International Council for the Exploration of the Sea

CM 1993/F:36
Mariculture Committee

VERTICAL TRANSMISSION STUDIES ON IPNV IN ATLANTIC SALMON (*Salmo salar* L.)

by

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SUMMARY

Two studies are reported on vertical (germ-line) transmission of Infectious Pancreatic Necrosis virus (IPNV) in Atlantic salmon. The first was an experimental study of virus adsorbed to milt and the effect on the progeny. It was found that virus could bind to sperm and enter the egg but complete transfer to the progeny was not detected. In a second study of IPNV-infected broodstock at a farm it was found that 17.5% of fish carried virus in the ovarian fluid but not in the kidney by virus culture. A new method to this laboratory is described for virus isolation from the ovarian fluid. However, virus was not detected in the progeny of three broodfish with detectable virus in the ovarian fluid. It is concluded that vertical transmission in Atlantic salmon is not proven to date by experiment or by sampling farm broodstock.

INTRODUCTION

The study of germ-line or vertical transmission of IPNV has been the focus of several workers on IPNV. Wolf *et al.* (1963) postulated egg transmission, Bullock *et al.* (1976) proved it experimentally in brook trout and more recently Bootland *et al.* (1991) confirmed the finding in this species. Brook trout in the United States, both growers and broodfish, have been shown to harbour IPNV and vertical transmission has been fairly easy to demonstrate. Novel isolation methods were described by McAllister *et al.* (1987) and this methodology has enhanced the isolation of IPNV from brood brook trout. By contrast, studies on Atlantic salmon (Smail and Munro, 1989) showed that vertical transmission was not proven experimentally in this host. However, Krogsrud *et al.* (1989) reported that vertical transmission took place in Atlantic salmon broodstock at a breeding centre in Norway from albeit virus negative parents to the progeny fry. There also the experimental studies in the literature (Ahne and Negele, 1985; Dorson and Torchy, 1985) which have shown degrees of vertical transmission in rainbow trout (*Oncorhynchus mykiss*). The first authors in particular have shown the importance of binding of IPNV to the hardened egg shell as a route of self-infection of the hatching sac-fry.

In this paper we report on two studies (a) and (b). (a) was an experimental cross of virus negative eggs with virus-adsorbed sperm to examine the effect of virion/sperm ratio and (b) was an assessment of possible vertical transmission at a farm with an IPN history. Study (a) showed only transient virus persistence in one group of eggs and study (b) showed that IPNV was concentrated in the ovarian fluid cell fraction from many of the fish examined. In both studies (a) and (b) it was not possible to demonstrate vertical transmission and possible reasons for this are discussed.

METHODS

Study (a)

Virus isolation procedures and experimental details.

i) Kidney

Head kidney was dissected aseptically, weighed and homogenised by Stomacher in nine vols HBSS (1x). The homogenate was clarified and the supernatant passed through a Millipore (0.45 µm) HV filter. 0.5 ml inoculum was inoculated to a CHSE monolayer in a 25 cm^2 culture flask. Cultures were read microscopically and passaged to fresh CHSE cells twice using a "straight pass".

ii) Green eggs

Eggs were rinsed thoroughly in distilled water and homogenised by Stomacher in one vol of HBSS 1(x) plus 10x concentration of antibiotics (10x kanamycin, 5x penicillin, 5x streptomycin and 2x fungizone). The homogenates were incubated overnight at 15°C to decontaminate bacteria. The homogenate was clarified and the supernatant passed through a Millipore filter (0.45 µm, HV) and a 25 cm^2 flask of CHSE cells inoculated.

iii) Microtitration of egg homogenate

25 µl of egg homogenate filtrate was inoculated to microwells in quadruplicate and four-fold dilutions of inoculum made directly in the medium overlying CHSE cells. Plates were read microscopically at five days post-inoculation for any cytopathic effect (CPE).

