FINAL REPORT

PROJECT TITLE:

NATIONAL DIAGNOSTIC TESTS FOR THE DETECTION OF EPIZOOTIC HAEMATOPOIETIC NECROSIS VIRUS (EHNV) AND CERTIFICATION OF EHNV-FREE FISH

A1 FRDC PROJECT NUMBER: 92/66

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1.0 EXECUTIVE SUMMARY

Results from this project have culminated in several important achievements for the identification and subsequent management of epizootic haematopoietic necrosis virus within Australian wild fish and the commercial fresh water fish industry.

The major objective of the project was to develop a national antigen capture ELISA for the detection of epizootic haematopoietic necrosis virus, a virus which impacts on the rainbow trout industry. At the onset of the project it was envisaged that a national test should be developed which had the potential to detect epizootic necrosis virus (EHNV) in field samples and subsequent infected tissue culture cells. The developed test which incorporates the best reagents from the Australian Animal Health Laboratory (AAHL) and the Elizabeth Macarthur Agricultural Institute (EMAI) satisfies this objective and has already been distributed to the Victorian Institute of Agricultural Research (VIAS), the Western Australian Animal Health Laboratory and various laboratories in Europe and North America. The positive control in this test is non-infectious virus; the inclusion of a non-infectious virus (antigen) is to minimise any possibility of inadvertent spread of the virus. The characteristics of the test have also been determined. Suggested cut-offs are given with the test such that the specificity is 99-100% (i.e. minimum false positives) and sensitivity is 60%. All reagents have been tested over a three year period and their recommended storage conditions are included with the test.

Research has also shown that the best tissues for testing in this EHNV antigen capture ELISA are liver, kidney and spleen. The ELISA "kits" have been compiled at EMAI and are now available for distribution.

Monoclonal antibodies have also been produced against EHNV. These antibodies are specific to a single epitope (ie a region on one of the viruses proteins) and are functional in the ELISA and other laboratory diagnostic assays such as immuno-fluorescence and electron microscopy. Of the panel of monoclonal antibodies some have shown the ability to differentiate between different iridoviruses. To understand the implications of this statement it must be realised that within Australia there exists at least two iridoviruses namely EHNV and Bohle iridovirus (BIV, a virus which infects frogs). It should also be noted that throughout the world there exists a range of other fish iridoviruses and frog/toad iridoviruses. Overall, these iridoviruses are very similar to EHNV and some such as sheatfish iridovirus can cause large scale mortalities (eg in Europe). The monoclonal antibodies developed in this project can differentiate between the Australian and exotic iridoviruses. However the use of these antibodies require specific laboratory skills and it is therefore recommended that these tests (EHNV antigen capture ELISA) be performed in specific laboratories AAHL and EMAI) by trained staff.

Results from this study have also shown that various strains of EHNV exist within the Victorian and NSW environment. Detailed studies revealed that a single "strain" of EHNV can infect both redfin perch and rainbow trout; i.e. a virus that infects redfin perch can also infect rainbow trout. It is therefore theoretically possible to track a specific strain of EHNV through the Australian environment utilising specific molecular techniques developed in this project.

Other tests which were developed throughout the course of this study (eg, polymerase chain reaction (PCR) assays, Western blots and restriction enzyme digests) offer the added advantage of differentiating EHNV from BIV.

It is now possible for the freshwater fish industry to test fish stocks for the presence of EHNV. The test is now standardised throughout Australia and has been adopted by the OIE. Refinements of the ELISA and other tests including restriction enzyme digests and the analyses of PCR products have also enabled imports to be tested for the presence of iridoviruses and to determine whether any isolated iridoviruses are exotic to Australia. The acceptance and implementation of the tests and knowledge developed and acquired throughout this project can be used by the industry to check their stock for iridoviruses and thereby maintain EHNV-free farms and minimise the chances of transporting the virus with the relocating/stocking of fish.

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5.0 INTRODUCTION

EHNV is an Iridovirus (Eaton et al,199O) which has been isolated from redfin perch and rainbow trout. The first isolation was reported by Langdon *et al.* (1986) from redfin perch collected from Lake Nillahcootie during November and December,1984.

The disease gained prominence because of the characteristic high mortalities of juvenile redfin and necrosis of the renal haematopoietic tissues including the liver, spleen and kidney in supposedly previously unchallenged adults (Langdon and Humphrey, 1987). To date, outbreaks in redfin perch have occurred in isolated Victorian farm dams, ovens River (Vic. December, 1985), Goulbourn River (Vic. 1986), Winneke Reservoir (Vic. 1988), Lake Nillahacootie (Vic, 1984,85), Lake Hume (Vic. 1984,85), Lake Makoan (1984,85,88), Blowering Reservoir (NSW, 1987), Mount Bold Reservoir (S.A., 1991), Lake Alexandrina (S.A., 1991) and Mount Albert Reservoir (S.A., 1991) Reports have indicated that the virus is now spreading upstream (as far as Renmark) from the lower reaches of the Murray river (Whittington *et al*, 1995). Prior to the more recent outbreaks of the disease, it was believed that EHN was restricted to waters of the upper Murray. As has been the case in other outbreaks the disease in S.A. has resulted in the near decimation (95%) of redfin populations over short periods of time (three weeks); outbreaks in redfin have been reported in the south west slopes, southern ranges and western plains regions of NSW, the virus is believed to be spreading progressively in NSW waterways. Outbreaks of EHNV have also occurred in rainbow trout farms in NSW (1986,87,90) and in Vic. (1988). Disease similar to that of EHN has been observed in diagnostic samples from trout farms in Vic. and W.A. during 1990,91. Although EHNV could not be isolated it was believed that the virus was the cause of the disease. The recent development of antibody capture ELISA s and PCR should, in the future, make it possible to confirm or refute the presence of the virus in such samples.

The virus has also been transmitted (under laboratory conditions) to a range of native and introduced teleosts including Macquarie perch (M. *australasica*), silver perch (*Bidyanus bidyanus*), mosquito fish (*Gambusia affinis)*, mountain galaxias (*Galaxias olidus)* and Murray cod (*Macullochella Peeli*), (Langdon *et al*, 1988). The susceptibility of native fish to EHNV may explain the decline in numbers of various species over the last decade. The disease posses serious consequences for the conservation of our native teleosts, the movement of fish throughout Australia and the export industry in eyed trout ova from south-eastern Australia to New Guinea, Europe and the Middle East.

The threat of the disease at both the national and international levels has been recognised by the OFFICE INTERNATIONAL DES EPIZOOTICS as a notifiable disease. Such classification places stringent conditions on the classification of EHNV free countries, zones and farms. That is, the movement (importation) of live fish of any species, or their spawning products (eggs, ova, milt) must be accompanied by an international animal health certificate from the exporting country certifying that the consignment originates from either a country declared to be EHNV-free or from a farm officially declared EHNV-free. The issue of such certificates will arise from nationally accepted standardised diagnostic tests and statistically valid sampling regimes.

REQUIREMENT FOR A STANDARDISED TEST AND A VALID SAMPLING SYSTEM

Over the past three years research into EHNV has been taken place at both AAHL and EMAI. The work has resulted in the development of diagnostic tests including an antigen detection ELISA. The antigen detection assays have already been used to detect EHNV in clinical samples from field and trout hatcheries.

There is now the need to adopt the antigen capture ELISA tests which show the greatest sensitivity and specificity and distribute these to state veterinary laboratories. These kits would contain inactivated virus for positive controls. It is important that only inactivated virus be used in diagnostic laboratories in non-endemic areas as live virus is extremely stable over long periods of time and is highly infectious. The possibility of accidental contamination of local environments would therefore be eliminated with inactivated (non-infectious) virus. Protocols for the inactivation and use of the virus therefore need to be completed. It should also be noted that the current antisera cross-reacts between Australian iridoviruses (refer below) and as such specific antibodies such as monoclonal antibodies are required for isolate differentiation.

The acceptance and use of the above ELISAs are of little use in certification of fish consignments unless a statistically valid sampling regime is established for the disease in each affected species. The standardised ELISA should therefore be trialed, in conjunction with conventional virus isolation techniques, to establish the frequency of infection (presence of virus) within cultured populations known to have been exposed to the virus. It is also of importance to use the same population to test for the presence of sero-conversion and the presence of virus within selected individuals. Such information is crucial for the formation of policies regarding the transport/export of fish and/or their products.

MONOCLONAL ANTIBODES CAN BE USED FOR DIFFERENTIATING DIFFERENT STRAINS OF EHNV AND OPTIMISING DIAGNOSTIC ASSAYS

The current available tests for the detection of EHNV utilise polyclonal antibodies. The problem generally associated with such antibodies is that they are highly heterogeneous in their antigen-binding affinities and are thus generally directed against a number of different epitopes on the immunising antigen. It therefore comes as no surprise that the polyclonal antibodies generated from purified EHNV reacts with EHNV isolated from both redfin perch ($EHNV_{RF}$), rainbow trout ($EHNV_{T}$) and from a closely related amphibian iridovirus isolated from Queensland frogs Bohle iridovirus, BIV). Monoclonal antibodies directed to strain specific epitopes are therefore required to identify the different strains of EHNV and to discriminate EHNV from other aquatic iridoviruses. These specific antibodies would be included in the diagnostic kits which would originally consist of an antigen capture ELISA and possibly later an antibody capture and/or blocking ELISA.

Monoclonal antibodies identify single epitopes. Therefore by characterising individual antibodies it is possible to differentiate between structural and non-structural proteins. Such discimination can be used to identify tissues/organs where the virus is transitory as opposed to those tissues/organs where the virus is replicating. Identification of tissues/organs in which EHNV replicates will

ultimately lead to correct sampling practices in non-specialised laboratories equiped with EHNV diagnostic kits. To this end it is imperative that some EHNV-free fish be inoculted with reference strains of EHNV.

OTHER TESTS CAN ALSO BE USED FOR DETECTING and DIFFERENTIATING DIFFERENT STRAINS OF EHNV.

Within Australia other iridoviruses have been found, including fish lymphocystis virus (Pearce *et al.*, 1990), an erythrocytic iridovirus (DPEV) from the diamond python (*Morelia spilota*) (unpublished) and Bohle iridovirus (BIV) which was isolated from the ornate burrowing frog *Limnodynastes ornatus* (Gray) in Queensland (Speare and Smith, 1992). The family Iridoviridae encompasses four genera namely *Iridovirus* (represented by type 1 Tipula iridescent virus), *Chloriridovirus* (e.g. type 2 mosquito iridoescent virus), *Ranavirus* (e.g. frog virus 3 (FV3)), *Lymphocystisvirus* (e.g. fish lymphocystis disease virus, (FLDV)) and a fifth proposed 'goldfish group' (Francki *et al.*, 1991). Recent studies have compared EHNV with iridoviruses from the sheatfish *Silurus glanis*, the catfish *Ictalurus* and FV3 (Hedrick *et al.*, 1992; Hengstberger *et al.*, 1993). These studies showed that EHNV, BIV and FV3 belonged to the genus *Ranavirus* as do the sheatfish and catfish viruses (Essani and Granoff, 1989; Hedrick *et al.*, 1992; Hengstberger *et al.*, 1993).

