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# Inoculation of Infectious Pancreatic Necrosis Virus Serotype Sp did not cause Pancreas Disease in Atlantic Salmon (*Salmo salar L.*)

By *E. Rimstad, T. Poppe, Ø. Evensen* and *B. Hyllseth*

Department of Microbiology and Immunology, Norwegian College of Veterinary Medicine, and National Veterinary Institute, Oslo, Norway.

**Rimstad, E., T. Poppe, Ø. Evensen and B. Hyllseth: Inoculation of infectious pancreatic necrosis virus serotype Sp did not cause pancreas disease in Atlantic salmon (*Salmo salar L.*). Acta vet. scand. 1991, 32, 503–510.** – Atlantic salmon were selected from a fish farm with no previous record of pancreas disease (PD) or infectious pancreatic necrosis virus (IPNV) infection. Groups of fish were inoculated with either IPNV (strain Sp) from cell culture, organ material from fish with PD or control material as phosphate-buffered saline (PBS). Virological, histological and immunohistochemical examinations were carried out throughout the experiment. None of the fish died or showed clinical symptoms of PD. Histological examination revealed no pathological changes, and immunohistochemical studies were negative. Virus was isolated only sporadically from the group inoculated with organ material, whereas it was isolated consistently from the group inoculated with virus propagated in cell culture, as well as from in-contact control fish after the first week. In a latent carrier test, changes were entirely lacking in the first mentioned group, and were only slight in the last mentioned group. The data suggest that PD is not a transmissible disease, and that IPNV isolated from a PD outbreak does not play any part in the etiology of this disease.

salmonids; pancreatic atrophy; transmission.

## Introduction

Pancreas disease (PD) affects postsmolts of Atlantic salmon (*Salmo salar*) during their 1st season in sea water (*Munro et al.* 1984). The disease is characterized by emaciation, cachectic appearance, pancreatic atrophy and fibrosis, and a degenerative myopathy, including the heart (*Ferguson et al.* 1986). The cause of the disease is unknown. Some authors have proposed that, in light of the associated lesions, the disease may have a basis in a vitamin E/selenium deficiency (*Ferguson et al.* 1986). Others have suggested an infectious etiology based on epidemiological investigations (*Munro et al.* 1984, *Kent & Elston* 1987, *McVicar* 1987, *McVicar* 1990). No infectious etiologic agent has, however, been isolated and propagated in vitro. In Norway, infectious pancreatic virus (IPNV) has frequently been isolated from fish with PD, but this virus is also regularly isolated from healthy fish populations. It is therefore difficult to evaluate the importance of IPNV in PD (*Poppe et al.* 1989). Infectious pancreas necrosis (IPN) is considered to be a disease of young salmonids (*Wolf et al.* 1960, *Reno et al.* 1978), especially rainbow trout (*Oncorhynchus mykiss*), brook trout (*Salvelinus fontinalis*) and Amago trout (*Oncorhynchus rhodurus*) to the age of about 6 months (*Frantsi & Savan* 1971, *Dorson & Torchy* 1981). IPN has been observed to a lesser extent in Altantic salmon (*Hill* 1982). There are, however, some re-

ports of stress-mediated recurrence of IPN in older fish (*Roberts & McKnight* 1976, *Willumsen & Brun* 1988). The sudden change from fresh water to sea water, often combined with transport of long duration, overcrowding and considerable handling constitutes severe stress that might possibly induce IPN in carrier fish. Though it could be expected that such stress-mediated recurrence of IPN would occur soon after transfer to sea water, PD does not normally appear until 2–3 months later.

The purpose of this study was to evaluate if PD is transmissible, and the potential role of IPNV in the etiology of the disease.