iv) Titration of virus for adsorption to milt

Five roux flasks of CHSE were inoculated at 0.1 pfu/cell with an Sp serotype isolate. At six days post infection the culture was harvested and virus precipitated from the clarified medium using polyethylene glycol by the method of Dixon and Hill (1983). The concentrated virus was resuspended in filtered modified Cortland saline (see reference below in method v) and titrated using agarose overlay. The titre was 7.6×10^9 pfu/ml.

v) Adsorption of virus to milt and experimental protocol

1. A broodfish was killed at a farm sea site previously well tested by SOAFD as virus negative. The eggs were stripped to a bucket and the kidney sampled for virus testing. The green eggs were transported to the SOAFD experimental site chilled in a vacuum flask.

2. Four male grilse were stripped individually, a pool of milt made and the fish killed. The kidneys were then sampled aseptically for virus testing.

3. An aliquot of the milt pool was then diluted 100x in tapwater and the motility checked under the microscope.

4. A sperm count was made on a 100x dilution of the milt in modified Cortland extender, a high K^+ buffer (Hoyle and Idler, 1968). The count was 6.5 x 10^9 cells/ml.

5. The milt was diluted to 7.6 x 10^7 cells/ml, sufficient to dilute with virus and fertilise four batches of eggs (see Table 1).

6. Virus was diluted in Cortland buffer from 10x to 1,000x, to give final volumes of 2.7 ml and added to the diluted milt as an equal volume.

7. Milt and virus dilutions were incubated at 4°C for 30 minutes for virus to adsorb.

8. 45 ml of eggs were measured into a bucket, the milt-virus added and gently mixed.

9. Fertilised eggs were washed three minutes in fresh water and a sample of 5 ml eggs (40 in number) taken into a universal tube for virus testing.

10. Egg batches 1-4 (virus/milt) and control batches 5 and 6, fertilised with normal milt, were carefully laid in hatching trays.

vi) **Eyed egg isolation**

Eyed eggs were washed in HBSS 1x twice to remove dirt etc and homogenised in two vols HBSS (1x). The homogenate was sonicated one minute at 180 W for 60 seconds in some cases only. 0.5 ml of Millipore filtered supernatant was inoculated to a number 25 flask of CHSE cells giving a minimum detectable titre of 6 pfu/ml eggs(= 10 eggs).

vii) **Sac-fry virus isolation**

Dead sac-fry were picked each day and stored at -20°C and transported to the laboratory for isolation when ready. Fry were homogenised by Stomacher in approximately 10 vols., the homogenate clarified and a further 5x dilution made in HBSS (1x), to give a 50x final dilution. Virus inoculation etc was as above for eyed eggs.

viii) **Live feeding fry**

Living and moribund fry were tested at three weeks after first-feeding (early fry) and at seven weeks (later fry), for egg lot 2 progeny only. For the early fry test, fry were pooled in numbers of 6-19 and mid-sections were homogenised in 10 ml HBSS (1x) and made up to 50x final dilution as above. The minimum detectable titre (MDT) from individual fry was therefore in the range 600-1,900 pfu/g. For the later fry test, 50 fry were taken from a surviving population of approximately 2,000, two pools of 25 fry (mid-sections) were dissected and processed for inoculation as above. This test gave a 95% confidence of detecting 5% virus prevalence. The minimum detectable titre from individual fry was therefore 2,500 pfu/g.

Study (b)

Kidney microtitration

The kidney was homogenised in 9 vols HBSS 1x, clarified and passed through a Millipore 0.22 µm filter to give a 10x dilution. 100 µl was inoculated to the first well of a microtitre plate and further 4x dilutions made across the plate directly in the medium overlying CHH-1 (chum heart) cells. The inoculum was removed at the lowest dilution only and the plates were incubated at 15°C for six days before being read microscopically for visible CPE.

