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# Enhanced cell culture isolation of Infectious Pancreatic Necrosis Virus from kidney tissue of carrier Atlantic salmon (*Salmo salar* L.) using sonication of the cell harvest.

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**Abstract**

IPNV in carrier Atlantic salmon parr was titrated in the supernatant after homogenisation of the kidney and compared with the titre in the supernatant of the sonicated cell pellet. In 17/20 fish the cell-associated and particulate-bound virus exceeded that in the clarified homogenate supernatant. In two method comparison experiments using pre- or post-smolt field samples, sonicated cell pellet after homogenisation yielded the highest number of isolations.

## Introduction

IPNV has been isolated from farmed Atlantic salmon in Scotland for 30 years both in freshwater and seawater life stages (Munro 1973;Smail *et al.*,1992). The prevalence of IPNV has recently been noted to increase in all regions of the Scottish mainland, the Western and Northern Isles (Murray *et. al.*,2003) . On the one hand, IPNV is very easy to isolate from post-smolts by standard OIE methods using the supernatant from homogenised kidney tissue (OIE, 2000) and high titres maybe obtained (Smail *et. al.*,1995). On the other hand, IPNV is not easily isolated in the covert carrier state in freshwater parr and extraction of the cell associated virus (CAV) must be used e.g. using sonication (Johanssen & Sommer, 1995). Where standard methods are used for extraction, IPNV isolation may not be successful from carrier fish, although RT-PCR indicates IPNV infection is in a covert carrier state (Taksdal et al.,2001). Other studies for brook trout broodstock (McAllister et al.,1987) indicated the benefit of sonication for isolation from ovarian fluid cell fractions. All these studies, led us to investigate the benefit of sonication for extracting IPNV CAV.

## Materials and Method

*Titration analysis in parr*

IPNV release after sonication was studied in a group of 20 carrier parr at a hatchery in the Shetland Isles. This population had previously been identified as carriers and IPNV Sp serotype confirmed by dot blotting (Ross et al.,1991).

Kidney tissue was sampled aseptically from 20 fish and transported chilled. The kidney was weighed and 19 vols. of Hanks balanced salt solution added. This gave a 20x w/v dilution. The tissue was homogenised in a Stomacher 80 lab blender for 1 min. at room temperature. The homogenate was centrifuged at 2000g for 15 min. Three fractions were assayed thereafter and titrated: soni-

cated supernatant, normal supernatant and sonicated cell pellet supernatant.

The supernatant from the kidney homogenate was divided into 2 equal aliquots. Half was sonicated for 1 min. at 70% amplitude of 550W capacity in a water sonicator (Heat Systems, Model XL2020) the other half was left untreated. The sonicate was centrifuged at 2000g for 15 min. to clarify. The pellet from the homogenate was weighed in a tared tube and 9 vols. of HBSS added. It was sonicated as above for 1 min. in the same apparatus. The cell sonicate was also clarified by centrifugation at 2000g for 15 min. Each fraction was sterile filtered using a 0.45µm disposable filter and titrated by virus plaquing on CHSE cells in 24-well plates using 0.75% carboxymethylcellulose overlay in Eagles MEM with 2% foetal calf serum. Plates were incubated for 5 days at 15°C then plaques fixed with buffered formol saline were stained with 1% crystal violet. Plaque counts were expressed as pfu/g kidney.

*Primary isolation experiments*

*Freshwater pre-smolts.* 50 fish from a hatchery in the Shetland Isles with a previous history of IPNV carriers were sampled individually. Approximately 1g of head kidney was sampled into 9 vols. of Eagles MEM transport medium containing 5% foetal calf serum. Thereafter fractions were processed for 3 methods as follows:-

*Method 1 = Sonicated homogenate*

The kidney was homogenised as above and one third aliquot of the homogenate was sonicated for 1 min. at 400W in an Ultrasonics VCX400 bath sonicator. This was centrifuged at 1500g for 15 min.

*Method 2= Homogenate supernatant*

The kidney was homogenised as above and a two third aliquot was centrifuged at 1500g for 15 min.