## Material and methods

*Experimental design*

The fish used in the infection experiment, Atlantic salmon (*Salmo salar*), were taken from a fish farm with no previous record of PD, IPN, or IPNV isolation. Kidney samples from 10 fish were examined virologically. No cytopathic effect (CPE) was observed after 3 weekly passages in CHSE-214 (Chinook salmon embryo) cultures. The fish used in the trial were acclimatized to the tanks for 3 weeks before the start of the experiment. The fish had an average weight of 110 grams, and had been transferred to sea water approximately 4 months earlier. They were kept in tanks of 2×2 m, filled to a depth of 0.5 m with sea water (giving a total of 2 $m^3$). The water was held at a constant temperature of 11°C throughout the experiment. There were 300 fish in tank A and 150 fish in tank B. The fish were sedated with chlorobutanol. Fish in tank A were then divided into 3 groups of 100 fish each, that were inoculated intraperitoneally (ip) with either: **a)** 1.0 ml of kidney material (treated as described below) from PD affected fish, or **b)** 1.0 ml heart homogenate from the same fish, or **c)** 1.0 ml phosphate-buffered saline (PBS: 0.14 M NaCl, 2.7 mM KCl, 0.88 mM $KH_2PO_4$, 7.6 mM $NaHPO_4$, pH 7.5) with 300 IU/ml penicillin and 300 µg/ml streptomycin. In tank B, 100 fish were each inoculated with 1.0 ml virus harvest (IPNV type Sp as described below, $10^{6.5}$ $TCID_{50}$), while the remaining 50 fish were inoculated with PBS containing 300 IU/ml penicillin and 300 µg/ml streptomycin. The different groups were identified by fin-clipping.

Two fish were collected from each group for histological (several organs) and virological (kidney) examination on day 1 and 7 after inoculation and thereafter at fortnight intervals. The organs were fixed in 4 % phosphate-buffered formaldehyde, and embedded in paraffin for light microscopic evaluation. Hearts and kidneys were prepared and assayed for virus infectivity as described below. The experiment lasted for 75 days. At day 67, the fish were submitted to a talent carrier test (LTD). Briefly, the fish were injected ip once with 20 mg/kg prednisolone acetate and the water temperature was increased to 16°C for 3 days.

*Cell culture propagated virus for inoculation*

Ten fish from an acute outbreak of PD in growers 3 months after sea water transfer, were collected for virological examination. The diagnosis of PD was based on histological findings, with degeneration and fibrosis of the exocrine portion of the pancreas, lack of peripancreatic fat and moderate to severe myopathy of the heart and skeletal muscles as the most prominent features. The farm in question had experienced PD sporadically in recent years. Pyloric ceca with pancreatic tissue, mid-kidney, heart, brain and gills were aseptically removed and homogenized separately. The various organ homogenates were prepared in 20 % suspension of PBS, and 10-fold dilutions were inoculated into CHSE-214 cell cultures (Chinook salmon

embryo cells, ATCC No. CRL 1681, American Type Culture Collection, Rockville, Maryland) to determine infectivity ($TCID_{50}$). Cultures showing no cytopathic effect (CPE) were harvested and inoculated into fresh cell cultures twice.

No CPE was observed in cultures inoculated with samples from the pyloric ceca, heart, brain or gills. In contrast, in cultures inoculated with material from the mid-kidney, CPE was observed in 5 of the 10 fish tested. End-point titers varied from $10^{3.0}$ to $10^{4.8}$ $TCID_{50}$/g organ. Neutralization tests (NT) were carried out using 3 different rabbit sera against the IPNV serotypes, i.e. Ab, Sp and VR-299. The viruses was efficiently neutralized by the serum against Sp, and there were minor cross-neutralizations with the other 2 sera used. By electron microscopy, carried out as described by *Grist et al.* (1979), a large number of virus particles, approximately 60 mm in size were seen in the cytoplasm. Other virus-like particles were not discernible. On the basis of these finding it was concluded that the isolated virus was IPNV serotype Sp.

Virus was propagated for experimental infection purposes in CHSE-214 cell cultures. The cultures were incubated at 15°C until complete CPE was seen (4–7 days). The harvest was frozen at –70°C, thawed, and then centrifuged (Digifuge, Heraus) at 1300 × g for 30 min. There were at most 4 passages of the virus from isolation until injection. The virus titer was $10^{6.5}$ $TCID_{50}$/ml. Virus isolation attempts from experimental fish were made in the same way.

*Organ material for inoculation*

Three weeks after the virus isolation described above, a total of 35 Atlantic salmon postsmolts with a clinical diagnosis of PD were taken from the same fish population. The diagnosis of PD was later confirmed by histopathology. The fish weighed 50–100 grams, and had been transferred to sea water approximately 4 months earlier. They had originated from a local hatchery. Kidneys were removed aseptically, homogenized in a sterile mortar, pooled and suspended in PBS to a final 20 % solution. This solution was centrifuged in a Digifuge (Heraus) at 1300 × for 30 min and the supernatant filtered through a 0.45 µm membrane. After addition of penicillin (300 IU/ml), and streptomycin (300 µg/ml) the material was then immediately put on dry ice where it was kept until inoculation of experimental fish about 40 h leter. Hearts from the same fish were treated in the same way. Virological examination showed that kidney material had a virus titer of $10^{2.1}$ $TCID_{50}$. Virus was detected in the heart material.