Ovarian fluid titration

The ovarian fluid was clarified by sedimentation at 3,000 g for 15 minutes and the supernatant passed through a Millipore HA filter (0.45 µm). An aliquot was titrated on CHH-1 cells on microtitre plates in duplicate rows. The CPE was read microscopically at six days post inoculation and the plates fixed and stained. The titre was calculated by the method of Reed and Muench (1938) and expressed as $TCID_{50}$/ml fluid.

Ovarian fluid cell titration

The cells were pelleted from each sample by sedimentation at 3,000 g for 15 minutes and resuspended in 1 ml HBSS. The cells were sonicated using 10 x 1 s bursts at 180 W in a waterbath sonicator (Ultrasonics Ltd, model A1809). The sonicate was passed through a Millipore (GV-0.22 µm) low protein binding filter and titrated by microtitration as above. Results are expressed as $TCID_{50}$/ml of ovarian fluid cell sonicate.

Virus isolation from eyed eggs, sac-fry and feeding fry

See the methods given for study (a)

Farm procedure and follow-up samples

Study (b) was planned to follow vertical transmission in the progeny of IPNV positive broodfish, at a site with a long history of IPNV in western Scotland. Forty 3+ brood fish were stripped of eggs, the gonadal fluids titrated and fertilised with previously tested IPN-negative milt by the fish farm staff. Virus was titrated from the ovarian fluid using the supernatant and also the cell pellet. Three progeny populations were then followed by virus testing. Dead eggs were picked and stored daily until virus testing could be carried out and lots of living eyed eggs, sac-fry and feeding fry were virus tested up to mid-July as feeding fry.

RESULTS

Study (a)

Kidney virus testing on four mature males showed all fish were negative after two passes. The milt pool was also virus tested but bacterial contamination from within the medium prevailed and that result was indeterminate. Virus was detected transiently in the egg lot 2 in fertilised eggs sampled 2-3 minutes after water hardening (Table 1). It should be

noted that these eggs had to be stored at 4°C for nine days until suitable cells were ready for virus testing. All further tests on eyed eggs, sac-fry, (including dead and alive), early fry and later fry were virus negative.

An estimate of the fertilisation rate of eggs was made at approximately 350 day degrees in late March (Table 2). Interestingly, egg lots 1 and 2 from the groups with high virion to sperm ratios showed very low fertilisation rates in a random egg sample. In groups 2 and 3 also, the embryos were visibly smaller eyed than the control groups.

Study (b)

IPNV was isolated in 22.5% of broodfish in the kidney, in 40% in both kidney and ovarian fluid and interestingly in 17.5% in the ovarian fluid only by the cell sonication method (Table 3). The sensitivity of the cell sonication method on the cells used, CHH-1, was striking. A range of low to moderate titres in the kidney was found and also in the ovarian fluid (Table 4). In fish numbers 2, 7 and 29 where the eggs were removed to the experimental site for further virus testing no virus was detected in any progeny either dead or alive (Table 5).

DISCUSSION

We have shown in these two studies that vertical transmission in Atlantic salmon was not proven by experiment. In the first study the transient persistence of virus in one lot of eggs showed that if sufficient virus were adsorbed to the fertilising sperm then IPNV could enter the egg at fertilisation. This agrees with the previous result of Smail and Munro (1989) which showed transient persistence of virus in a group of eggs at one virion per sperm. It was surprising that no virus was recovered in the lots of eggs at 100 virions/sperm and one virion/sperm but it is important to note that the fertilised eggs were stored nine days at 4°C. Storage for such a time could decrease the infectivity of virus slightly. This was clearly a difference to the previous methodology. Also it is most unlikely that the group of eggs fertilised with sperm adsorbed with 100 virions/sperm was actually successfully fertilised, as no eyed embryos were visible in late March. It must be concluded that the conditions of fertilisation were therefore unsuitable.