EHNV has been isolated from both redfin perch and rainbow trout (Langdon *et al.*,1986, 1988). Antibodies have been raised against each of these isolates in addition to BIV (Hyatt *et al.*, 1991; Steiner *et al.*, 1991; Hengstberger *et al.*, 1993; Whittington and Steiner, 1993) and used in diagnostic tests such as antigen capture ELISA and immunoelectron microscopy. When polyclonal antibodies against EHNV are applied to Western blots they can demonstrate differences in the molecular weights of some of the structural proteins (e.g. the 38kD and 45kD proteins) of trout and redfin isolates; whether these differences are attributable to strain or isolate differences is yet to be determined (Hengstberger *et al.*, 1993). Although these antibodies also interact with BIV (Hengstberger *et al.*, 1993) they do not react with FLDV or the recently isolated DPEV (unpublished data), nor can they detect concentrations of virus less than $10^{3.5}$ $TCID_{50}$/ml (Hyatt *et al.*, 1991, Steiner *et al.*, 1991, Whittington and Steiner, 1993).

A test which can detect low levels of virus and/or its nucleic acid is the polymerase chain reaction (PCR). This method generates detectable amounts of DNA from only a few copies of the target nucleic acid sequence by repeated cycles of DNA synthesis using a thermostable DNA polymerase and two sequence-specific primers that span a segment of the target genome. It would therefore be beneficial to the fish industry to develop a PCR test which has the capacity to rapidly detect DNA from EHNV and BIV and differentiate the piscine from amphibian viruses.

Collectively the research will provide a comprehensive range of EHNV diagnostic tests. With the standardisation of the tests, and a basic understanding of the cell biology and pathogenesis, it should be possible to offer the industry a service whereby the status of EHNV in cultured stock and expensive breeding stock can be assessed. This information could then be used to develop and maintain disease-free stock.

6.0 OBJECTIVES

1 **Selection and use of EHNV detection protocols**.

(a) Select optimum EHNV detection protocols.

(b) Investigate the use of inactivated antigen in antigen capture ELISAs which would facilitate the distribution of the diagnostic tests.

(c) Use diagnostic tests to determine minimum sampling sizes and types of samples required for disease-free certification of commercial stock.

2 **Produce monoclonal antibodies to:**

(a) Production of monoclonal antibodies for use in antigen capture ELISA.

(b) Identify tissues\organs within host organisms where the virus replicates

(c) Differentiate the major strains of Australian iridoviruses (e.g. EHNV that infects redfin perch (*Perca fluviatlis*) and rainbow trout (*Salmo gairdneri*)).

7.0

ACHIEVEMENTS

7.1 Production Of Monoclonal Antibodies For Use In Antigen Capture ELISAs

METHODS

Cells and Viruses.

Bluegill fry (BF-2, ATCC CCL 91), Chinook salmon embryo (CHSE-214, ATCC CRL 1681), rainbow trout gonad (RTG-2, ATCC CCL 55) and fathead minnow (FHM, ATCC CCL 42) cell monolayers were grown at 22°C in Eagle's minimal essential medium (EMEM) supplemented with 10% foetal calf serum(FCS) and 10mM HEPES.

EHNV originally isolated from cultured rainbow trout EHNV$_T$ *Oncorhynchus mykiss (Walbaum)* EHNV, (Langdon *et al.*, 1988), was plaque purified three times in RTG cells, passed three times in RTG cells and then passed eight times in BF2 cells. BIV, originally isolated from the ornate burrowing frog *Limnodynastes ornatus* (Speare *et al.*, 1992), was plaque purified three times, passed four times in BF2 cells and six times in CHSE cells. FV3 obtained from ATCC (567-VR), was also plaque purified three times in FHM cells and passed twice in FHM cells. The clarified supernatant from virus infected cell cultures of EHNV, BIV, or FV3 displaying advanced cytopathic effect (CPE) was used as neat, released stock supernatant.

Purification of Viruses.

Sixty BF2 cell monolayers in 150 cm^2 polystyrene culture flasks (Corning) were infected with released stock EHNV at a multiplicity of infection (MOI) of approximatley 0.1 PFU $cell^{-1}$. At 48 h post infection (p.i.) cells were collected by pelleting in a bench-top centrifuge at x 2600 *g* for 10 min and the media discarded. The cell pellet was resuspended in an equal volume of buffer (10mM Tris(pH 7.4), 10mM NaCl, 1.5 mM $MgCl_2$) and half the volume 1,1,2- Trichlorofluoroethane (Genitron). The pellet mixture was then dounced in a motor driven homogeniser and virus purified as described by Eaton *et al.*, (1991). The purified preparation was estimated to have a protein concentration of ca 1.4 mg/ml.

Monoclonal Antibodies (MAbs)

Groups of female balb/C mice, 6 weeks old, were each inoculated with preparations of (a) purified virus, (b) purified virus pretreated with 1% (w/v) SDS and boiled for 2 mins, or (c) EHNV infected tissue culture supernatant (released virus preparation) containing approximately 1.8 x 10^7 plaque forming units (pfu) per ml. Two hundred microlitres of an equal mixture of Freund's complete adjuvant and virus preparation (refer above) was inoculated intraperitoneally (i.p.) into mice. An i.p. inoculation of 200 ml of an equal mixture of Freund's incomplete adjuvant and each virus preparation was given one week later. A further week later mice received an i.p. inoculation of 200 ml of virus

preparation alone. Four to five days prior to a fusion experiment, mice were anaesthetised by an intra-muscular inoculation of 50 ml of a mixture of four parts of a 20 mg/ml solution of xylanine-HCl (Rompun. Bayer AG, Germany) to one part of a 10 mg/ml solution of ketamine-HCl (Ketavet. Delta Vet. Labs, NSW, Australia). The spleen was then exposed by a small excision through the peritoneum and approximately 40 ml of virus preparation inoculated directly into the spleen using a 27 gauge needle (Terumo) after the method of Spitz *et al.*, (1984). Following inoculation, mice were immediately sutured with coated vicryl thread (Ethicon, Johnson and Johnson Medical, Sydney. Cat no. J442). On the day of fusion, spleen lymphocytes were isolated and fused to myeloma cells (Sp2/0-Ag14) at a ratio of 5:1 using 1500 MW polyethylene glycol (BDH), as previously described (Eaton *et al.*, 1987). Resultant hybridoma cultures were selected with hypoxanthine-aminopterin-thymidine medium (HAT, Sigma, cat no. N0262) and maintained in Dulbecco's modification of Eagle's medium (DMEM) (ICN, cat no. 73-142-54) incorporating 20% foetal calf serum (Hyclone Laboratories Inc., Utah).

Characterisation of MAbs.

(i) Direct cell culture ELISA

Hybridoma supernatants were screened neat for the presence of virus reactive MAbs. Cells were grown in the bottom of ELISA plates and infected with EHNV. Infected cells, upon indication of a cytopathic effect, were fixed with 100% methanol. The cells were then incubated with hybridoma supernatants follwed by sheep anti-mouse Ig HRP conjugate (Silenus, Melbourne, Australia. Cat. No. DAH) was diluted 1:5000 in PBS containing 2% (w/v) skim milk powder and 0.05% (v/v) Tween-20 (PBST). The substrate was 42mmol/L tetramethyl benzidine (TMB) in DMSO (10 min) and stopped with 1 M sulphuric acid. Plates were read in a Labsystems Multiskan MS photometer with a 450 nm filter.

Individiual hybridoma cultures producing MAbs which reacted positively were cloned by limiting dilution and the MAb they secreted was further characterised according to the procedures described below.

(ii) Antigen capture ELISA

MAbs were further screened by their ability to react in an antigen capture ELISA as described by Hyatt *et al* (1991) with the following changes. The capture antibody used was a hyper-immune rabbit antiserum raised to $EHNV_T$ diluted at 1:8000 in coating buffer (0.05M Tris HCL, NaCl 0.15, pH 9.0). Sheep anti-mouse Ig HRP conjugated (Silenus, Melbourne, Australia, cat no. DAH) was diluted 1:5000 in PBS containing 2% (w/v) skim milk powder and 0.05% (v/v) Tween 20 (PBST). The substrate was 42mmol/L tetramethyl benzidine (TMB) in DMSO (10 min) and stopped with 1 M sulphuric acid. Plates were read as described above.

(iii) Immunofluorescence & Immunoperoxidase Test.

Virus reactive MAbs were screened in a fluorescent antibody test. BF2 cells were seeded on glass coverslips to obtain a confluency of approximately 70% and infected with released $EHNV_T$ stock. At 18 h post infection (p.i.) the infected cells were fixed in 100% methanol at -20°C for 3 min, 100% acetone at -20°C for 3 min and then air dried. The coverslips were incubated with neat Mab supernatant for 30 min at 37°C in a humid chamber, washed three times with 1% BSA in PBSA

(PBSAB) and incubated with sheep anti-mouse Ig-FITC conjugate (Silenus) diluted at 1:100 in PBSAB for 30 min at 37°C in a humid chamber. After washing an additional three times in the diluent and mounting in glycerol, the coverslips were analysed for fluoresence.

The MAbs were also tested for their ability to react with formalin-fixed tissues. The protocol is outlined in section 8.

(iv) Electron microscopy.

Grid cell culture.

BF2 cells were grown on gold electron microscope grids as described by Hyatt *et al.*, (1987). Cells were infected with released $EHNV_T$ and at approximately 18h p.i. treated with 0.1% gluteraldehyde in EMEM (10% FCS, 10mM Hepes), either with or without 1% NP40 for 2 min, fixed with 0.5% gluteraldehyde in PBS for 2 min and blocked with 5% (w/v) cold water fish gelatin (FG) in PBS for 10 min.

Immunoelectron-microscopy.