*Method 3 = Sonicated cell pellet.*

The cell pellet from method 2 aliquot above was resuspended in 3ml of EMEM with 5% foetal calf serum and sonicated as for method 1. The sonicate was centrifuged at 1500g for 15 min.

*Isolation and titration.*

For all methods 1-3 above 0.5ml of the final supernatant was inoculated to a 25 $cm^2$ flask of CHSE cells, incubated at 15°C and then observed microscopically for CPE at 7 days. Passage by freeze thawing was carried out at day 7 to fresh CHSE cells, using a 1:10 dilution of the primary culture medium and any viral CPE confirmed by ELISA as follows:

The ELISA used was a double antibody sandwich ELISA based on the studies of Ross (1991). The ELISA uses polyclonal antibody to the A2 (Sp) serotype to capture antigen and either of the monoclonal antibodies CC3 or CE4, which cross react to A2, A3 (Ab) and A5 (Te) serotypes (Ross *et al.*,1991) as the secondary antibody.

The solid phase was coated with purified rabbit antibody to the A2 serotype at a coating concentration of 1.4 µg/ml in carbonate/bicarbonate coating buffer (Sigma) overnight at 37°C. Unbound antibody was washed off using three rinses of PBS-tween 20 (0.1%v/v) (PBSt) and unbound sites blocked by incubation for 1h at 37°C with heat-inactivated 3% normal goat serum in tris-buffered saline. Excess blocker was washed off with PBSt as above. Then virus in cell culture medium di-

| Fish No. | PFU/g | | | | % virus | | | %virus | |
|---|---|---|---|---|---|---|---|---|---|
| | s/n | Son.s/n[a] | Son. cells | Total | s/n | Son s/n | Son cells | RV[b] | CAV[c] |
| 1 | 1374 | - | 1078 | 2452 | 56.0 | 0 | 44.0 | 56.0 | 44.0 |
| 2 | 2050 | 946 | 2302 | 5294 | 38.6 | 17.9 | 43.5 | 38.6 | 61.4 |
| 3 | 1262 | 3856 | 8538 | 13657 | 9.2 | 28.2 | 62.6 | 9.2 | 90.8 |
| 4 | 316 | 842 | 2648 | 3806 | 8.3 | 22.1 | 69.6 | 8.3 | 91.7 |
| 5 | 892 | - | 2772 | 3664 | 24.3 | 0 | 75.7 | 24.3 | 75.7 |
| 6 | 315 | 842 | 4554 | 5712 | 5.5 | 14.7 | 79.7 | 5.5 | 94.5 |
| 7 | 4384 | - | 2554 | 6939 | 63.2 | 0 | 36.8 | 63.2 | 36.8 |
| 8 | <105 | - | 5666 | 5666 | 0 | 0 | 100 | 0 | 100 |
| 9 | 681 | 5285 | 1771 | 7744 | 8.9 | 68.1 | 23.0 | 8.9 | 91.1 |
| 10 | 527 | - | 2108 | 2636 | 20.0 | 0 | 80.0 | 20.0 | 80.0 |
| 11 | <108 | - | 43 | 43 | 0 | 0 | 100 | 0 | 100 |
| 12 | 315 | 630 | 1010 | 1957 | 16.1 | 32.2 | 51.6 | 16.1 | 83.9 |
| 13 | 4134 | 1320 | 3400 | 8825 | 46.9 | 14.6 | 38.5 | 46.9 | 53.1 |
| 14 | 843 | - | 2623 | 3466 | 24.3 | 0 | 75.6 | 24.3 | 75.6 |
| 15 | 2056 | 628 | 1040 | 3725 | 55.1 | 17.0 | 27.9 | 55.1 | 44.9 |
| 16 | <105 | 105 | 610 | 716 | 0 | 7.2 | 92.8 | 0 | 100 |
| 17 | <106 | - | 315 | 315 | 0 | 0 | 100 | 0 | 100 |
| 18 | 211 | 738 | 548 | 1497 | 14.0 | 49.4 | 36.6 | 14.0 | 86.0 |
| 19 | <105 | 105 | 72 | 177 | 0 | 59.4 | 40.6 | 0 | 100 |
| 20 | <105 | 105 | 166 | 272 | 0 | 38.6 | 61.4 | 0 | 100 |

