a thermomixer (Eppendorf, Germany) at room temperature. When most of the debris had dissolved, 200 µl chloroform were added and the tube was roughly shaken for half a minute. After centrifugation (10.000 rpm, 15 min, 4°C), the clear supernatant was transferred into a new Eppendorf tube contained 0.5 ml isopropanol for RNA precipitation. The resulting pellet was washed twice in 75 % ethanol and pelleted by centrifugation (8500 rpm, 5 min, 4°C). The pellet was allowed to dry for 10 min at room temperature after complete removal of the supernatant. The RNA was dissolved in 50 µl RNase free water and the content was measured at 260 nm in a spectrometer (Beckmann, Germany).

For extraction of RNA from S49P infected and non-infected cells, the virus was propagated in CHSE 214 cells for seven days in 12.5 cm^2 flasks (Nunc, Denmark), frozen at –20°C, thawed on ice, centrifuged at 1200 g for 20 min at 4°C. The cell pellet was treated as described above.

Extracted total RNA (1 – 10 µg, respectively) was used for RNA electroporation into CHSE 214. Cells from a 125 cm^2 flask (Greiner, Germany) were trypsinised, washed twice in sterile phosphate buffered saline (PBS^-) and resuspended in 1.2 ml PBS^-. Aliquots of 200µl were prepared into which were added the RNA from infected and non-infected cells as well as from organ samples of infected fish and the cells were pulsed at 250 to 325 V in an Easyjec™ electroporation system (Eurogentech, Belgium). The cells were then cultivated in six-well cell culture plates (Greiner, Germany) at 15°C. The plates were observed daily for cpe. Any virus isolated was identified by immunofluorescence assay (IFA) and reverse transcription polymerase chain reaction (RT-PCR).

RT-PCR for detection of SDV and IPNV

One step RT-PCR kit (Qiagen Germany) was used to perform RT-PCR according to Villoing et al. (2000) for detection of SDV/SPDV and for detection of IPNV according to Taksdal et al. (2001). A 306 bp fragment for SDV/SPDV strains and a 223 bp fragment for IPNV were expected.

Indirect Immunofluorescence assay (iIFA)

CHSE 214 cells were cultivated in 96 well plates (Greiner, Germany) for 24 hours at 26°C. After 24 h, the confluent monolayer was infected with 10-fold dilutions of the viruses. Five days later, medium was removed, cells were air-dried and then fixed with an ice-cold methanol-acetone-mixture (ratio 80 : 20) for 10 min at – 20°C and the plates stored at –20°C until used.

For iIFA, the plates were incubated with antiserum against SDV (rabbit anti-SDV no. 7, dilution 1:500, AFSSA, France) in PBS^- for 1 h at room temperature. The plates were then washed three times with PBS^- and incubated with anti-rabbit-IgG-FITC conjugate (Sigma, Germany) according to manufacturer's instructions. To identify contaminating IPNV,

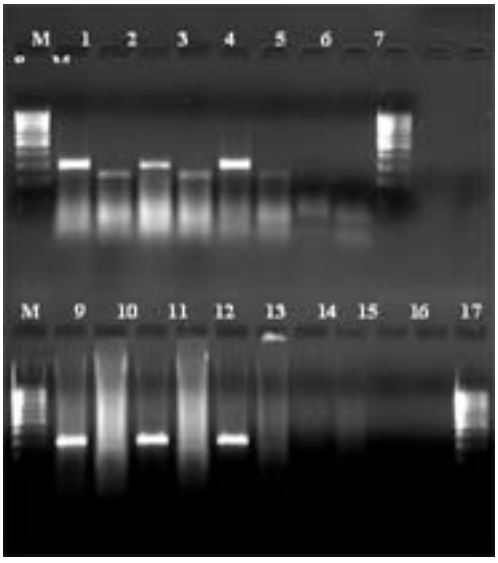

Figure 1. RT-PCR from tissue materials of the isolates G 1 and G 2.