Immunoelectron-microscopy was also performed using $EHNV_T$, purified as described above, directly adsorbed to carbon coated, parlodion filmed, gold grids. Grids were then blocked with 1% FG in PBSA (PBSFG) for 10 min followed by incubation at room temperature with normal goat sera diluted 1:20 in PBSA for 20 min. Immediately after blocking both grid cell culture and purified preparations were treated as follows for MAb screening and gold labelling. MAbs were incubated neat for 1 h at 37°C, washed with PBSFG (X3) and detected via addition of protein A-gold (9nm) diluted 1:100 in PBSFG for 1h at 37°C. The grids were then washed in PBS (X6) and fixed with 2.5% gluteraldehyde in PBS. After washing three times in water the grids were stained in 2% phosphotungstic acid (pH 6.8) and viewed with a Hitachi H600 scanning transmission electron-microscope at 50kV. MAbs which did not radioimmune precipitate were examined by electron microscopy. Specifically the localisation of gold probes were correlated to the ultrastructure of the virus thereby indicating the virus substructure and protein it was directed against. The procedure is outlined in section 8.

(v) Radioimmune precipitation.

Virus particles were adsorbed to BF2 cells at a MOI of 1 pfu $cell^{-1}$ in 25 cm^2 tissue culture flask for 1 h at 24° C. At 10 h post-infection (p.i.) with EHNV, the growth medium was removed from infected and uninfected BF2 cell cultures and replaced with methionine- free EMEM containing 50 μCi ml^{-1} [^{35}S]-methionine. After labelling for 2 h at 24°C, infected cells were scraped from the flask, pelleted and then resuspended in 1 ml of PBS. The preparation was pelleted in an Eppendorf microfuge for 1 min, the supernatant discarded and the pellet stored at -20°C. After thawing, the pellet was resuspended in 500 ul of lysis buffer (500mM NaCl, 1% (v/v) NP40, 0.01% (w/v) SDS, 50mM Tris (pH 8.0)). Five microlitres of 100mM phenylmethylsulfonylfluoride (PMSF) in iso-propanol and 50 ul of x10 extraction buffer (5% (w/v) sodium deoxycholate, 10% (v/v) Triton-X100, 1% (w/v) SDS and 50mM Tris-HCl (pH 8.0) was then added to the preparation and mixed thoroughly. The preparation was then microfuged at 13000 g for 2 mins to remove any insoluble material. For the immunoprecipitation reaction, 50 ul of test MAb was mixed with an equal volume of labeled virus preparation and incubated for 90 mins at 37°C with constant agitation. Approximately 30 ul of

agarose-protein A (Sigma P-2670) or rabbit anti-mouse Ig labeled immunobeads (Biorad Cat no. 170-5104) was added to the reaction mixture and incubated for 1 hr at 37°C with constant rocking. Following incubation the mixture was briefly microfuged and the pellet washed x4 with lysis buffer. The immune complex was then eluted from the precipitant by resuspension and incubation in sample buffer (0.2M Tris, 4% dithiothreitol (w/v), 2% SDS (w/v), 0.2% glycerated bromophenol blue, pH 6.8) for 45 mins at room temperature with occasional agitation. Samples were then microfuged briefly immediately prior to loading onto a 10% Laemmli PAGE gel system. Samples of labeled infected and uninfected cell culture were incorporated as controls.

(vi) *Western Blotting.*

EHNV derived from rainbow trout (isolate B86:8774) was purified (Eaton et al., 1991) and the protein concentrated by acetone precipitation at -20°C overnight. The acetone was removed (supernatant) via centrifugation at 4°C and the pellet dried using vacuum evaporation. The pellet was reconstituted in 10ul 10% SDS, 20ul H_2O, 10ul DDT mix (10mg DTT, 100ul 1M Tris-HCl pH 6.5, 100ul 0.5% bromophenol blue/90% glycerol) and run on 10% poly acrylamide gels (Laemmli 1970). Markers used were Bio-rad Kaleidoscope Prestained Standards (Catalog 161-0324).

The gel was transferred to nitro-cellulose and then immediately blocked with 5% Skim milk in TBST for 30 min. The membrane was then incubated with the MAb of interest at neat or diluted 1:2 (MAb:5% skim milk) for 1hr at room temperature. A polyclonal antibody control was also run with each trial using rabbit anti-EHNV (AAHL) at 1:300 in 5% skim milk in TBST (20mM Tris base, 137mM NaCl, 3.8mM HCL, 0.1% Tween 20) for 1hr at room temperature. The membrane was washed 3 times in 5% skim milk in TBST and then incubated with conjugate, anti-mouse IgG(Fc) Alkaline Phosphatase (Promega 1mg/ml) for MAbs or anti-rabbit IgG(Fc) Alkaline Phosphatase (Promega 1mg/ml) for polyclonal control, at 1:2000 in 5% skim milk in TBST for 1hr at room temperature. After washing in AP buffer (100mM Tris pH9.5.100mM NaCl, 5mM $MgCl_2$) the membrane was developed for 10 min. in the dark with 10ml AP buffer, 33µl BCIP, 66µl NBT(Protoblot System Promega Substrate) and finally washed in H_2O.

(vii) Isotyping.

All MAbs were isotyped using a mouse MAb isotyping kit and protocol as supplied by Amersham (RPN 29).

Competitive Binding Assay.

To determine whether different MAbs were binding to the same or to different epitopes, a competitive assay was performed.

The dilution levels of each MAb which resulted in saturation of viral antigen in the capture ELISA were first determined by titration. To determine the level of competition for virus binding between individual MAbs, an additivity ELISA was employed (Friguet *et al.*, 1983; Collins *et al.*, 1995). An appropriate dilution of the primary MAb (MAb 1°) was first incubated in the capture ELISA for 90 mins at 37°C. The MAb solution was then carefully removed from each plate well and the secondary MAb (MAb 2°) was added (ie. there was no wash step between) and incubated for a further 90 mins at 37°C. Appropriate controls included MAb 1° and MAb 2° incubated alone (ie. assay diluent only added instead of MAb 1° or MAb 2°) and where MAb 1° and MAb 2° represented the same MAb. Following the incubation of MAb 2°, conjugate and substrate addition steps followed the same procedure detailed for the capture ELISA. The level of competition between two particular MAbs was then determined by summing the individual absorbance values for MAb 1° and MAb 2° alone and subtracting the absorbance gained with the same MAbs sequentially incubated in the same well.

The residual value was then expressed as a percentage of the absorbance value obtained with MAb 2^{o} alone to provide the level of blocking of MAb 2^{o} by MAb 1^{o}.

Virus Differentiation by ELISA.

MAbs that yielded positive results in the capture ELISA described previously were assayed using the same protocol comparing antigens $EHNV_T$, BIV, and FV3 released stock supernatants.

RESULTS & DISCUSSION

Characterisation of MAbs to EHNV.

Of the hundreds of "parent" MAbs produced 97 were selected for further work. From these parent lines 22 clones were selected for detailed examination by immunofluoresence, EHNV-antigen capture ELISA, antibody competitive ELISA and electron microscopy; these clones were designated 230 (29), 21(45), 27(25), 3C1/B12), 33(335), 122(281), 3C4/B7, 6(111), 196(136), 180(319),3B1/A7, 186(319), 7H10/D4, 7A4/D1, E10(56), 7F6/A), 7G7/C1, 182(26), 22(28), 7G2/B5, 79(11) and 7D12/A3.

Of these clones 12 reacted in EHNV-capture ELISA and 16 were positive in immunofluoresence (FAT). The clones listed below reacted in ELISA's where the polyclonal antibodies used for coating the plates were rabbit anti-$EHNV_{RF}$ (AAHL), or rabbit anti-$EHNV_T$ (AAHL) or sheep anti-$EHNV_T$ (AAHL) or rabbit anti $EHNV_T$ (EMAI). These MAbs were 28(181), D4(29)*, 335(10), 48(22)*, 7(131)*, D1(53), 22(28), 111(55)*, 2(192)*, E10(56), 154(48) and 79(11). The strongest reactors are indicated (*). Those clones which reacted in FAT were 230(29), 21(45), 27(25), 2(192), 28(181), 6(111), 196(136), 180(319), 7(131), D4(29), D1(53), E10(56), 335(10), 22(28), 48(222) and 111(55). All clones with the exception of 33(335), 196(136) and 180(319) reacted with EHNV via immuno-electron microscopy.

(i) *ELISA*

Nine MAbs were selected for use in the capture ELISA. Table 2 details the properties of the MAb panel. To standardise names/numbers of MAbs all MAbs were renamed; to avoid any present and future confusion of "names" the reference names/numbers for the nine MAbs used in the project are given in Table 1. All the MAbs were derived from Group (a) inoculated mice ie, inoculated with purified virus preparation. The MAbs were routinely diluted out three fold and assayed against $EHNV_T$ or BF2 cell supernatant and selected on the basis of no significant prozone effect or increased background being evident.

Group 2 and 3 mice also produced MAbs that reacted to $EHNV_T$. However these MAbs also displayed extensive cross-reaction with a BF2 cell negative control preparation in the capture ELISA

(results not shown) and were thus unacceptable reagents for virus detection and differentiation. The background/cross-reactions are most likely attributable to the presence of the cell derived outer virus membrane.

(ii) *Light microscopy.*

Eight of the nine MAbs selected in the capture ELISA were also positive in the fluoresent antibody test (Table 2). Fluoresence extended throughout the cytoplasm becoming heavier in regions thought to be inclusion bodies. Fine extracellular dots were noted on the glass substrate representing released virus. No activity was noted in the nucleus. The eight MAbs gave similiar fluoresence patterns. The MAbs were also tested for their ability to react with formalin fixed material. None of the MAbs reacted with the formalin fixed material, yet they reacted with glutaraldehyde fixed viruses and ultra-thin sections.

Table 1: Names of the selected nine MAbs for ELISA and other diagnostic protocols.

Current MAb Reference Name	Equivalent Working Name (1)	Equivalent Working Name (2)
3C1 or 3C1/B12	2C1/B12	2(192)
7F6 or 7F6/A1	7F610/A1	154(48)
3B1 or 3B1/A7	7A7	7(131)
7G2 or 7G2/B5	7G27/B5	48(22)
3C4 or 3C4/B7	3C4/B7	28(181)
7H10 or 7H10/D4	7H10(4)0.5/D4	D4(29)
7A4 or 7A4/D1	7A4(1)0.5/D1	D1(53)
7G7 or 7G7/C1	7G75/C1	335(10)
7D12 or 7D12/A3	7D12(2)/A3	111(55)

(iii) *Electron microscopy.*

Labelling of infected cells, via grid cell culture, was confined to detergent treated preparations where the outer plasma membrane derived envelope was disrupted. No interaction was observed between a MAb and the outer envelope in either budding or released virus where the envelope remained intact (non detergent treated grids). Polyclonal and MAb antibodies also reacted with ultra-thin sections of EHNV-infected cells (Table 2, Fig5).

Purified virus preparations used in immunoelectron-microscopy were derived from cell associated virus sheared from the cytoskeleton and hence contained no enveloped virus. Specific gold labelling on purified virus preparations was observed with all members of the capture ELISA MAb panel (Table 2).