This result, failure of virus to persist, agrees with the finding of Ahne and Negele (1985). They reported that fertilisation of rainbow trout eggs with virus adsorbed milt (probably a low but unstated virion/sperm ratio of 1/10 or less) gave rise to detectable virus only up to six minutes after water hardening. However, Dorson and Torchy (1985) showed that complete vertical transmission in rainbow trout was achievable using virus adsorbed milt, at a ratio of approximately one virion per three sperm. In that case virus was detected only after hatching and in dead fry with clinical symptoms.

In the farm study (b) no virus was transmitted through to the eggs or to the feeding fry. This result reflects the commercial conditions used for farmed broodfish rather than attempted transmission by experimental infection of the gametes. It supports the supposition of Wolf (1963) for brook and rainbow trout that vertical transmission may occur only when the ovarian fluid titre is very high or exceeds $\log_{10}6.0$ $TCID_{50}$ per ml in the ovarian fluid.

In this connection, an enhanced method for IPNV isolation from ovarian fluid was successful using the sonicated cell fraction of ovarian fluid. This was based on the method described by McAllister *et al.* (1987) and this is now integral to this laboratory's procedure for broodstock IPNV testing. Interestingly, 17.5% of the broodstock were ovarian fluid positive and kidney negative, which emphasises the importance of always including an ovarian fluid sample in IPNV broodstock testing of Atlantic salmon.

The proof of vertical transmission of IPNV in Atlantic salmon remains conjectural. Smail and Munro (1989) showed that virus could be detected transiently after fertilisation when either virus was adsorbed to milt or green eggs were virus bathed. The farm study here shows that a high proportion of broodstock may carry virus in the ovary (with or without kidney detection). However, by analogy to brook trout, vertical transmission may take place only in a very small proportion of infected broodstock (Wolf, 1963) and only at very high titres in the ovarian fluid. As Mulcahy (1985) has pointed out for IHNV vertical transmission, experiments with small numbers of broodfish may mean that the probability of detecting vertical transmission is very slight. This may explain why Krogsrud, Hastein and Ronningen (1989) claimed vertical transmission at a Norwegian breeding station in large numbers of broodstock.

Another important consideration is there are probably species differences with respect to the ease of vertical transmission in farmed salmonids. Could it be that the egg proteins and yolk phospholipids of different species confer different degrees of resistance and inactivation to viruses surviving in the newly fertilised egg? There is some evidence along this line of logic. For example there is evidence from several salmonid species that eggs contain lectins (Voss *et al.*, 1978; Bildfell *et al.*, 1992) and chemically similar protectins have been described from salmonid roe extracts (Voak *et al.*, 1974). The bactericidal properties of one these carbohydrate-binding egg lectins has been described for four bacterial fish pathogens (Voss *et al.*, 1978). However, lectins are also capable of precipitating glycoproteins and it is possible that if a virus comprises glycoproteins amongst the structural external proteins (and this is likely for IPNV) then an egg lectin could bind to and inactivate virus. Yolk phospholipids are another source of possible virus inactivation and Yoshimizu *et al.* (1988) showed that the anti-viral activity of masu and chum salmon eggs to IHNV was attributed to a yolk phospholipid.

Vertical transmission of IPNV in Atlantic salmon is a topic of ongoing investigation which deserves a larger farm sampling research exercise. Of particular interest will be answering the following questions in farm IPN-infected broodstock: 1) How much detectable virus must be present in the ovarian fluid at the time of stripping for virus to penetrate at least a significant proportion of eggs?; 2) Does the antibody status of broodfish correlate with the titre of virus in the ovarian fluid?; 3) Is the antibody status at the time of stripping critical to the prevention of vertical transmission?; and 4) Does the outer layer, the egg shell, play a role in trapping and preserving virus infectivity such that sac-fry and fry are infected by lateral transmission from the discarded egg shell, thus by-passing intra-ovum infection?

ACKNOWLEDGEMENTS

We thank Elaine Rough and Karen Haddo for technical assistance. We thank E Aris and Sally Clements for technical assistance at the SOAFD experimental unit.