Table 1. Virus titres in 20 carrier parr analysed for RV and CAV. (a)Additional titre after sonication. (b) Released virus in first supernatant. (c) Cell associated virus released by sonication.

luted 3.5x in the above coating buffer was added and incubated for 1h at 37°C followed by standard washing. Bound virus was identified by addition of either of the monoclonal antibodies CC3 or CE4 (Ross *et al.*,1991) freshly diluted to 1:100 in coating buffer, incubated 1h at 37° C, followed by standard washing. Bound monoclonal was detected by addition of goat anti-mouse whole molecule alkaline phosphatase conjugate (Sigma) freshly diluted to 1:2500 in PBS for 1h at 37°C, followed by standard washing. Bound conjugate was visualised by the addition of Sigma *FAST*™ p-nitrophenyl phosphate in tris-buffered saline and the colour reaction developed for up to 30 min. at room temperature. Absorbance of the ELISA was read at 405nm using an MRX II ELISA plate reader (Dynex) calibrated for use at this wavelength.

Positive controls used were A2 and A5 reference isolates and the negative control was normal cell culture supernatant. All samples were set up in duplicate. The test sample was positive if the mean absorbance of both duplicate wells was more than three times that of the negative control wells.

| | Fish sampled | Method 1 | Method 2 | Method 3 |
|---|---|---|---|---|
| Pre-smolts | 50 | 3 | 3 | 16 |
| Post-smolts | 50 | 6 | 4 | 8 |
| Total | 100 | 9 | 7 | 24 |

Table 2 Numbers of fish isolations from the 3 methods tested: all isolations were confirmed by ELISA

*Seawater post-smolts.*
50 fish from a seacage site in the Shetland Isles with a previous history of IPNV were sampled individually exactly as per the freshwater fish and all 3 methods were carried out likewise.

## Results

For calculating the total CAV, the additional virus released from sonication of the supernatant, is included in the CAV titre. This is taken to be a broad measure of the virus either within whole cells which can be pelleted or associated with membanes and organelles which will remain suspended in the first supernatant. The CAV is thus a measure of the virus not free in aqueous suspension in the homogenate.

The sonicated pellet yielded the highest titres in a low range of titres, where the highest titre was 8538 pfu/g (Table 1). All fish yielded viral plaques using the sonicated cell pellet. Using the first supernatant, 6/20 fish did not show viral plaques and were not detectable. The sonicated supernatant yielded additional virus infectivity in 12/20 fish. In 17/20 fish the CAV from sonicated pelleted cells plus supernatant yielded more virus infectivity than the released virus from the first supernatant. The exceptions were fish nos. 1, 7 and15.

In the primary isolation experiments, method 3, using the sonicated cell pellet, yielded the highest number of isolations for both pre- and post-smolts (Table 2). This indicates that washing and resuspension of the cell pellet, removing non-bound particles such as protein, increases sensitivity and maximises IPNV isolations as well as reducing cytotoxicity in primary cultures. All the culture supernatants showing CPE were confirmed by virus ELISA.

The results here demonstrate that a majority of the virus infectivity in carrier Atlantic salmon is cell-associated after homogenisation using a Stomacher apparatus. This apparatus has been validated against other blenders e.g. mortar and pestle. It was found to be effective and on a par with other blenders (Hedrick *et al.*1986). This result corroborates with other studies indicating the leucocyte-associated carrier state of IPNV in Atlantic salmon (Knott & Munro,1986).

The result has significance for the diagnostic surveillance for IPNV in carrier fish. The OIE manual for fish disease testing advises using a supernatant of homogenised carrier tissue for direct inoculation to cell cultures. These results indicate that critical individual testing of carrier Atlantic salmon will require the

head kidney tissue to be sonicated for maximum virus extraction. This result agrees with the results of Johannsen & Sommer (1995) who showed that sonicated kidney tissue and macrophage cultures yielded low virus titres from individually tested fish. By contrast, the results of Taksdal *et al*.(2001) using plain supernatant yielded no cultured virus which bears out this main contention.

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