M 100 bp marker (peqlab); **upper part G1:** lane 1 sample 107/1 SDV primers, lane 2 sample 107/1 IPNV primers, lane 3 sample 107/2 SDV primers, lane 4 sample 107/2 IPNV primers, lane 5 sample 107/3 SDV primers, lane 6 sample 107/3 IPNV primers, lane 7 negative preparation control SDV primers, lane 8 negative preparation control IPNV primers; **lower part G 2:** lane 9 sample 134/1 SDV primers, lane 10 sample 134/1 IPNV primers, lane 11 sample 134/2 SDV primers, lane 12 sample 134/2 IPNV primers, lane 13 sample 134/3 SDV primers, lane 14 sample 134/3 IPNV primers, lane 15 sample 135 SDV primers, lane 16 sample 135 IPNV primers, lane 17 negative preparation control SDV primers, lane 18 negative preparation control IPNV primers.

monoclonal antibodies against IPNV (Bio X, Belgium) were used. Fluorescence was visualised by microscope (Olympus, Germany).

Histology

After fixation in neutral buffered formalin (10%) for at least 24 hours, muscle tissue was dehydrated and paraffin embedded. Sections were cut (5 –10 µm) and stained with haematoxylin and eosin. The occurrence and the absence of degenerative changes in muscle tissue were assessed only.

Results

Virus isolation in cell cultures

Homogenised tissue pools (G 1 three pooled tissue samples 107/1-3, G 2 four pooled tissue samples 134/1-3, 135) were inoculated onto CHSE 214 and RTG-2 cell monolayers. After 3 days incubation at 15°C, cpe occurred in CHSE 214 and RTG-2 but also in EPC, FHM and BF-2. The virus was identified as IPNV by iIFA and by RT-PCR (Figure 1). An absorption of the organ material with a-IPNV serum from rabbit (rabbit a -Spjarup 1:50, incubation over night at 4°C) did not neutralize the IPNV completely. After three passages in CHSE 214 or RTG-2 an IPNV induced cpe occurred again. IPNV was identified by IFA and RT-PCR (Figure 2). The German SDV isolates G1 and G2 did not grow in EPC, FHM or BF-2 cells at 15, 20 or 26°C but with the reference viruses S49P and P42P a limited cpe was observed in EPC and BF-2 cells at 15°C.

RNA transfection by electroporation

After transfection of the purified RNA from organ tissue pools by electroporation into CHSE 214, a small discrete cpe occurred after five to seven days. Infected cells appeared round or irregular in morphology and became vacuolated but no cytolysis was detected. Following subculture in CHSE 214 and RTG-2 cells at 15°C CPE was observed regularly after five to ten days. For transfection by electroporation, 1 – 20 µg of total RNA was used. One, 5 and 10 µg RNA from a P42P infected CHSE 214 served as positive transfection control. Whereas 1 µg appeared

Isolate	Sample	Detection by RT-PCR				In CHSE-214 cells			
		SDV		IPNV		SDV		IPNV	
		Organ	Transf.*	Organ	Transf.	Organ	Transf.	Organ	Transf.
G 1	107/1	+	+	+	-	-	+	+	-
	107/2	+	+	+	-	-	+	+	-
	107/3	+	+	+	-	-	+	+	-
G 2	134/1	+	+	+	-	-	+	+	-
	134/2	+	+	+	-	-	+	+	-
	134/3	+	+	+	-	-	+	+	-
	135	-	-	-	-	-	-	-	-

* Post transfection by electroporation.

Table 2. Detection of SDV and IPNV in samples of the isolates G 1 and G 2.

not to be sufficient RNA, the use of 5 and 10 µg RNA was an adequate amount of nucleic acid to show the expected fragments in the gel. For transfection of CHSE 214 cells by RNA from tissues, 5 to 20 µg RNA was used and 10 µg was a sufficient amount of RNA.

Virus identification by RT-PCR

Original organ material, infected and non-infected cells as well as transfected cells were analysed by RT-PCR to identify and discriminate the viruses. In total, 2 to 5 µg RNA emerged as a necessary RNA amount. The extracted RNA's from the German SDV isolates G 1 and G 2 or the RNA's obtained from the reference viruses were positive in the RT-PCR using SDV primers but not with IPNV primers (Table 2). While IPNV is detectable in original organ tissue and in the third cell culture passage after neutralization with a-IPNV serum by RT-PCR, it disappeared completely after transfection directly from the organs as well as from cell culture extracted viral RNA.

