

## NOTE

## Determination of buoyant density and sensitivity to chloroform and freon for the etiological agent of infectious salmon anaemia

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**ABSTRACT:** Plasma was collected from Atlantic salmon *Salmo salar* with acute infectious salmon anaemia (ISA) and used to challenge Atlantic salmon parr by intraperitoneal injection. Treatment of plasma with the lipid solvent, chloroform, showed that the etiological agent of ISA contained essential lipids, probably as a viral envelope. Some infectivity remained following treatment with freon. Injection challenges using fractions from equilibrium density gradient centrifugation of plasma from fish with acute ISA revealed a band of infectivity in the range 1.184 to 1.262 g cm<sup>-3</sup>. The band was believed to contain both complete ISA-virus particles and infectious particles lacking a complete envelope, nucleocapsid or genome. Density gradient centrifugation of infectious plasma for enrichment of the putative ISA virus appeared to offer a suitable method for obtaining virus-specific nucleic acid for use in the construction of cDNA libraries.

Infectious salmon anaemia (ISA) was first observed in 1984 among farmed Atlantic salmon *Salmo salar* reared at 2 locations in Norway (Thorud & Djupvik 1988, Thorud 1989). The disease has now spread to more than 150 Norwegian fish farms. Typical signs of acute ISA are anaemia, ascites and haemorrhagic necrosis of the liver (Evensen & Thorud 1991, Thorud 1991b). Mortality due to ISA varies between 15 and 100 %. While the etiological agent of ISA is unknown, the disease is transmissible by injection of bacteria-free 220 nm filtrates of tissue homogenates and is believed to be caused by a virus (Thorud et al. 1990, Christie et al. 1991). Fish that have recovered from ISA are less susceptible to reinfection and their sera contain factors that neutralize the ISA-agent (K. Falk & K. Thorud pers. comm.).

Both infectious pancreatic necrosis (IPN) virus and erythrocytic inclusion body syndrome (EIBS) virus are often detected in fish suffering outbreaks of ISA

(Thorud et al. 1990). These viruses are widespread in Norway and usually not associated with high mortality in Atlantic salmon (Christie et al. 1990, Lunder et al. 1990). In our laboratory, IPN-free Atlantic salmon parr injected with IPN virus isolated from ISA-infected fish developed no signs of ISA and absorption of ISA-infectious tissue homogenate with anti-IPN coated magnetic beads did not reduce the ISA-infectivity of the homogenate (unpubl. data). While the EIBS virus may cause severe anaemia in coho salmon *Oncorhynchus kisutch* and chinook salmon *Oncorhynchus tshawytscha*, only minor signs of gross pathology or mortality appear to be caused by the virus alone (Leek 1987, Piacentini et al. 1989). Inclusion bodies ranging in size from 1 to 8 µm containing virus particles 70 nm in diameter with a 38 nm electron-dense center are frequently detected in the erythrocytes of fish naturally or artificially infected with EIBS-virus (Leek 1987, Arakawa et al. 1989). In our laboratory, no EIBS inclusion bodies or EIBS-virus particles were detected by transmission electron microscopy (TEM) of blood cells from fish artificially infected with ISA for 2, 3 and 4 wk (unpubl. data). The results suggested that IPN or EIBS viruses were not involved in ISA, but the existence of a particularly virulent mutant strain of EIBS virus cannot be ruled out.

Intraerythrocytic virus-like particles of 80 to 100 nm in diameter have been observed within Atlantic salmon blood cells by electron microscopy (Thorud et al. 1990). We have reported that ISA infectivity remained following passage of plasma through a 220 nm pore size membrane filter and electron microscopy (EM) of pellets from fractions by centrifugation of plasma on sucrose gradients showed virus-like particles in the

range of 50 to 100 nm (Christie et al. 1991). The causative agent of ISA has not been grown in cell culture and no methods for purification of the virus have been described. It can be detected only by *in vivo* injection experiments and the diagnosis of ISA is based on clinical signs and histopathological findings. Development of a specific diagnostic method and a protective vaccine against ISA may be facilitated using recombinant DNA technology following enrichment of the virus particles from infected fish.

Injection trials have shown that the infectious agent of ISA is present both in blood cells and plasma of infected fish (Christie et al. 1991). Although the concentration of virus appeared lower in plasma than in tissue homogenates, the purity of virus-specific nucleic acid extracted from plasma was anticipated to be higher than from the more heterogeneous tissue homogenate. To develop methods for purification of the ISA virus from plasma, for improved visualization of the virus by EM, and for preparation of good quality viral nucleic acid, we examined the sensitivity of the ISA-agent to chloroform and freon, and estimated the buoyant density of the infectious agent in CsCl gradients. A preliminary infectivity experiment using 6 fractions collected following density gradient centrifugation of ISA-infectious plasma indicated a buoyant density for ISA virus to be in the range 1.2 to 1.3 g cm<sup>-3</sup> (data not shown).