(iv) *Radioimmuneprecipitation.*

Seven of the MAb panel precipitated the 50 kD major core protein of EHNV from infected cells. Results of the RIPA with eight of these MAbs using agarose-protein A as the precipitant are shown

in Fig. 2. Monoclonal antibody 7A4 failed to precipitate any protein with agarose-protein A but precipitated the 50 kD protein when rabbit anti-mouse immunobeads were used as the precipitant. MAb 3C4 failed to precipitate any specific protein in the RIPA when either of the precipitants were used. This MAb was found to be of the IgM class and also exhibited a relatively low affinity for viral antigen in the capture ELISA, however when examined by immuno electron microscopy the labelling was associated with the capsid i.e. the 50 kD major core protein.

(v) *Western Blotting.*

None of the MAb panel members reacted with virus-specified proteins in the western blot assay.

Table 2: Characterisation details for the MAb panel. Strong reaction (4+); weak reaction (+). FAT, fluorescent antibody assay; IEM NCEM, immuno-electron microscopy on negative contrasted samples; IEM CRYO, immuno-electron microscopy on cryo-sections; IEM K4M, immuno-electron microscopy on plastic (K4M) sections of EHNV-infected cells.

	SPECIFICITY					
MAb	ISOTYPE	FAT	IEM NCEM	IEM CRYO	IEM K4M	RIP
3B1/A7	IgG1	4+	+	4+	-	50kD
7F6/A1	IgG1	4+	-	?	+	50kD
7G2/B5	IgG1	4+	2+	4+	3+	50kD
3C1/B12	IgG2a	4+	3+	4+	3+	50kD
7A4/D1	IgG2a	4+	2+	?	+	50kD
7D12/A3	IgG2b	4+	2+	?	2+	50kD
7G7/C1	IgM	4+	4+	2+	2+	50kD
7H10/D4	IgM	4+	3+	4+	2+	50kD
3C4/B7	IgM	-	3+	4+	3+	?

Epitope Characterisation on the 50kD Protein.

All possible paired permutations of eight of the nine MAbs comprising the ELISA reaction panel were tested in the competition assay (Fig. 3) (MAb 7F6 was not included). Five MAbs (7H10, 3B1, 3C1, 7G2 and 7D12), designated as group I, displayed high to absolute levels of mutual blocking and all precipitated the 50 kd viral core protein (Table 2; Fig. 2) thus indicating these MAbs were directed to the same, or very closely associated epitopes, on this protein. Two other MAbs specific for the 50kd protein, 7A4 and 7G7 (group II), displayed strong levels of mutual blocking of antigen binding (although 7A4 appeared to block 7G7 more efficiently than vise versa) and were also highly blocked by group I MAbs. However, these MAbs displayed a relatively poor ability to block the group I MAbs, with only MAb 7H10 being sensitive to moderate blocking levels by both the group II MAbs. The non-reciprocal nature of binding site blocking between the two MAb groups indicated the group 1 MAbs may induce conformational changes to the 50 kd protein which affect the efficiency of binding of group II MAbs. Alternatively the results may simply reflect a one-way stearic hindrance phenomenom between two discrete antigenic regions. Binding of the remaining MAb,

3C4, was also strongly inhibited by Group I MAbs and 3C4 showed more persistent blocking of Group I Mabs (especially 7H10) than did the Group II MAbs. In addition, 3C4 strongly inhibited the binding of Group II MAbs, yet both these MAbs only displayed a moderate ability to block 3C4. The combined competition data indicated at least two immunogenic regions exist on the 50 kd protein of EHNV. The ability of Mab 3C4 to compete for binding with MAbs shown to be specific for the protein, coupled with the unique properties this Mab possesses, may indicate a third discrete region exists on the 50 kd protein. However the viral protein specificity of this MAb was unable to be confirmed by radioimmuneprecipitation (Fig. 2).

Differentiation Between Iridoviruses.

The ability of the MAbs to differentiate between $EHNV_T$, BIV and FV3 in the capture ELISA format was investigated (Fig. 4). The MAbs 3C1/B12 and 7F6/A1 gave near equivalent O.D. values with all three virus isolates, thus they served as a positive non-differentiating control to ensure equivalent

Fig. 1. Flourescence light micrograph of EHNV-cells with MAb 7A4

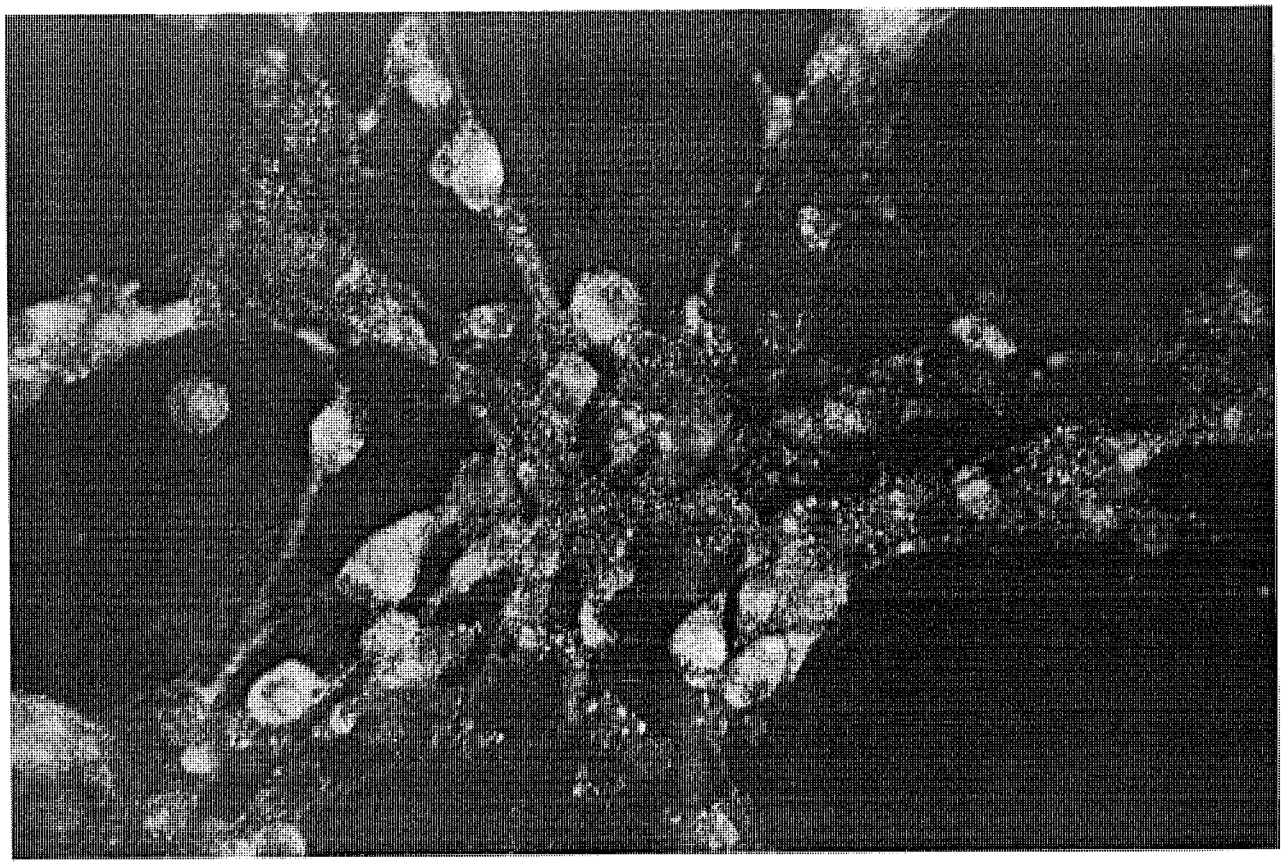
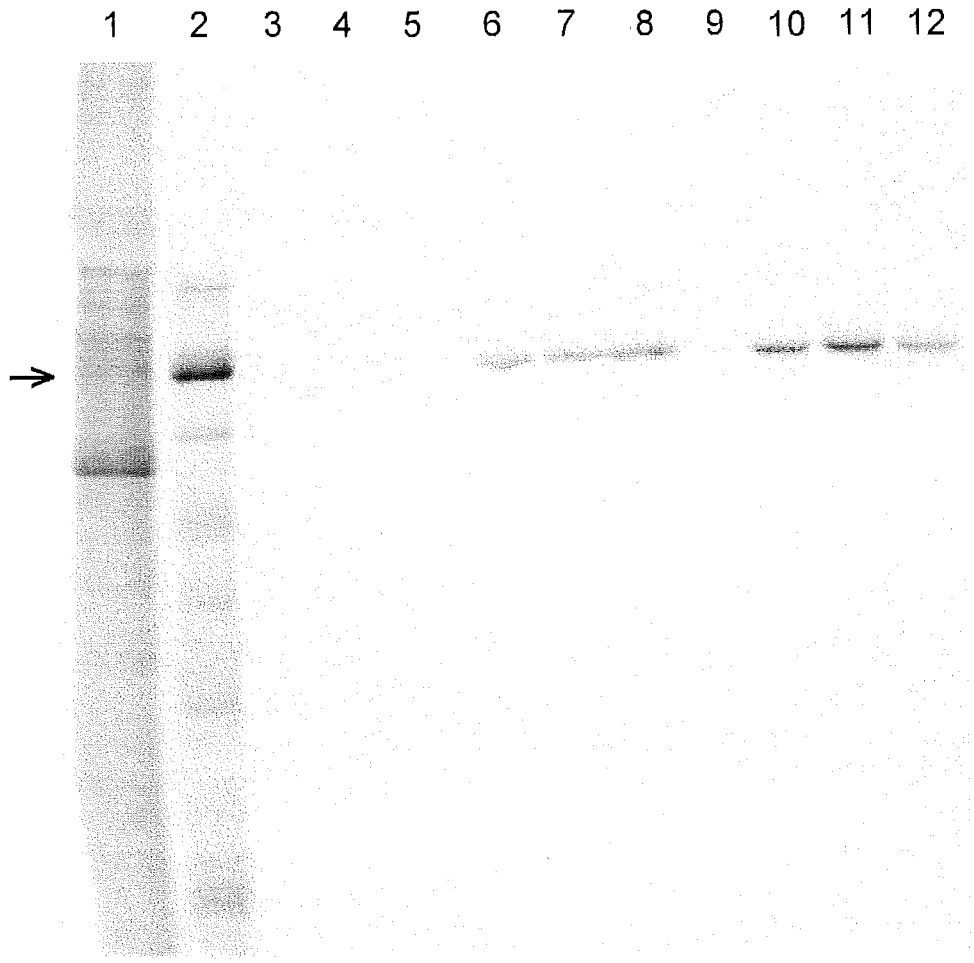
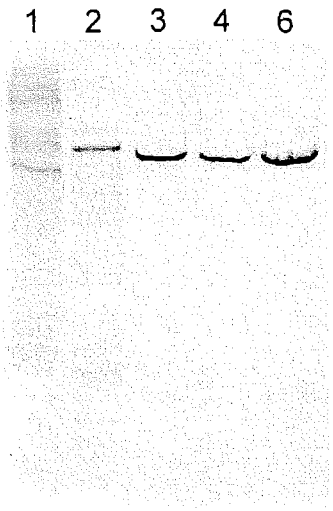


Fig. 2 Radioimmneprecipitation precipitation of MAbs using agarose-protein A (Sigma). (A) Uninfected cells (lane 1), virus-infected cells (lane 2), virus-infected cells reacted with a negative MAb (lane 3), uninfected cells reacted with Mab 3C1 (lane 4), MAb 7A4 (lane 5), MAb 7G7 (lane 6), MAb 7H10 (lane 7), MAB 3C4 (lane 9), MAb 3B1 (lane 10), MAb 3C1 (lane 11). MAb 7D12 (lane 12).



