FINAL REPORT

IMPROVED DIAGNOSIS OF THE INFECTIOUS DISEASE EPIZOOTIC HAEMATOPOIETIC NECROSIS (EHN) IN SALMONID FISH IN AUSTRALIA

FISHERIES RESEARCH AND DEVELOPMENT CORPORATION

1st July 1989 to 30th June 1992

NSW AGRICULTURE

ELIZABETH MACARTHUR AGRICULTURAL INSTITUTE

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NON-TECHNICAL SUMMARY

Epizootic haematopoietic necrosis virus (EHNV) was the first virus isolated from finfish in Australia. It causes outbreaks of disease in wild redfin perch (*Perca fluviatilis*) and also in hatchery reared rainbow trout (*Onchorhynchus mykiss*).

The principal aim of this project, which was to develop a rapid, cheap and accurate immunodiagnostic test for EHN virus (EHNV) infection, was achieved. The new test, an enzyme-linked immunosorbent assay (ELISA) will replace the traditional technique of virus isolation for routine diagnosis of EHNV and will be utilised as a certification test. The ELISA detects the presence of EHNV components (known as antigens) in the tissues of infected fish. The test has a sensitivity of 80%, a level which is considered to be more than adequate for evaluation of samples in fish disease diagnosis. The specificity of the ELISA is greater than 98%.

Blood samples were collected from a population of rainbow trout on a commercial hatchery known to be endemically infected with EHNV. Antibodies (substances produced by an infected fish in response to infection) specifically directed against EHNV were not detected in any of these fish. However, anti-EHNV antibody was detected in the blood of wild redfin perch from Victoria. The potential role of serology in the diagnosis of EHNV infection cannot be assessed at this time because of incomplete understanding of the behaviour of the disease in infected populations of rainbow trout and redfin perch.

Further information is required on the epidemiology of EHNV in hatchery populations of rainbow trout and in wild populations of redfin perch. Acquisition of this data using the tests developed during this study is essential for the formulation of rational protocols for disease certification. In addition, further data is needed on possible differences between strains of EHNV and other iridoviruses that have been isolated in Australia.

ACKNOWLEDGEMENTS

This project could not have been undertaken without the cooperation and support of staff in the Virology Section, EMAI. Thanks are due in particular to Dr P. Kirkland, Mr. T. Batty, Mrs C. Hornitzky, Miss C. Williams, Mrs R. Thurston, Mrs A. Moyle and Mr. S. Wilson. The provision of laboratory facilities, a constant supply of reliable cell cultures and access to the Electron Microscopy unit is gratefully acknowledged.

Fish used for experimental inoculations and evaluation of laboratory tests during this project were kindly provided by the following: ACT Parks and Conservation Service (Mr. T. Rutzo); NSW Fisheries, Gaden Hatchery (Mr. D. Stock), Snowy Mountains Trout Farm (Mr. S. McGowan) and the University of Tasmania (Dr. B. Munday). Staff from the Fisheries Research Institute, Cronulla provided expert assistance with fish collections in the field.

Mr. G. Reddacliff (NSW Agriculture, Regional Veterinary Laboratory, Menangle) and Mr. A. Philbey (Regional Veterinary Laboratory, Wagga Wagga) provided support in collection of samples from commercial hatcheries. Dr. B. Munday, University of Tasmania, collaborated in experiments involving immunisation of rainbow trout with model antigens.

The assistance of Fisheries Inspectors employed by NSW Fisheries in the notification of outbreaks of EHN in redfin perch during the course of this study contributed greatly to the success of this project.

Miss Kirsten Steiner (Technical Officer, Scientific) and Mrs Claudia Kristo (Temporary Assistance) undertook technical work described in this report.

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BACKGROUND INFORMATION

Viral diseases are among the most important of the diseases affecting fin fish. Diseases such as infectious pancreatic necrosis, viral haemorrhagic septicaemia and infectious haematopoietic necrosis are responsible for significant economic losses in salmonid aquaculture in the northern hemisphere but do not occur in Australia (Langdon et al 1986; Wolf, 1988). Australia's freedom from these diseases has conferred important advantages in husbandry options, productivity and marketability of our fish products.

Langdon *et al* (1986) reported the first isolation of a virus from fin fish in Australia. The virus, epizootic haematopoietic necrosis virus (EHNV) (Langdon and Humphrey 1987), appeared to be the cause of widespread mortalities in the redfin perch (Perca fluviatilis), an introduced fish favoured by recreational fishermen in rivers and impoundments in southern Australia.

EHNV was tentatively assigned to the Iridoviridae based on size, icosahedral morphology, presence of viral inclusion bodies and virions in the cytoplasm of infected cells and the sensitivity of the virus to ether (Langdon *et al* 1986); following further studies of ultrastructure, methylation of the DNA and polypeptide profiles this classification was confirmed (Eaton *et al* 1991). Iridoviruses are a diverse group that infect invertebrates (insects and crustaceans) and vertebrates (fish and amphibians). Piscine iridoviruses have been isolated from goldfish (goldfish virus, GFV, Berry *et al* 1983), carp (carp gill necrosis virus, Wolf 1988), sheatfish (icosahedral cytoplasmic deoxyribovirus, Ahne *et al* 1989) and other species (erythrocytic necrosis virus, Wolf 1988; fish lymphocystis disease virus, Flugel 1985). Only EHNV and the sheatfish virus have been associated with epizootics in finfish.

Clinical EHNV infection is known to occur in Australia in southern NSW, Victoria and South Australia, but is unknown in other countries (Langdon *et al* 1986). As redfin perch were originally imported from England in 1861 (Cadwallader and Backhouse 1983) and the disease EHNV has only been recognised in recent years (Langdon and Humphrey 1987; Langdon 1989) there are serious questions concerning the origin and normal host of this virus.

The significance of EHNV to the Australian fisheries industries escalated dramatically with the report of outbreaks of a disease in cultured rainbow trout on two commercial hatcheries (Langdon *et al* 1988). A virus indistinguishable from EHNV was isolated from affected fish. Quarantine was imposed on the affected hatcheries and procedures for certification testing of salmonid fish were implemented. EHNV has proven to be a recurrent problem on infected hatcheries, necessitating continued quarantine, a ban on the sale of live fish and closure of a fish out facility.

Of teleosts studied to date, redfin perch appear to be the species most susceptible to EHNV. Langdon (1989) studied the susceptibility of other teleosts to EHNV and found that Macquarie perch *Macquaria australasica*, mosquito fish *Gambusia affinis*, silver perch *Bidyanus bidyanus*, and mountain galaxias *Galaxias olidus* were highly susceptible to experimental infection by a natural route of exposure. Murray cod *Maccullochella peeli*

was regarded as being moderately susceptible as it became infected after bath exposure and succumbed after injection of EHNV. Golden perch *Macquaria ambigua* and Australian bass *Macquaria novemaculeata* could only be infected by injection of EHNV and were regarded as being resistant to the virus.

The occurrence of EHNV on several salmonid hatcheries and its demonstrated virulence for other species, including several native species that are endangered or farmed commercially is a regrettable situation. As well as considerations of movement of potentially infected fish from these hatcheries to uninfected areas in Australia, there are important considerations of disease translocation to the fisheries industries of our overseas trading partners. Regulatory authorities in Australia have been required to accurately assess the distribution of EHNV on commercial hatcheries in Australia and to devise methods for certification of fish and fish products as being free of this infectious agent.

The only method of diagnosis currently available for EHNV is virus isolation and tentative identification by electron microscopy. This does not provide a definitive identification of a virus causing cytopathic effect and is time consuming, expensive, and inefficient. Other types of assay, for example, immunoassays, are routinely used in laboratory diagnosis and are advantageous due to their specificity, low cost, reagent stability, safety, sensitivity, and ease of procedure.

FIRDTF provided grants to NSW Agriculture & Fisheries (Elizabeth Macarthur Agricultural Institute) and CSIRO (Australian Animal Health Laboratory) to collaboratively develop efficient diagnostic tests for EHNV. This report documents research carried out by NSW Agriculture. A complementary report is being prepared by CSIRO.

OBJECTIVES OF PROJECT

The objectives of this project are:

1. To develop a rapid, cheap and accurate immunodiagnostic test for EHN virus (EHNV) infection.

2. To apply the test for diagnosis of EHNV and certification for freedom from the disease in salmonids.

3. Using the test, to determine the distribution of EHNV in salmonid fish and redfin perch in Australia.

4. To use immunodiagnostic methods for infectious diseases by applying the technology developed in 1. to formulate disease control strategies.

INTRODUCTORY TECHNICAL INFORMATION

Diagnosis of viral diseases of fish

There are several standard methods of confirming the presence and identity of viruses in animal populations and tissue samples. The methods are based on detecting the virus or its components in fish or by detecting the specific immune response of the fish to the presence of the virus. Both direct viral detection and assay of the immune response of fish were investigated in this project as means of diagnosis of EHNV.

Methods of detecting the presence of viruses

Direct detection of the virus in fish

1. Culture of the virus in living cells in tissue culture. The virus often may cause death or morphological change of the cells which is detected visually as a cytopathic effect (CPE). The identity of the agent causing CPE is confirmed using an immunological procedure such as a neutralisation or fluorescent antibody test.

2. Direct detection of the virus in tissue samples. This may be achieved by:

    a. Direct observation using electron microscopy, or

    b. Immunological tests. Reagents (antibodies) that specifically attach to the parts of the virus (antigens) can be labelled with a marker to enable their visualisation. Labels may be enzymes or fluorescent dyes. Common assays include the enzyme linked immunosorbent assay (ELISA) and the fluorescent antibody test (FAT).

    c. Molecular (DNA) tests. The nucleic acid of the virus may be detected using nucleic acid probes, polymerase chain reaction and other procedures. These methods will be addressed by CSIRO.

Detection of the immune response of the fish to the virus

Following infection by a virus, the immune system of the host may respond by secreting antibodies (immunoglobulins) which bind specifically to that virus. Antibodies can be detected using a variety of laboratory assays including:

1. Neutralisation assay
2. Double immunodiffusion assay
3. Enzyme linked immunosorbent assay (ELISA)

Of these assays, the ELISA is the most sensitive. Neutralisation assays for fish viruses are generally considered to be difficult or unreliable in comparison to similar assays in

mammals and were not investigated in detail in this project. Similarly, double immunodiffusion assays are less well suited to investigation of immune responses in fish.

Production of laboratory reagents for detection of EHNV

In order to carry out this work in the key species rainbow trout and redfin perch it was necessary to produce the necessary reagents, there being none available at the time the project commenced. These reagents can be classified according to the aim of the test:

Direct detection of EHNV

1. Rabbit antibodies specific for purified EHNV
2. Sheep antibodies specific for purified EHNV

Detection of the immune response of fish to EHNV

1. Rabbit antibodies specific for rainbow trout immunoglobulin (Ig)
2. Rabbit antibodies specific for redfin perch Ig
3. Sheep antibodies specific for redfin perch Ig

Pre-requisite information also needed to be generated to enable the above reagents to be manufactured:
1. Reliable methods for growth and purification of EHNV
2. The nature of immunoglobulins of the redfin perch, there being data only for rainbow trout

Following the production of the necessary reagents it was possible to formulate tests that efficiently detected EHNV directly in the tissues of infected redfin perch and rainbow trout and also to detect specific immune responses in these species. The technical basis of this work is described in detail in subsequent sections of this report.

RESEARCH METHODOLOGY, RESULTS AND DISCUSSION

SECTION 1 GROWTH AND PURIFICATION OF EHNV AND ITS DETECTION BY ELISA AND IMMUNOFLUORESCENCE

SECTION 2 IMPROVEMENT AND TECHNICAL ASSESSMENT OF THE ELISA FOR DIRECT DETECTION OF EHNV IN FISH TISSUES

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SECTION 5 STORAGE OF TISSUES AND *IN-VITRO* GROWTH CONDITIONS FOR DIAGNOSIS OF EHNV

SECTION 1

GROWTH AND PURIFICATION OF EHNV AND ITS DETECTION BY ELISA AND IMMUNOFLUORESCENCE

The aim of this work was to develop a protocol for growth and purification of EHNV and to evaluate the potential of solid phase, antigen-capture ELISA and other immunological assays for specific detection of the virus.

Materials and Methods

Growth and purification of EHNV

Confluent monolayers of BF-2 (Bluegill Fry, American Type Culture Collection CCL 91) cells were produced after 3-4 days incubation at 24°C in minimal essential medium (MEM) supplemented with 10% foetal calf serum (FCS), 100 U/ml penicillin, 100 ug/ml streptomycin and 2 ug/ml Fungizone. Confluent monolayers in 120 cm^2 tissue culture bottles were inoculated with EHNV strain 86/8774 stock virus at a multiplicity of infection of $1.2x10^{-3}$. After adsorption for 1 h at room temperature (RT) with rocking every 10 min, 100 ml of medium (as above with 2% foetal calf serum) was added. The bottles were incubated at 24°C until complete lysis of the cell monolayer was evident, typically after 3 days. Cells and media were centrifuged at 12,000 x g for 30 min at 4°C. The supernatant (SN1) was stored at 4°C while the pellet was resuspended in a small volume of SN1 and subjected to three rapid freeze/thaw cycles using liquid nitrogen and a 37°C water bath. Following ultrasonication for 3 one minute cycles at 4°C, the virus-cell debris suspension was centrifuged at 6,500 x g for 15 min at 4°C. The supernatant (SN2) was pooled with SN1 and stored overnight at 4°C, while the pellet was discarded. Virus was pelleted from the pooled supernatant by centrifugation at 10,000 x g for 8 h at 4°C and resuspended in 2 ml final volume of 100 mM Tris-HCl buffer, pH 8.6. The suspension was equally divided and loaded onto 2 pre-formed gradients of 15-60% W/V sucrose, and ultracentrifuged at 150,000 x g for 45 min at 4°C. Resulting bands were harvested separately by aspiration, diluted in 100 mM Tris-HCl buffer, pH 8.6, until the concentration of sucrose was about 10%, then pelleted through a cushion of 20% W/V sucrose at 80,000 x g for 1 h at 4°C. Pellets were resuspended in 200 µl of 100 mM Tris-HCl buffer, pH 8.6, and stored at 4°C.

Viral purity was assessed by electron microscopy, SDS-PAGE and Western blotting.

Electron microscopy

Gradient purified virus (10 µl) was negatively stained with an equal volume of 2% phosphotungstic acid pH 6.8 and applied to a parlodion coated copper grid for 30 sec then allowed to dry for 5 min, before being viewed with a Phillips 201 transmission electron microscope.

SDS–PAGE and Western blotting

SDS–PAGE was performed as previously described (Laemmli, 1970) using a 12% resolving gel and a 4% stacking gel. Molecular weight standards (Biorad, low range) were included with each gel. Proteins were stained with 0.2% coomassie brilliant blue (CBB) or transferred to Immobilon–P membrane (Millipore) using a Semiphor TE70 semi–dry transfer unit (Hoefer) at 100 mA constant current for 40 min. Transfer membranes were blocked with 1% gelatin in Tris–saline buffer (TS) (100 mM Tris–HCl pH 7.4, 0.15 M NaCl) for 30 min at RT, before being washed for 3 x 10 min in wash buffer (WB) (TS containing 0.05% Tween 20). Rabbit or sheep antiserum was then reacted for 2 h, followed by washing in WB and reaction of swine anti–rabbit (Dako) or donkey anti–sheep (Silenus) horse radish peroxidase conjugates as appropriate for 2 h. After final washing in WB, bound conjugate was demonstrated with 4–chloro–1–naphthol (Biorad) and H_2O_2 substrate.

Production of antisera in rabbits and sheep

Antiserum to BF–2 cells BF–2 cells (6.46×10^6) were harvested following trypsinisation and washed twice with phosphate buffered saline (PBS), pH 7.2. The cell pellet was resuspended in a final volume of 1.5 ml PBS, pH 7.2, ultrasonicated for 3 one min cycles to give a BF–2 cell lysate, then emulsified with 1.5 ml of Freund's complete adjuvant (Sigma) (FCA) and stored at 4°C. This preparation was injected into a rabbit (intramuscularly) (0.75 ml) at each of two sites in the hind legs and immunisation was repeated after 3 and 6 weeks by subcutaneous injection of 0.8 ml of the same preparation.

Anti – EHNV antisera Sucrose gradient purified EHNV (B2 – see results) was suspended in 100 mM Tris–HCl, pH 8.6, 0.15 M NaCl buffer and emulsified in an equal volume of FCA. This was administered subcutaneously to a sheep and a rabbit that had been selected on the basis of minimum reactivity against EHNV antigens by Western blotting of prebleed serum. The sheep was given a total of 190 µg of viral protein in 2 ml of vaccine (0.5 ml at each of four sites); this was repeated after 3 weeks. The rabbit was given a total of 143 µg of viral protein (0.5 ml at three sites) in the first dose and 190 µg of viral protein (0.5 ml at four sites) in the second dose, 3 weeks later. Immune serum was designated as anti–B2 serum.

Blood was collected prior to immunisation and thereafter at fortnightly intervals, allowed to clot for 2 h at RT, centrifuged at 2000 rpm for 15 min and serum was harvested and stored at –20°C. Antibody production was evaluated by agarose gel immunodiffusion against the appropriate antigen.

Antigen–capture ELISA

Microtitre plates (Linbro) were coated with 100 µl/well of rabbit–anti–B2 serum in borate coating buffer, pH 8.4 (100 mM boric acid, 25 mM disodium tetraborate, 75 mM NaCl), incubated for 16 h at 4°C, then washed 5 times in distilled water + 0.05% Tween 20

(DWT). Remaining free binding sites were blocked with 100 µl/well of 1% W/V ovalbumin (Sigma) in PBS + 0.05% Tween 20 (PBST) for 30 min at RT. After washing in DWT, EHNV antigen and controls were added (100 µl/well) and incubated for 90 min at RT. After washing, adsorbed (see results) sheep-anti-B2 serum diluted in PBST + 0.1% ovalbumin (PBSTO) was added (100 µl/well) and incubated for 90 min at RT. After washing in DWT, monoclonal mouse-anti sheep IgG conjugated to horse radish peroxidase (Selenus Laboratories, Australian Monoclonal Developments Bi2) diluted 1:500 in PBSTO was added (100 µl/well) and incubated for 90 min at RT. After washing, 100 µl/well of 1 mM 2,2'-azino-di (3-ethyl-benzthiazolin-6-sulphonate) (ABTS) in 100 mM citrate phosphate pH 4.2, 2.5 mM hydrogen peroxide was added. The reaction was stopped after 20 min at RT by the addition of 50 µl of 0.01% sodium azide in 0.1 M citric acid and optical density (OD) was measured at 405 nm.

Preparation of viral antigen for ELISA

A mature 170 mm redfin perch, maintained in a non-recirculating aquarium was injected intraperitoneally with $2x10^{3.6}$ $TCID_{50}$ of EHNV strain 86/8774 according to the method of Langdon (1989). After the fish succumbed to infection, organs were removed aseptically, homogenised, clarified by centrifugation and inoculated onto BF-2 cell monolayers incubated at 24°C. Culture supernatants were pooled.

Indirect immunofluoresence assay for detection of EHNV in cell culture

BF-2 cells grown in tissue culture chamber slides were inoculated with EHNV culture supernatant. After appearance of CPE, cells were fixed and incubated with RxB2 serum. After washing and incubation with FITC conjugated swine anti-rabbit immunoglobulin, slides were viewed with a UV microscope.

Immunofluoresence assay for detection of EHNV in tissue sections

Formalin fixed tissues from experimentally infected fish were placed on gelatin coated slides and incubated with RxB2 serum followed by FITC conjugated swine anti-rabbit immunoglobulin. Slides were viewed with a UV microscope.

Titration of EHNV

Titration of EHNV was performed in BF-2 cells. End points were calculated using the method of Reed and Muench (1938).

Determination of protein concentration

The coomassie blue dye binding method of Bradford (1976) was used to measure protein concentration. Calibration curves were prepared using bovine serum albumin (Sigma).

Results

Growth and purification of EHNV

Total virus yield (per 10 120 cm^2 culture bottles) from culture supernatant plus that released by physical disruption of cells was $10^{7.9}$ $TCID_{50}$. Two prominent bands were obtained on 15–60% sucrose gradients after ultracentrifugation. A diffuse band (B1) was located at about 40% sucrose concentration, while a discrete band (B2) was located at about 50% sucrose concentration. B1 yielded $10^{6.13}$ $TCID_{50}$ total virus (0.45 mg total protein) while B2 yielded $10^{5.38}$ $TCID_{50}$ total virus (0.15 mg total protein).

Variations to the protocol described in Materials and Methods were assessed. In pelleting virus from pooled supernatant, longer low speed spins (10,000 x g for 8 h) resulted in greater yield of virus than shorter high speed spins (35,000 x g for 45 min), which appeared to result in clumping of the virus, causing it to move straight through subsequent gradient centrifugation steps. Pelleting of viral particles onto a 60% sucrose cushion was attempted to reduce clumping, followed by dialysis and concentration of virus with carboxymethyl cellulose. This was, however, relatively unsuccessful. Sonication of viral suspensions after high speed pelleting steps was also unsuccessful in dislodging clumped virions and appeared to disrupt virus particles as they would no longer form discrete bands on gradients. Although 10–40% sucrose gradients gave similar yields to 15–60% gradients, the bands were better separated and more discrete in the 15–60% gradients and thus easier to collect. Excessive handling of the virus also seemed to reduce yields. If gradient bands were rerun on fresh gradients, the resulting bands were reduced greatly in density.

The purity of the virus recoverd from sucrose gradients was assessed by electron microscopy, SDS–PAGE and Western blotting. In electron micrographs, B1 contained viral particles together with amorphous material, membranous structures and other cell debris, while B2 contained viral particles and was apparently free of other material (Fig 1.1).

B1, B2 and BF–2 cell lysate were analysed by SDS–PAGE (Fig 1.2). B2 was resolved into approximately 8 major bands and 5–7 minor bands. These bands were also present in B1, however, B1 had many additional bands. BF–2 cell lysate was resolved into innumerable bands, very few of which appeared to be common to B2, while many were common to B1.