*Immunohistochemistry*

The tissues used in the immunohistochemical studies were taken both from fish with clinical PD as well as from other fish selected and removed during the experiment (all fish from the same farm as above). Tissue samples from *Salmo salar* fry collected during an outbreak of IPNV infection in a hatchery were used as positive controls. Samples of liver, pancreas (pyloric ceca) and kidney tissues were treated in 1 of the following 2 ways: **1)** Tissue specimens were fixed in 4 % phosphate-buffered formaldehyde for 24 h and then embedded in paraffin by conventional techniques. After being sectioned in a microtome (4–5 µm), they were transferred to glycerine coated slides; **2)** Tissue specimens were frozen in liquid nitrogen and kept at –70°C until further preparation, when samples were allowed to equilibrate to –20°C to –25°C before being sectioned in a microtome (6–7 µm). The sections thus obtained were transferred to gelatin-coated glass slides, and air dried at room tempera-

ture before being immersed in 4 % formol-calcium at 4°C for 10 min and then again air dried. Rabbit antisera to IPNV serotypes Sp and Ab and control rabbit serum (all diluted 1/500), were used separately as primary antibodies to test formaldehyde fixed and liquid nitrogen fixed sections. The immunohistochemical procedure were as previously described (*Evensen & Rimstad* 1990).

## Results

No clinical symptoms of pancreas disease were observed in the groups of fish in tank A that had been inoculated with kidney or heart homogenate. During the 1st 4 days of the experiment, 7 fish died in the group (n = 100) inoculated with kidney homogenate, while 3 fish died in the group (n = 100) inoculated with heart homogenate. After thorough examination (autopsy, bacteriology and virology) of these fish, it was concluded that the cause of death was probably a toxic effect of the inoculum combined with trauma from handling. No further deaths occurred in tank A during the experiment. No deaths occurred among the fish in tank B inoculated with virus material from cell cultures, nor were clinical signs of disease observed.

Virological examination by tissue culture technique of heart tissue failed to show any CPE-producing agents in any of the groups in either of the tanks. Attempts to isolate virus from kidney tissue from fish in tank A were successful on 3 occasions, once from the group inoculated with heart tissue and twice from the kidney-inoculated group. All fish in the group inoculated with cell culture virus in tank B tested, harboured virus, and the same was true for the control group in this tank after day 7. In tank A, virus titers were always relatively low, i.e. below $10^3$ $TCID_{50}$/ml of a 20 % kidney suspension. In tank B, on the other hand, average virus

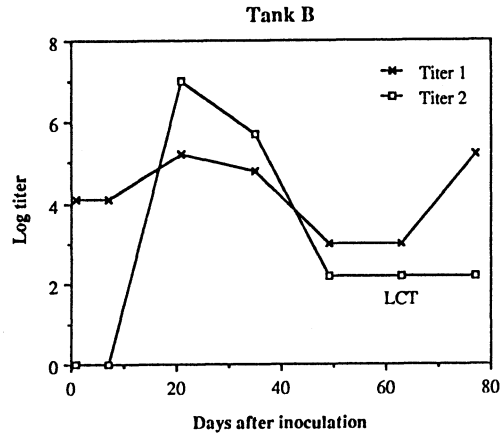


Figure 1. Virus titers found in the group after inoculated with cell culture virus (*Titer 1*), and in control group inoculated with PBS (*Titer 2*).

titers found in kidney samples throughout the experiment were relatively high (Fig. 1). There was only a slight increase in the virus titer in the group inoculated with cell culture virus in tank B after the latent carrier test (LCT) was performed at day 67. There was no change in the titer in the control group. In tank A, there were no observable changes following the LCT, and no virus was isolated subsequently.