**Materials and methods.** Heparinized blood was collected from adult farmed Atlantic salmon during natural outbreaks of ISA showing typical signs of the disease. The blood was transported on ice to the laboratory. Samples from 8 fish were pooled and centrifuged at 800 × *g* for 10 min. Plasma was collected, centrifuged at 10 000 × *g* for 30 min and the supernatant passed through a membrane filter with a pore size of 220 nm. Plasma was stored at 4 °C for no more than 24 h.

Two ml plasma was layered over each of 4 step gradients made of 0.5 ml 50 %, 1 ml 40 %, 1 ml 30 % and 1 ml 20 % (W/V) CsCl in TE-buffer (0.05M Tris-HCl, 0.01M EDTA, pH 7.8). The gradients were centrifuged for 18 h at 114 000 × *g* in a SW50.1 rotor. For each gradient, 9 fractions were collected by bottom puncture of the tube and the refractive index of each fraction was measured. The estimated buoyant density of parallel fractions did not differ more than 0.008 g cm<sup>-3</sup>. Parallel fractions were pooled and dialyzed overnight against TE-buffer and the protein concentration of the dialyzed fractions was estimated using a commercial assay (Bio-Rad Laboratories) with mouse immunoglobulin serving as a protein standard.

Infectious 220 nm-filtered plasma was treated with chloroform to determine if the ISA virus had a lipid-containing envelope and with freon to determine if the virions could be better visualized by electron micros-

copy. Plasma was mixed 1:1 with chloroform or freon (C<sub>2</sub>Cl<sub>3</sub>F<sub>3</sub>), vortexed, centrifuged at 2000 × *g* for 5 min, and the upper aqueous phase collected for each preparation. The freon treatment was repeated twice. Untreated plasma served as a control for the infectivity trials.

Atlantic salmon S1 parr (30 to 60 g) were used for the injection trials. The fish were kept in 200 l tanks and provided with 1.2 to 1.4 % flowing UV-treated salt water at 6 to 8 °C with a flow rate of 4 l min<sup>-1</sup>. The fish were anesthetized with benzocain, individually marked by visible implant tags (VIT) (Fish Eagle) and i.p. injected with 200 µl samples. Groups of 10 fish were injected with each of 9 pooled fractions and kept in the same tank. In a second tank, groups of 10 fish were injected with ISA-infectious plasma treated with chloroform or freon. Fifteen fish were injected with non-treated ISA-infectious plasma and kept in a third tank as positive controls. Ten non-infected fish were added to each tank as negative controls.

Signs of ISA and mortality were recorded each day and dead fish were examined for gross and histopathological changes. The challenge studies were terminated at 45 d post-injection (p.i.) when the negative control fish first began to acquire ISA by waterborne exposure to the virus being shed by the experimental fish in the same tank.

**Results and discussion.** The first signs of ISA were observed 24 d p.i. among fish in the positive control group and in the group injected with Fraction 3 (density 1.262 g cm<sup>-3</sup>) (Fig. 1). The fish stopped feeding, darkened, became lethargic and started to die. Dead and moribund fish exhibited haemorrhages on the eyes, ascites, had pale gills and heart, petechiation on the visceral fat and enlargement of the liver and spleen. Histopathological inspection of livers revealed extensive haemorrhages and zonal necrosis. All fish that died during the experiment showed typical signs

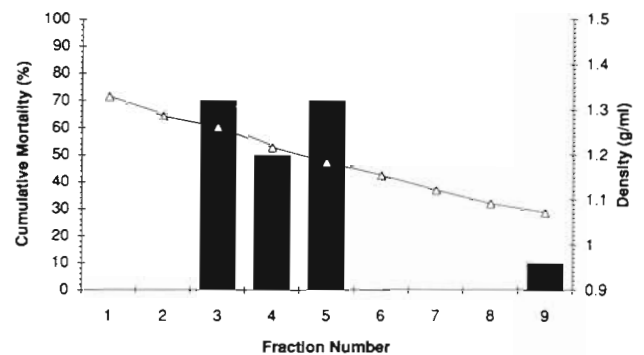


Fig. 1. *Salmo salar* infected with ISA. Buoyant density ( $\Delta$ ) of 9 fractions collected following centrifugation of 2 ml plasma from ISA-infected Atlantic salmon on a 20 to 50 % CsCl gradient. Percent cumulative mortality at 40 d p.i. among groups of fish injected with each fraction is shown