In Western blots, rabbit anti–BF–2 immune serum recognised a host of antigens in BF–2 cell lysate and B1. In B2, however, only 2 bands were recognised faintly (Fig 1.3). Pre–immune serum from this rabbit failed to detect any antigens in BF–2 cell lysate, B1 or B2 (data not shown). These data confirmed that B2 consisted predominantly of EHN virus.

Production and assessment of anti-viral antisera

Pre-immune serum from 3 sheep faintly recognised some antigens in BF-2 cell lysate, B1 and B2 in Western blots, however, no pre-existing recognition of these antigens was found in 2 rabbits (data not shown). The sheep with least pre-immune reactivity and one rabbit were subsequently immunised with B2. Agarose gel precipitation reactions of sheep and rabbit serum against B2 were positive 5 weeks after the first inoculation, while reactions against BF-2 cell lysate remained negative. In Western blots, rabbit and sheep anti-B2 serum reacted strongly with antigens in B1 and B2 (Figs 1.4 and 1.5). In addition, sheep anti-B2 serum recognised a number of antigens in BF-2 cell lysate (Fig 1.5). These data indicate that rabbit anti-B2 serum was specific for EHNV while sheep anti-B2 sera contained antibodies with reactivity for BF-2 cells.

Antigen-capture ELISA

Chequerboard titrations of capture antibody (rabbit anti-B2 serum), viral antigen ($10^{6.8}$ $TCID_{50}$/ml in undiluted stock) and second antibody (sheep anti-B2 serum) were undertaken. Capture antibody and second antibody were used at a dilution of 1:200 in subsequent experiments. Significant reactivity of BF-2 cell lysate as antigen was detected, however, there was no reactivity in other control wells (second antibody with capture antibody, or conjugate with viral antigen).

Due to the reaction caused by BF-2 cell lysate, diluted sheep-anti-B2 serum was pre-adsorbed with cell culture/media ingredients (1900 µl sheep anti-B2 serum at 1:200 + 100 µl media or foetal calf serum), BF-2 cell lysate (1900 µl sheep anti-B2 serum at 1:200 + BF-2 cell lysate, 0.17 mg protein in 100 µl) or BF-2 cell lysate plus MEM or FCS (1900 µl sheep anti-B2 serum at 1:200 + BF-2 cell lysate, 0.09 mg protein in 50 µl + 50 µl MEM or FCS). Results are given in Table 1.1. Reactivity against BF-2 cell lysate was markedly reduced by pre-adsorption with BF-2 cell lysate, BF-2 cell lysate + MEM and BF-2 cell lysate + FCS, but not by MEM or FCS alone. No reactivity of MEM or FCS was observed.

The sensitivity of the ELISA format was estimated by titrating viral antigen (Fig 1.6). The background OD of undiluted BF-2 cell lysate was 0.18. The OD dropped to a level approaching background when virus was diluted to a concentration below $10^{5.8}$ $TCID_{50}$/ml.

Immunofluorescence assays

Experiments indicated a favourable role for indirect immunofluorescence in the detection of EHNV in cell culture. In addition, EHNV was demonstrated in the liver and kidney of experimentally infected redfin perch. High levels of background staining have confounded attempts to use this technique in rainbow trout to date.

Discussion

EHNV is a large (148-167 nm) icosahedral virus believed to be a member of the Family Iridoviridae (Langdon et al, 1986). Iridoviruses, which are known from a number of invertebrates, fish and amphibians, are a diverse group ranging in size from the small

insect iridoviruses (130 nm) to fish lymphocystis disease virus (300 nm) (Devauchelle et al, 1985). Protocols for the purification of viruses are often based on the properties of size and mass of the virus. Frog virus 3 (FV3) is a vertebrate Iridovirus of similar size to EHNV. FV3 has been extensively studied and purification protocols have been described by several authors (Tan and McAuslan, 1971; Aubertin et al, 1973; Braunwald, 1979; Aubertin et al, 1980). We adapted these protocols, modifying centrifugation intensity and time to maximise yield, for purifying EHNV. A high degree of purity was desired in order to produce specific anti-EHNV antisera for use in immunoassay.

EHNV was separated from cellular material by cell disruption, differential centrifugation and density gradient centrifugation in sucrose. Efforts to achieve further purification by physical means resulted in dramatic reductions in yield and were therefore not undertaken in preparative experiments. Two bands were obtained in sucrose gradients; the lower band (B2) contained virions, with only traces of cellular antigen being detected in Western blots with anti-BF-2 serum. This B2 material was subsequently used to immunize a rabbit and a sheep. While the upper band (B1) also contained virions, a considerable amount of cellular material was detected by electron microscopy, SDS-PAGE, and Western blotting with anti-BF-2 cell serum. The association of infective virus with cell debris in B1, after the application of disruptive physical forces during viral purification, suggests that EHNV is highly associated with cell constituents.

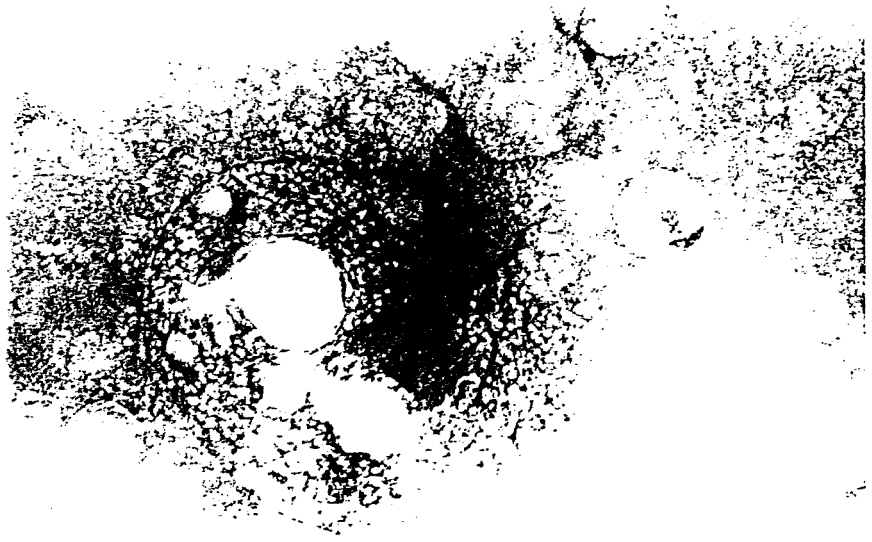
Rabbit and sheep antisera against B2 were assessed by Western blotting of purified B2 virus and BF-2 cells. The sheep antiserum recognised a greater range of viral antigens compared to the rabbit antiserum, but BF-2 cell antigens were also recognised by the sheep antiserum. Three of 3 sheep screened prior to immunisation recognised both cellular and viral antigens. Immunisation of one of these sheep appeared not to greatly increase reactivity with cellular antigens in Western blots, while recognition of viral antigens was heightened. The rabbit-anti B2 serum did not react against BF-2 cells. These results indicated that B2 EHNV was substantially free of contaminating cellular antigen and that immunocapture of EHNV would be more specific if rabbit anti-B2 was used. Further, use of sheep anti-B2 serum in immunoassay would be likely to require pre-adsorption with BF-2 cell antigens.

An indirect ELISA formatted with rabbit anti-B2 serum as capture antibody and sheep anti-B2 serum as second antibody successfully detected EHNV antigen in cell culture supernatant. When diluted sheep-anti-B2 serum was pre-adsorbed with BF-2 cell lysate, the OD in BF-2 cell control wells was markedly reduced, thus increasing the specificity of the assay. The same approach was used by Hsu and Leong (1985) and McAllister and Schill (1986) to reduce cross reactivity in immunoassay to detect other viral pathogens of fish.

Further development of the ELISA and immunofluorescence assays will be required to achieve greater sensitivity and specificity. Improvement of the ELISA is described in the next section of this report. The high degree of specificity of rabbit anti-EHNV antisera produced during this study will enable the development of other immunoassays for use in rapid diagnosis of EHNV.

Fig. 1.1. Electron micrograph of sucrose gradient purified EHNV. (a) Band 1 (b) Band 2. Magnification x 43,000.

(a)



(b)

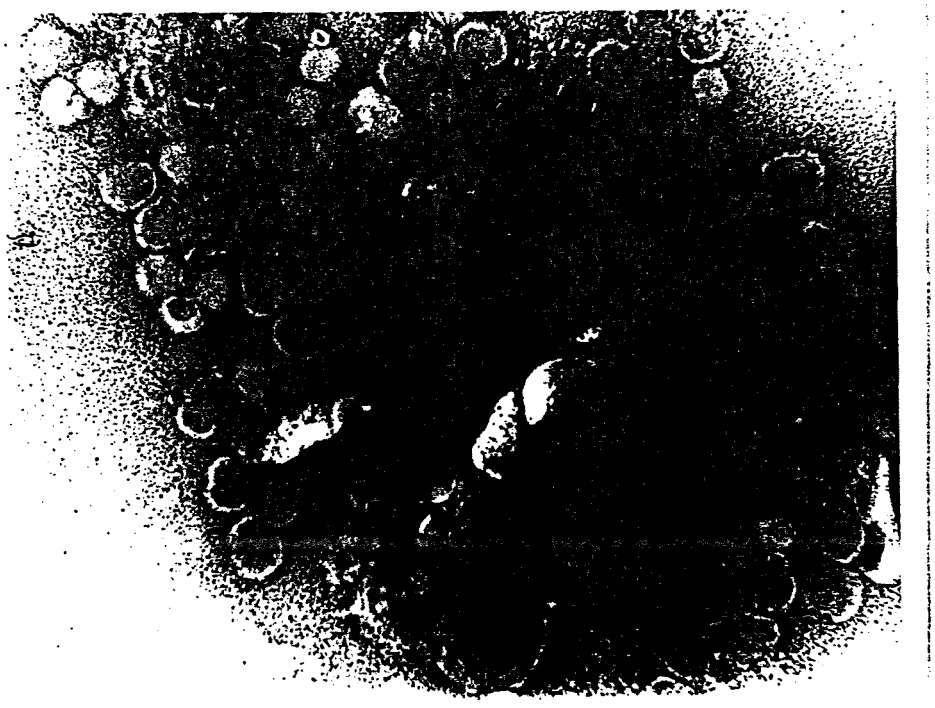


Fig. 1.2. SDS-PAGE analysis of BF-2 cell lysate (lane 2), sucrose gradient purified EHNV Band 2 (lane 3) and sucrose gradient purified EHNV Band 1 (lane 4); molecular weight markers (lane 1).

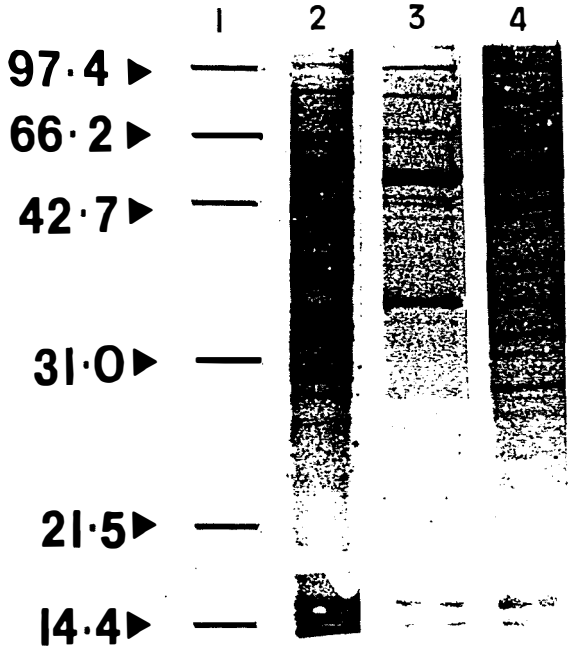


Fig. 1.3. Western blot analysis of the same material as Figure 2, using rabbit anti BF-2 serum.

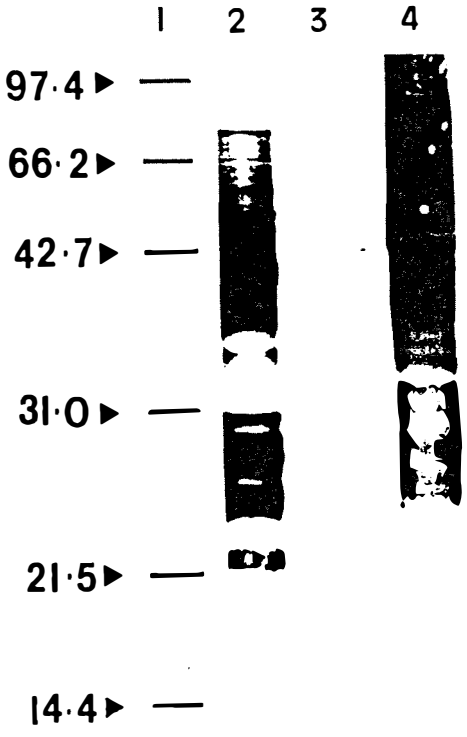


Fig. 1.4. Western blot analysis of the same material as Figure 2, using rabbit anti B2 serum.

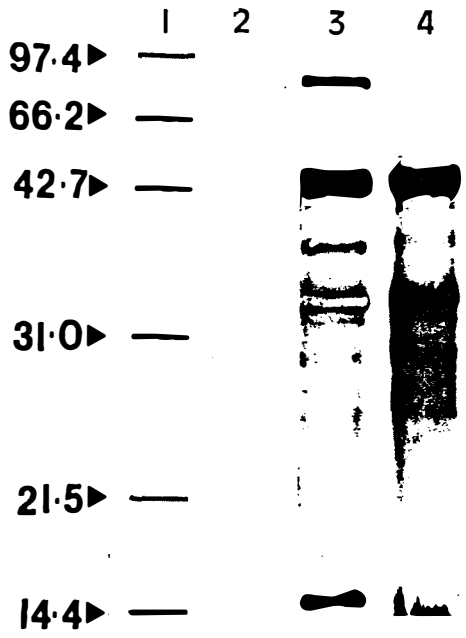


Fig. 1.5. Western blot analysis of the same material as Figure 2, using sheep anti B2 serum (pre–bleed, lanes 2–4; immune bleed, lanes 6–8).

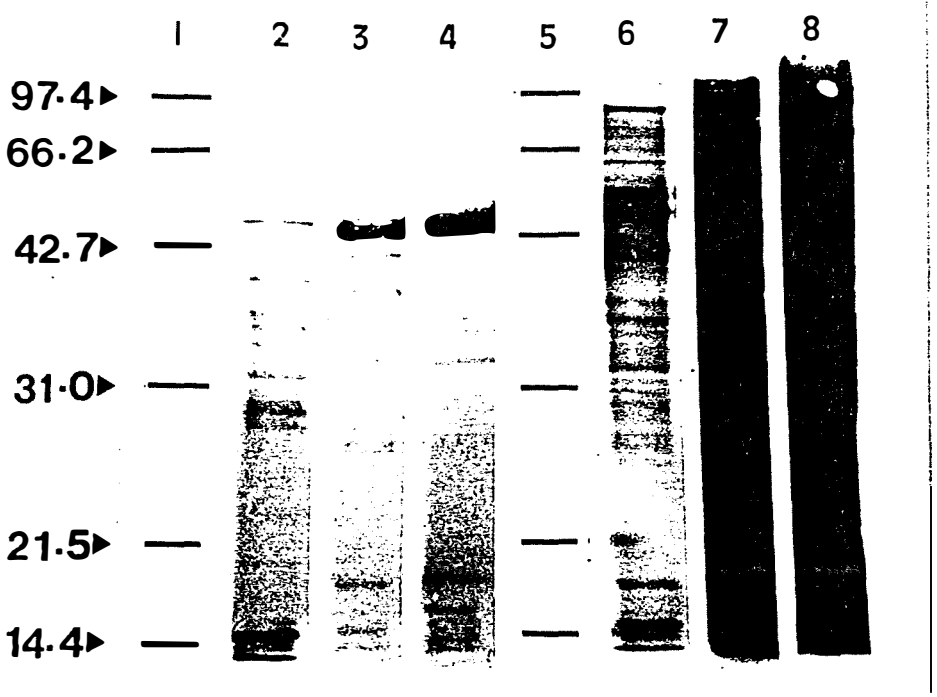


Fig. 1.6. Titration of EHNV viral antigen in cell culture supernatant using antigen–capture ELISA in which the second antibody was pre–adsorbed with BF–2 cell lysate.

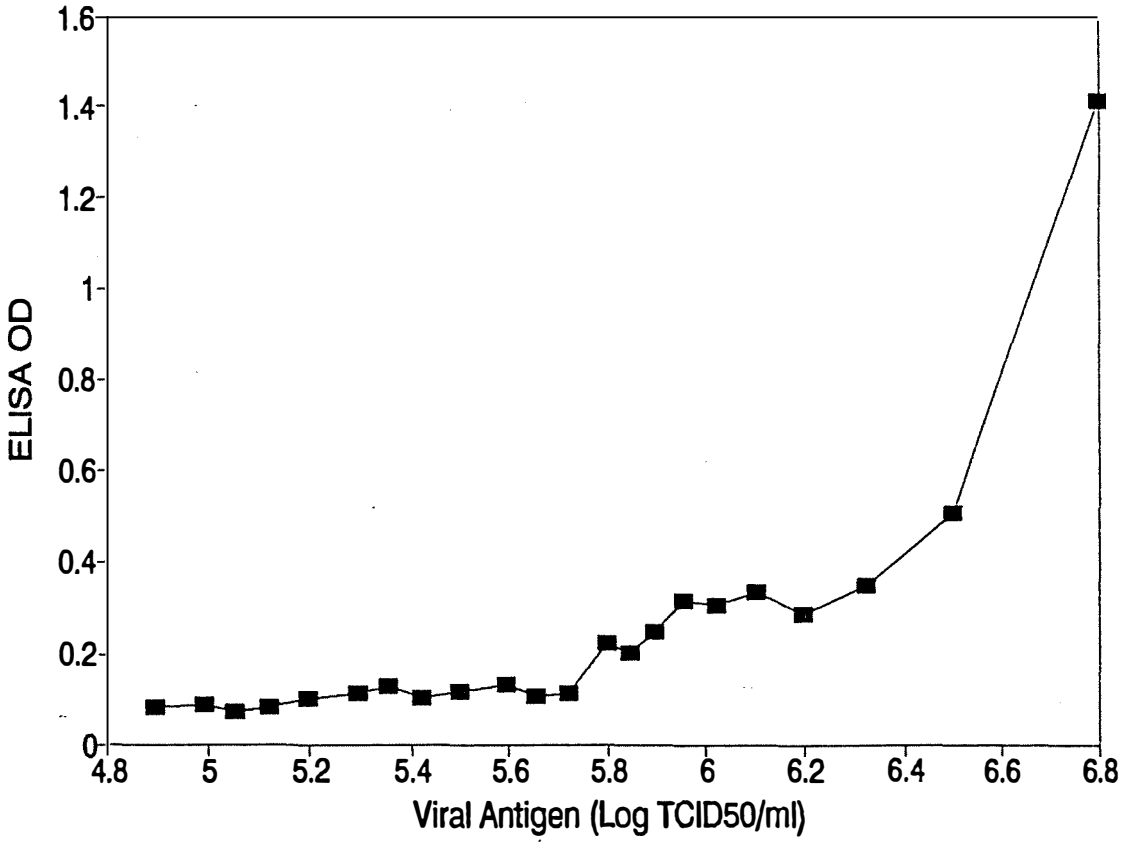


Table 1.1

The effect of pre-adsorption of diluted sheep anti-B2 serum (second antibody) on non-specific binding in antigen-capture ELISA for EHNV. MEM - minimal essential medium; FCS - foetal calf serum; BF-2 - Bluegill fry cell lysate.

Adsorption agent:	Nil	MEM	FCS	BF-2	BF-2 +MEM	BF-2 +FCS
Antigen	ELISA OPTICAL DENSITY					
Virus	0.92	0.56	0.53	1.02	1.01	0.86
BF-2 neat	0.69	0.52	0.45	0.16	0.20	0.25
BF-2 1:10	0.10	0.09	0.09	0.08	0.09	0.09
MEM neat	0.08	0.08	0.07	0.07	0.08	0.08
FCS neat	0.08	0.07	0.07	0.07	0.08	0.08

SECTION 2

IMPROVEMENT AND TECHNICAL ASSESSMENT OF THE ELISA FOR DIRECT DETECTION OF EHNV IN FISH TISSUES

Introduction

Following the development of a prototype ELISA for the detection of EHNV in tissue culture supernatants (Section 1, this report) it was desirable to increase the sensitivity of the assay to facilitate its use with infected tissues where fewer viral particles may exist than in culture supernatants. The results of experiments aiming to improve the prototype ELISA are described in this section.

For direct examination of tissue samples from fish there must be an efficient method for releasing viral antigen from those tissues. Tissue homogenisation in a blender or mortar and pestle is a classic method of achieving release of virus particles (Dixon and Hill, 1983; Langdon et al, 1986), but is time consuming, expensive and relatively impractical when large numbers of samples need to be processed together, a common event in the diagnosis of fish diseases. Other methods of releasing EHNV from tissues were developed and assessed during this study. A statistical evaluation of the improved ELISA procedure on a large number of tissue samples is then given.

Materials and methods

Preparation of rabbit and sheep antisera

Sheep anti-EHNV (SxB2), rabbit anti-EHNV (RxB2), rabbit anti-bluegill fry (BF-2) cell (RxBF-2) and rabbit anti-rainbow trout gonad cell (RxRTG) antisera were prepared according to methods described in Section 1 (this report).

Affinity purification of rabbit antisera

Protein A Sepharose CL-4B (Pharmacia) (1.5 g) was swollen in 20 ml of starting buffer (25 mM Tris-HCl, pH 7.2, 0.15 M NaCl, 0.01% sodium azide), for 15-20 min. The swollen gel was poured smoothly into an emptied PD-10 column (Pharmacia) and packed using 150 ml of starting buffer. Serum (1-2 ml) was centrifuged at 3000 g for 10 min in a microcentrifuge. The supernatant was mixed with an equal volume of starting buffer, loaded onto the column and washed through with 25 ml of starting buffer at a flow rate of 10 ml/hr. Immunoglobulins (Ig) were eluted with 30 ml of 0.1 M glycine-HCl pH 3.0, 0.15 M NaCl, at a flow rate of 30 ml/hr. Fractions of 2.5 ml were collected using an LKB fraction collector and immediately neutralised by the addition of 35 ul of 2 M Tris. Pooled fractions were dialysed overnight against starting buffer, and concentrated by dialysis

against Aquacide II (Calbiochem) to the original volume of serum. Purification of Ig was confirmed by SDS-PAGE. Purified Ig were diluted 1:10 in 25 mM Tris-HCl pH 7.4, 0.15 M NaCl, 50% glycerol and stored at -20°C. Purified antiserum was termed affinity purified rabbit anti-B2 immunoglobulin (RxB2 Ig).