(B) Immuno complexes precipitated using immunobeads (Bio-Rad); lanes 1 & 2 as per above, 3C1 (lane 3), 7A4 (lane4) and 7H10 (lane 5)



amounts of viral antigen were used in the ELISA. Of the remaining MAbs, six (3C4, 3B1, 7H10, 7A4, 7G7 and 7G2) all displayed distinct, yet varying abilities to distinguish $EHNV_T$ from the other two viruses, BIV and FV3 . Each MAb reacted with BIV and FV3 at a similar level although this varied between MAbs. Monoclonal antibodies 7A4 and 7G7 displayed similar and relatively low O.D. values with homologous virus which exceeded the values obtained with the most reactive heterologous virus isolate (FV3 with both MAbs) by approximately 0.30 O.D. units. Monoclonal antibodies 3C4, 7H10, 3B1 and 7G2 exhibited O.D. values ^{3} 1.10 units with the homologous virus isolate which were also at least 0.55 (7G2) and up to 1.10 (7H10) O.D. units greater than the most reactive heterologous virus isolate. However MAbs 7H10 (O.D. approx. 0.20 units) and, in particular, 3C4 (O.D. approx 0.06 units) reacted comparatively poorly with the two heterologous virus isolates whereas 3B1 and 7G2 exhibited significant reactivity levels with BIV and FV3 (O.D. 0.65). The latter two MAbs also showed some limited capacity to differentiate between BIV and FV3. However MAb 7D12 showed the greatest ability to differentiate between these viruses. This MAb gave an O.D. reading of 1.28 units with $EHNV_T$ and 0.69 units with BIV, yet barely reacted with FV3 (0.08 O.D. units).

Based upon preliminary antigen capture ELISA data and the results of FAT and IEM investigations, a panel of nine MAbs [2(192), 28(181), 7(131), D4(29), D1(53), 335(10), 22(28), 48(22) and 111(55)] raised to a purified preparation of $EHNV_T$ was assembled for the purpose of differentiating the homologous virus from other related iridoviruses. Eight of the nine MAbs were found to be specific for a 50 kd virus-specified protein (Table 2, Fig. 2), which immunoelectron-microscopy on purified, non-enveloped particles proved to be the major capsid protein of the virus. The uncharacterised MAb (3C4) displayed a significant degree of interaction with other panel members in a competitive binding assay (Fig.3). It is therefore likely this MAb, although failing to precipitate or western blot a virus-specified protein, may also be specific for an epitope on the same protein (this interpretation is supported by IEM results). Hybridoma fusions undertaken with lymphocytes from

Fig. 3.

Figure 2 shows the ability of any one MAb (primary) to compete against binding of all other Mabs(secondary). Percentage levels of inhibition of secondary Mab binding are designated as ≤ 40 (white); 40-49 (half stipled); 60-79 (full-stipled); ≥ 80 (black).

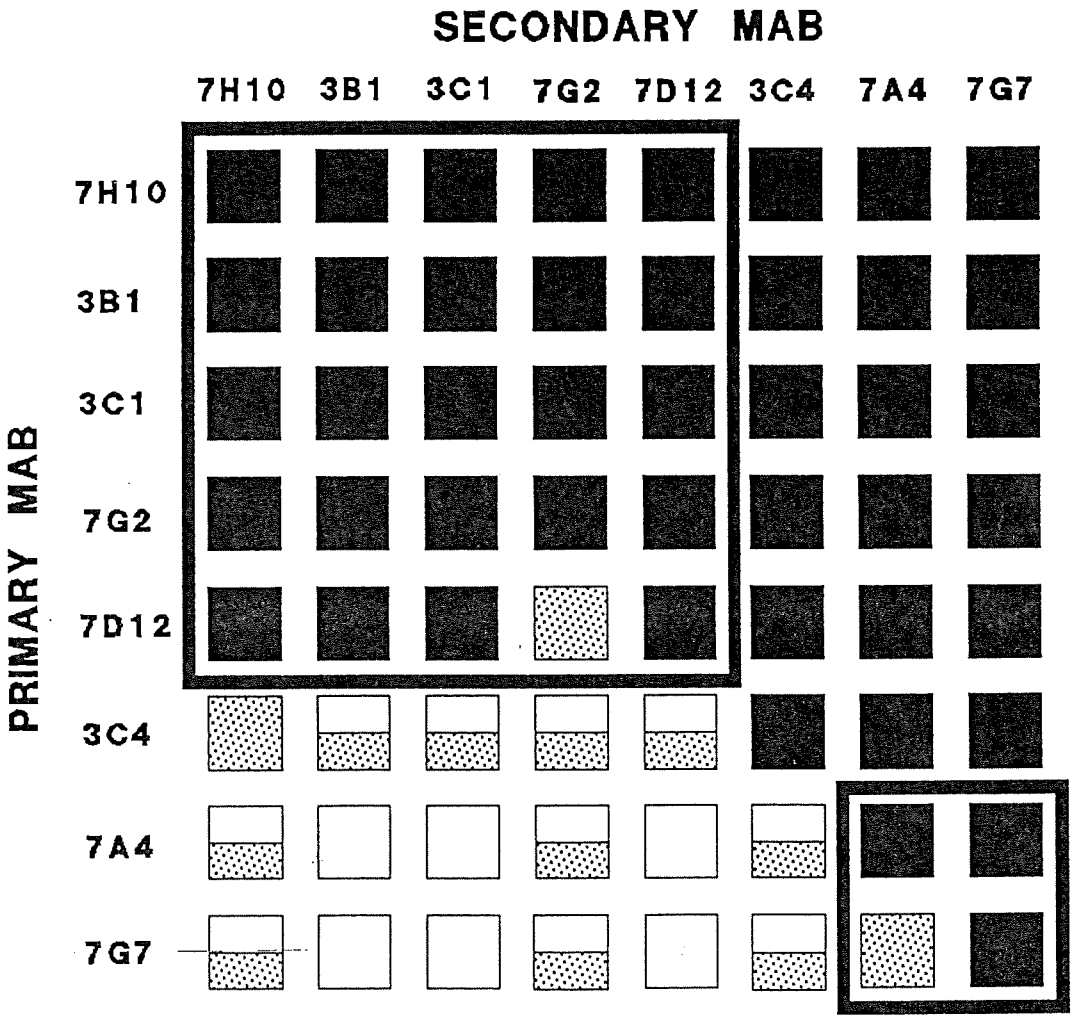
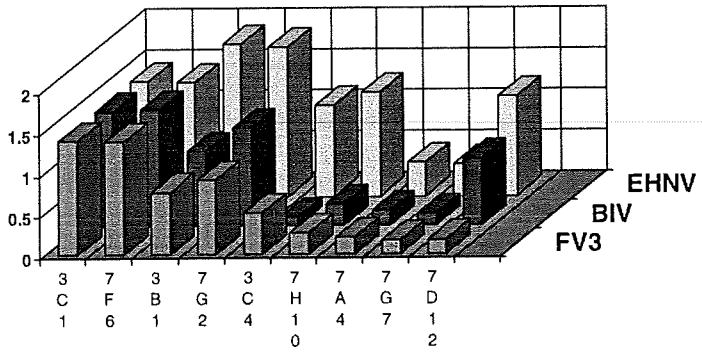


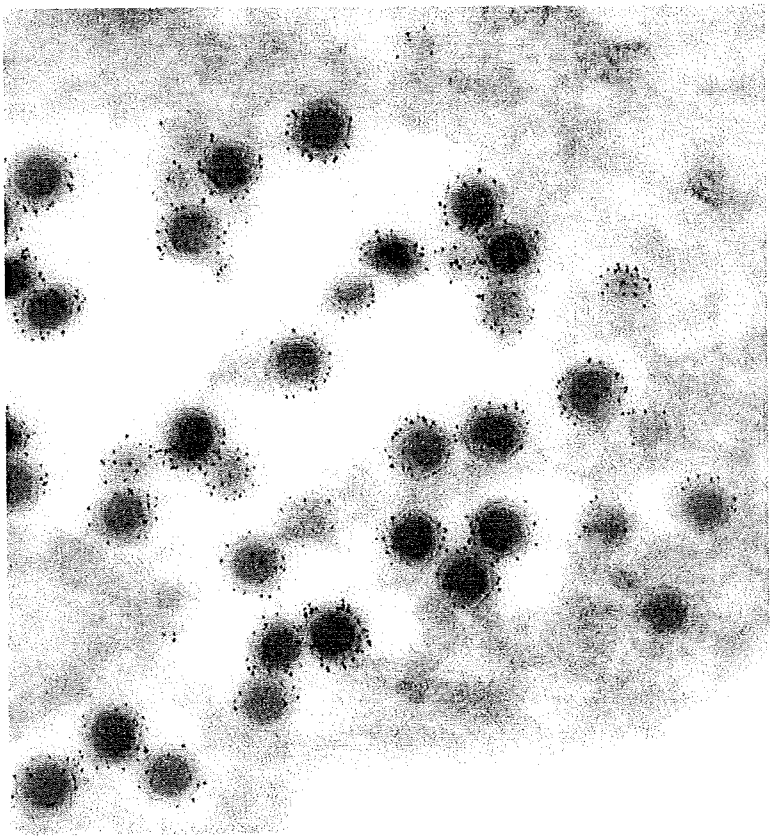
Fig. 4.

Differentiating Ability of Monoclonal Antibodies



Graph shows the ability of MAbs to differentiate between piscine and amphibian iridoviruses.