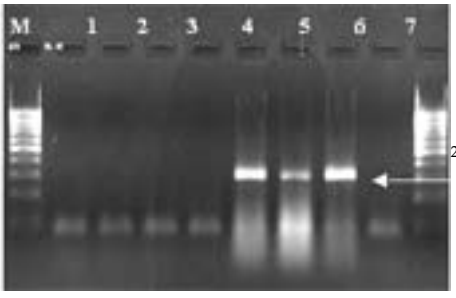

Figure 2. RT-PCR of the third CHSE 214 passage of G 1 after IPNV neutralisation by a rabbit antiserum against IPNV (Sp type).
M 100 bp marker (peqlab); lane 1 sample 107/1 SDV primers, lane 2 sample 107/2 SDV primers, lane 3 sample 107/3 SDV primers, lane 4 negative preparation control SDV primers, lane 5 sample 107/1 IPNV primers, lane 6 sample 107/2 IPNV primers, lane 7 sample 107/3 IPNV primers, lane 8 negative preparation control IPNV primers.

Virus identification by iIFA

SDV or SPDV infected CHSE 214 cells including subcultures of the transfected cells showed a specific fluorescence with polyclonal antibodies against SDV (Figure 3). While SDV and SPDV did not react with a-IPNV monoclonal antibodies (Bio X, Belgium) in iIFA, the co-isolated IPNV did show a typical fluorescense in the cytoplasma of the infected cells.

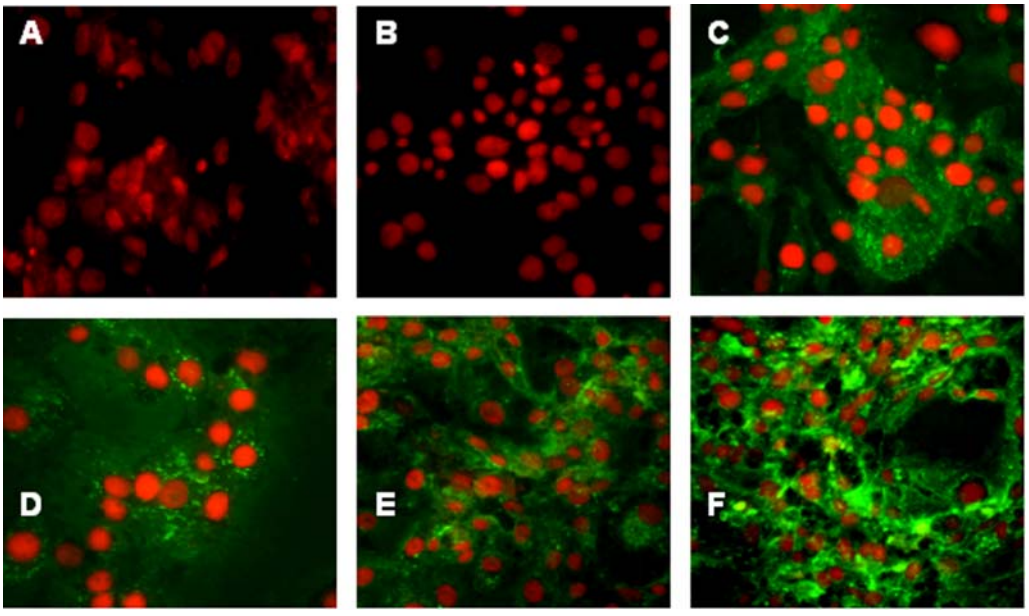

Figure 3. Identification of SDV by iIFAT in second cell culture passage after RNA electroporation.
A negative control with anti-SDV serum; **B** conjugate control of infected cell culture; **C** SDV strains S49P; **D** SPDV strain P42P; **E** SDV strain G 1 (DF 11/03); **F** SDV strain G 2 (DF 18/03).

Histology

Sections of muscle tissue obtained from diseased fish were examined, assessed and used for this study only. Fish with different stages of degeneration in muscle tissue were observed (Figure 4). Weaker degenerative changes were also found in pancreas and heart muscle.

Discussion

This is the first report of SDV in Germany and the fourth report of the isolation of SDV in Europe. In agreement with other reports (Castric et al., 1997; Villoing et al., 2000 and Graham et al. 2003) SDV was difficult to isolate using traditional cell culture isolation methods. Only a limited, focal cpe was seen in initial subcultures on susceptible cell monolayers and SDV is easily overgrown by other viruses or bacteria. *Alphavirus* RNA is known to be infectious (Frolov et al., 1996) and in this investigation the virus was isolated by transfection of CHSE-214 cells with RNA purified from infected tissue samples using an easy to perform electroporation method. The advantage of this method is that the isolate is free of any contaminating viruses or bacteria. Transfection by electroporation is a reliable method for isolation of SDV. In all tissue samples, in which SDV RNA was detected by RT-PCR in this investigation, the virus was also isolated in cell cultures by transfection. One disadvantage of the method used here is that electroporated CHSE 214 cells show poorer growth of virus compared to non transfected cells. However, this may be because the temperature range for isolation of SDV is between 10°C and 15°C and the electroporated CHSE-214 cells do not grow