Antigens for ELISA evaluation

Pooled 2-7 day old EHNV culture supernatants in BF-2 cells (EHNV $TCID_{50}$ $10^{6.47}$/ml) and BF-2 cell lysate (1-1.5 mg total protein/ml) (Section 1, this report) were used as control antigens in all experiments unless otherwise stated.

Basic ELISA protocol

The prototype ELISA used in experiments for detection of EHNV was described in Section 1 (this report). Chequerboard board titrations of capture antibody, second antibody and conjugate were performed to optimise the concentrations of each reagent used in the basic protocol.

Configuration and Capture antibody

The effect of reversing the type of capture and second antibodies was assessed. SxB2 serum was coated to a plate overnight at 4°C. Viral antigen was used neat. RxB2 serum or affinity purified-RxB2 Ig was used as second antibody with swine anti-rabbit-Ig-HRP (Dako) at a dilution of 1:500 as conjugate.

Affinity purified-RxB2 Ig was titrated as capture antibody against viral antigen. The efficiency of detection of viral antigen without a capture antibody and using 1:800 dilutions of RxB2 serum or affinity purified RxB2 Ig as capture antibody were compared.

Comparison of blocking agents

After overnight incubation of capture antibody and washing, the plate was blocked with either: 1% w/v ovalbumin in PBS/0.05% Tween 20 (PBST), 1% w/v gelatin in PBST, 1% w/v skim milk in PBST, or control with no block. After incubation for 30 min at RT and washing, antigen was added.

Comparison of conjugates

The following conjugates were compared at their optimal dilutions in titration against antigen: polyclonal rabbit anti-sheep IgG conjugated to horse radish peroxidase (HRP) (KPL-HRP, KPL Laboratories); monoclonal mouse anti-bovine IgG conjugated to HRP (AMD Bi2-HRP, Silenus Laboratories); monoclonal mouse anti-bovine IgG conjugated to alkaline phosphatase (AP) (AMD Bi2-AP, Silenus Laboratories); monoclonal mouse-anti-sheep Ig conjugated to HRP (ARIMAB-HRP, EMAI Microbiology and Immunology Laboratory), and; monoclonal mouse-anti-sheep Ig conjugated to AP (ARIMAB-AP,

EMAI Microbiology and Immunology Laboratory). The chromogen 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) was used with HRP conjugates while alkaline phosphatase nitrophenyl phosphate (PNPP) was used with AP conjugates. To develop PNPP substrate, the plate was incubated in the dark for 30 min prior to stopping the reaction with 3M NaOH and reading at 405 nm.

Comparison of HRP chromogens

The chromogens ABTS, tetramethylbenzidine (TMB) and o-phenylenediamine (OPD) were evaluated by titration against antigen with HRP conjugates used at their optimal dilution. To develop TMB substrate the plate was placed in the dark for 10 min, the reaction stopped with an equal volume of 1 M H_2SO_4, and read at 450 nm. To develop OPD substrate, the plate was shaken for 15 min in the dark, the reaction stopped with 0.5 volume of 2.5 M H_2SO_4, and read at 495 nm.

Comparison of ELISA protocols

The original format of the ELISA (Section 1, this report) and the format derived after further experimentation were evaluated on the same plate by titration of viral antigen. The culture supernatant contained $10^{6.47}$ $TCID_{50}$/ml prior to dilution.

Preparation of EHNV infected tissue

A mature 27 cm forklength redfin perch was infected with EHNV (strain 86:8774) by bath exposure (1 x 10^0 $TCID_{50}$/ml) on day 1 and intraperitoneal injection ($10^{5.05}$ $TCID_{50}$) on day 16. The fish died on day 18. Multifocal hepatic necrosis was observed grossly and microscopically in the liver which was sliced into 2 mm^3 pieces. These were mixed well, divided into 8 groups of about 0.4 g total weight each and frozen at -20°C until required.

Protocols for release of EHNV from tissues

Homogenisation

Liver (0.4 g) was roughly minced with fine scissors in a 10ml glass homogeniser and 2.5 ml of PBS was added. The suspension was homogenised on a laboratory blender for 30 seconds and then transferred to a 10 ml glass tube.

Nonidet P-40 (NP-40)

Liver (0.2 g) was roughly minced with fine scissors in a 10 ml glass tube and 1.25 ml of PBS with 1% NP-40 was added to the tube and mixed.

Sonication with large glass beads

Liver (0.2 g) was roughly minced with fine scissors in a 10 ml glass tube, 1.25 ml of PBS and five 3 mm diameter glass beads were added. The suspension was sonicated for three 1 min cycles on ice.

Grinding

Liver (0.2 g) was roughly minced with fine scissors in an eppendorf tube and ground with a fitted pestle (Edwards Instrument Company). PBS (1.25 ml) was added and mixed.

Snap freezing and grinding with small glass beads

Liver (0.2 g) was roughly minced with fine scissors in an eppendorf tube, snap frozen in liquid nitrogen with 0.94 mg of 106 um diameter glass beads and then ground with a fitted pestle. PBS (1.25 ml) was added and mixed.

Grinding and large glass beads in a plastic tube

Liver (0.2 g) was roughly minced with fine scissors in an eppendorf tube and ground with a fitted pestle. PBS (1.25 ml) and three 3 mm diameter glass beads were added and mixed.

Grinding and small glass beads in a plastic tube

Liver (0.2 g) was roughly minced with fine scissors in an eppendorf tube and ground with a fitted pestle. PBS (1.25 ml) and 0.75 mg/ml of 106 um glass beads were added and mixed.

Grinding and large glass beads in a glass tube

Liver (0.2 g) was roughly minced with fine scissors in an eppendorf tube and ground with a fitted pestle. The suspension was transferred to a 10 ml glass tube, PBS (1.25 ml) and three 3 mm diameter glass beads were added and mixed.

The resulting suspensions were placed at 4°C and vigorously vortexed for 20 sec every 30 min for 2 hr. Debris was pelleted by centrifugation at 900 g for 10 min. Supernatants were transferred to fresh eppendorf tubes and stored at −20°C.

Supernatants were diluted and evaluated using the improved ELISA protocol (see results).

Selection and processing of specimens for evaluation of the sensitivity and the specificity of the improved ELISA

Tissues from infected and uninfected rainbow trout and redfin perch were evaluated for the presence of EHNV using both virus isolation in BF−2 cell monolayers and the improved ELISA protocol. BF−2 cell culture supernatants from each inoculation were also evaluated by ELISA.

Selection and testing of infected fish

Rainbow trout fingerlings were collected from natural outbreaks of EHN on a commercial hatchery. Redfin perch were collected from natural outbreaks of EHN in impoundments in NSW and the ACT as well as from two experiments where fish were infected by bath exposure to EHNV.

Tissues (liver, alone or pooled with kidney, spleen and gonad) of individual fish from

which EHNV was detected by the development of cytopathic effect following inoculation of BF–2 cell culture monolayers were tested using the improved ELISA protocol.

Selection and testing of uninfected fish

Clinically healthy fish from populations believed to be free of EHNV were selected. Rainbow trout were obtained from the Key Centre for Teaching and Research in Aquaculture, University of Tasmania, and from Gaden hatchery, Jindabyne, NSW. EHNV is unknown in Tasmania and has not been recovered from Gaden hatchery during annual certification tests by virus isolation since 1988. Redfin perch were obtained from Khancoban Pondage, NSW and Googong Reservoir, ACT where EHN has not been observed.

Tissues (liver, alone or pooled with kidney, spleen and gonad) from 1 to 5 fish were pooled and tested for the presence of EHNV by inoculation of BF–2 cell monolayers with negative results after 3 passes. The same tissues of individual fish were tested using the improved ELISA protocol.

Tissue processing for virology and ELISA

Organs were removed aseptically from freshly killed or frozen fish and stored in duplicate in sterile plastic tubes at −20°C until required. Approximately 0.1 g of tissue was transferred to an eppendorf tube and ground with a fitted pestle. Homogenising medium (Minimal Essential Medium with 200 U/ml penicillin, 200 ug/ml streptomycin, 4 ug/ml amphotericin B) (1.0 ml) was added together with three 3 mm diameter glass beads. The suspension was vortexed vigorously as described above and the supernatant after centrifugation was termed tissue homogenate. A 200 ul volume of each homogenate was inoculated neat, in duplicate, into tissue culture tubes (Kimex, Flow) containing a monolayer of BF–2 cells, and 200 ul of homogenate diluted 1:10 in homogenising media was inoculated into a third tube. Tubes were incubated at 22°C and checked periodically until day seven. If no cytopathic effect developed the tubes were frozen overnight and 100 ul from each tube was passed to fresh tubes of BF–2 cells. This was repeated again if CPE was suspected but not clearly observed.

Homogenates were stored at −20°C after inoculation of cell cultures for use in ELISA. Homogenates of samples received after November, 1991 were tested neat and at a 1:10 dilution in PBST with 0.1% W/V ovalbumin. The dilution was carried out in the microtitre plates using an eight channel pipette. Eight controls were used on each ELISA plate: a tissue culture supernatant diluted 1:10, 1:100, 1:200, 1:500, 1:1000 and 1:10000, and two rainbow trout liver homogenates determined to be free of EHNV by virus isolation and polymerase chain reaction (PCR) (PCR carried out at CSIRO, Australian Animal Health Laboratory, Geelong).

Analysis of results

The sensitivity of the ELISA was calculated from individually tested samples where

virus isolation on these individual samples was positive in the first, second or third pass in BF–2 cells. The specificity of the ELISA was calculated from individually tested samples from EHNV free populations where virus isolation was negative on pools of 1–5 fish. The data were analysed using Minitab statistical software on an IBM computer. Frequency distributions of the OD results from infected and non–infected populations were determined in order that the specificity and sensitivity of the ELISA could be determined. Comparisons of ELISA results from infected and uninfected populations were made with Student's t test. The individual data used for these analyses is listed in Appendix A.

Rapid ELISA

Affinity purified RxB2 was coated to a plate for 2 hrs at RT. BF–2 cell culture supernatants containing EHNV were added in duplicate, along with MEM as a negative control, and left for 30 min. Second antibody and conjugate were incubated for 15 min. ABTS chromogen was reacted for 20 min. The plate was washed three times between each of the above steps. A block was not used.

In a second experiment, a plate that had been pre–coated by overnight incubation with affinity purified RxB2 was used. After blocking for 30 min, antigen was added and incubated for 45 min. Second antibody, conjugate and substrate were reacted for 40 min, 70 min and 12 min respectively.

Reduction of background reactions detected in ELISA of rainbow trout tissue homogenates

Several means of reducing the background OD of EHNV negative rainbow trout tissue homogenates were investigated. Attempts were made to block rainbow trout tissue and BF–2 cell reactivity by including RxRTG cell and RxBF–2 cell antisera in the second antibody solution to compete for binding sites. The following second antibody solutions were used: SxB2 serum 1:400; SxB2 serum 1:400 + RxBF–2 cell serum 1:200; SxB2 serum 1:400 + RxBF–2 cell serum 1:400; SxB2 serum 1:400 + RxBF–2 cell serum 1:800 and; SxB2 serum 1:400 + BF–2 cell lysate.

RxRTG cell and RxBF–2 cell antisera were used also as a separate blocking step incubated for 90 min, after incubation and washing of antigen. The blocks were: RxBF–2 cell serum used at 1:100, 1:200, and 1:400; RxRTG cell serum used at 1:100, 1:200, and 1:400; RxBF–2 cell serum + RxRTG cell serum, with both sera used at 1:100, 1:200, and 1:400 respectively, and control with no block.

In order to investigate the possibility that anti–mycobacterial antibodies inherent in the capture and second antibody reagents by virtue of use of Freund's complete adjuvant in immunisation protocols may be responsible for background reactivity, the second antibody SxB2 serum was diluted 1:400 in a 0.6 mg/ml suspension of *Mycobacterium phlei* in PBS/0.05% Tween 80/0.1% gelatin, incubated overnight at 4°C, then used in comparison with non–adsorbed SxB2 immune serum.

Results

Titration of all reagents

Titration of reagents present in the original ELISA format (Section 1, this report) did not significantly increase the sensitivity of the assay, but did enable the amount of immune serum being used in each assay to be decreased. The optimal dilutions based on signal to noise ratios (OD viral antigen/OD cell antigen) were 1:800 for RxB2 immune serum (capture antibody), 1:400 for SxB2 immune serum (second antibody) and 1:500 for AMD Bi2–HRP conjugate. Unless otherwise stated these dilutions were used in subsequent experiments.

Configuration of the ELISA and nature of the capture antibody

RxB2 serum was more efficient as capture antibody than SxB2 serum (Table 2.1 and 2.2). In chequerboard titration, RxB2 serum consistently resulted in greater OD and S/N than SxB2 serum. With RxB2 serum as capture antibody the optical densities increased with increasing capture antibody dilutions to 1:400 indicating a prozone effect (Table 2.1). Optical densities remained stable with increasing SxB2 serum second antibody dilutions to 1:400, but at 1:800 the optical densities started to fall.

Use of affinity purified RxB2 Ig as capture antibody resulted in increased sensitivity. OD plateaued at a dilution of 1:3200–1:6400. In contrast to results for RxB2 serum, no prozone effect was evident (Table 2.3). Although it was possible to detect EHNV antigen without using a capture antibody by directly coating antigen (culture supernatant) to the plate, the sensitivity of the procedure was low (Table 2.4). RxB2 serum enabled detection of antigen to 1:20, while affinity purified RxB2 Ig enabled detection of antigen to 1:400. The cell background reaction was about 25% greater where no capture antibody was used.

Comparison of blocking agents

A blocking agent was necessary to reduce non–specific binding of antigenic components to the microtitre plate. The OD of BF–2 cell lysate control antigen was 25% greater in the absence of a blocking agent. There was no difference in the efficacy of 1% W/V ovalbumin, gelatin or skim milk as blocking agent (Table 2.5).

Comparison of conjugates

Polyclonal conjugate (KPL–HRP) used at an optimal dilution of 1:4000 resulted in greater sensitivity than monoclonal conjugate AMD Bi2–HRP used at its optimal dilution of 1:500 in detection of EHNV in culture supernatant, however, reactivity with BF–2 cells was greater and signal noise ratios were consequently slightly lower with the polyclonal conjugate (Table 2.6). Although the conjugates were similar, the monoclonal conjugate was chosen for further evaluation because of its greater potential specificity.

Different enzymes conjugated to the same anti–ruminant monoclonal antibodies were compared. Both conjugates of AMD Bi2 resulted in greater sensitivity compared to ARIMAB conjugates. Use of AMD–HRP appeared to result in greatest assay sensitivity

(Figure 2.1).

Comparison of HRP chromogens

At high concentrations of antigen, ABTS was the most sensitive chromogen, however, at antigen dilutions beyond 1:400 the S/N ratios of the three chromogens were similar (Figure 2.2). The dose response of TMB chromogen was relatively unresponsive to antigen concentration from neat to 1:400.

Definition of an improved ELISA method

Following evaluation of the above results a protocol was selected for maximisation of S/N ratios, minimisation of background reactivities, ease of performance and availability and safety of reagents.

Polystyrene 96 well plates (Linbro Cat # 76:381:04, ICN Flow) were coated with 100 μl/well of affinity purified rabbit–anti–B2 Ig diluted 1:800 in borate buffer (100 mM boric acid, 25 mM disodium tetraborate, 75 mM NaCl pH 8.4), incubated overnight at 4°C, then washed 5 times in purified water + 0.05% Tween 20 (DWT). Remaining free binding sites were blocked with 100 μl/well of 1% W/V ovalbumin (Sigma) in PBS + 0.05% Tween 20 (PBST) for 30 min at RT. After washing in DWT, tissue or culture supernatant samples and controls were added (100 μl/well) and incubated for 90 min at RT. After washing, sheep–anti–B2 serum diluted 1:400 in PBST + 0.1% ovalbumin (PBSTO) and preadsorbed for 1 hour with 100 ul of a 0.85 mg/ml solution of BF–2 cell lysate per 2 ml of diluted serum was added (100 μl/well) and incubated for 90 min at RT. After washing in DWT, mouse monoclonal anti bovine IgG conjugated to horse radish peroxidase (AMD Bi2, Silenus Laboratories) diluted 1:500 in PBSTO was added (100 μl/well) and incubated for 90 min at RT. After washing, 100 μl/well of ABTS chromogen in 100 mM citrate phosphate pH 4.2, 2.5 mM hydrogen peroxide was added. The reaction was stopped after 20 min at RT by the addition of 50 μl of 0.01% sodium azide in 0.1 M citric acid and optical density (OD) was measured at 405 nm.

Comparison of Original and Improved ELISA Methods

The improved method was found to be approximately 2 logs more sensitive than the method originally proposed (Section 1, this report) and detects EHNV in tissue culture supernatant at concentrations as low as $10^{3.5}$ $TCID_{50}$/ml (Figure 2.3).

Release of EHNV antigen from tissue samples

Homogenisation of tissue in a blender, extraction of tissues with NP–40 and grinding of tissue, all followed by vigorous vortexing, and grinding of tissues with or without snap freezing followed by vigorous vortexing with small glass beads were relatively less efficient in releasing EHNV antigen from tissue compared with other methods (Table 2.7). Sonication or grinding followed by vortexing in glass or plastic tubes appeared to result in

release of the most antigen. With these methods ELISA OD remained above 0.5 at an antigen dilution of 1:2000. The most practical method was grinding and vortexing with large glass beads as the procedure could be accomplished with minimal equipment, in disposable plastic tubes and with centrifugation in a microcentrifuge.

Comparison of ELISA OD from infected and uninfected populations

The OD of undiluted and diluted homogenates from infected fish were significantly greater ($p = 0$) than those from uninfected fish and there was no difference ($p = 0.14$) between the OD of the homogenates from infected rainbow trout or redfin perch (Table 2.8). Homogenates from uninfected rainbow trout (mean OD = 0.35) had significantly greater OD than those from uninfected redfin perch (mean OD = 0.12) ($p = 0$). Dilution of homogenates from uninfected rainbow trout resulted in a decrease in mean OD from 0.35 to 0.17.

The OD of tissue culture supernatants from individual fish from which EHNV was isolated were significantly higher than those from EHNV free fish ($p = 0$) (Table 2.9).

Four infected rainbow trout and 4 infected redfin perch had low ELISA OD in tissue homogenate and/or first pass culture supernatant. The development of cytopathic effect in BF–2 cell cultures from each of the rainbow trout and 3 of the redfin perch did not occur until the second or third pass, and were then accompanied by high ELISA OD. This suggests that apparent false negative results in the ELISA of both homogenates and supernatants were due to low numbers of infectious EHNV particles in some infected fish (Tables 2.8 and 2.9).

Sensitivity and specificity of the ELISA

Analysis of frequency distributions of the ELISA data enabled selection of positive–negative cutoff (P/N) OD to maximise sensitivity and specificity. For all homogenates tested neat, a P/N cutoff of 0.95 was selected, resulting in a sensitivity of 81.2% and specificity of 98.9%. It was possible to select a lower P/N cutoff for redfin perch because tissue homogenate background OD was lower in this species than in rainbow trout. Dilution of homogenates resulted in a lower estimate of sensitivity for rainbow trout, however, specificity was increased to 100%.

At a P/N cutoff of 0.75, sensitivity and specificity for culture supernatants were 96.2% and 99.9% (Table 2.9) respectively.

Rapid ELISA

In the first experiment the assay was completed in 3.5 hr. Two undiluted tissue culture supernatants gave OD of 2.87 and 2.63, while a negative control (culture medium) had an optical density of 0.23. The second experiment was completed in a similar time with OD results for 13 individual culture supernatant antigens ranging from 1.20 to 1.57.

Reduction of background reactions of rainbow trout tissues

Addition of RxBF-2 immune serum to the second antibody solution (SxB2) resulted in an increased OD for wells containing EHNV culture supernatant and a reduced OD for wells containing BF-2 cell lysate (Table 2.10). There was no effect on the OD of wells containing EHNV free tissue homogenates from rainbow trout. Adsorbing with BF-2 cell lysate alone considerably decreased the OD of wells containing BF-2 cell lysate as expected from previous work.

Use of RxBF-2 immune serum and/or RxRTG immune serum as a block after incubation of antigen, did not reduce the optical densities of the EHNV culture supernatant, BF-2 cell lysate, or RT tissue homogenate (Table 2.11).

Adsorption of second antibody solution with *M. phlei* did not reduce the OD of EHNV negative or positive rainbow trout tissue homogenates. However, the OD of EHNV culture supernatants were lower when the *M. phlei* adsorbed SxB2 serum was used (Table 2.12).

Discussion

This section described the development of an indirect, antigen capture ELISA for the detection of EHNV in the tissues of fish. The sensitivity of this assay for infectious viral particles was about $10^{3.5}$ $TCID_{50}$ EHNV/ml, its statistical sensitivity was about 80% while its specificity was > 98%. A comparison of the prototype ELISA method with the improved method confirmed that assay sensitivity was increased by 2 logs. In assessing the format of this ELISA and the types and concentrations of reagents used, we were primarily concerned with maximisation of assay sensitivity under conditions where antigen was limiting. The crucial factor in the ELISA system turned out to be the nature of the capture antibody.

Rabbit-anti B2 immune serum was a more competent capture antibody than sheep-anti-B2 immune serum; this was assumed to be due to the higher specificity for EHNV antigens that the former reagent demonstrated in immunoblots (see Section 1, this report). It is possible that the rabbit immunoglobulin has greater affinity for EHNV itself and/or that the rabbit immunoglobulin has a greater affinity for the polystyrene microtitre plates used as the solid phase in the ELISA. Kemeny and Challacombe (1988) and Cantarero, et al. (1980) have both stated that different proteins have different binding capacities for the polystyrene solid phase.