Fig. 5. Electron micrograph of a cryo section of a cell infected with EHNV. The section has been incubated with Mab 7G2 (X30000).



mice previously inoculated with virus particles released from infected cells (ie. enveloped particles) only yielded MAbs which reacted with host proteins, presumed to be those incorporated in the cell plasma membrane. It is likely the immunodominant nature of epitopes on these proteins made the isolation of MAbs, specific for virus-specified proteins on the surface of enveloped particles, hard to achieve.

Number of epitopes recognised by MAbs.

At least two separate immunogenic regions (designated I and II) were identified on the protein based upon data obtained from competitive binding assays (Fig. 2). Although group I MAbs actively blocked group II MAbs, the correlation of capture ELISA data on virus differentiation (Fig. 3) with the competition data provided further evidence for the discrete nature of these two regions and the minimum number of epitopes present within each region. At least four epitopes exist in region I, MAb 7D12 clearly differentiates between EHNV, BIV and FV3, whereas 3C1 and 7F6 cross-react with all three viruses to the same extent. Monoclonal antibodies 7G2 and 3B1 show very similar reaction patterns in the capture ELISA for virus differentiation, with a predominant homologous reaction yet still substantial heterologous reaction with FV3 and BIV. However MAb 7H10, whilst also reacting strongly with $EHNV_T$, had a greatly reduced capacity to react with both heterologous viruses. This MAb was of further interest, as its reaction pattern with the three different virus isolates in the capture ELISA was very similar to that of MAb 3C4 and also more similar to the reaction patterns seen with the Group II MAbs. However the competition assay data, although placing this MAb in Group I, also indicated the 7H10 epitope was more interactive with 3C4 and region II than were the other four Group I MAbs. Presumably this epitope is sited in region I at a point which has some interaction or overlap with region II. The presence of at least two separate epitopes in region II can be also infered from the combined data presented in this study.

Note: (RF) represents redfin perch; (T) represents rainbow trout and O.D. represents optical density.

CONCLUSIONS

1. Nine MAbs were selected for use in ELISA

2. Eight MAbs gave specific positive results in fluoresence antigen tests where the antigen was acetone/methanol fixed whole cells.

3. All nine MAbs do not react on conventional formalin fixed tissues.

4. Most MAbs label glutaraldehyde fixed viruses. Enveloped viruses must be disrupted with detergent (0.1% glut in 1% NP40, 2mins) for successful labelling.

5. Seven of the nine MAbs immunoprecipate the 50kD major core protein; the other two react via electron microscopy to the major capsid i.e. the 50kD. Thus all nine MAbs are to the major 50kd protein.

6. Three of the MAbs can distinguish between EHNV and two related iridoviruses BIV and FV3 in an antigen capture ELISA.

7.2 Identification Of Optimum Tissues For Testing By Antigen Capture ELISA

METHODS

Fish and Husbandry

Fish were housed in a biological security facility at EMAI in 120 L aquaria with external biological canister filters containing 5 mm gravel, glass wool and crushed limestone or shell grit. Water temperature was controlled by regulation of air temperature, submersible heaters and a refrigerated water cooler, which circulated water through a stainless steel heat exchange coil immersed in each tank. Total-ammonia and nitrite levels were measured weekly using commercial test kits and remained below the detectable thresholds of 0.1 ppm and 0.25 ppm, respectively. Water changes of 50% were routinely conducted fortnightly.

Adult redfin perch (1 to > 3 years old, estimate) measuring 130-300 mm forklength (FL) and weighing 50-414 g were obtained from Googong Reservoir, an impoundment in New South Wales, in which EHNV infection had not been observed. Rainbow trout fingerlings (O+, 55-145 mm FL, 3-45 g) were obtained from a farm which was believed to be free of EHNV based on absence of clinical signs of EHNV infection and annual certification testing by virus isolation since 1988. Fish were fed chicken liver (redfin perch) or commercial pellets (rainbow trout) three times weekly.

Preparation of Viral Inocula

EHNV isolate 86/8774, which was recovered originally from rainbow trout, and isolate ME90/24, which was recovered originally from redfin perch and propagated in bluegill fry (BF-2) cells (22^0C) were used. After destruction of the monolayer, tubes or flasks were shaken to dislodge adherent cells and the culture supernatant (SN) was dispensed in 5 ml aliquots and stored at 4^0C for $\leq$ 10 days. For each inoculum of EHNV, the titre of infectious virus as the 50 percent endpoint tissue culture infective dose ($TCID_{50}$) was determined retrospectively in a microplate assay with BF-2 cells.

Intraperitoneal Inoculation

Fish were anaesthetised in benzocaine (40 mg/ml) and held in dorsal recumbency. A volume of 0.1 ml of viral inoculum was administered from a tuberculin syringe with a 26 g needle by shallow IP injection in the ventral midline immediately anterior to the pelvic fins.

Bath Exposure

Viral inoculum was added to the tank from a syringe placed below the surface of the water and mixed by currents induced by the filtration equipment. Fish were not handled. The amount of virus was expressed as initial dose, calculated from the total $TCID_{50}$ of the inoculum and the volume of the tank.

Analysis of Fish Tissues

Liver, spleen and kidney were removed aseptically from every fish within 12 h of death, placed in sterile tubes and stored at -20°C. Virus isolation and antigen capture enzyme-linked immunosorbent assay (ELISA) for detection of EHNV were undertaken as described previously (Whittington and Steiner 1993).

Histopathology

Freshly dead fish, or moribund fish killed by immersion in 200 mg/l benzocaine, were preserved whole in 10% buffered neutral formalin after opening the abdominal wall to allow inspection of the viscera and the collection of tissues for virologic examination. Individual organs were dissected from large fish and placed in fixative. Small rainbow trout were serially sliced transversely in preparation for embedding, so that organs could subsequently be examined *in situ*. Tissues were routinely parrafin-embedded, sectioned at 5 µm, stained with haematoxylin and eosin and examined by brightfield microscopy.

Immunoperoxidase (IP) Staining

A commercial kit (Dako LSAB K680, Dako Corporation, California, USA) with peroxidase-labelled streptavidin and a mixture of monoclonal blotinylated goat anti-rabbit/anti-mouse IP as the link antibody was used for IP staining. The primary affinity purified rabbit polyclonal anti-EHNV antibody was stored as a 1:10 solution in 25mM Tris-HCl, pH 7.4, 0.15 M NaCl, 50 % glycerol 20°C, and diluted in phosphate buffered saline, pH 7.4 (PBS) to 1:3000 immediately prior to use. Selected sections were recut at 5µm from the parrafin blocks, and mounted on poly-l-lysine-coated slides. Staining was then completed on the same day. Sections were dried at 60°C for 30-45 mins, immediately deparrafinised routinely to water, and the remaining procedures undertaken without allowing the slides to dry. A diamond pencil was used to mark out selected areas on or around the section to limit the spread of reagents. Proteolytic digestion was performed with a 20 minute incubation in 0.1% trypsin/0.1% $CaCl_2$ at 22 °C. Slides were then washed for 5 mins in deionised water and 5 mins in PBS. Subsequent incubations with H_2O_2, blocking serum, primary antibody, link antibody, labelled streptavidin and chromagen were performed according to the manufacturer's directions, except that the incubations with the primary and link antibodies were at 37°C for 30 mins, and the intermediate washing steps were 3 consecutive 5 min baths in PBS. Slides were counterstained with Meyer's haematoxylin and mounted with an aqueous mounting gel. A negative reagent control was prepared for each section examined, using an irrelevant affinity purified primay rabbit antibody against the bacterium *Dichelobacter nodosers.* Known positive and negative tissue controls were included in each batch of slides.

Experimental Design

(i) *Redfin perch*

In preliminary trials with EHNV in adult fish (data not shown) fatal disease was invariably produced within 28 days across the range of ambient temperatures recorded in our facility (12-19°C) regardless of the route of inoculation or isolate of EHNV. In this study we chose bath exposure as it is considered the more natural route of infection. Large adult redfin perch were used to enable repeated blood sampling, but this restricted group size in the temperature-controlled tanks. Groups of 3 redfin perch were considered adequate because of their extreme susceptibility to EHNV infection, and because confirmation of the cause of death was made by histopathology and virological examination in every fish. All fish were maintained in the laboratory at 18°C for 5 months before the commencement of the experiment and thus were considered to be EHNV-free and, based on serology, not to be immune to EHNV infection.

(ii) Rainbow trout

Because of the difficulty of reproducing disease due to EHNV in preliminary trials with this species (data not shown), both IP and bath exposure were employed to evaluate the effect of water temperature. For IP exposure experimental groups of fish (n = 13) were acclimatised at either 19-21°C or 8-10°C for one month before inoculation with EHNV (isolate 86/8774, $10^{5.6}$ $TCID_{50}$). Two additional groups of fish (n = 13) were acclimatised at 19-21°C or 8-10°C for one month before bath exposure to EHNV (isolate 86/8774, $10^{2.2}$ $TCID_{50}.mL^{-1}$). Controls (n = 13) not exposed to EHNV were kept at 16-17°C. Fish from both IP and bath-exposed groups were observed for 63 days, at which time blood was collected from survivors immediately before euthanasia.

In a second experiment, fish (n = 30) were kept at 18-20°C and bath-exposed to EHNV (isolate 86/8774, 3.6 $TCID_{50}.mL^{-1}$). On day 28 after exposure prednisolone acetate (20 mg/kg) was administered IP to all fish to simulate stress and the tank was reinoculated with EHNV (isolate 86/8774, 1 $TCID_{50}.mL^{-1}$). Observations were continued until day 66 when surviving fish were bled and euthanased. Control fish (n = 10) not exposed to EHNV but injected IP with prednisolone acetate were kept at 18-20°C.

During each experiment with rainbow trout, one or more redfin perch were given the same inocula by bath or IP routes to confirm the virulence of the inoculum.

RESULTS and DISCUSSION

Redfin perch

All fish which were exposed to EHNV at water temperatures greater than approximately 12^0C died. In each dead fish, EHNV was detected by both culture and antigen capture ELISA and histopathological changes consistent with EHNV infection were present. Control fish remained clinically normal throughout the experiments.

Rainbow Trout

(i) IP inoculation

All fish in the 19-21°C group died within 3-10 days PE. EHNV was cultured from each fish and detected also by antigen capture ELISA in pooled liver, kidney and spleen; histopathological lesions were consistent with active EHNV infection. Nine of 13 (69%) fish in the 8-IO'C group died between days 14-47 PE. Seven fish died before day 32 PE, had histopathological signs of active EHNV infection and EHNV was detected in 5 by virus isolation and antigen capture ELISA. The two fish that died after day 32 PE had died of other causes. Of the 4 fish that survived and were euthanased at day 63 PE, EHNV was cultured after 2 passes in BF-2 cells from 1 (#53) but was not detected by antigen capture ELISA of tissue homogenates of any. Grossly, the anterior kidney of fish #53 was enlarged and nodular, and microscopically, the normal architecture was obscured by a dense infiltration of large mononuclear cells. EHNV was not recovered from 13 control fish that were kept at 16-17'C and euthanased at day 63 PE and these fish were free of histopathological abnormalities.