In neutralization tests, all virus isolates from the experiment were serotyped as Sp, i.e., the same serotype as used for inoculation. Histologically, no pathological changes were seen in any group at any time during the experiment. The results of immunohistochemical examination were negative both with regard to the formaldehyde-fixed organ tissues and the tissues fixed in liquid nitrogen, no matter whether they were obtained from fish with clinical PD or from any fish inoculated in the experiment. Positive control material from an IPN outbreak (see materials and methods) showed specific staining of single cells of the exocrine pancreas (Fig. 2) when using antiserum against serotype SP of

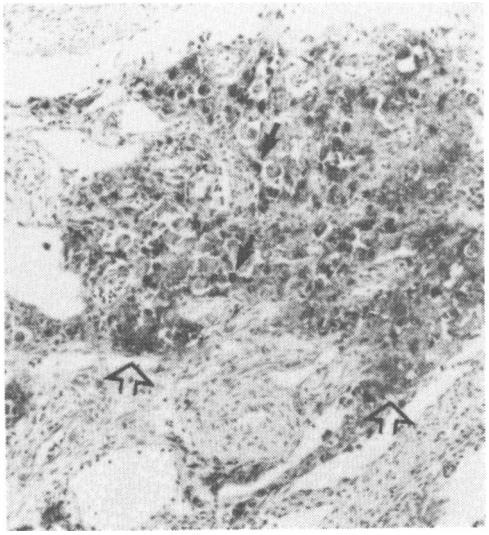

Figure 2. IPNV infection in fry. Incubation with anti IPNV-Sp serum shows strong cytoplasmic reaction in exocrine pancreatic cells (solid arrows). A diffuse coloration is observed in degenerated cells (open arrows). (× 250).

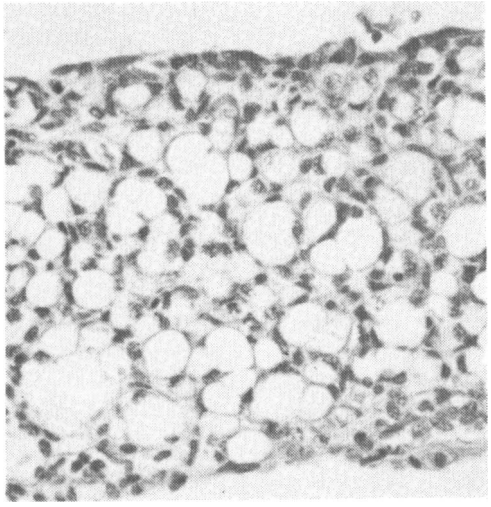

Figure 3. Photomicrograph of lesions in the pancreas of severely affected Atlantic salmon with pancreas disease (PD). Note extensive vacuolation of the exocrine portion of the pancreas. (× 400).

IPNV, but not when using antiserum to serotype Ab.

**Discussion**

The described experiment failed to provide any evidence that pancreas disease (PD) is a transmissible disease, or that IPNV isolated from fish involved in a PD outbreak plays any part in the etiology of this disease.

In Atlantic salmon, it has been well documented that there is a factor of disease resistance to IPN that is age dependent. In 2 studies on the phagonesis of IPN, *Swanson & Gillespie* (1979) and *Swanson et al.* (1982) were unable to induce disease by intraperitoneal injection of the IPNV serotype VR-299 into yearling Atlantic salmon or into 14 month-old brook trout. This could also explain why no disease symptoms were observed in our experiment. However, most cases of PD occur in fish belonging to this age group, this also being the reason why fish of similar age were chosen for the purposes of the present experiment. Furthermore, the sporadic IPNV isolations in the different groups in tank A could indicate that the amount of virus inoculated was insufficient to ensure that all inoculated fish in fact became infected. The kidney material used for inoculation had a rather low titer when tested in cell cultures, and virus was not detected from the heart material. On the other hand, fish in tank B that were inoculated with cell culture virus, apparently became carriers without developing disease symptoms. Considered together, these observations seem to favor the view that IPNV is unable to induce symptoms typical of PD in Atlantic salmon.

The question of serotype-related virulence and possible attenuation in cell cultures also deserves attention. The Sp serotype of IPNV was used in our experiment since this serotype was isolated from a PD out-

break, and previous experiments by other workers had shown that the Sp serotype was associated with higher mortalities in rainbow trout fry under field conditions that the Ab serotype (*Vestergård Jørgensen & Kehlet* 1971). It has been shown that IPNV becomes avirulent for rainbow trout fry after a relatively low number of serial passages in cell cultures (*Dorson et al.* 1978). On the other hand, *Hill & Dixon* (1977) found that serotype Sp was still pathogenic for rainbow trout fry after up to five passages in cell cultures. We passaged the virus no more than 4 times in cell cultures and assumed that its virulence was not lost.