Rabbit antisera were affinity purified to remove unwanted activities and irrelevant proteins. Use of affinity purified rabbit anti-B2 immunoglobulin increased the sensitivity of the ELISA by at least a hundred fold. This effect is most easily explained in terms of the density of anti-EHNV antibodies bound to the solid phase. When whole serum was used as a source of capture antibody, a plethora of irrelevant serum proteins would have bound to the solid phase along with antibodies. In contrast, only immunoglobulins were bound to the solid phase when affinity purified serum was used as a source of capture antibody.

A comparison of blocking agents showed that the use of a block decreased background effects, but there was little difference between the blocks used. Without a block there may be a tendency for extraneous material in the antigen solution to bind to the unoccupied sites on the solid phase and to be detected by the second antibody (Spencer, 1988).

Choice of conjugate was important in formatting this antigen capture ELISA. Use of a monoclonal conjugate (AMD) resulted in slightly greater sensitivity and specificity for EHNV antigens when compared to the use of a polyclonal conjugate (KPL). This is intuitively due to the greater specificity of monoclonal antibody for the sheep second antibody and a lower tendency for non-specific binding of the monoclonal conjugate to the rabbit capture antibody. When the two enzymes, HRP and AP were compared there was no real difference in sensitivity or specificity. HRP was used to facilitate compatibility with other assays undertaken in the laboratory. AMD Bi2 monoclonal antibody was selected for routine use because it is commercially available and used widely. Polyclonal KPL conjugate could be substituted for the monoclonal conjugate if necessary.

ABTS, OPD, and TMB are three chromogens used in ELISA which are compatible with HRP conjugates. Heytman (1988), demonstrated that TMB was the most efficient in detecting low levels of enzyme, followed by OPD and then ABTS. When these chromogens were compared in the EHNV ELISA, no major difference was found in sensitivity between ABTS and TMB when antigen was present in low concentration, and OPD was only slightly more sensitive. As well as being a sensitive detection method for enzyme, the substrate must also be cheap, safe and easy to use. OPD is considered to be a possible carcinogen, and OPD and ABTS are thought to be mutagenic (Heytman, 1988). ABTS is relatively safe if handled carefully, and is also the easiest to use, as it is not light sensitive and can be prepared in advance and stored for long periods in frozen aliquots. Thus it was the chosen substrate for this system.

It was shown that this ELISA could be reformatted in several ways to enable the entire assay to be completed in less than 4 hours. This is a very useful feature for a diagnostic pathology laboratory.

An efficient technique for preparation of tissue samples for ELISA was developed during this study. The method, which can be completed without complex equipment and using small disposable tubes, was more effective than traditional homogenisation in releasing EHNV antigens from tissue samples. It is possible for a single operator to process 150-200 individual tissues for subsequent inoculation of tissue culture or assessment by ELISA, in less than a day.

In statistical terms, the sensitivity and specificity of the ELISA are high enough to enable its use in routine diagnosis and certification of EHNV. In infected fish, EHNV is readily demonstrated in 80% of individuals. The 20% false negative rate is attributable to there being relatively few virus particles in individuals in the early stages of infection. Tissue culture is inherently more sensitive than ELISA in detecting these infected individuals because the virus multiplies to detectable levels in tissue culture. Diagnosis of EHNV by ELISA would require the testing of more than one infected fish, which is consistent with current laboratory practice for the diagnosis of fish diseases in any case.

Using the tissue preparation method and ELISA described in this section, certification test results on large samples of fish could be available within 24 hours of receipt of specimens at a diagnostic laboratory. This compares more than favourably with the present turn around time of about 2 weeks for certification based on tissue culture. In addition, when fully costed, the new technique will be proportionately cheaper than tissue culture techniques.

There is still opportunity for improving the sensitivity of the newly improved ELISA. EHNV free rainbow trout tissues characteristically give a high background signal with this assay. The reason is uncertain, but must related to non–specific reactivity with components of the polyclonal capture and second antibody reagents. This non–specific reactivity could not be reduced using standard adsorption and blocking techniques. The development of monoclonal antibodies specific for EHNV should enable the reduction of these background reactions. This would permit the positive–negative test threshold to be lowered, and thereby increase the statistical sensitivity of the assay.

TABLE 2.1

ELISA OD of EHNV culture supernatant and BF-2 cell lysate in a titration of Rx-B2 serum capture antibody SxB2 serum second antibody.

Dilution of SxB2 serum second antibody	Antigen					
	Supernatant					BF-2 cell
	Dilution of RxB2 serum capture antibody					
	1:100	1:200	1:400	1:800	1:1600	1:200
1:100	0.74	0.99	0.98	1.12	1.48	0.23
1:200	0.73	0.98	1.16	1.06	1.36	0.21
1:400	0.77	0.92	1.08	1.11	1.49	0.17
1:800	0.6	0.66	0.93	1.0	0.98	0.13

TABLE 2.2

ELISA OD of EHNV culture supernatant and BF-2 cell lysate in titration of SxB2 serum as capture antibody and RxB2 serum as second antibody.

Dilution of RxB2 serum second antibody	Antigen					
	Supernatant					BF-2 cell
	Dilution of SxB2 serum capture antibody					
	1:100	1:200	1:400	1:800	1:1600	1:200
1:100	0.89	0.48	0.54	0.57	0.70	0.52
1:200	0.34	0.38	0.37	0.50	0.41	0.45
1:400	0.31	0.36	0.34	0.40	0.36	0.72
1:800	0.51	0.29	0.29	0.33	0.32	0.33

TABLE 2.3

ELISA OD of EHNV culture supernatant in titration with affinity purified RxB2 Ig capture antibody.

Dilution of affinity purified RxB2 Ig capture antibody	Dilution of supernatant		
	Neat	1:10	1:100
1:200	2.89	2.66	0.49
1:400	3.15	2.74	0.49
1:800	2.73	0.81	0.29
1:1600	2.84	1.38	0.44
1:3200	2.83	1.09	0.26
1:6400	2.81	0.52	0.12
1:12800	2.75	0.31	0.1
1:25600	2.24	0.21	0.1
1:51200	2.52	0.33	0.08
1:102400	1.32	0.16	0.08

TABLE 2.4

ELISA OD of EHNV supernatant titrated with and without capture antibody.

Dilution of supernatant	Capture antibody		
	Nil	RxB2 serum 1:800	Affinity purified RxB2 Ig 1:800
1:1	0.71	2.92	3.10
1:10	0.20	0.77	3.00
1:20	0.38	0.50	2.85
1:40	0.22	0.28	2.46
1:80	0.21	0.21	1.78
1:100	0.17	0.19	1.54
1:200	0.15	0.18	0.84
1:400	0.14	0.14	0.47
1:800	0.11	0.11	0.27
1:1000	0.11	0.1	0.28
1:10000	0.09	0.11	0.13
BF-2 cells	0.42	0.33	0.34

TABLE 2.5

ELISA OD of EHNV supernatant tested using different blocking agents

Dilution of supernatant	Blocking agent			
	Ovalbumin	Gelatin	Skim milk	No block
neat	2.99	2.80	2.90	2.76
1:10	2.83	2.76	2.94	2.81
1:20	2.47	2.76	2.76	2.78
1:40	2.29	2.34	2.2	2.39
1:80	1.33	1.62	1.43	1.41
1:100	1.01	0.97	1.15	1.03
1:200	0.59	0.76	0.61	0.64
1:400	0.41	0.43	0.42	0.35
1:800	0.22	0.3	0.28	0.24
1:1000	0.25	0.25	0.25	0.21
1:10000	0.11	0.14	0.13	0.12
BF-2 cells	0.30	0.31	0.33	0.41

TABLE 2.6

Comparison of polyclonal and monoclonal conjugates. KPL–HRP was reacted at a dilution 1:4000 while AMD Bi2–HRP was used at 1:500 in titration against viral antigen and BF–2 cells.

Antigen	Optical density		Signal – noise ratio	
	KPL–HRP	AMD Bi2–HRP	KPL–HRP	AMD Bi2–HRP
1:10	2.41	1.19	6.3	8.5
1:100	1.16	0.33	3.1	2.4
1:200	0.70	0.22	1.8	1.6
1:500	0.44	0.17	1.2	1.2
1:1000	0.30	0.14	0.8	1.0
1:10000	0.20	0.12	0.5	0.9
BF–2 cells	0.38	0.14		

TABLE 2.7

ELISA OD of tissue homogenates treated to release EHNV.

Viral antigen dilution	Method of treatment of tissue							
	Homogenise	NP–40	Sonicate + large glass beads	Grind	Snap freeze + grind with small glass beads	Grind + large glass beads, plastic tube	Grind + small glass beads, plastic tube	Grind + large glass beads, glass tube
1:100	2.07	2.09	2.68	2.35	2.38	2.42	1.94	2.48
1:1000	0.53	0.41	0.99	0.50	0.60	0.99	0.67	1.08
1:2000	0.29	0.27	0.55	0.33	0.29	0.58	0.43	0.66
1:4000	0.23	0.20	0.34	0.24	0.23	0.32	0.25	0.39
1:6000	0.20	0.18	0.27	0.20	0.19	0.26	0.2	0.29
1:8000	0.15	0.16	0.23	0.17	0.18	0.2	0.17	0.23
1:10000	0.15	0.17	0.19	0.20	0.16	0.17	0.14	0.2

TABLE 2.8

ELISA OD results of tissue homogenates from rainbow trout and redfin perch populations infected and unaffected by EHNV.

Species	Status	Dilution of homogenate	n	ELISA OD					No. positive	No. negative	Sensitivity	Specificity
				Mean	s.d.	min	max	P–N cutoff				
Both	infected	neat	55	2.10	0.98	0.17	3.13	0.95	45	10	81.2	
		1:10	21	2.05	1.08	0.11	3.10	0.55	17	4	81.0	
	uninfected	neat	348	0.29	0.25	0.06	1.12	0.95	4	344		98.9
		1:10	250	0.17	0.04	0.07	0.45	0.55	0	250		100
Rainbow trout	infected	neat	26	2.31	0.96	0.17	3.13	0.95	22	4	84.6	
		1:10	11	1.39	1.14	0.11	2.97	0.55	7	4	63.6	
	uninfected	neat	261	0.35	0.26	0.06	1.12	0.95	4	257		98.5
		1:10	250	0.17	0.04	0.07	0.45	0.55	0	250		100
Redfin perch	infected	neat	29	1.92	0.97	0.23	2.97	0.55	25	4	86.2	
		1:10	10	2.78	0.19	2.41	3.10	0.55	10	0	100	
	uninfected	neat	87	0.12	0.05	0.06	0.33	0.55	0	87		100

TABLE 2.9

ELISA OD results for EHNV positive and negative culture supernatants. with EHNV and free of EHNV.

Status	Pass	n	ELISA OD					No. positive	No. negative	Sensitivity	Specificity
			mean	s.d.	min	max	P–N cutoff				
Infected	1	80	2.15	0.75	0.20	2.99					
	2	67	2.46	0.48	0.28	3.28	0.75	152	6	96.2	
	3	11	2.70	0.35	1.75	3.04					
Uninfected	1	142	0.23	0.11	0.10	1.05					
	2	126	0.22	0.05	0.15	0.52	0.75	2	353		99.9
	3	87	0.39	0.15	0.21	0.70					

TABLE 2.10

Effect of addition of anti-cell competitive antibodies to the second antibody solution (ELISA OD).

Second antibody SxB2 serum 1:400 plus	Antigens		
	Supernatant	BF-2 cell lysate	Rainbow trout homogenate
nil	0.89	0.41	0.30
RxBF-2 cell serum 1:200	1.23	0.33	0.34
RxBF-2 cell serum 1:400	1.16	0.37	0.32
RxBF-2 cell serum 1:800	1.01	0.33	0.29
BF-2 cell lysate	0.78	0.17	0.29

TABLE 2.11

Effect of anti-cell antisera as blocking agents after incubation of antigen (ELISA OD).

Block	Antigens		
	Supernatant	BF-2 cell lysate	Rainbow trout homogenate
nil	0.83	0.57	0.26
RxBF-2 cell serum 1:100	1.06	0.44	0.37
RxBF-2 cell serum 1:200	1.06	0.47	0.34
RxBF-2 cell serum 1:400	0.74	0.47	0.32
RxRTG cell serum 1:100	1.14	0.61	0.38
RxRTG cell serum 1:200	1.14	0.54	0.36
RxRTG cell serum 1:400	1.04	0.60	0.34
RxBF-2 cell serum + RxRTG cell serum 1:100	1.00	0.57	0.39
RxBF-2 cell serum + RxRTG cell serum 1:200	0.90	0.53	0.40
RxBF-2 cell serum + RxRTG cell serum 1:400	0.86	0.50	0.38

TABLE 2.12

ELISA OD of EHNV cell culture supernatants, and EHNV positive and negative tissue homogenates from rainbow trout compared using SxB2 second antibody adsorbed with *M. phlei*.

Antigens	Second antibody	
	SxB2 serum	SxB2 serum + *M. phlei*
Supernatants		
1	2.09	1.82
2	1.73	1.30
3	2.20	1.68
4	2.52	1.88
5	2.39	1.42
6	3.27	1.94
7	3.24	2.26
8	1.49	1.05
9	2.71	2.11
10	1.79	0.96
11	3.22	2.26
EHNV positive homogenates		
1	1.30	1.29
2	0.30	0.30
3	0.55	0.49
4	1.77	1.76
5	0.95	0.91
EHNV negative homogenates		
1	0.28	0.26
2	0.27	0.25
3	0.25	0.25
4	0.25	0.26

Fig. 2.1. Comparison of four monoclonal antibody conjugates by titration against EHNV tissue culture supernatant.

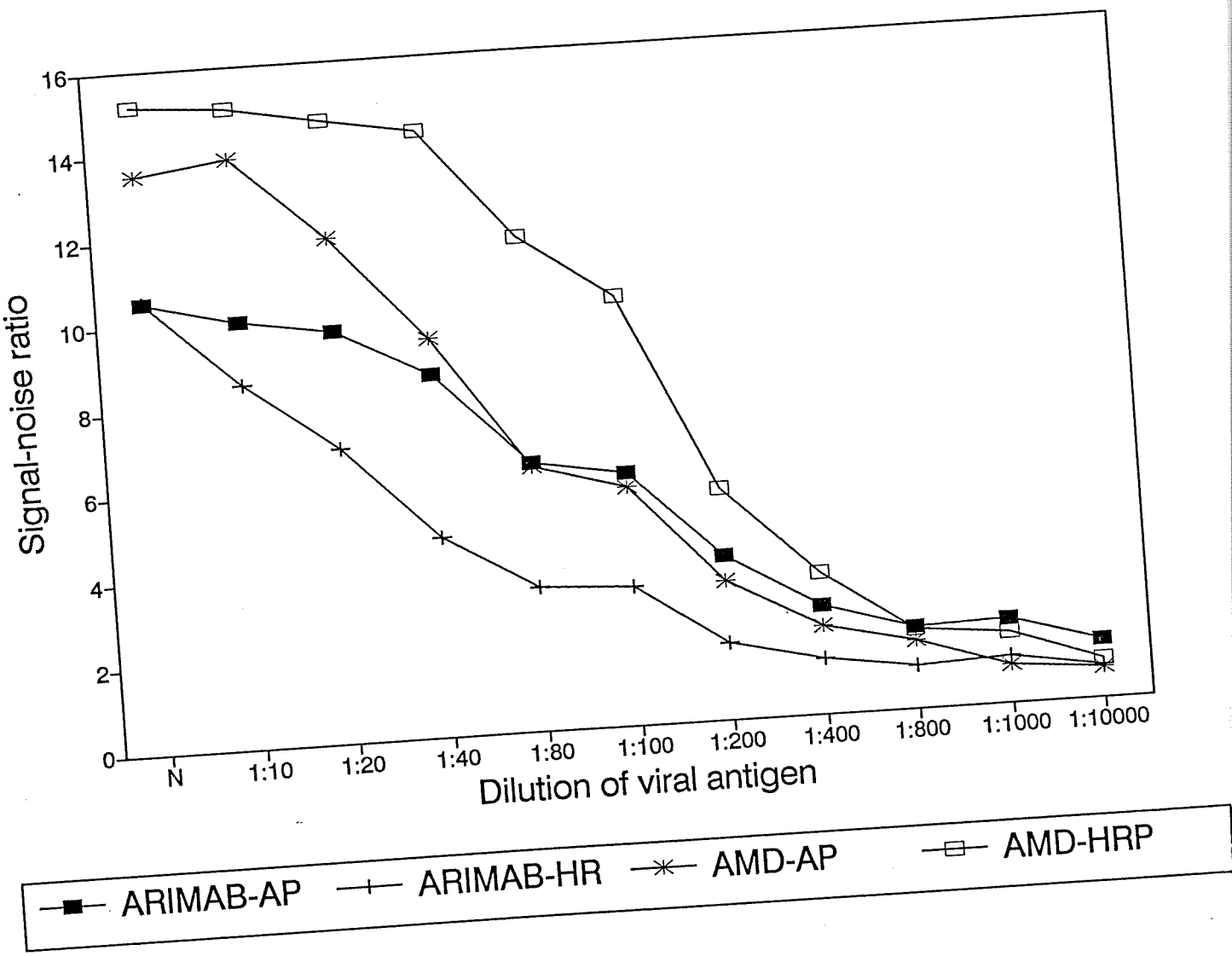


Fig. 2.2. Comparison of three chromogens using AMD–Bi2 conjugate by titration against EHNV tissue culture supernatant.

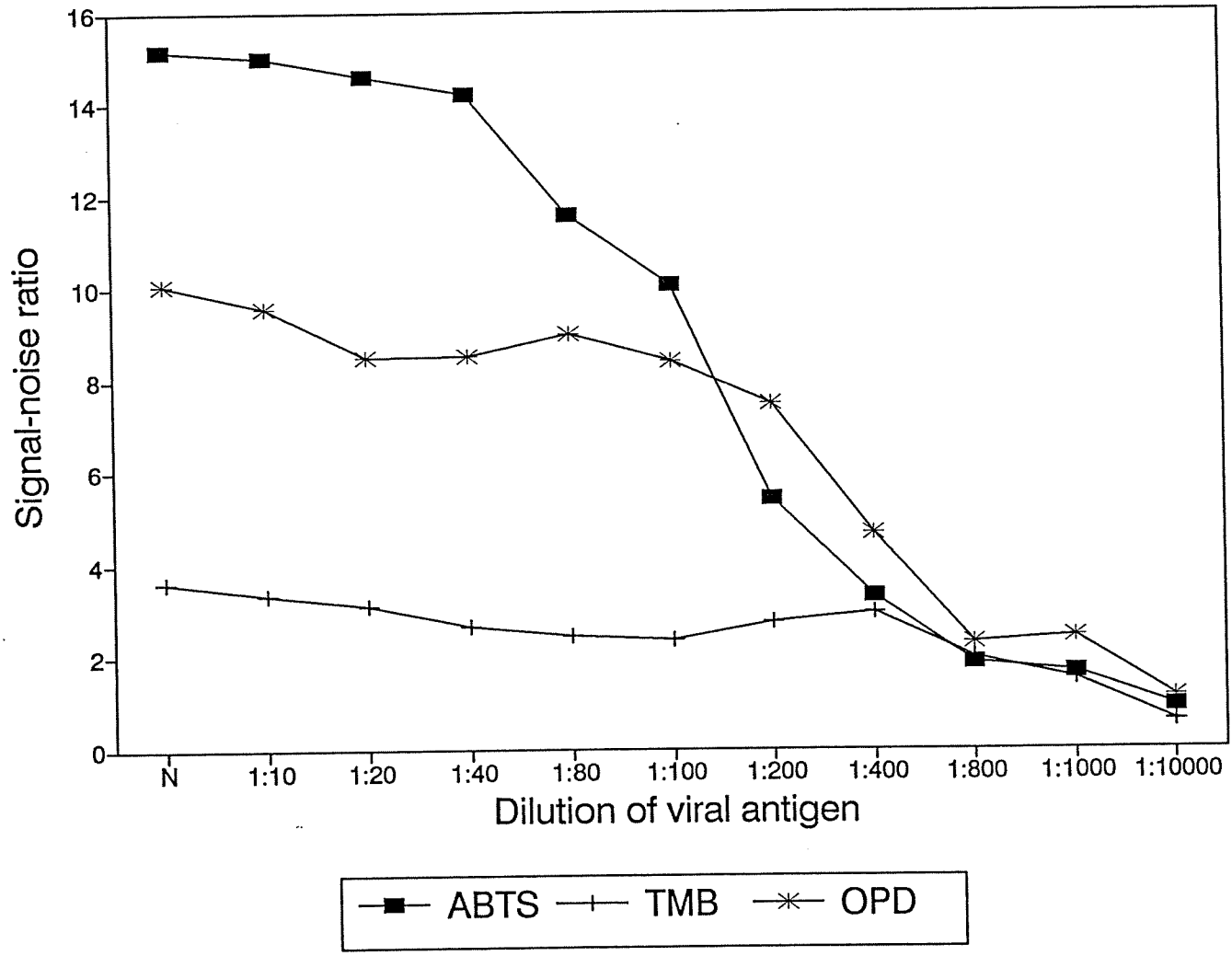
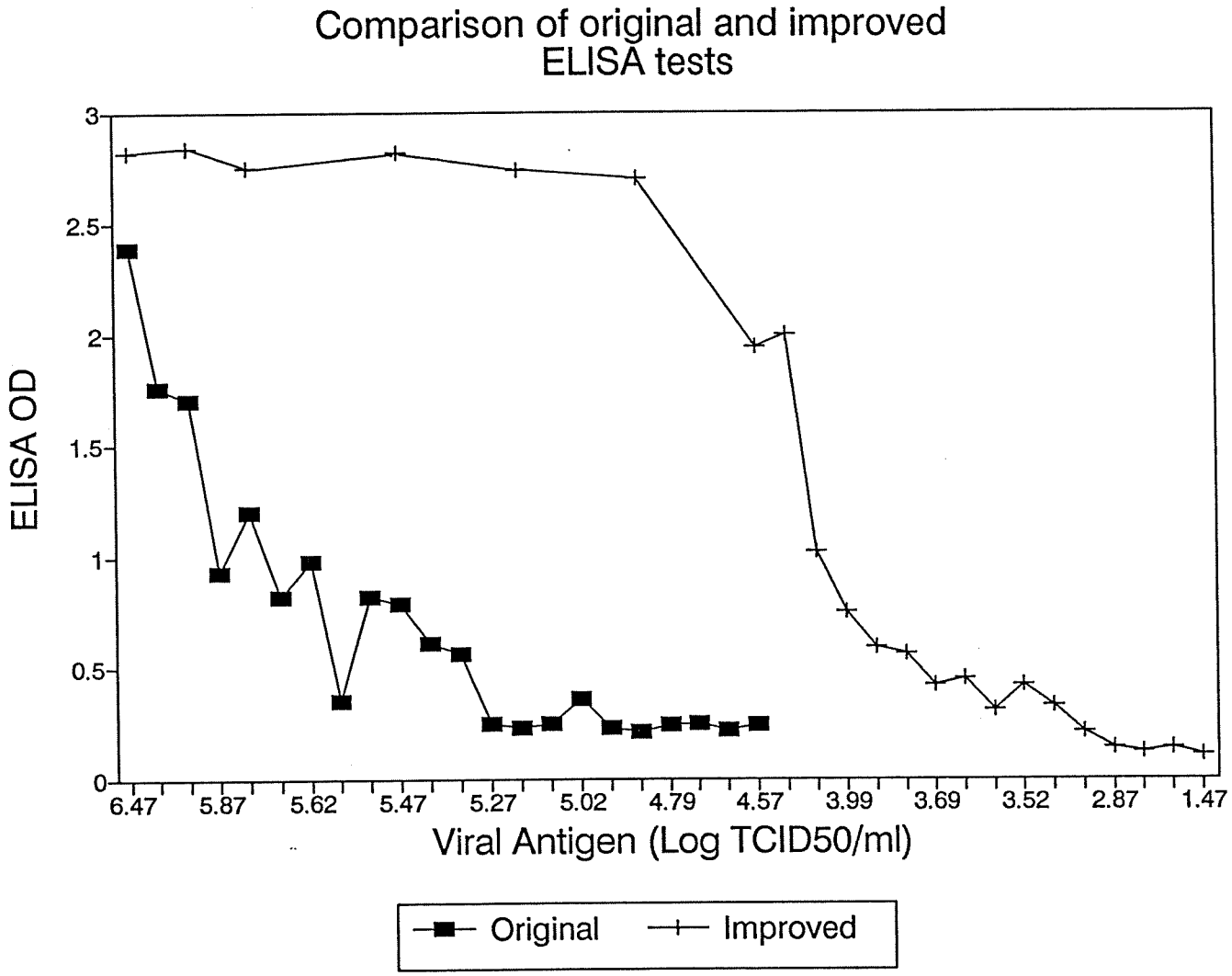


Fig. 2.3. Comparison of assay sensitivity of the prototype ELISA and the improved ELISA by titration against EHNV tissue culture supernatant.