(ii) Bath inoculation

Morbidity or mortality due to EHNV did not occur, EHNV was not detected by virus isolation or antigen capture ELISA and histopathological changes consistent with EHNV infection were not detected in any fish. In the second bath inoculation experiment in which fish were injected IP with prednisolone acetate to induce a stress response, none of the fish died due to EHNV infection and EHNV was not detected by culture or antigen capture ELISA in any.

Assessment of tissues containing EHNV

Specimens from natural outbreaks of EHNV were also included in this section. Forty one dead or moribund rainbow trout fingerlings infected with EHNV were included, comprising 17 from experimental intraperitoneal infections and 24 from field outbreaks. Thirty nine dead or moribund redfin perch were included. These were all experimentally infected by the bath method. Microscopic lesions were similar in both species, so are described here together.

(i) *Kidney*

Acute renal haematopoietic necrosis was present in 35/36 redfin perch & 35/40 rainbow trout, The necrosis varied from widely scattered necrosis of individual cells (more often seen in rainbow trout), through multifocal, to locally extensive, sometimes coalescing areas of necrosis. In redfin perch, the discrete melanomacrophage centres were involved, if they happened to occur within an area of necrosis. The more diffuse melanomacrophage tissue of the rainbow trout kidney was similarly affected, and scattered melanin granules were prominent within necrotic areas. In both species foci of necrosis had a characteristic eosinophilic background, with scattered basophilic debris, and the normal sinusoidal stucture was rorally obscured. Fibrin thrombi were present within sinusoids adjacent to necrotic foci. In larger blood vessels, few normal erythrocyte were present, and there was considerable granular basophilic debris within clumps of eosinophilic fibrinoid material. Large blast-like cells were also frequent. Such material was common within blood vessels tn tissues throughout the body. Dark, basophilic intracytoplasmic inclusions were seen in large intact cells within the haematopoletic kidney, usually markedly displacing the nucleus, in about half the cases in both species, although abundant necrotic debris often made recognition of inclusions difficult. In the posterior kidney, the excretory tubules and glomeruli were mainly unaffected. Occasional necrotic cells were seen within the tubular epithelium from some affected fish, and these were also present in some uninfected control fish. IP staining consistently revealed granular intracytoplamic staining of scattered intact haematopoietic cells, with inclusions staining intensely. Staining was more intense around the periphery of the lesions compared to the area containing necrotic debris. Occasional necrotic cells within the tubules did not stain with IP.

In the one EHNV carrier rainbow trout with gross kidney lesions there was diffuse hypercellularity of the anterior kidriey which obscured the normal sinusoidal structure. The cells were blast-like and rnitotic fig'ures were frequent. Similar cells infiltrated the interstitium of the posterior kidney, but the excretory tubules were unaffected. All other tissues were normal. The three carrier rainbow trout which had no gross lesions also had no microscopic lesions and no viral antigen was detected in any tissue of' any carrier fish with the IP stain.

(ii) *Liver*

Multifocal acute hepatocellular necrosis was present in 20/33 rainbow trout & 38/39 redfin perch. Lesions were randomly distributed throughout the liver. In rainbow trout the lesions were often very small and not readily seen at low power, whilst in redfin perch the lesions were typically large, 1 mm or more in diameter. Small lesions consisted of only a few necrotic cells. Larger lesions showed coagulative necrosis with pyknosis or fragmentation of nuclei, and the largest lesions had a pale liquefactive centre. In a few rainbow trout there was also mild mononuclear cell infiltration in the portal areas. Usually there was a rim of hyperchromatic hepatocytes surrounding each necrotic focus. Basophilic intracytoplasmic inclusions were present in the hyperchromatic hepatocytes of all but one redfin perch and in 70% of the rainbow trout with liver lesions. Often there was more than one inclusion per cell. The inclusions were paler than in the kidney, and had an almost hyaline appearance. Necrotic foci consistently stained with IP. There was intense

granular intracytoplasmic staining of hepatocytes in the hyperchromatic and necrotic areas, and inclusions were strongly stained. Especially in the rainbow trout, many more, foci of infection were revealed with the IP stain than were apparent on the H & E sections. Some scattered sinusoidal lining cells also stained with IP.

(iii) *Spleen*

Multifocal to diffuse acute necrosis of the spleen, often with congestion, was present in 19/25 rainbow trout & 31/33 redfin perch. A particular feature of splenic lesions in redfin perch was marked thickening of the walls of the ellipsoids, with deposition of pale, amorphous eosinophilic material. Usually the endothelial lining cells were intact. In IP stained sections, there was scattered staining, especially around the periphery of necrotic lesions. However, the ellipsoids were compicuous in not staining.

(iv) *Heart*

Necrotic debris and degenerate haematopoietic cells were present in the the cardiac chambers of most fish. Myocardial lesions were present in 11/27 rainbow trout & 20/37 redfin perch. In redfin perch these lesions were primarily acute focal necrosis and swelling of the atrial trabeculae with little associated inflammation. In some fish the mural phagocytces were prominent, and some were were degenerate with pyknotic nuclei and fragmenting cytoplasm. Similar lesions were present in rainbow trout, but both atrial and ventricular trabeculae and the compact myocardium were involved. In addition, a mononuclear inflammatory cell infiltrate was a feature of cardiac lesions in many rainbow trout. Some necrotic lesions failed to stain with IP, while others, even on the same section, stained strongly. Granular cytoplasmic staining of scattered mural phagocytes was observed in most hearts with lesions.

(v) *Gastrointestinal tract*

Lesions were present in 8/35 rainbow trout & 6/26 redfin perch. Affected rainbow trout, all from field outbreaks (8/24), had scattered mild focal necrosis of the crypt epithelium at varying levels of the gastrointestinal tract. Focal necrosis of the lamina propria was seen in one rainbow trout, but was the major gastrointestinal lesion seen in redfin perch, sometimes extending through the muscle layers to the overlying serosa. Scattered cells and some debris within the necrotic areas stained well with IP.

(vi) *Gills and Pseudobranch*

Debris and necrotic cells were prominent in the vessel lumens in the gills of both species. Mild bronchial epithelial hyperplasia, often with lamellar oedema was present in 31/33 rainbow trout. In mildest cases there was simple hypertrophy of the epithelial cells; in more severe cases there

was early fusion at the bases of the secondary lamellae. Similar but milder lesions were present in unaffected cohorts from field outbreaks, but there were no lesion controls from inoculation experiments. Nineteen infected rainbow trout had a mild scattered focal or individual cell necrosis of pilar cells or other connective tissue cells within the filaments. Only 12/22 redfin perch had lesions in the gills, when present these were usually severe, comprising focal to locally extensive necrosis associated with thrombosis, as well as lamellar oedema and epithelial hyperplasia. Pseudobranch was examined from 21 rainbow trout, and apart from presence of necrotic debris within the vessels only 2 fish had lesions of mild focal necrosis. IP stains revealed viral antigen associated with the areas of necrosis in both gill and pseudabranch, and also in scattered endothelial and connective tissue cells in these organs, even when necrosis was not obvious on H&E sections.

(vii) *Pancreas*

16/27 redfin perch had focal to locally extensive necrosis of pancreatic parenchyma. Small focal lesions involved only a few adjacent parenchymal cells whereas the more extensive lesions were centred on blood vessels. Basophilic intracytoplasmic inclusions were seen in cells within and adjacent to necrotic foci. IP staining was intense in and around the areas of necrosis, and inclusions were strongly stained. Similar lesions were seen in only 1/31 rainbow trout.

(viii) *Central nervous system*

One of 20 redfin perch had marked meningeal congestion. Minor vacuolation was present in large neurones of the ventral horn of the spinal cord in some, affected rainbow trout, but similar changes were occasionally seen in unaffected control fish. Vacuolation of the neuropile, especially white matter tracts was a consistent finding in affected and control fish of both species, and was regarded as an artefact. Occasional rainbow trout, from both infected and control groups had multifocal epidural infiltration by granulocytes. No staining with IP was seen within nervous tissue of either species, although occasional fish had some mild scattered staining of vascular endothelial and connective tissue in the meninges.

(ix) *Skin*

Skin from redfin perch was not examined histologically. Histologic changes were seen in 7/41 rainbow trout, all from the one field outbreak in which ulcers were seen grossly. Ulcerated areas had superficial necrosis and underlying interstitial and myofibre oedema. The interstitium also contained scattered necrotic debris, and macrophages were prominent in some fish. Epidermis adjacent to ulcers had spongiosis, sometimes with a mononuclear inflammatory cell infiltrate. The dermis extending considerable distance from the ulcer was oedematous with abundant necrotic cellular debris. Cells and necrotic debris in dermis and muscular interstitium stained intensely with IP.

(x) *Swim bladder*

This was not examined in redfin perch. There was focal to diffuse oedema and congestion, often with scattered necrotic cells, in the submucosa and serosa of 23/31 rainbow trout. Focal lesions consisted of frank necrosis with abundant basophilic debris against a background of eosinophilic fibrinous material. Walls of blood vessels in these areas were often necrotic. Necrotic debris and scattered connective tissue cells stained strongly with IP.

(xi) *Other tissues*

Occasional lesions included focal necrosis of thymus (1/29 rainbow trout) and thyroid (1/5 trout). Gonads were unaffected, except occasionally by direct extension of peritoneal lesions involving swim bladder or gut serosa.

In both species of fish the most consistent finding was the presence of degenerate haematopoietic cells in many tissues and damage to vascular endothelium in most organs. These findings suggest that EHNV is endotheliotrophic. Most lesions in rainbow trout and redfin perch can be explained by vascular damage. However, the parenchymal lesions in liver and pancreas, with abundant viral inclusions in parenchymal cells indicate a tropism for cells other than endothelium. Gill lesions were a consistent finding in rainbow trout and occurred in about half the redfin perch and these were associated with the presence of virus. The vacuolating encephalopathy reported previously in rainbow trout with EHNV was not apparent in our cases and we considered vacuolation of neuropil to be artefacts.

While EHNV can be detected in many organs of affected fish, some organs are more suited to collection for laboratory examination. The kidney, liver and spleen are the organs of choice as they become routinely infected with virus and generally can be removed aseptically from fish with little difficulty. The gills may also be worthwhile for examination if fish are collected freshly dead, but this organ tends to degenerate rapidly and may be heavily contaminated with faecal material and detritus. There is little or no value in examination of brain, skeletal muscle or gastrointestinal tract as these organs are either not routinely infected with the virus (the former two) or degenerate too rapidly after death to enable meaningful sampling (the latter one).