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TABLE 1

Virus testing of a broodfish kidney, the eggs crossed with virus-adsorbed sperm at various virion per sperm doses and the subsequent virus tests on the progeny of four egg lots

Egg lot	K*	Eggs**	Virion/ sperm	Eggs fert	Eyed eggs	Early fry	Later fry
1	<20	<4	100/1	-ve	-ve	-ve	NT
2	<20	<4	10/1	+ve***	-ve	-ve	-ve
3	<20	<4	1/1	-ve	-ve	-ve	NT
4	<20	<4	1/10	-ve	-ve	-ve	NT

* - Titre = pfu/g; ** - Titre = pfu/ml eggs (eight in number); ***- >1 pfu/4 eggs but <40 $TCID_{50}$/egg; NT - Not tested

TABLE 2

The assessment of fertilisation rate in the egg lots from the sperm-virus adsorption experiment by observation at approximately 350 day degrees

Egg lot	Nos eyed eggs in sample	% eyed	Comments
1	0/20	0	No embryos visible
2	3/20	15	Small eyed only
3	11/20	55	Some small eyed only
4	7/20	35	Normal
Controls			
5	13/20	65	Normal
6	12/20	60	Normal

TABLE 3

Broodfish virus isolations: % of fish virus positive by kidney and ovarian fluid testing methods

Tissue/fluid tested	Nos +ve	Fish tested	% +ve
a) kidney and ovarian fluid cell sonicate	16	40	40
b) kidney only	9	40	22.5
c) ovarian fluid cell sonicate only	7	40	17.5

TABLE 4

Virus titres in the kidney and ovarian fluid (fluid and cells) of 40 broodfish at a commercial site with a history of IPNV (Titres in kidney are $\log_{10}$ TCID50/g and ovarian fluid $\log_{10}$ TCID50/ml fluid or cell sonicate)

Fish no	Kidney	OF	OFC	Fish no	Kidney	OF	OFC
1	2.92	<1	3.41	21	3.20	<1	2.51
2	4.79	3.11	3.11	22	4.11	<1	3.11
3	<2.0	<1	<1	23	3.20	<1	<1
4	3.51	<1	3.11	24	<2	<1	3.70
5	<2	<1	<1	25	3.80	<1	<1
6	<2	<1	3.11	26	<2	<1	<1
7	5.0	<1	3.11	27	5.44	<1	<1
8	3.80	<1	3.11	28	3.51	<1	<1
9	2.00	<1	2.51	29	<2	<1	2.51
10	4.70	<1	3.11	30	<2	<1	<1
11	2.92	<1	<1	31	4.11	<1	<1
12	3.80	<1	3.11	32	<2	<1	2.51
13	2.92	<1	2.51	33	3.51	2.50	3.11
14	4.70	<1	3.11	34	<2	<1	<1
15	3.51	<1	2.51	35	4.11	<1	<1
16	4.70	<1	<1	36	3.51	1.90	3.11
17	3.80	<1	<1	37	4.11	<1	<1
18	<2	<1	2.51	38	<2	<1	<1
19	<2	<1	2.51	39	2.92	<1	<1
20	<2	<1	<1	40	<2	<1	<1

OF = ovarian fluid; OFC = ovarian fluid cell sonicate

TABLE 5

Kidney and ovarian fluid titres (by two methods) in three broodfish (numbers 2, 7 and 29) and virus testing of the progeny crossed with virus negative milt. Titres are $\log_{10}$ values as in Table 4

Fish no	Kidney	OF	OFC	Eggs	Sac-fry	Fry
2	4.79	3.11	3.11	-ve	-ve	-ve
7	5.0	<1	3.11	-ve	-ve	-ve
29	<2	<1	2.51	-ve	-ve	-ve

OF = ovarian fluid; OFC = ovarian fluid cell sonicate