SECTION 3

PURIFICATION AND CHARACTERISATION OF SERUM IMMUNOGLOBULIN OF THE REDFIN PERCH IN ORDER TO PRODUCE SPECIFIC REAGENTS FOR ELISA

Introduction

In order to detect specific immune responses against EHNV in redfin perch it is necessary to produce reagents capable of binding to redfin immunoglobulins in an ELISA assay. Although much is known in general terms about fish immunoglobulins, there has been no critical assessment for redfin perch, and no reagents are available for use with this species. The serum Ig of the redfin perch, was investigated by Richter & Ambrosius (1972) who identified only a high m.w. (HMW) Ig. The aim of this study was to purify, partially characterise and raise specific polyclonal antisera to perch serum Ig to enable the study of immune responses by ELISA during infection with EHNV. Methods were chosen to maximise the chance of detecting HMW and low m.w. (LMW) Ig populations.

Many studies of the Ig of fish have confirmed intergeneric variation and fewer isotypes than are present in higher vertebrates. Elasmobranchs have pentameric and monomeric forms of IgM in serum. Teleosts have tetrameric IgM in serum, but the presence of monomeric IgM is unclear (Corbel, 1975). While most teleosts do not have Ig in low molecular weight (m.w.) fractions of serum there is unequivocal evidence that some do (Clem, 1971; Lobb & Clem, 1981; Clem & McLean, 1975; Warr, 1983). In addition, H and L chain isotypes have been found in some teleosts (Lobb & Clem, 1981; Lobb & Clem, 1983; Lobb *et al.*, 1984; Havarstein *et al.*, 1988; Lobb & Olsen, 1988; Sanchez *et al.*, 1989; Sanchez and Dominguez, 1991).

Materials and Methods

Immunisation of fish

Two 340 mm forklength perch obtained from a population believed to be free of EHN virus were acclimatised in 120 L aquaria at 16°C, fed earthworms and cockroaches and immunised by intraperitoneal injection with 1 mg of ovalbumin (Sigma, St Louis) in Freund's complete adjuvant (FCA) (Sigma). Immunisation was repeated 4, 6 and 8 weeks later. Blood was collected from the caudal vein at 2 week intervals, allowed to clot overnight at 4°C, centrifuged at 2000 g for 15 min and serum was collected and stored at −20°C until required.

Immunisation of rabbits and sheep

Antiserum against perch whole serum (RAPS) was prepared by immunising a rabbit with 2 ml of 1:1 FCA:perch pre-immune serum by subcutaneous injection. This was repeated after 21 days and followed by a second booster with 2 ml 1:1 FCA:perch immune serum after a further 28 days.

Antiserum against perch HMW Ig (RAPI) was similarly prepared in rabbits and sheep. An emulsion of 1:1 FCA:240 ug affinity purified HMW Ig from gel filtration pool 1a (see below) in 1 ml sterile PBS pH 7.4 was administered and repeated after 21 days.

Blood was collected from the jugular vein of each rabbit two weeks after the last immunisation, allowed to clot at room temperature for 2 hours, and serum was harvested and stored at −20°C.

Enzyme linked immunosorbent assay

Microtitre plates (Flow, McClean) were coated with ovalbumin (Sigma) (1mg/ml solution in borate buffer pH 8.4) for 18−h at 4°C then washed five times in distilled water containing 0.05% V/V Tween 20 (Sigma). Perch serum (diluted 1:200) or chromatography fraction (diluted 1:10) in PBS pH 7.2, gelatin 0.1% W/V, Tween 20 0.05% V/V (PBSGT) was added to duplicate wells and incubated for 90 min at room temperature (RT). After washing, RAPS diluted 1:200 in PBSGT was added and incubated for 90 min. After washing, protein A conjugated to horse radish peroxidase (Biorad, Richmond) diluted 1:3000 in PBSGT was added and incubated for 90 min. After washing, 1 mM 2,2'−azino−di(3−ethyl −benzthiazolin−6−sulphonate) (Sigma) in 100 mM citrate phosphate pH 4.2, 2.5 mM H_2O_2 was added. The reaction was stopped after 20 min at RT by the addition of 50 ul of 0.01% NaN_3 in 0.1 M citric acid and optical density (OD) was measured at 405 nm. Control positive and negative samples determined independently by immunodiffusion in agarose were included on all plates.

Agarose gel immunodiffusion test

Perch serum (undiluted) was diffused overnight at room temperature against ovalbumin (1 mg/ml solution) in agarose (1% W/V in borate buffer pH 8.5).

Gel filtration

Perch serum was chromatographed on a 100 x 2.6 cm column containing Sephacryl S300 (Pharmacia, Uppsala) with 25 mM Tris/HCl pH 7.2, 0.15 M NaCl, 0.01% NaN_3 buffer (TS), a flow rate of 30 ml/h and a fraction size of 5ml. Protein content was monitored in the eluent by absorbance at 280 nm. Pooled fractions were concentrated by dialysis against Aquacide II (Calbiochem, San Diego) and stored at −20°C.

Affinity chromatography

Ovalbumin (15 mg) was coupled to CNBr activated Sepharose 4B (Pharmacia) (3 g) using procedures recommended by the manufacturer, packed in a 10 ml column and equilibrated with 100 ml of 0.1 M Tris/Hcl pH 8, 0.15 M NaCl. A flow rate of 60 ml/hour was used throughout. Bound material was eluted with 0.1 M glycine, 0.5 M NaCl, pH 3 and fractions of 5 ml were immediately neutralised by adding 50 mg of Tris base (Sigma), pooled and dialysed overnight at 4°C against TS prior to concentration by dialysis against Aquacide II and storage at −20°C.

Ion−exchange chromatography

A 40 x 2.6 cm column containing DEAE−Sepharose CL−6B (Pharmacia) with 25 mM Tris/HCl pH 7.2 equilibration buffer and 25 mM Tris/HCl pH 7.2, 0.5 M NaCl as limit buffer in a linear gradient at a flow rate of 30 ml/hour was used. Fractions of 5 ml

were pooled and concentrated against Aquacide II, aliquoted and stored at 4°C and at -20°C.

Electrophoresis and western blotting

SDS-PAGE under reducing conditions was performed according to Laemmli (1970) with a 12% resolving gel and a 4% stacking gel containing 29:1 acrylamide:bis acrylamide. SDS-PAGE under non-reducing conditions was similarly performed in 3% gels, however, samples were diluted in 62.5 mM Tris/HCl pH 6.8, 10% V/V glycerol, 2% W/V SDS and incubated for 15-60 min at room temperature prior to electrophoresis. The apparent m.w. of native Ig and subunits were estimated from the mid-point of peak intensity of staining of bands. Calibration curves for reduced gels were prepared using low range m.w. standards (Biorad, Richmond). Calibration curves for non-reduced gels were prepared with the following standards: human IgM (Calbiochem), thyroglobulin and catalase (Pharmacia), and ovine IgG (Silenus, Hawthorn).

Proteins were stained with 0.2% Coomassie brilliant blue (CBB) or electrophoretically transferred to Immobilon-P membrane (Millipore, Bedford). The membrane was immunostained after blocking in 1% gelatin in 10 mM Tris/HCl pH 7.4, 0.15 M NaCl (BTS), using RAPI diluted 1:200 in BTS, 0.1% gelatin, 0.05% Tween 20 (BTSGT), followed by swine anti-rabbit IgG-horse radish peroxidase (KPL, Gaithersburg) diluted 1:250 in BTSGT. Binding of conjugate was demonstrated with 4-chloro-1-naphthol (Biorad). The position of m.w. markers (Biorad) was detected by staining excised lanes of the membrane with 0.2% CBB.

Immunoelectrophoresis

Serum and Ig samples were electrophoresed at 60 mA constant current in 1% agarose in Tris barbitone buffer pH 8.6 on Gelbond film (FMC Bioproducts, Rockland) and then diffused overnight against RAPS (neat or diluted 1:2) or ovalbumin (0.1 mg/ml). Gels were dried and stained with 0.1% CBB.

Electron microscopy

Ig preparations were diluted to 30 ug/ml in TS. Collodion coated copper grids were immersed in dilute Ig for 5 min., allowed to dry then immersed in 1% uranyl acetate pH 5 for 5 min. Some grids were overlayed with a solution of 90 µm diameter latex beads for size calibration. After drying, grids were examined in a Philips 201 transmission electron microscope.

Determination of protein concentration

The coomassie blue dye binding method of Bradford (1976) was used to measure protein concentration. Calibration curves were prepared using bovine serum albumin (Sigma).

Results

Response of fish to immunisation

Ovalbumin binding and precipitating activity was detected in the serum of both fish

at 6–8 weeks after the first immunisation. Pre–immune serum gave ELISA OD readings of 0.19–0.22 and lacked precipitating activity. Positive agarose gel immunodiffusion reactions were correlated with ELISA OD readings greater than 0.8.

Purification of serum Ig

Immune perch serum was resolved into 5 major peaks by gel filtration (Figure 3.1). Fractions with binding activity for ovalbumin eluted from the column after human IgM indicating a m.w. less than 900–kDa. These fractions were pooled as shown in Figure 3.1 (pools 1a, 1a+1b and 3). Affinity or ion exchange chromatography was used to further purify Ig. Ig in pools 1a and 1a+1b will be referred to as HMW Ig while that in pool 3 will be referred to as LMW Ig.

The HMW Ig pools and the LMW Ig pool were each resolved into two distinct peaks by affinity chromatography. Only the second peak, which was eluted by glycine–HCL pH 3, contained ovalbumin binding activity. The yields of affinity purified Ig from pools 1a and 3 were approximately 300 μg and 70 μg per ml of immune serum respectively. Insufficient pool 3 Ig was purified to enable detailed analysis.

In ion–exchange chromatography of pool 1a HMW Ig, fractions eluted by 0.25 – 0.35 M NaCl possessed ovalbumin binding activity and were pooled, yielding approximately 1.4 mg Ig per ml immune serum. This method isolated Ig regardless of its antigenic specificity, accounting partly for the greater yield.

Purification of perch Ig was confirmed by immunoelectrophoresis. In diffusion against RAPS, a single arc of precipitation was observed in affinity purified HMW Ig while one major and a second minor arc was observed in ion–exchange purified HMW Ig. The location of these arcs corresponded that obtained by diffusion of perch immune serum against ovalbumin. These results fulfil the usual criteria for purity of an Ig preparation; ion–exchange chromatography was less effective than affinity chromatography in purifying perch HMW Ig.

Analysis of reduced immunoglobulins

In SDS–PAGE under conditions in which disulfide bonds had been cleaved, bands in perch samples were identified as H and L chains on the basis of their mobilities being similar to those of H and L chains from human, bovine and ovine Ig run on the same gel (Figure 3.2). In HMW Ig, the predominant H chain had an apparent m.w. of 72–kDa. It was of similar apparent m.w. to human μ chain, lighter than bovine μ chain and markedly heavier than ovine γ chain, justifying its classification as μ–equivalent. A faint band with an apparent m.w. of 64–kDa was present also (Figure 3.2); this band was prominent in Western blots (see below). Resolution of L chains was variable, however, four bands in the range 27–kDa to 30–kDa were evident, a 28–kDa species being present in greatest concentration. Apart from heterogenous polypeptides that were visible in ion–exchange purified HMW Ig, results for affinity and ion–exchange purified HMW Ig were similar.

LMW Ig contained four bands with similar electrophoretic mobility (72–kDa, 64–kDa, 28–kDa and 27–kDa) to H and L chains in the HMW Ig, together with three additional bands (56–kDa, 51–kDa and 39–kDa) (Figure 3.2).

The antigenic specificity of Ig preparations was evaluated by Western blotting (Figure 3.4). Anti-serum raised against affinity purified HMW Ig (RAPI) recognised H and L chains in HMW Ig and whole serum. All seven bands in LMW Ig were strongly recognised by this anti-serum (lane 5). This indicates antigenic homology between HMW Ig and LMW Ig. Several additional bands, representing traces of proteins not detectable in CBB stained gels, were demonstrated in the HMW Ig samples (lanes 3 and 4).

Analysis of unreduced immunoglobulin

Affinity and ion-exchange purified HMW Ig dissociated into up to four bands in non-reducing SDS-PAGE while pentameric mammalian IgM samples that were prepared in the same buffer migrated as single bands in the same gels. In the experiment depicted in Figure 3.5, ion-exchange purified HMW Ig (lane 2) was resolved into two bands (HMW1, HMW3) while affinity purified HMW Ig (lane 4) was separated into four bands (HMW1, HMW2, HMW3, HMW4). Under the same conditions, LMW Ig (lane 3) was resolved into three components (LMW1, LMW2, LMW3), the heaviest of which was present in greatest concentration and co-migrated with HMW4.

Warr (1982) reported that Ig standards give straight line calibration curves in non-reducing SDS-PAGE, while other types of standards of m.w. less than 100-kDa give lines with a different slope that are shifted to the right. In this study, calibration curves prepared with Ig and non-Ig standards (>100-kDa) approximated straight lines (Figure 3.6, data derived from Figure 3.5), however, there was slight run-to-run variation in the mobility of the standards. Where a stringent straight-line calibration curve was not achieved, a second calibration curve was prepared by ignoring the non-Ig standards on that gel. This resulted in upper and lower estimates of m.w. of the unknowns for some gels.

Based on examination of several gels, estimates of apparent m.w. for HMW1, HMW2, HMW3 and HMW4 were 730-800-kDa, 600-kDa, 390-kDa and 210-225-kDa respectively. These estimates are consistent with the identification of the components of HMW Ig as being tetramers, trimers, dimers and monomers of H_2L_2 IgM with a unit m.w. of about 200-kDa. The major component of LMW Ig (LMW1) had an apparent m.w. of 220-kDa, while the minor components LMW2 and LMW3 had apparent m.w. of 185-kDa and 150-kDa respectively.

Electron microscopy

Affinity purified pool 1a Ig consisted of coarse filaments that tended to clump to form a lattice. Individual Ig molecules were rare but appeared to be tetramers (Figure 3.7). They had an apparent diameter of about 50 nm measured across the longest axis.

Discussion

Ig was purified from immune serum of perch by exploiting its ability to bind to the immunising antigen, ovalbumin, in ELISA and affinity chromatography. The perch has two populations of Ig in immune serum that can be distinguished by difference in m.w. The predominant population has a m.w. of about 800-kDa. By deduction it has a tetrameric structure: it elutes later than pentameric human IgM in Sephacryl S300 gel

filtration, it migrates faster than pentameric mammalian IgM in 3% polyacrylamide gels under non-reducing conditions, it dissociates into four subunits in buffers containing SDS and it appears to have a four-armed structure in electron micrographs. The LMW Ig (about 200-kDa) population is present in much smaller amounts in immune serum and is antigenically related to the HMW population. Co-migration of LMW Ig with the lightest component of HMW Ig and the presence of H and L chains of similar m.w. in both populations suggests that the LMW Ig population contains monomers of the HMW Ig H_2L_2 unit. These results extend the work of Richter & Ambrosius (1972) who found only 14S to 15S HMW Ig in the serum of immunised perch.

Several H chain isotypes have been reported in the serum of the sheepshead (*Archosargus probatocephalus*) (Lobb & Clem, 1981), the toadfish (*Spheroides glaber*) (Warr, 1983), the channel catfish (*Ictalurus punctatus*) (Lobb & Olson, 1988) and the rainbow trout (Sanchez *et al.*, 1989). In this study of perch, proteolysis of the 72-kDa H chain could account for subsidiary species of lower m.w. such as the 64-kDa species, but there is also the possibility that both Ig populations contain H chain isotypes. The 64-kDa species was present in lower concentration than the 72-kDa H chain in the HMW Ig population, however this situation was reversed in the LMW population (assessed by the intensity of CBB staining in SDS-PAGE gels, Figures 3.2 and 3.3). In the sheepshead, a 45-kDa H chain occurs in LMW Ig while a 70-kDa H chain occurs in HMW Ig (Lobb & Clem, 1981).

Based on molecular size and/or antigenic differences, several L chain isotypes have been identified in channel catfish (Lobb *et al.*, 1984), Atlantic salmon (*Salmo salar*) (Havarstein *et al.*, 1988) and rainbow trout (Sanchez *et al.*, 1989; Sanchez and Dominguez, 1991). Up to four m.w. variants of L chain in the range 27-kDa to 30-kDa were found in the perch HMW Ig population, while only the lightest two L chains were found in the LMW Ig population.

In estimating the m.w. of perch Ig subunits from SDS-PAGE gels during this study it was noted that m.w. values determined for human, bovine and ovine Ig standards were greater than those usually quoted. Anomalous migration of Ig subunits in polyacrylamide gels has been reported previously (Butler, 1983). Richter & Ambrosius (1972) determined the m.w. of perch H chains to be 65-kDa by disc gel electrophoresis in polyacrylamide.

Perch HMW Ig tended to dissociate in buffers containing SDS under conditions that did not reduce covalent bonds or cause dissociation of mammalian pentameric IgM. This observation is consistent with four 200-kDa H_2L_2 units being non-covalently linked in a proportion of the tetrameric HMW Ig population. Non-covalent linkage of multimeric IgM has been reported in the sheepshead (Lobb & Clem, 1981), the chum salmon (*Onchorhynchus keta*) (Kobayashi *et al.*, 1982), the channel catfish (Lobb & Clem, 1983) and the toadfish (Warr, 1983).

The nature of the components of LMW Ig that were resolved by non-reducing SDS-PAGE was not determined due to the small amount of material available. Analysis of these components under reducing conditions will be required to determine if each component is an H_2L_2 unit differing in the m.w. of the respective H chain. If this was

found to be the case it could account for the 56-kDa and 51-kDa bands resolved in the LMW Ig population in SDS-PAGE under reducing conditions.

The finding of Ig in both high and low m.w. fractions of perch serum in this study is noteworthy as LMW Ig was not found in an earlier study of perch (Richter & Ambrosius, 1972). Considering that similar methods of immunisation of fish and affinity purification of serum were used in both studies, the discovery of LMW Ig in the present study is explicable only in terms of the method used to identify Ig in fractions of serum during purification. A primary antibody-antigen binding assay (ELISA) was used here, while Richter & Ambrosius (1972) used less sensitive, secondary binding assays (passive haemagglutination and haemagglutination). Similarly, Clem & McClean (1975) were able to detect margate LMW Ig by radioimmunoassay but not by haemagglutination. Sensitive Ig detection methods were used also in the only other studies where LMW Ig has been found in teleosts: radiolabelling (giant grouper, *Epinephelus itaira*) (Clem, 1971); radioimmunoassay (toadfish) (Warr, 1983); and immunoprecipitation with specific antiserum raised against purified Ig (sheepshead) (Lobb & Clem, 1981). In general, LMW Ig has not been found in studies of teleosts where non-enhanced secondary antibody-antigen binding assays (Fletcher & Grant, 1969; Marchalonis, 1971; Cisar & Fryer, 1974; Ingram & Alexander, 1979; Ourth & Phillips, 1981; Fukuda & Kusuda, 1982; Vilain *et al.*, 1984; Kusuda *et al.*, 1987), or no immunological criteria (Kobayashi *et al.*, 1982; Lobb & Clem, 1983; Havarstein *et al.*, 1988) have been used to identify Ig containing fractions of immune serum. Consideration of this literature and the results of the present study lends support to the view that highly sensitive detection systems should be used to rule out the presence of LMW Ig in teleosts.