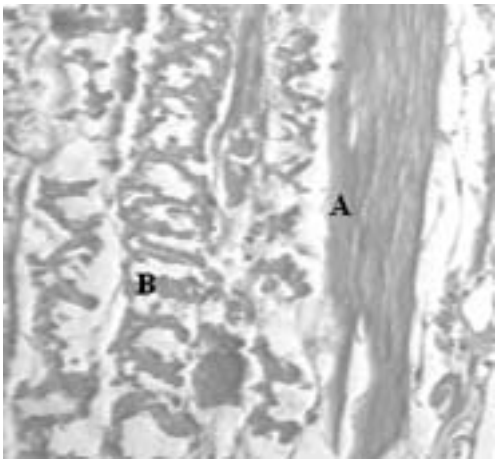

Figure 4. HE staining of muscle tissue of SDV infected rainbow trout fry.
A: normal muscle fibres. **B**: degenerated muscle fibres.

well at low temperatures. The CPE induced by SDV in CHSE 214 or RTG-2 cells rarely affected the full monolayer. Changes in the morphology of the infected cells were seen after three days post infection (p.i.) by light microscopy. Cytolysis was observed over 20 to 50% of the cell monolayers at seven to ten days p.i. but results from the iIFA show that many more of the cells were infected.

The published RT-PCR methods of Villoing et al. (2000) and Graham et al. (2003) are powerful tools for the detection of the SDV or SPDV in organs of salmonids. Villoing et al. (2000) described a specific 284 bp fragment and referred to a gene bank sequence (AJ238578). In contrast, we found that the fragment size produced was 303 bp when the published primer pair SD3/SD5 was used, which fits the gene bank sequence size better (Figure 5) as well as other published SDV sequences (AJ316246, AJ007631). All of our amplified fragments of the reference strains and the German isolates had an estimated size of 303 bp and not 284 bp as illustrated in Figure 1.

Diagnosis of SDV / SPDV in cell culture after transfection by one or two identifying PCRs can be achieved by applying an iIFA or immunoperoxidase assay (Graham et al., 2003) using monospecific polyclonal antisera or monoclonal antibodies against SDV/SPDV which have recently been produced in our institute by Dr. Malte Dauber. These laboratory tests, which allow identification of the agent, along with clinical observations of

SDV5' (start nt 1857)

1801 ccacccggta ccacagtgaa attcgataag aggtgcaaga acgctgccaa agagac**cgtc**
1861 **accttcacca gcgactccca gacg**tttacg tgcgaggagc cggtcctaac ggccgccagc
1921 atcacccagg gcaagccgca cctcagatcg tcaatgttgc ccagcggagg caaagaggtg
1981 aaagcgagga ttccattccc gttcccgcca gagactgcga cttgcagagt gagcatcgcc
2041 ccactgccat cgattaccta tgaggaaagc gatgttctgc tggccggcac tgcgaaatac
2101 cccgtgctgc taactacacg gaaccttggt tt**ccatagca acgccacatc tgaatggatc**

SDV3' (end nt 2160)

Figure 5. Comparison of published genomic region, primer fitting and amplicon size (acc. number AJ238578).

disease in the field will increase the reliability of SDV/SPDV diagnosis.

Furthermore, the transfection method with RNA directly extracted from the infected organs developed for this study allows the detection of SDV in clinically healthy fish. This is in high demand for vaccine experiments in terms of a reliable virus isolation rate Graham et al. (2003) followed by discrimination between vaccine and wildtype virus using RT-PCR.

Acknowledgements

For expert assistance we thank Irina Werner, Kerstin Wink, Kathrin Giesow and the technical staff of the *CCLV*. For critical review of the manuscript we thank Dr. Keith Way from CEFAS in Weymouth.

References

Boucher P & Baudin Laurencin F (1994). Sleeping disease and pancreas disease: comparative histopathology and acquired cross-protection. *Journal of Fish Diseases* **19**, 303-310.

Boucher P & Baudin Laurencin F (1996). Sleeping disease (SD) of salmonids. *Bulletin of the European Association of Fish Pathologists* **14**(5), 179-180.