of ISA. At 40 d p.i., the mortality in the group injected with Fraction 3 and in the group injected with Fraction 5 (density  $1.184 \text{ g cm}^{-3}$ ) was 70 %. In the group injected with Fraction 4 (density  $1.216 \text{ g cm}^{-3}$ ), mortality at 40 d p.i. was 50 %. Among the fish in the group injected with the Fraction 9 (density  $1.070 \text{ g cm}^{-3}$ ), 1 fish (10 %) died with typical signs of ISA at 4 wk p.i. This low level of infectivity at the top of the gradient may have been due to aggregated or membrane-bound virus that did not reach equilibrium with the free virions. At 40 d p.i., no mortality or disease signs were observed for fish in the negative control group or in groups injected with the other fractions. The fish were necropsied and no pathological findings were observed. The mortality among fish in the positive control group was 93 %.

At 40 d p.i. no mortality or ISA symptoms were observed in the group injected with chloroform-treated plasma. In the group injected with freon-treated plasma, typical signs of ISA and 30 % mortality were observed (Fig. 2). The results showed that infectious virus in the plasma was inactivated by chloroform treatment indicating that the ISA virion had a lipid-containing envelope. The results verify the previously reported sensitivity of the ISA virus to ether (Thorud et al. 1991a). Treatment of infectious plasma with freon, a compound often used in the preparation of non-enveloped viruses for EM, resulted in a reduction in infectivity suggesting that the envelope was partly removed and that freon was not useful for preparation of the ISA virus for electron microscopy.

The results of the density gradient experiment were in good agreement with the preliminary results and suggested that the ISA virions have a buoyant density in CsCl in the range  $1.184$  to  $1.262 \text{ g cm}^{-3}$ . The infectious fractions probably contained both complete virus particles and particles lacking a complete genome, nucleocapsid or envelope. The results suggest that ISA is caused by an enveloped virus of the Togaviridae or Flaviviridae family (density  $1.22$  to  $1.24 \text{ g cm}^{-3}$ ) or the Hepadnaviridae family (density  $1.24$  to  $1.26 \text{ g cm}^{-3}$ ) (Francki et al. 1991). No virus-like particles were observed by EM of pelleted material from the infectious fractions. The negative results may indicate that too few virus particles were present in the plasma or that degradation of the virus particles occurred during preparation for EM. Although the ISA-agent was not specifically identified, the infectivity trials provided a good approximation of the buoyant density of the ISA-

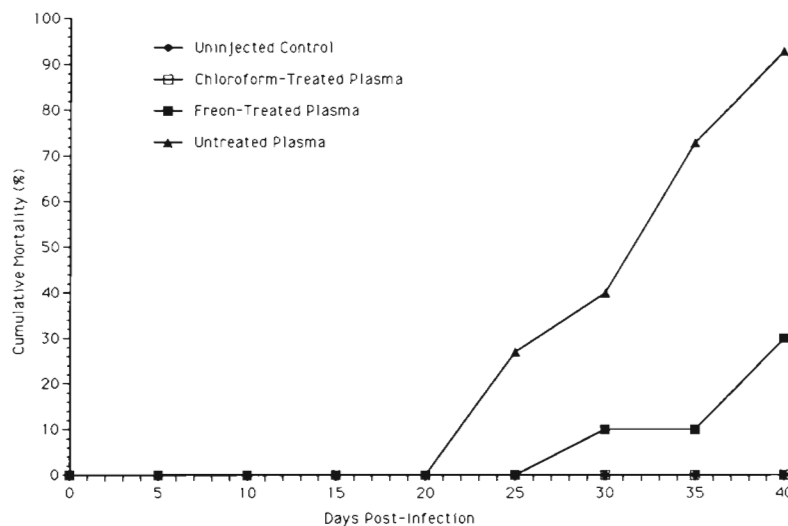


Fig. 2. *Salmo salar* infected with ISA. Percent cumulative mortality occurring over 40 d among 10 uninjected control fish, among groups of 10 fish injected with ISA-infected Atlantic salmon plasma that had been treated with 50 % chloroform and 50 % freon, and among a group of 15 fish injected with untreated infectious plasma

agent that will assist in further purification of the putative virus.

Our results indicated that the ISA virus can be concentrated by equilibrium centrifugation of plasma from infected fish suggesting that enriched amounts of ISA-specific nucleic acid could be extracted from the pellets obtained by centrifugation of the fractions having a density of  $1.18$  to  $1.26 \text{ g cm}^{-3}$ . These methods are being used to extract ISA-specific nucleic acid from plasma for construction of cDNA libraries. The method may also be used for identification of other non-culturable viruses.

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