Although the results of this investigation have confirmed the presence of HMW and LMW Ig populations in serum of perch and suggested H and L chain isotypic differences within and between the two populations, further studies are required to elucidate the precise structure of these Ig molecules and their functions in the life history of the perch.

The anti-redfin perch immunoglobulin antisera produced in sheep and rabbits will be applied to the detection of specific immune responses against EHNV. This is discussed in the next section.

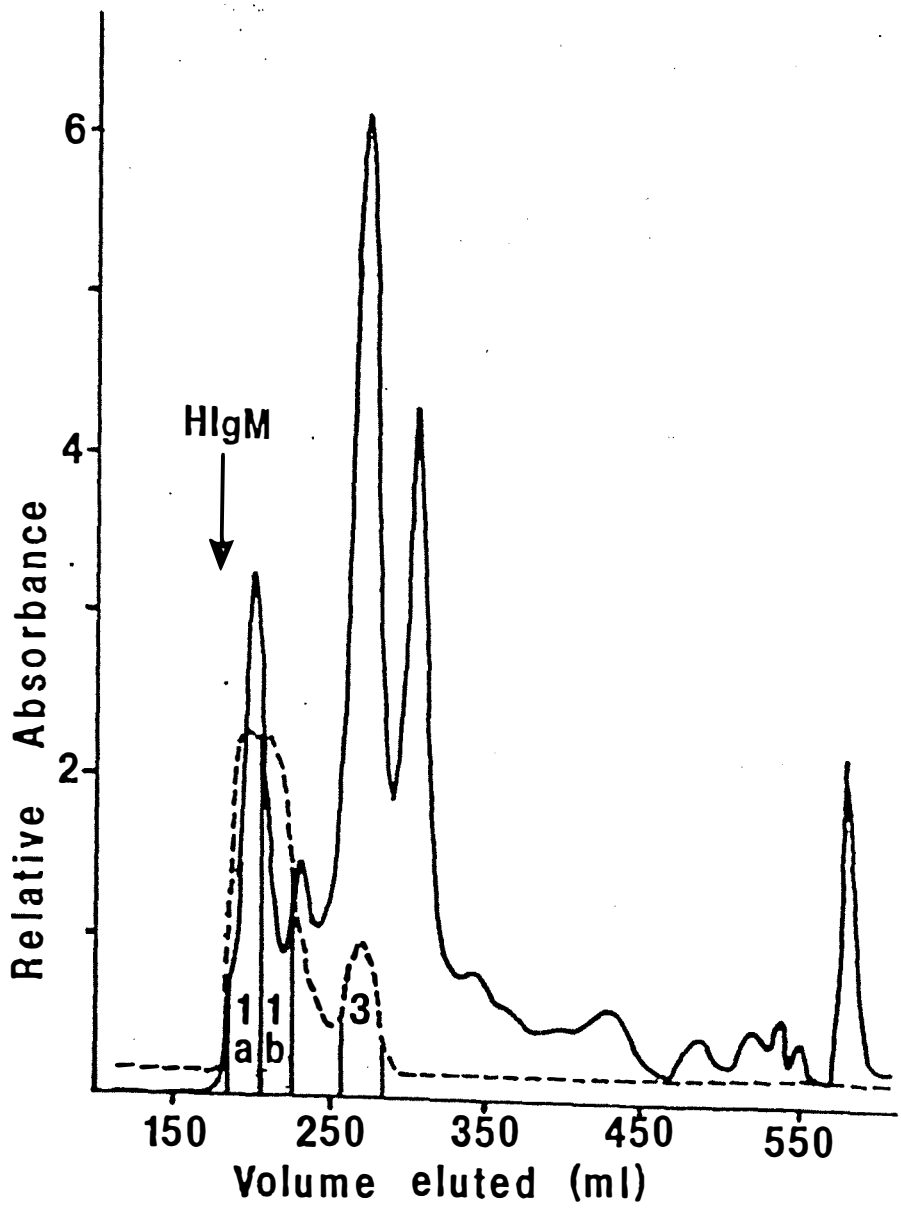


Figure 3.1. Chromatogram (relative absorbance 280 nm _____) of perch immune serum on Sephacryl S300 and relative ELISA absorbance (-------) of chromatographic fractions (arbitrary units). Fractions containing ovalbumin binding activity were pooled (1a, 1a+1b, 3). The position of peak elution of human IgM (HIgM) is indicated by the arrow.

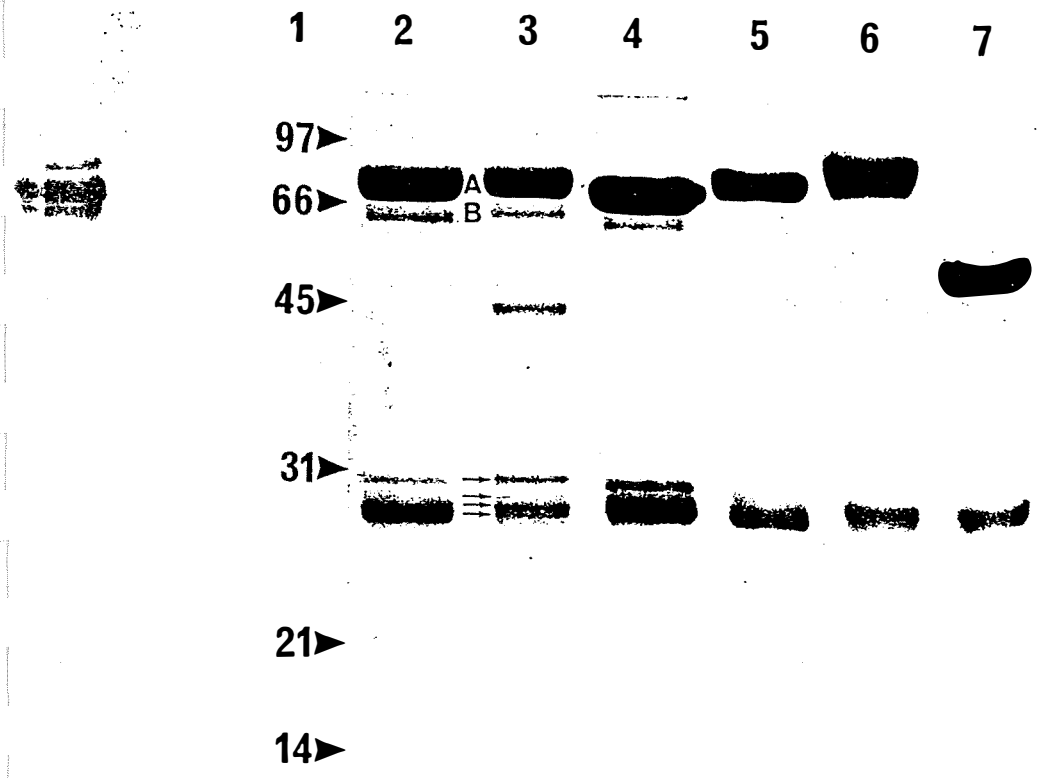


Figure 3.2. SDS-PAGE analysis of perch Ig in relation to mammalian Ig under reducing conditions in a 12% gel stained with CBB. The 72-kDa H chain of perch (A) and a 64-kDa band (B) are indicated. The positions of four putative L chains are indicated by arrows. Molecular weights in kDa (lane 1), affinity purified pool 1a HMW Ig (lane 2), affinity purified pool 1a+1b HMW Ig (lane 3), ion-exchange purified pool 1a HMW Ig (lane 4), human IgM (lane 5), bovine IgM (lane 6), ovine IgG (lane 7).

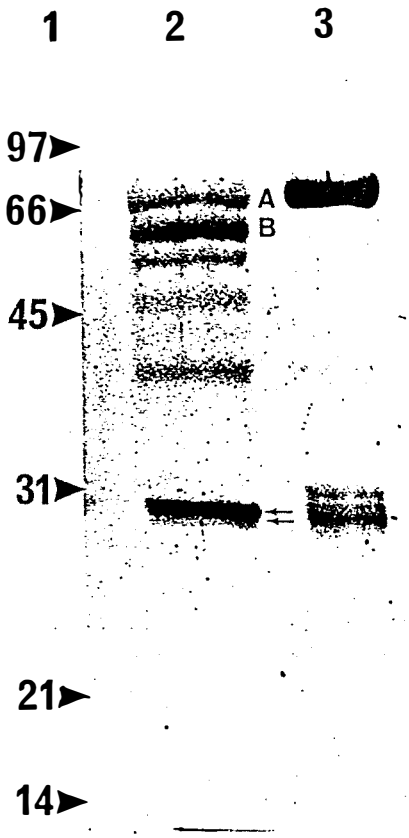


Figure 3.3. SDS-PAGE analysis comparing perch LMW and HMW Ig under reducing conditions in a 12% gel stained with CBB. Bands of 72-kDa (A) and 64-kDa (B) and putative L chains of 28-kDa and 27-kDa are indicated (arrows). MW in kDa (lane 1), affinity purified LMW Ig (lane 2), affinity purified pool 1a HMW Ig (lane 3).

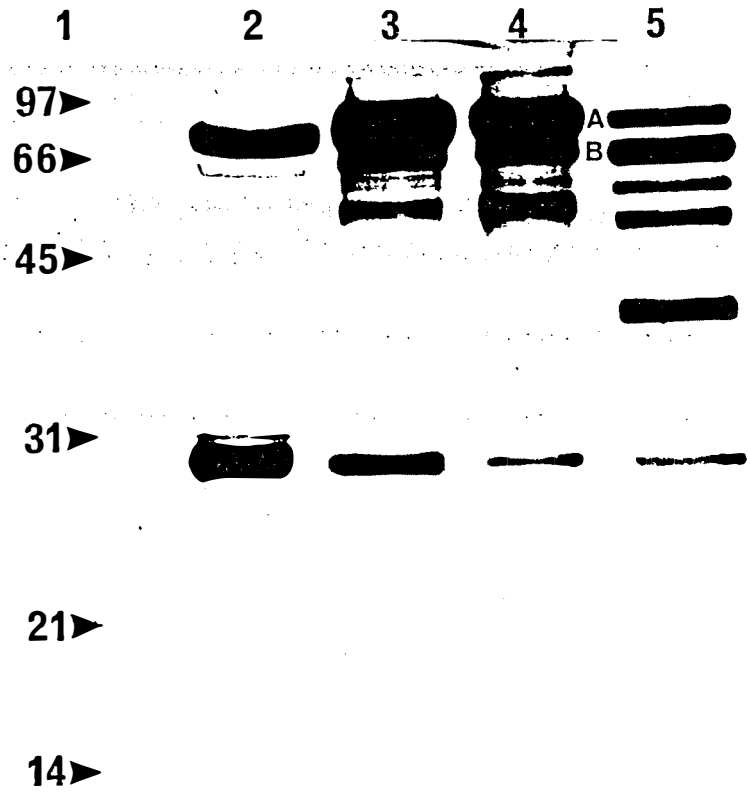


Figure 3.4. Western blot analysis of the antigenic relatedness of HMW and LMW perch Ig using antiserum (RAPI) prepared against affinity purified HMW Ig. Molecular weights in kDa (lane 1), immune perch serum (lane 2), affinity purified pool 1a HMW Ig (lane 3), affinity purified pool 1a+1b HMW Ig (lane 4), affinity purified LMW Ig (lane 5). The 72–kDa H chain (A) and the 64–kDa band (B) are indicated.

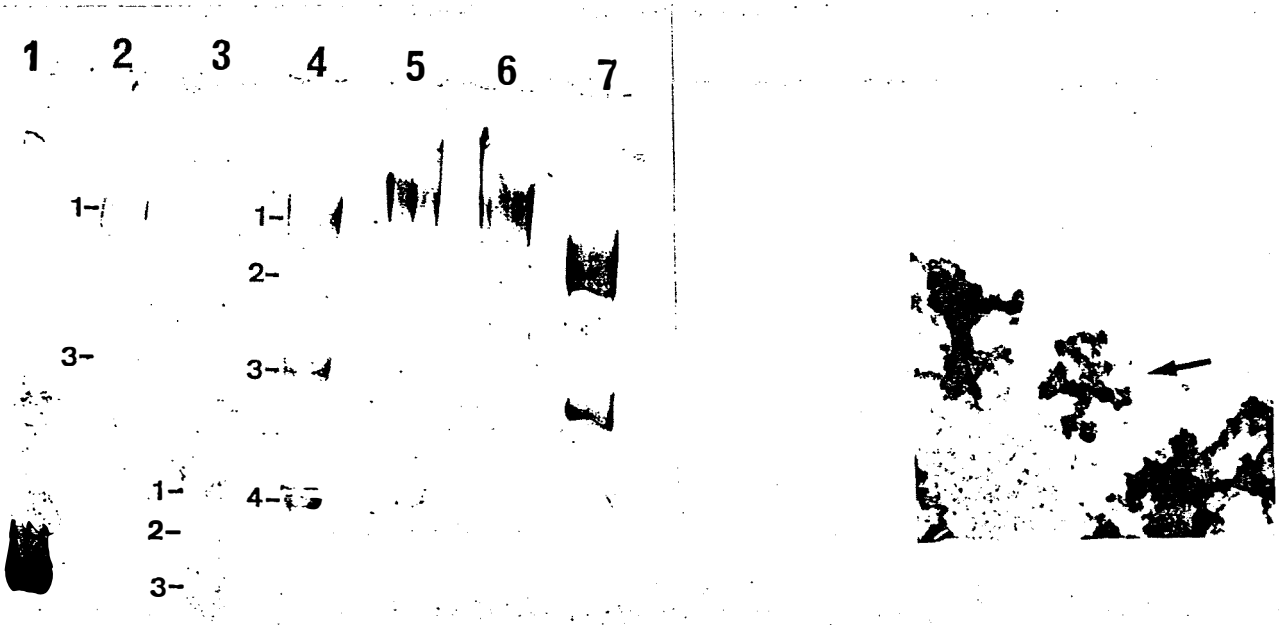


Figure 3.5. SDS–PAGE analysis of unreduced perch Ig in a 3% gel stained with CBB. Separated components of perch Ig are indicated (–). Ion–exchange purified pool 1a HMW Ig (lane 2) was resolved into two components (HMW1, HMW3), LMW Ig (lane 3) was resolved into 3 bands (LMW1, LMW2, LMW3) while affinity purified pool 1a HMW Ig (lane 4) was resolved into 4 bands (HMW1, HMW2, HMW3, HMW4). Protein standards (lane 1), Ovine IgG; (lane 5), bovine IgM; (lane 6), human IgM; (lane 7), thyroglobulin.

Figure 3.7. Electron micrograph of affinity purified pool 1a HMW Ig. Individual Ig molecules about 50 nm in diameter (arrow) were observed adjacent to a lattice of Ig molecules. Magnification X 205,000.

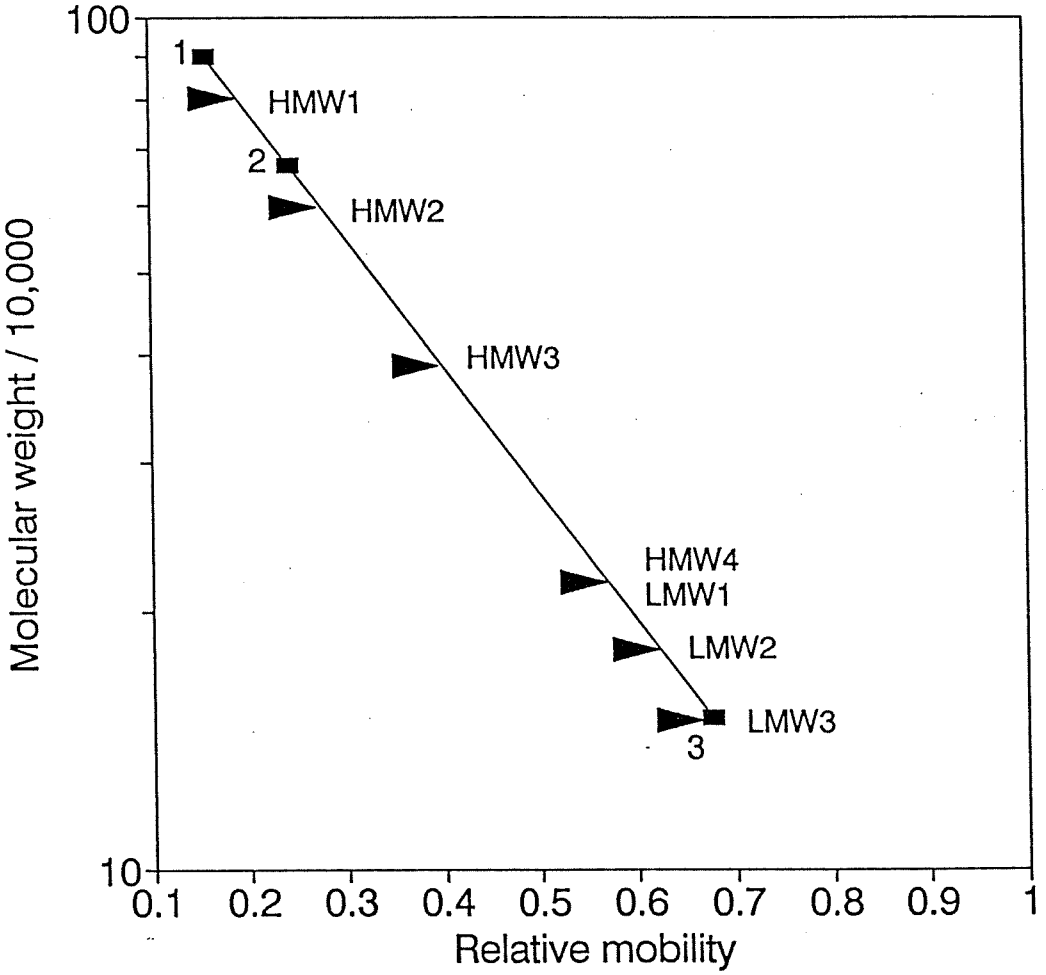


Figure 3.6. Plot of relative mobility vs. molecular weight from Fig. 5 using the following protein standards: (1), human IgM; (2), thyroglobulin; (3), ovine IgG. Remaining captions as for Figure 3.5.

SECTION 4

DETECTION OF SPECIFIC IMMUNE RESPONSES TO EHNV IN RAINBOW TROUT AND REDFIN PERCH

Introduction

In earlier sections of this report the detection of EHNV particles in the tissues of infected fish was detailed. Work aiming to detect the immune response of infected fish to EHNV is detailed in this section.

In general, following infection with any pathogenic virus there are a number of possible outcomes for individual hosts:

1. Death
2. Elimination of infection and recovery
3. Recovery but with persistent infection

In the case of the latter outcomes it may be difficult to detect the presence of the virus, which may exist in low numbers if at all. To overcome this problem it is often advantageous to seek indirect evidence of viral infection in the form of circulating antibody in serum. Most infectious agents stimulate production of specific antibody by the host's immune serum which can be detected by a number of serological assays including double immunodiffusion, complement fixation, viral neutralisation, fluorescent antibody and ELISA. The ELISA is favoured because of its sensitivity.

Immune responses have not been studied in detail in redfin perch and data for rainbow trout under Australian conditions is non-existent. There is no information concerning the immune response of fish to EHNV. Consequently in this project the general nature of the immune response to model protein antigens was studied in both species in order to optimise assay conditions and to assess the feasibility of detecting responses to EHNV. Specific responses to EHNV were then assessed.

Materials and methods

Model antigens for assessment of immune responses

The following antigens were used to model the immune responses of rainbow trout and redfin perch: ovalbumin (Sigma), a soluble protein and weak immunogen and *Vibrio anguillarum* whole cells, a bacterial particulate antigen and strong immunogen.

Inactivation of EHNV for inoculation of fish

EHNV was inactivated by several means to permit inoculation of fish without induction of disease. This was done to produce specific high titred antisera against EHNV

in rainbow trout and redfin perch to determine the kinetics of the immune response and to act subsequently as control reagents for diagnostic testing.

Formalin

a. Inactivation in a small volume. EHNV (sucrose gradient purified B2) in a volume of 500 ul of 100 mM Tris–HCL pH 8.6 buffer was inactivated by the addition of formalin to a final concentration of 0.1% and left for 7 days RT; following neutralisation with metabisulphite at a rate of 1 mole per 2 moles of formalin, inactivation was not evident by BF–2 cell culture inoculation and the procedure was repeated to achieve inactivation.

b. Inactivation in a large volume. EHNV (sucrose gradient purified B2) was made up to 100ml in 100 mM Tris–HCL pH 8.6 buffer and inactivated with 0.1% formalin at RT for 25 days. Following concentration by centrifugation, the preparation was inactive as determined by inoculation of BF–2 cells; the virus was stored at 4oC for 12 weeks prior to vaccination of fish.

Gamma irradiation

EHNV (cell culture supernatant) was inactivated with 9 megarad of gamma radiation while another lot of EHNV (sucrose gradient purified B2) was treated with 6 megarad of radiation. Virus was stored at 4°C prior to inoculation of fish.

Immunisation of fish

Rainbow trout

Ovalbumin

Experiment 1. Rainbow trout fingerlings (25) were injected intraperitoneally with 1mg of ovalbumin in Freund's complete adjuvant; a further 20 fingerlings were revaccinated 4 weeks later. There were 30 control fish. Blood was collected from subgroups of fish 4, 6 and 8.5 weeks after initial immunisation.

Experiment 2. Rainbow trout fingerlings (5) were injected with 10 ug ovalbumin, 4 fingerlings were injected with 1 mg ovalbumin and 5 fingerlings were given saline. Adjuvant was not used. Serum was collected prior to immunisation and at 6 and 9 weeks later.

Vibrio anguillarum

Experiment 3. Rainbow trout fingerlings (25) were immunised with 1 mg of whole bacterial cells in FCA; a further 20 fingerlings were revaccinated 4 weeks later. There were 30 control fish.