Boucher P, Castric J & Baudin Laurencin F (1994). Observation of virus-like particles in rainbow trout *Oncorhynchus mykiss* infected with sleeping disease virulent material. *Bulletin of the European Association of Fish Pathologists* **14**(6), 215-216.

Branson E (2002). Sleeping disease of trout. *Veterinary Record* **150**(24)**,** 759-760.

Castric J, Baudin-Laurencin F, Brémont M, Jeffroy J, Le Ven A & Béarzotti M (1997). Isolation of the virus responsible for sleeping-disease in experimentally infected rainbow-trout *Oncorhynchus mykiss. Bulletin of the European Association of Fish Pathologists* **17**, 27-30.

Desvignes L, Quentel C, Lamour F & le Ven A (2002). Pathogenesis and immune response in Atlantic salmon (Salmo salar L) parr experimentally infected with salmon pancreas disease virus (SPDV). *Fish and Shellfish Immunology* **12**(1), 77-95.

Frolov I, Hoffman TA, Pragai BM, Dryga SA, Huang HV, Schlesinger S & Rice CM (1996). Alphavirus-based expression vectors: Strategies and applications. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 11371-11377.

Graham DA, Rowley HM, Walker IW, Weston JH, Branson EJ & Todd D (2003). First isolation of sleeping disease virus from rainbow trout, *Oncorhynchus mykiss* (Walbaum) in the United Kingdom. *Journal of Fish Diseases* **26**, 691-694.

Lopez-Doriga MV, Smail DA, Smith RJ, Domenech A, Castric J, Smith PD & Ellis AE (2001). Isolation of salmon pancreas disease virus (SPDV) in cell culture and its ability to protect against infection by the wild-type agent. *Fish and Shellfish Immunology* **11**, 505–522.

Kent ML & Elston RA (1987). Pancreas disease in pen-reared Atlantic Salmon in North America. *Bulletin of the European Association of Fish Pathologists* **7**, 29-31.

Munro ALS, Ellis AE, McVicar AH, McLay HA & Needham EA (1984). An exocrine pancreas disease of farmed Atlantic salmon in Scotland. *Helgoländer Meeresuntersuchungen* **37**(1-4), 571-586.

Murphy TM, Rodger HD, Drinan EM, Gannon F, Kruse P & Korting W (1992). The sequential pathology of pancreas disease in Atlantic salmon farms in Ireland. *Journal of Fish Diseases* **15**, 401-408.

Nelson RT, McLoughlin MF, Rowley HM, Platten MA & McCormick JI (1995). Isolation of a toga-like virus from farmed Atlantic salmon Salmo salar with pancreas disease. *Diseases of Aquatic Organisms* **22**, 25-32.

Poppe T, Rimstad E & Hyllseth B (1989). Pancreas disease in Atlantic salmon (*Salmo salar*) post smolt infected with infectious pancreatic necrosis virus (IPNV). *Bulletin of the European Association of Fish Pathologists* **9**, 83-85.

Raynard RS, Houghton G & Munro ALS (1992). Pancreas disease of Atlantic salmon: proceedings of a European Commission workshop Scottish office Aquaculture Report No 1 ,The Scottish Office Aquaculture and Fisheries, Aberdeen, p 2-4.

Taksdal T, Dannevig BH & Rimstad E (2001). Detection of infectious pancreatic necrosis (IPN)-virus in experimentally infected Atlantic salmon parr by RT-PCR and cell culture isolation. *Bulletin of the European Association of Fish Pathologists* **21**(5), 214- 219.

Villoing S, Castric J, Jeffroy J, Le Ven A, Thiery R & Bremont M (2000a). An RT-PCR-based method for the diagnosis of the sleeping disease virus in experimentally and naturally infected salmonids. *Diseases of Aquatic Organisms* **40**, 19-27.

Villoing S, Bearzotti M, Chilmonczyk S, Castric J & Bremont M (2000b). Rainbow trout sleeping disease virus is an atypical alphavirus. *Journal of Virology* **74**(1), 173-83.

Weston J, Villoing S, Bremont M, Castric J, Pfeffer M, Jewhurst V, McLoughlin M, Rodseth O, Christie KE, Koumans J & Todd D (2002). Comparison of two aquatic alphaviruses, salmon pancreas disease virus and sleeping disease virus, by using genome sequence analysis, monoclonal reactivity, and cross-infection. *Journal of Virology* **76**(12), 6155-63.