The influence of water temperature on the virulence of IPNV has been described by several authors (*Frantsi & Savan* 1971, *Dorson & Torchy* 1981). Their results indicate that rainbow trout fry are susceptible to disease development at temperatures of about 10°C. Accordingly, the temperature chosen for the present experiment was 11°C. The failure to induce disease is therefore not considered to be an effect of temperature.

Studies by *Ferguson et al.* (1986) showed that fish from the same lot that had been transferred to 2 geographical sites only developed PD at the site where the disease had occurred before, and that fish transported to a site with no history of the disease did not develop the disease. Therefore, if PD is indeed caused by an infectious agent, this is most likely to be of marine origin. Such an agent would also seem to be widespread, as indicated by the geographical distribution of the disease. IPNV is widespread in the aquaculture industry in Norway (*Krogsrud et al.* 1988), and is almost a constant finding in fish with PD as well as other disease conditions. Nevertheless, our various attempts to identify an etiological agent by means such as histology, light microscopy and bacteriological and virological investigations, have so far proved unsuccessful, and this is an experience shared by other workers (*Munroe et al.* 1984). Furthermore, histological examination of pancreatic tissue from PD-affected fish usually reveals diffuse necrosis of the exocrine cells (Fig. 3) (*Kent & Elston* 1987), whereas the pancreatic necrosis observed in fry with IPN disease is often of a multifocal nature, although extensive areas may be involved (*Wolf* 1988). These observations thus give further support to our view that PD is not an IPNV-associated disease.

The possibility that vitamin-E/selenium deficiency is involved in the etiology of PD has been raised in the light of the similarity to morphological lesions seen in mammals with such deficiencies, and those appearing in association with PD, e.g. cardiomyopathy (*Ferguson et al.* 1988). However, it should be noted that outbreaks of PD have occurred, in which the same commercial feed has been fed to fish in both affected and unaffected farms.

In conclusion, the present study indicates that PD is probably not a transmissible disease. If IPNV does indeed play a role in the development of this disease, it would seem to be in conjunction with other factors.

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**Sammendrag**

*Inokulering av Atlantiske laks (Salmo salar L.) med infeksiøst pankreas nekrose virus, serotype Sp frambrakte ikke pancreas disease.*

Pancreas diseases (PD) er en sykdom på oppdrettslaks som har hatt en betydelig spredning i de senere år. En infeksiøs etiologi har blitt foreslått, men det er ikke identifisert noe infeksiøst agens. Atlantisk laks i mottagelig alder for PD ble brukt i et smitteforsøk. Laksen kom fra et oppdrettsanlegg uten noen forhistorie med PD eller isolasjon av IPNV. Tre grupper med fisk ble enten inokulert med a) IPNV type Sp (isolert fra fisk med PD), b)

organmateriale fra fisk med PD, eller c) negativt kontrollmateriale. Virologiske, histologiske og immunohistokjemiske undersøkelser ble utført gjennom hele forsøket. Ingen av forsøksfiskene døde eller viste tegn på patologiske forandringer, videre var immunohistokjemiske undersøkelser negative. IPNV ble isolert fra gruppen som ble inokulert med virus og fra kontrollfisk som var i samme kar som disse under hele forsøket som totalt varte i 75 dagar. Fra gruppen som ble inokulert med organmateriale ble IPNV kun isolert sporadisk. En latent carrier test utført på dag 67 ga ingen forandringer hos fisk inokulert med organmateriale eller hos fisk inokulert med virus, mens en moderat stigning i IPNV titeret ble funnet i kontrollene til sistnevnte gruppe. Konklusjonen fra smitteforsøket ble at det ikke var funnet bevis for at pancreas disease er en smitsom sykdom. IPNV isolert fra fisk under et PD utbrudd synes heller ikke å ha noen etiologisk betydning for denne sykdommen.

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Reprints may be restricted from: E. Rimstad, Department of Microbiology and Immunology, Norwegian College of Veterinary Medicine, P. O. Box 8146, N-0033 Oslo 1, Norway.