EHNV

Experiment 4. Two mature rainbow trout were immunised with 10 ug EHNV inactivated with formalin (small volume method) in FCA followed by a second dose of 10 ug EHNV inactivated with formalin (large volume method) in FCA after 7 weeks. Doses of 39 ug and 200 ug of non–inactivated EHNV (sucrose gradient purified B2) in FCA were given at 19 and 24 weeks respectively. All immunisations were by the intraperitoneal route.

Experiment 5. Rainbow trout fingerlings (4) were injected with EHNV ($10^{6.56}TCID_{50}$ in cell culture supernatant) without adjuvant. Serum was collected from these fish and from controls prior to immunisation and at 6 and 9 weeks after immunisation.

Redfin perch

Ovalbumin
Experiment 6. Two mature redfin perch were given 1 mg ovalbumin in Freund's complete adjuvant and this was repeated 4, 6 and 8 weeks later. One mature redfin perch was immunised with 10 ug of ovalbumin without adjuvant.

EHNV
Experiment 7. Two mature redfin perch were immunised with a single dose of 10ug of EHNV inactivated with formalin (small volume method) in FCA while 3 were immunised with EHNV inactivated with formalin (large volume method) in FCA.

Experiment 8. One mature 270 mm redfin was inoculated with EHNV ($2x10^7$ $TCID_{50}$ gamma irradiated cell culture supernatant) in FCA in divided doses given 4 weeks apart.

Experiment 9. Two mature 260 mm FL redfin perch were inoculated with EHNV (500 ug of gamma irradiated sucrose gradient purified B2) in FCA.

Collection of serum samples from the field

Rainbow trout
Sera were collected from fish from an EHNV free hatchery (University of Tasmania), and from an endemically infected hatchery in NSW.

Redfin perch
Sera were collected from redfin perch acquired from populations believed to be free of EHNV (Googong Reservoir, Khancoban Pondage and Mulwala) as well as from endemically infected populations in Victoria. The latter samples were provided by Dr Alex Hyatt, CSIRO.

Serological tests
Indirect ELISA using species specific anti-immunoglobulin conjugates produced during the project and purchased from commercial sources and double immunodiffusion assays were used to assess immune responses to each antigen.

Results

Immune responses in rainbow trout

Ovalbumin

Experiment 1. When tested by ELISA, a small increase in antibody level was noted 4 weeks after immunisation with ovalbumin in FCA, but by week 6 mean increases greater than 9 fold were evident (Figure 4.1). There was no significant difference in antibody levels between single dose and revaccinated fish at week 6. By week 8.5 revaccinated fish had a 23 fold mean increase in antibody level and this was significantly greater ($P<0.01$) than the 13 fold mean increase in the single dose group.

When tested by double immunodiffusion, one of 20 sera at week 6 and 4 of 20 sera at week 8.5 produced a visible line of precipitation close to the serum well. These sera were all from the revaccinated group. Ovalbumin was used at a concentration of 0.5 mg/ml and 1 mg/ml while serum was used neat.

Experiment 2. Six and 9 weeks after immunisation with ovalbumin (without adjuvant) there were no specific antibody responses against ovalbumin detected by indirect ELISA in any of the fish (Table 4.1).

Vibrio anguillarum

Experiment 3. A 10 fold mean increase in antibody level was evident 4 weeks after immunisation with *V. anguillarum*, increasing to a 16–18 fold mean increase at week 6, and declining slightly from this level by week 8.5. (Figure 4.2). There was no significant difference in antibody level between single dose and revaccinated fish at week 6 or week 8.5.

EHNV

Experiment 4. A strong antibody response was detected 14 days after the fourth dose of EHNV in one fish and 31 days after the fourth dose of vaccine in the second fish (Table 4.2). It is unclear whether this response was due to the third or fourth doses of vaccine or to both. It appears that there was no immune response attributable to the first two doses of EHNV.

Experiment 5. Following immunisation with EHNV without adjuvant an increase in serum antibody specific for EHNV was detected by ELISA in two of the 4 fish at 6 and 9 weeks (Table 4.3). Mean antibody levels in controls did not change over this period. ELISA OD in the four fish immunised with EHNV were 0.40, 0.88, 0.18 and 0.29 at 6 weeks and 0.32, 1.04, 0.13 and 0.14 at 9 weeks.

Immune responses in redfin perch

Ovalbumin

Experiment 6. Strong antibody responses were detected in two fish following

immunisation with ovalbumin in FCA. One fish was monitored for more than 12 months without evidence of a decline in antibody levels (Table 4.4). Weak antibody responses occurred after immunisation with 10ug ovalbumin without adjuvant and the response was delayed until 15 weeks after immunisation (Table 4.5).

EHNV

Experiment 7. All 5 fish immunised with formalin inactivated EHNV died within 4 weeks without evidence of an increase in antibody levels specific for EHNV. EHNV was recovered from each fish confirming active viral infection as the cause of death.

Experiment 8. ELISA OD prior to immunisation with gamma irradiated EHNV cell culture supernatant in FCA, and at 4 and 8 weeks after initial immunisation were 0.13, 0.37 and 0.39 respectively.

Experiment 9. Both fish died within 11 days of immunisation and EHNV was recovered from their tissues, confirming active viral infection. Retrospective examination of the immunising preparation by inoculation of BF-2 cells confirmed the presence of live virus.

Serological testing of EHNV infected and free populations of fish

Rainbow trout

A total of 449 sera were collected from rainbow trout from an endemically infected hatchery in NSW. Of these sera, 40 were from 3-4 y.o. broodstock, while the remainder were from 0+ fingerlings from cohorts that had suffered from clinical EHN within the last 12 months.

A total of 179 sera were collected from 0+ fingerlings from an EHNV free population of rainbow trout in Tasmania.

The results of testing by indirect ELISA are given in Table 4.6. There was no significant difference between the ELISA OD of the endemically infected and the disease free population. The mean, median, 75th percentile and maximum ELISA OD, and frequency distributions of ELISA OD (Figure 4.3) from the two populations were also similar.

Redfin perch

A total of 39 sera were collected from wild redfin perch from populations believed to be free of EHNV. The mean (range) ELISA OD of these samples was 0.18 (0.13-0.31). Six sera were available from fish from endemically infected redfin perch populations in Victoria. The ELISA OD of these samples are given in Table 4.7. The mean ELISA OD of samples from the infected populations was significantly greater ($p=0$) than the ELISA OD from EHNV free populations. Examination of ELISA OD from individual fish suggests that 3 of the 6 fish were positive for antibody against EHNV.

Discussion

Techniques were developed during this study to enable detection of specific humoral responses in the serum of both rainbow trout and redfin perch. As it was unknown whether fish could mount an immune response following infection with EHNV, model antigens were employed to validate the indirect ELISA tests that were developed. Fish were immunised with ovalbumin and *Vibrio anguillarum* antigens and blood samples were collected at key intervals after immunisation. Specific responses against these antigens were detected in both species (redfin were not immunised with *V. anguillarum*). The humoral responses were of high magnitude and persisted for months in the fish immunised with antigen in adjuvant. Responses to non-adjuvanted antigen were poor; no responses were detected against ovalbumin in rainbow trout, while the response in a redfin perch was weak. Evaluation of responses to model antigens enabled ELISA techniques to be adapted for EHNV with confidence that the test formats optimised conditions for detection of serum antibody in these species of fish. ELISA was found to be more sensitive than double immunodiffusion in the detection of specific serum antibody in rainbow trout. Few responses were detected in rainbow trout using double immunodiffusion of immune serum against ovalbumin as the antigen. However, double immunodiffusion was a useful technique for detection of responses against ovalbumin in redfin perch.

Rainbow trout humoral responses against EHNV administered in adjuvant were similar in magnitude to those induced against ovalbumin and *V. anguillarum*. Specific anti-EHNV antibody was detected 48 days after primary immunisation with 39 ug of EHNV (14 days after a booster with 200 ug of EHNV) in one fish and 65 days after primary immunisation in a second fish. Previously, doses of 10 ug of inactivated EHNV in adjuvant had failed to induce a detectable response in either fish 19 weeks after primary immunisation (12 weeks after booster). Significantly, a single dose of EHNV administered without adjuvant resulted in seroconversion in 2 of 4 fingerlings after 6 weeks.

Efforts to stimulate humoral responses against EHNV in redfin perch were hampered by lack of a reliable method for inactivation of the virus in two experiments. Traditional inactivation protocols using formalin did not eliminate infectivity for redfin perch, even though infectivity for BF-2 cells in vitro could not be demonstrated. Rigorous procedures for assessment of infectivity in vitro will be required if this study is repeated. Gamma irradiation (6 megarad) did not inactivate one lot of EHNV, which was fully virulent when inoculated into redfin perch. Apparent seroconversion was demonstrated in a single redfin perch immunised with a second gamma irradiation (9 megarad) inactivated lot of EHNV. The serological response was weak (ELISA OD increased from 0.13 to approximately 0.38) 4 and 8 weeks after primary immunisation.

Using serum from experiments where rainbow trout had been immunised with EHNV as positive and negative controls in ELISA, the sera of a large number of rainbow trout from EHNV free and infected populations were examined. No difference in the levels of specific antibody against EHNV could be demonstrated between these two groups of fish. This observation is consistent with several possible patterns of disease behaviour:

a. EHNV does not stimulate a humoral response that can be detected by indirect ELISA following natural infection in rainbow trout

b. The prevalence of EHNV infection in an endemically infected population of rainbow trout is very low (less than 2% at the 95% confidence level given the sample sizes used), or

c. Infection with EHNV in rainbow trout is uniformly fatal, no fish surviving to mount a humoral response.

Evaluation of humoral responses in wild caught redfin perch from populations free of EHNV and endemically infected with EHNV were not possible on a large scale during this study. Firstly it was difficult to catch redfin perch from sites believed to be free of the disease at the times field trips were undertaken for this purpose. Secondly, because of the difficulties experienced in immunising redfin perch with EHNV in the laboratory, high titred reference positive and negative sera were not available. Nevertheless, specific antibody activity was demonstrated in the sera of 3 of 6 redfin obtained from endemically infected populations in Victoria. These results were confirmed by Dr Alex Hyatt, CSIRO AAHL, using an independent serological test. The limited data suggest that some redfin perch do survive natural infection with EHNV and mount a humoral response that can be detected by indirect ELISA.

Fig. 4.1. Serum antibody responses (ELISA OD) in rainbow trout fingerlings immunised with one or two doses of ovalbumin in adjuvant.

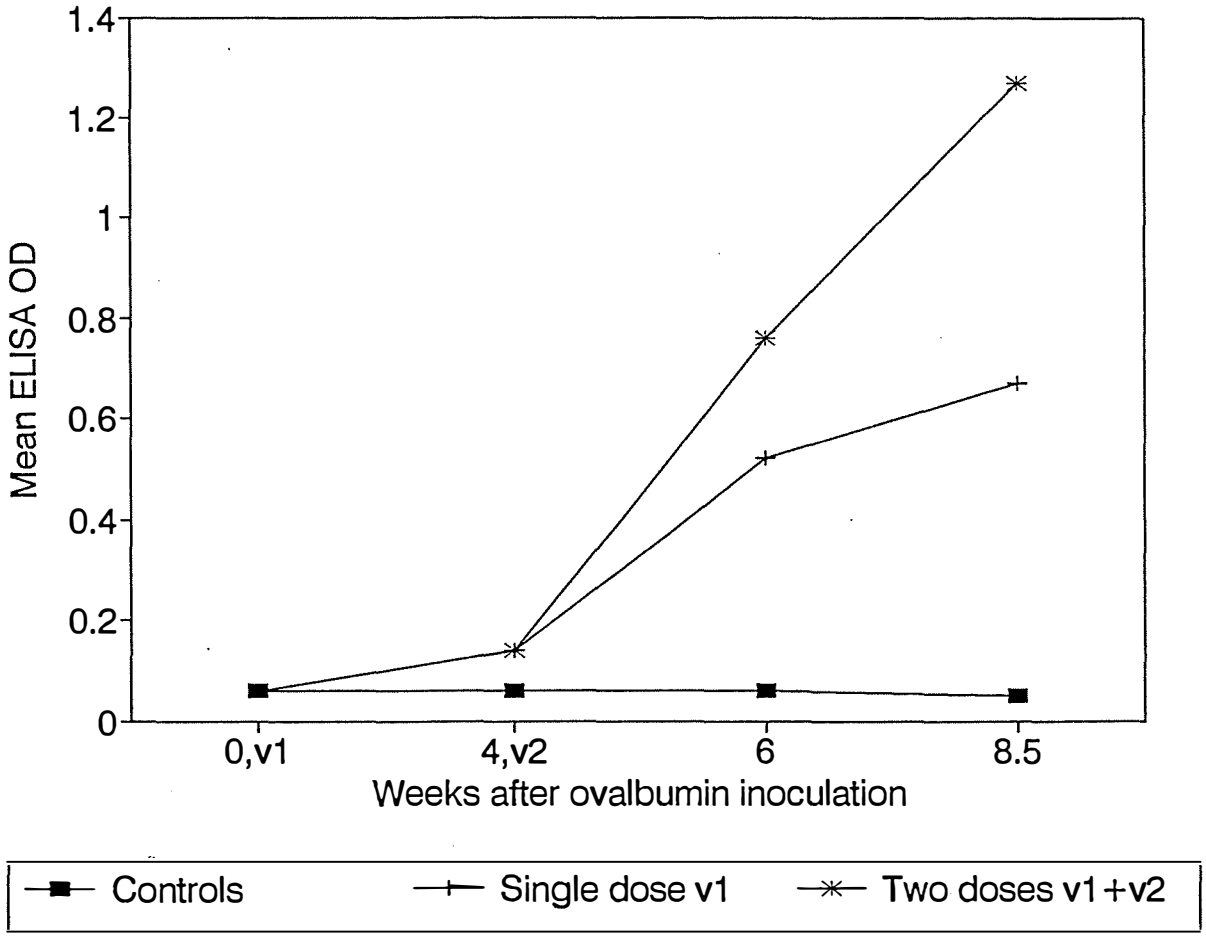


Fig. 4.2. Serum antibody responses (ELISA OD) in rainbow trout fingerlings immunised with one or two doses of *Vibrio anguillarum* in adjuvant.

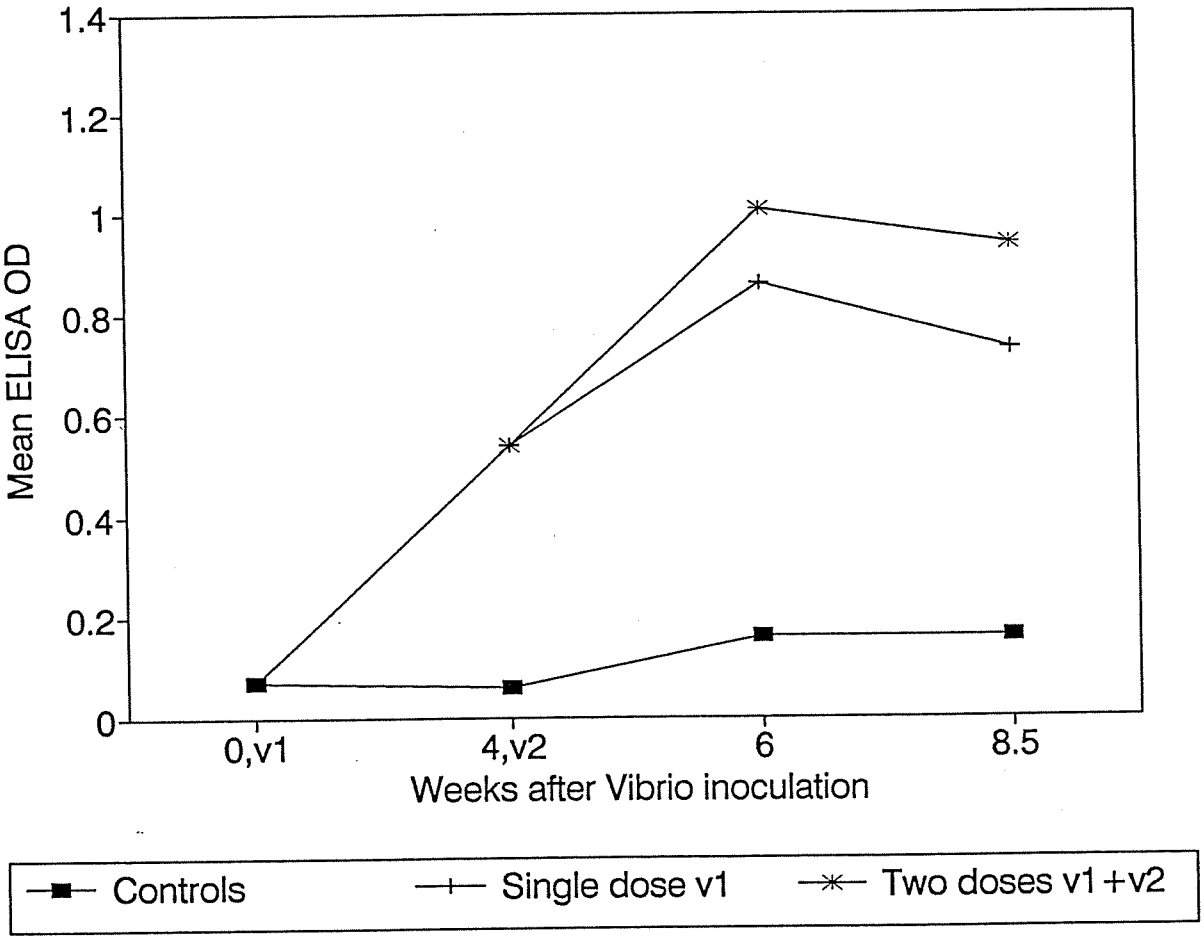


Fig. 4.3. Frequency distributions of anti-EHNV antibody levels in rainbow trout from EHNV free (tasOD) and infected (smtOD) populations.

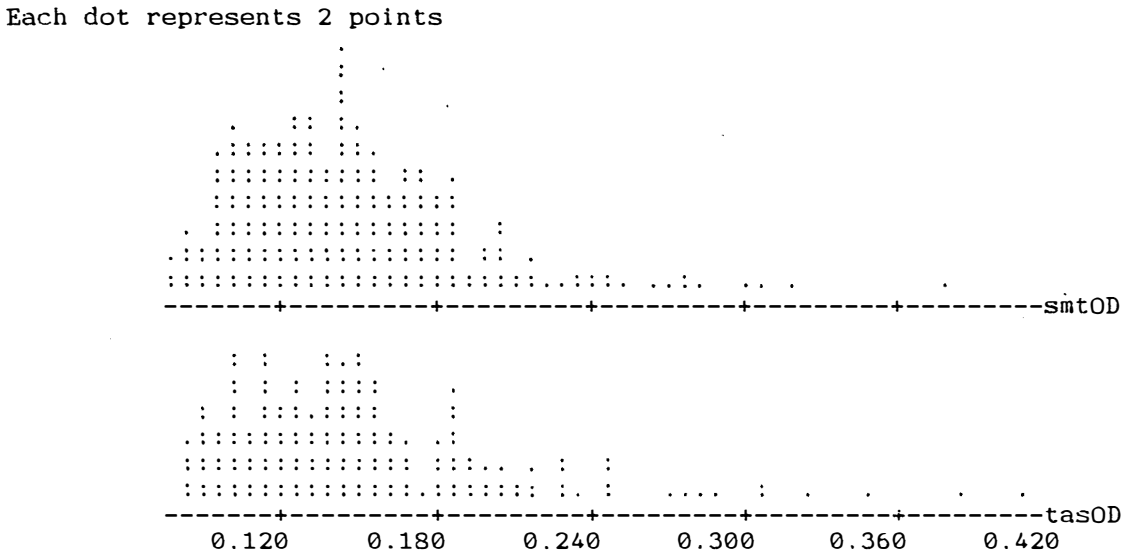


TABLE 4.1

ELISA OD in rainbow trout fingerlings immunised with ovalbumin without adjuvant (Experiment 2)

Group	Week 0	Week 6	Week 9
Control	0.08	0.09	0.06
	0.07	0.08	0.07
	0.07	0.08	0.08
	0.06	0.07	0.07
	0.07	0.08	0.06
Ovalbumin 10 ug	0.06	0.07	0.06
	0.08	0.07	0.07
	0.08	0.07	0.07
	0.07	0.10	0.06
	0.07	0.10	0.06
Ovalbumin 1 mg	0.08	0.10	0.09
	0.06	0.10	0.07
	0.06	0.08	0.06
	0.10	0.06	0.06

TABLE 4.2

Serological responses (ELISA OD) in two rainbow trout immunised with four doses of EHNV in FCA.

Bleed	Days after 3rd dose	Days after 4th dose	ELISA OD	
			Fish 1	Fish 2
1			0.098	0.086
2			0.162	0.135
3			0.175	0.153
4			0.150	0.122
5			0.157	0.148
6			0.127	0.112
7			0.116	0.139
8			0.112	0.110
9	–8	–42	0.124	0.152
10	20	–14	0.086	0.162
11	34	0	0.105	0.148
12	48	14	0.701	0.183
13	65	31	1.328	0.792
14	75	41	1.391	1.339
15	84	50	1.066	1.243
16	111	77	1.213	1.543
17	130	96	1.233	1.680
18	168	134	1.730	
19	209	175	1.704	
20	258	224	1.491	

TABLE 4.3

ELISA OD (mean ± s.d.) in rainbow trout fingerlings immunised with EHNV without adjuvant.

Group	Week 0	Week 6	Week 9
Control	0.15 ± 0.05, n=37	0.14 ± 0.04, n=14	0.12 ± 0.02, n=10
EHNV		0.44 ± 0.31, n= 4	0.41 ± 0.43, n= 4

TABLE 4.4

Serological response measured by ELISA and double immunodiffusion (GDPT) in redfin perch following immunisation with ovalbumin in FCA.

WEEK	Fish 1		Fish 2	
	ELISA OD	GDPT	ELISA OD	GDPT
0*	0.16	–	0.13	–
4*				
6*	1.24	+	0.13	–
8*	0.27	–	1.44	+
10	0.74	+	1.53	+
12	0.84	+	1.58	+
14	0.63	+	1.52	+
16	0.65	+	1.52	+
18	0.54	+	1.62	+
20	1.18	+	1.48	+
22	1.13	+	1.48	nt
24	1.22	+	1.29	+
29			1.62	nt
68			1.53	nt

* Vaccination with ovalbumin nt = not tested

TABLE 4.5

Serum antibody response detected by ELISA in a redfin perch immunised with ovalbumin without adjuvant.

Week	ELISA OD
0	0.09
2	0.12
4	0.09
6	0.14
9	0.14
11	0.17
15	0.55
17	0.53

TABLE 4.6

EHNV antibody levels (ELISA OD) in rainbow trout populations with and without the disease

Population	n	ELISA OD				
		Mean	s.d.	Median	75th percentile	Maximum
EHNV free	179	0.15	0.06	0.14	0.18	0.41
EHNV Infected	449	0.15	0.04	0.14	0.17	0.38

TABLE 4.7

EHNV antibody levels (ELISA OD) in redfin perch from endemically infected populations in Victoria.

Accession	Source	Date collected	Fish	ELISA OD
92/32	Lake Mokoan	08.04.92	1	0.21
	Lake Mokoan	06.02.92	2	0.93
	Lake Mokoan	08.04.92	3	0.24
	Lake Mokoan	08.04.92	4	0.71
91/88	Lake Mokoan	10.04.91	1	1.23
	Lake Nillahcootie	10.05.91	2	0.18
Mean				0.58

SECTION 5

STORAGE OF TISSUES AND *IN-VITRO* GROWTH CONDITIONS FOR DIAGNOSIS OF EHNV

Introduction

Information provided by Langdon (1989) suggested that EHNV was an extremely resistant agent. In fish tissues stored at −20°C the virus remained viable for at least 2 years, although the development of CPE was delayed. Viability was similar in tissue culture supernatants stored at 4°C or at −20°C. This data and the ability of EHNV to withstand harsh conditions such as drying onto a surface for 100–200 days suggested that there were unlikely to be any specific requirements for handling and storage of diagnostic specimens. Consequently, simple protocols were set up during this study to enable acquisition, dissection and processing of tissue specimens. Tissues were generally stored at −20°C while tissue culture supernatants were stored at 4°C or −20°C.

Results of virus isolation and ELISA obtained from specimens handled and stored as outlined above were consistent with the history of each accession, i.e. EHNV was demonstrated in populations with known exposure to the virus. Late in the study, however, results obtained from fish that had been stored frozen at −20°C for a prolonged period suggested that the survival of EHNV may not be guaranteed in frozen tissues.

Although EHNV was generally found to grow vigorously *in-vitro* during this study, results were sometimes variable, particularly during summer. Consequently, the temperature tolerance of EHNV in BF-2 cells was specifically investigated. Langdon (1989) had previously found that a temperature of 40°C resulted in complete inactivation in 24 hours.

Materials and methods

Handling and storage of tissues

Whole fish submitted for examination for EHNV were stored frozen at −20°C. After a period of frozen storage, fish were thawed and organs were dissected using aseptic techniques. Organs were transferred to sterile vials and stored frozen at −20°C until required for further examination. After preparation of tissue homogenates for inoculation of cell cultures, remaining homogenates were frozen at −20°C until required for ELISA. Similarly, tissue culture supernatants were harvested, transferred to sterile vials and stored frozen until required for ELISA.

Results from three experiments conducted for another primary aim, in which tissues of EHNV infected fish had been stored frozen for a prolonged period prior to testing, were available for assessment of survival of EHNV in frozen storage at −20°C.

Virus isolation and antigen capture ELISA

Virus isolation and antigen capture ELISA were conducted as described in Sections 1 and 2 of Research Methodology, Results and Discussion.

Temperature tolerance of EHNV in BF-2 cells

In order to assess the importance of incubation temperature on growth of EHNV *in vitro* the following experiments were undertaken:

Experiment 1
Six 25 cm^2 tissue culture flasks were seeded with BF-2 cells to give 90% confluence and were incubated over night at 25°C. The media was then removed and 300 ul of EHNV tissue culture supernatant (86:8774) was adsorbed in each of the flasks for 1 hr, before 50 ml of fresh media was added. Two flasks were placed at 25°C, two at 20°C and two at 15°C. Flasks were left for 24 hrs, and then 200 ul aliquots were taken twice daily from each. Each aliquot was freeze/thawed three times in liquid nitrogen and a 37°C water bath and the supernatant was then titrated. The monolayers were examined by light microscopy at the time each aliquot was taken.

Experiment 2
Two stock EHNV tissue culture supernatants were titrated in triplicate in BF-2 cells at three temperatures: 22°C, 27°C and 30°C. Plates were read after 6 days.

Experiment 3
Near confluent monolayers of BF-2 cells were inoculated with five stock EHNV tissue culture supernatants in triplicate. Tubes were incubated at 22°C, 27°C and 37°C and read 6 days later.

Results

Handling and storage of tissues

Group A specimens
Adult redfin perch (27 sick or dead fish) were obtained from a natural outbreak of the disease. Chilled whole fish were received after 24 hours in transit and were placed at -20°C on arrival at the laboratory. The fish were thawed 8 months later and dissected. Organs from groups of 3 fish were pooled and examined.

EHNV was demonstrated by antigen capture ELISA in 6 of 9 tissue homogenate pools but it was recovered from only 3 of 9 of these pools when virus isolation was used.

Group B specimens
Whole rainbow trout (12 fingerlings) with known history of exposure to EHNV were stored frozen for 20 months prior to dissection. Organs were stored frozen for a further 6 weeks prior to homogenisation and testing by virus isolation and ELISA.

Organs from the fish were combined into 2 pools. One pool was positive for EHNV by virus isolation. Antigen capture ELISA results were positive for one fish in this pool.

Group 3 specimens
Redfin perch were infected with EHNV by bath exposure. When each fish died, organs (liver, kidney, spleen) were removed and frozen at −20°C. Samples were thawed and homogenised and the homogenates were frozen prior to testing of all samples as a group. The amount of viable EHNV in each sample was determined by dilution assay ($TCID_{50}$). Duplicate tissue samples were retested after a further 18 weeks of storage at −20°C. Tissues of all fish contained viable virus when tested by inoculation of tube cultures. Organs of all fish contained similar histological lesions.

The tissues of fish 13 and 14, which had been frozen for only 7 days, contained the greatest amount of viable EHNV (Table 5.1). The titre of viable virus in these tissues declined with further storage. The titre of viable virus in the tissues of fish 1 was below the detectable limit in this assay.

Temperature tolerance of EHNV in-vitro

Experiment 1
At each of the three temperatures focal rounding of cells appeared after 20 hrs and scattered foci of CPE were present after 46 hrs. After 70 hrs, half the monolayer in the flasks incubated at 20°C was disrupted by CPE (50% CPE), while flasks incubated at 25°C had 90% CPE. At 75 hrs, flasks at 15°C had 50% CPE, while CPE was complete in flasks at 20°C and 25°C (Table 5.2). The titre of EHNV (Table 5.3) paralleled the development of CPE in each flask.

Experiment 2
The titration of two different EHNV stocks at three temperatures confirmed distinct differences in the growth of EHNV. At 30°C CPE did not develop with either stock. At 27°C, CPE did not develop in wells containing stock 1, while wells containing stock 2 had CPE ($TCID_{50}$ $10^{4.46}$/ml). At 20°C CPE was evident in wells containing either stock ($TCID_{50}$ $10^{4.78}$/ml and $10^{7.56}$/ml respectively).

Experiment 3
Growth of EHNV stocks in tubes containing BF-2 cells was also affected by incubation temperature (Table 5.4). Growth at 27°C was not detected for 2 of 4 stocks that grew at 20°C. Only 1 of 4 stocks grew at 30°C.

Discussion

Survival of EHNV in frozen tissues and tissue homogenates

These experiments do not provide conclusive evidence for the stability of EHNV in frozen fish/tissues but do suggest that viability of the virus may be impaired over time by storage at −20°C. Further critical experiments are required to confirm ideal conditions for storage of tissues prior to processing for virus isolation and antigen capture ELISA. In addition, storage of tissue homogenates and tissue culture supernatants must be assessed. An issue not addressed in this study is the retention of antigenicity of EHNV under various conditions of storage; understanding of antigenic stability is also important in the development of sampling and testing protocols for disease certification.

Growth conditions in BF−2 cells

The temperature of incubation of BF−2 cells appears to be critical for the development of CPE and optimal replication of EHNV. Growth at temperatures greater than 25°C is unreliable. Some strains of the virus may be more tolerant of high incubation temperatures than other strains. The use of a thermostatically controlled refrigerated incubator for routine culture of EHNV in diagnostic specimens is mandatory. Incubation at room temperature or in non−refrigerated incubators set to 22°C cannot be guaranteed to support growth of the virus if ambient temperatures fluctuate above 22°C, as they do in many laboratories, for example at night and on weekends when air conditioning systems are "down regulated".

Table 5.1

Effect of sample storage on the titre of EHNV detected in redfin perch tissues

Fish #	Initial test			Retest		
	Duration of storage of organs (weeks at –20°C)	Duration of storage of homogenate (days at –20°C)	Titre (Log_{10} $TCID_{50}$/ml)	Duration of storage of organs (weeks at –20°C)	Duration of storage of homogenate (days at –20°C)	Titre (Log_{10} $TCID_{50}$/ml)
1	23	6	0.00	42	13	0.00
2	22	6	2.46	41	13	0.00
7	6	6	3.38	25	13	1.53
8	4	6	0.00	23	13	2.61
13	1	0	6.90	19	13	4.59
14	1	0	8.02	19	13	5.92

Table 5.2

Presence of CPE in BF-2 cells at intervals following inoculation with EHNV and incubation at the temperatures shown.

Time (hrs)	Presence of CPE		
	15°C	20°C	25°C
0	–	–	–
4	–	–	–
20	FR	FR	FR
24	FR	FR	FR
28	FR	FR	FR
46	CPE	CPE	CPE
49	CPE	CPE	CPE
53	CPE	CPE	50%CPE
70	CPE	50%CPE	90%CPE
75	50%CPE	100%CPE	100%CPE

FR = Focal rounding
– = CPE absent

Table 5.3

Titre of EHNV at intervals following inoculation of BF-2 cells with EHNV and incubation at the temperatures shown.

Time (hrs)	Log_{10} $TCID_{50}$/ml		
	15oC	20oC	25oC
4	–	3.15	3.98
20	–	3.98	4.15
28	2.69	4.46	4.15
46	3.56	4.46	4.98
53	3.69	5.46	5.46
70	4.15	7.46	7.69
75	6.15	7.46	7.98

Table 5.4

Presence of CPE in BF-2 cells following inoculation with 5 strains of EHNV and incubation at the temperatures shown.

Stock virus #	Presence of CPE		
	20°C	27°C	30°C
1	+	+	–
2	+	–	–
3	+	+	+
4	cont	cont	cont
5	+	–	–

+ = CPE present
– = CPE absent
cont = contaminated, no result

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IMPLICATIONS AND RECOMMENDATIONS

1. **Use of antigen capture ELISA for diagnosis and certification of EHNV**

a. The sensitivity and specificity of the antigen capture ELISA developed during this study are high enough to enable its use in routine diagnosis and certification of EHNV. In infected fish, EHNV is readily demonstrated in 80% of individuals. The 20% false negative rate is attributable to there being relatively few virus particles in individuals in the early stages of infection. Tissue culture is inherently more sensitive than ELISA in detecting these infected individuals because the virus multiplies to detectable levels in tissue culture. Diagnosis of EHNV by ELISA would require the testing of more than one infected fish, which is consistent with current laboratory practice for the diagnosis of fish diseases.

b. Accurate costings for antigen capture ELISA and conventional virus isolation in cell culture cannot be provided at the time of writing, however, the newly developed ELISA is likely to be significantly cheaper than the existing test.

c. The antigen capture ELISA is rapid and practical. Using the tissue preparation method and ELISA described in this report, certification test results on large samples of fish could be available within 24 hours of receipt of specimens at a diagnostic laboratory. This compares more than favourably with the present turn around time of about 2 weeks for certification based on tissue culture.

2. **Detection of specific anti-EHNV antibody in blood samples**

a. Anti-EHNV antibody was not found in the serum of rainbow trout on an endemically infected hatchery. This observation is consistent with several possible patterns of disease behaviour: a. EHNV does not stimulate a humoral response that can be detected by indirect ELISA following natural infection in rainbow trout, or; b. The prevalence of EHNV infection in an endemically infected population of rainbow trout is very low (less than 2% at the 95% confidence level given the sample sizes used), or; c. Infection with EHNV in rainbow trout is uniformly fatal, no fish surviving to mount a humoral response. Additional research is required in rainbow trout to evaluate the epidemiology of the infection. The potential usefulness of antibody detection ELISA is not yet clear.

Limited data suggest that some redfin perch survive natural infection with EHNV and mount a humoral response that is detectable by indirect ELISA.

3. **Certification protocols for rainbow trout**

Research on the behaviour (spread, persistence, re-introduction, carrier fish etc) of EHNV in populations of rainbow trout in endemically infected hatcheries should be

supported. Knowledge of these factors is required to define appropriate protocols for specimen collection for disease certification.

4. Collection and handling of tissues for disease certification

Critical experiments are required to confirm ideal conditions for storage of tissues prior to processing for virus isolation and antigen capture ELISA. In addition, storage of tissue homogenates and tissue culture supernatants must be assessed. Retention of antigenicity of EHNV under various conditions of storage must also be assessed in the development of sampling and testing protocols for disease certification.

5. Laboratory culture of EHNV in BF-2 cells

The use of a thermostatically controlled refrigerated incubator for routine culture of EHNV in diagnostic specimens is mandatory. Incubation at room temperature or in non-refrigerated incubators set to 22°C cannot be guaranteed to support growth of the virus if ambient temperatures fluctuate above 22°C, as they do in many laboratories, for example at night and on weekends when air conditioning systems are "down regulated".

6. Additional laboratory studies

a. The need to reduce non-specific background reactions in antigen capture ELISA for rainbow trout tissues can be addressed by the development of monoclonal antibodies specific for EHNV. Successful incorporation of monoclonal antibodies in the antigen capture ELISA would permit the positive-negative threshold in the ELISA to be lowered, and thereby increase the statistical sensitivity of the assay above the existing figure of 80%.

b. The possibility that several distinct strains (pathotypes) of EHNV exist requires assessment. Again, monoclonal antibodies can be produced for this purpose, but experimental infection of fish will also be required. EHNV isolates must be compared with similar iridoviruses recovered from amphibians in Australia.

c. A reliable method for inactivation of EHNV is needed in order to produce non-infectious control reagents for use in ELISA. In addition, inactivated virus is required to produce control positive antisera in redfin perch for further evaluation of an antibody detection ELISA in this species.

INTELLECTUAL PROPERTY

The intellectual property developed during this project comprises:

1. Method for purification of EHNV

2. Method for production of specific antisera against EHNV in rabbits, sheep and fish

3. Method for antigen capture ELISA for detection of EHNV in tissue culture supernatant and fish tissues

4. Method for tissue preparation for laboratory assay for virus

5. Immunofluorescence method for detection of EHNV in cell culture and in fish tissues

6. Method for detection of specific antibodies against EHNV in serum of redfin perch and rainbow trout

While several novel approaches were taken during this project, the underlying technology must be regarded as generic. The commercial potential of the findings is considered to be negligible at this time. This would need to be reviewed if EHNV became an important disease in aquaculture industries in the northern hemisphere.

The principal value of this research is in making new diagnostic techniques available for disease control.

In addition to any contractual arrangements between NSW Agriculture and FRDC, the role of the University of Wollongong must be mentioned. Miss Kirsten Steiner, Technical Officer Scientific was employed on the project while also enrolled as a MSc student (part-time) at the University of Wollongong. All work related to the project was carried out on NSW Agriculture premises (Glenfield and EMAI), under the direct supervision of staff employed by NSW Agriculture. The role of staff of the University of Wollongong was limited to the administration of Miss Steiner in her capacity as a student.

TECHNICAL SUMMARY

1. **Purification of EHNV**
EHNV was separated from cellular material by cell disruption, differential centrifugation and density gradient centrifugation in sucrose. Efforts to achieve further purification by physical means resulted in dramatic reductions in yield and were therefore not undertaken in preparative experiments. Two bands were obtained in sucrose gradients; the lower band (B2) contained virions, with only traces of cellular antigen being detected in Western blots with anti-BF-2 cell serum. This B2 material was subsequently used to immunise a rabbit and a sheep. While the upper band (B1) also contained virions, a considerable amount of cellular material was detected by electron microscopy, SDS-PAGE, and Western blotting with anti-BF-2 cell serum. The association of infective virus with cell debris in B1, after the application of disruptive physical forces during viral purification, suggests that EHNV is highly associated with cell constituents.

2. **Indirect ELISA**
An indirect ELISA formatted with rabbit anti-B2 serum as capture antibody and sheep anti-B2 serum as second antibody successfully detected EHNV antigen in cell culture supernatant. When diluted sheep-anti-B2 serum was pre-adsorbed with BF-2 cell lysate, the OD in BF-2 cell control wells was markedly reduced, thus increasing the specificity of the assay.

3. **Immunofluorescence**
Experiments indicated a favourable role for indirect immunofluorescence in the detection of EHNV in cell culture. In addition, EHNV was demonstrated in the liver and kidney of experimentally infected redfin perch. High levels of background staining have confounded attempts to use this technique in rainbow trout to date.

4. **Technical assessment and improvement of indirect ELISA**
Rabbit antisera were affinity purified to remove unwanted activities and irrelevant proteins. A comparison of the prototype ELISA method with the improved method confirmed that assay sensitivity had been increased by 2 logs. The sensitivity of the assay for infectious viral particles was about $10^{3.5}$ $TCID_{50}$ EHNV/ml. The statistical sensitivity was approximately 80% while the specificity was > 98%.

5. **Rapid ELISA**
The ELISA was reformatted in several ways to enable the entire assay to be completed in less than 4 hours.

6. **Preparation of tissue samples for ELISA**
An efficient technique for preparation of tissue samples for ELISA was developed. The method, which can be completed without complex equipment and using small disposable tubes, was more effective than traditional homogenisation in releasing EHNV antigens from tissue samples. It is possible for a single operator to process 150-200 individual tissues for subsequent inoculation of tissue culture or assessment by ELISA, in less than a day.

7. **Background reactivity of rainbow trout tissues in ELISA**
EHNV free rainbow trout tissues characteristically give a high background signal with the ELISA. The reason is uncertain, but must relate to non–specific reactivity with components of the polyclonal capture and second antibody reagents. This non–specific reactivity could not be reduced using standard adsorption and blocking techniques and necessitated a relatively high positive–negative cut–off optical density.

8. **Preparation of immunological reagents for antibody detection ELISA**
Immunoglobulin (Ig) was purified from immune serum of perch by exploiting its ability to bind to the immunising antigen, in ELISA and affinity chromatography. The redfin perch was found to have two populations of Ig in immune serum that could be distinguished by difference in molecular weight (m.w.). The predominant population had a m.w. of about 800–kDa. By deduction it had a tetrameric structure. The low m.w. Ig (about 200–kDa) population was present in much smaller amounts in immune serum and was antigenically related to the high m.w. population. Antisera against redfin perch Ig were prepared in rabbits and sheep for use in ELISA to detect anti–EHNV antibody.

9. **Detection of specific immune responses against model antigens in fish**
Techniques were developed to enable detection of specific humoral responses in the serum of both rainbow trout and redfin perch. Model antigens were employed initially to validate indirect ELISA tests as it was unknown whether fish were capable of mounting an immune response against EHNV. Specific immune responses were detected in both species. The humoral responses were of high magnitude and persisted for months in the fish immunised with antigen in adjuvant. Responses to non–adjuvanted antigen were poor. Evaluation of responses to model antigens enabled ELISA techniques to be adapted for EHNV with confidence. ELISA was found to be more sensitive than double immunodiffusion in the detection of specific serum antibody in rainbow trout.

10. **Detection of specific immune responses against EHNV**
Rainbow trout humoral responses against EHNV administered in adjuvant were similar in magnitude to those induced against model antigens. Specific anti–EHNV antibody was detected 48 days after primary immunisation with 39 ug of EHNV (14 days after a booster with 200 ug of EHNV) in one fish and 65 days after primary immunisation in a second fish. Previously, doses of 10 ug of inactivated EHNV in adjuvant had failed to induce a detectable response in either fish 19 weeks after primary immunisation (12 weeks after booster). Significantly, a single dose of EHNV administered without adjuvant resulted in seroconversion in 2 of 4 fingerlings after 6 weeks. Efforts to stimulate humoral responses against EHNV in redfin perch were hampered by lack of a reliable method for inactivation of the virus. Traditional inactivation protocols using formalin did not eliminate infectivity for redfin perch, even though infectivity for BF–2 cells *in vitro* could not be demonstrated. Rigorous procedures for assessment of infectivity in vitro will be required if this study is repeated. Apparent seroconversion was demonstrated in a

single redfin perch immunised with gamma irradiated EHNV. The serological response was weak (ELISA OD increased from 0.13 to approximately 0.38) 4 and 8 weeks after primary immunisation.

11. **Evaluation of EHNV antibody in an endemically infected trout population**
The sera of a large number of rainbow trout from EHNV free and infected populations were examined. No difference in the levels of specific antibody against EHNV could be demonstrated between these two groups of fish.

12. **Evaluation of EHNV antibody in wild caught redfin perch**
Evaluation of humoral responses in wild caught redfin perch from populations free of EHNV and endemically infected with EHNV were not possible on a large scale during this study. Anti–EHNV antibody activity was demonstrated in the sera of 3 of 6 redfin perch obtained from endemically infected populations in Victoria.

13. **Collection and storage of tissue specimens for diagnosis of EHNV**
Evidence for the stability of EHNV in frozen fish/tissues was not conclusive but suggested that viability of the virus may be impaired over time by storage at –20°C.

14. ***In–vitro* growth conditions for EHNV**
The temperature of incubation of BF–2 cells appears to be critical for the development of CPE and optimal replication of EHNV. Growth at temperatures greater than 25°C is unreliable. Some strains of the virus may be more tolerant of high incubation temperatures than other strains.