Note: When examining tissues for IP staining it is important to differentiate between specific and the occassional non-specific staining. Specific staining is characterised by particulate-granular staining within necrotic cells or associated with such areas. If the staining is uniform throughout the cells and if there is a lack of particulate staining then it may represent non-specific staining or endogenous peroxidase activity. In these situations caution is advised with interpretaion.

CONCLUSION:

Kidney, liver and spleen are the organs of choice for testing of EHNV within infected fish. These organs consistently were sites for replication of EHNV in both rainbow trout and redfin perch. In addition the collection of these organs is straight forward.

7.3 Application and Evaluation of Monoclonal and Polyclonal Antibodies in Antigen Capture ELISAs and Electron Microscopy

(As determined for EHNV-infected tissue culture cells)

METHODS & RESULTS

General

Antibodies produced at AAHL were irradiated and sent to EMAI for investigation. Combinations of AAHL and EMAI reagents were compared.

Antigen for ELISA

Cell culture grown EHNV strains B86/8774(RT-X852) and B85/3707(RF-X853) were initially plaque purified and large quantities of each were then prepared, dispensed in 5ml aliquots and stored at 4^0C. The titres of EHNV in these supernatants were $10^{5.51}$ $TCID_{50}$/ml for X852 and $10^{4.87}$ $TCID_{50}$/ml for X853.

Cell culture control for ELISA

BF-2 cells were cultured in tubes with MEM growth medium. The tubes were shaken to detach the cells, pooled in 5ml lots and sonicated for 3 x 1 min. cycles and stored at 4^0C.

Antibodies for ELISA

Polyclonal anti-EHNV antisera (All prepared against trout isolate B86/8774)	
1. Rabbit anti-EHNV(EMAI) (R@B2).	Rabbit Icabod, affinity purified IgG.
2. Rabbit anti-EHNV(AAHL) (R@EHNV).	ME 94/24.
3. Sheep anti-EHNV(AAHL) (S@EHNV).	ME 94/23.
4. Sheep anti-EHNV(EMAI) (S@B2).	Sheep 5924, IB2

Monoclonal anti-EHNV antibody (MAb) culture supernatants (All prepared against trout isolate B86/8774)		
1. MAb anti-TEHNV (C1)	2C1/B12 clone	ME 94/25
2. MAb anti-TEHNV (C4)	3C4/B7 clone	ME 94/26
3. MAb(1) - IgG2a	7(192)	ME 94/38
4. MAb(2) - IgG2a	107(192)	ME 94/38
5. MAb(3) - IgG2a	7A4/D1	ME 94/38
6. MAb(4) - IgG1	24(45)	ME 94/38
7. MAb(5) - IgG1	3B1A7	ME 94/38
8. MAb(6) - IgG1	E10(56)	ME 94/38
9. MAb(7) - IgM	24(181)	ME 94/38
10. MAb(8) - IgM	D1(29)	ME 94/38
11. MAb(9) - IgM	7H10/D4	ME 94/38

Optimisation of ELISA using different combinations of anti-EHNV antibodies

To evaluate the operating characteristics of the antigen capture ELISA employing different combinations of the above antisera it was necessary to perform a large number of optimisation experiments. These are summarised below. The aim of optimisation was to maximise the signal:noise ratio of the assay, that is, the ratio of optical density (OD) obtained from antigen compared with cell culture control medium.

(i) ***Titration of Rabbit-anti-EHNV (AAHL) as capture antibody and Sheep-anti-EHNV (AAHL) as second antibody using Sheep-anti-EHNV (EMAI) and Rabbit-anti-EHNV (EMAI) as reference antibodies at predetermined optimal concentrations.***

All material exported from the secure area of AAHL was irradiated to comply with the strict microbiological requirements of AAHL. Rabbit-anti-EHNV (AAHL), however, did not react with antigen although post-irradiation tests at AAHL showed no decrease in activity. As a consequence this polyclonal antibody could not be assessed. Sheep-anti-EHNV (AAHL) was not affected by irradiation. The optimal dilution for this second antibody was found to be 1:16000-1:32000.

(ii) ***Evaluation of Sheep-anti-EHNV (AAHL) as capture antibody.***

A checkerboard titration of Sheep-anti-EHNV (AAHL) vs Rabbit-anti-EHNV (EMAI) was performed. Sheep-anti-EHNV (AAHL) did not function as a capture antibody under the experimental conditions used at EMAI.

(iii) ***Optimising the concentration of Rabbit-anti-EHNV (EMAI) vs Sheep-anti-EHNV (EMAI) and Rabbit-anti-EHNV (EMAI) vs Sheep-anti-EHNV (AAHL).***

Checkerboard titrations of Rabbit-anti-EHNV (EMAI) vs Sheep-anti-EHNV (EMAI) and Rabbit-anti-EHNV (EMAI) vs Sheep-anti-EHNV (AAHL) were performed. The dilutions of Rabbit-anti-EHNV (EMAI) and Sheep-anti-EHNV (EMAI) which gave optimal OD readings and signal to noise ratios were 1:3200 and 1:3200, respectively when antigen was diluted 1:10 and cell culture control was used neat. To avoid a reduction in sensitivity a lower dilution of Rabbit-anti-EHNV (EMAI), 1:1600, is recommended with Sheep-anti-EHNV (EMAI) remaining at 1:3200, as this dilution resulted in the greatest signal to noise ratios at all Rabbit-anti-EHNV (EMAI) dilutions (Fig.1). Dilutions of 1:3200 and 1:8000 for Rabbit-anti-EHNV (EMAI) and Sheep-anti-EHNV (AAHL), respectively gave optimal results. Again to avoid a reduction in sensitivity and to maintain Rabbit-anti-EHNV (EMAI) at a constant dilution for all combinations of ELISA's, a dilution of 1:1600 is recommended for Rabbit-anti-EHNV (EMAI). The recommended dilution of Sheep-anti-EHNV (AAHL) is 1:16000 (Fig.2).

(iv) ***Comparing the effectiveness of a polyclonal ELISA using Rabbit-anti-EHNV (EMAI) + Sheep-anti-EHNV (EMAI) with that of a polyclonal ELISA using Rabbit-anti-EHNV (EMAI) + Sheep-anti-EHNV (AAHL) with all antibodies at their optimised dilutions.***

Both ELISA's showed similar signal to noise ratios when antigen and cell culture control were titrated (Fig 3).

(v) ***Determining the effectiveness of monoclonal antibodies 1-11 in detecting EHNV with Rabbit-anti-EHNV (EMAI) as capture antibody.***

Of the 11 MAbs tested only 4 were shown to be effective in detecting antigen. These results are in agreement with those from a capture ELISA conducted at AAHL using the same monoclonals after they were irradiated but before they were sent to EMAI. The lack of activity of most of the monoclonals is therefore due to the irradiation. The exception is MAb(6) which was shown to have a high OD in AAHL's results but showed a low OD in the EMAI ELISA.

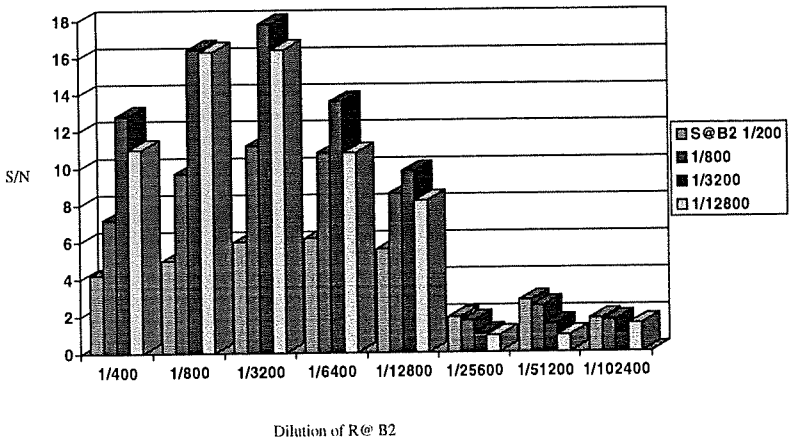
From these initial experiments, the dilution shown to give optimal results for 3 active monoclonals was 1:8 while the fourth had an optimal dilution of 1:32. There were some severe fluctuations in the OD's of diluted cell culture controls, particularly in the lower dilutions, which resulted in irregular signal to noise patterns for some monoclonals, especially for MAb 1.

(vi) ***Titration of polyclonal rabbit-anti-sheep Ig-HRP conjugate (KPL laboratories) and rabbit-anti-mouse IgG-HRP conjugate (Dakopatts).***

The optimal dilution of Rabbit-anti-sheep-HRP conjugate was 1:3000 for both Rabbit-anti-EHNV (EMAI) + Sheep-anti-EHNV (EMAI) and Rabbit-anti-EHNV (EMAI) + Sheep-anti-EHNV (AAHL) ELISA's. For the monoclonal antibodies, a dilution of 1:750 for the conjugate Rabbit-anti-mouse-HRP provided the best signal to noise response (Figs 4, 5 and 6).

(vii) ***Definition of working ELISA protocols and determination of analytical sensitivity.***

Six functional ELISA configurations were identified and optimised (Table 1). The analytical sensitivity of each assay was determined by titration of antigen. The monoclonal antibody-based ELISAs have an analytical sensitivity approximately half that of the polyclonal antibody-based assays. Both polyclonal antibody-based assays had similar analytical sensitivity as did all four MAb based assays .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8
6
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1/12800
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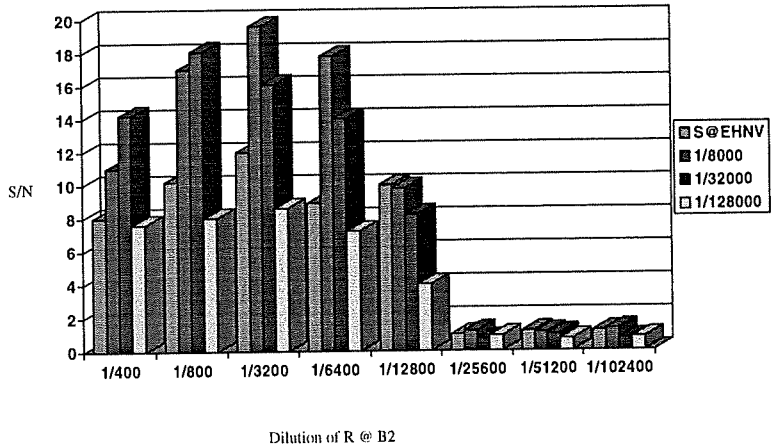
S@B2 1/200
1/800
1/3200
1/12800
Dilution of R@ B2

Fig. 1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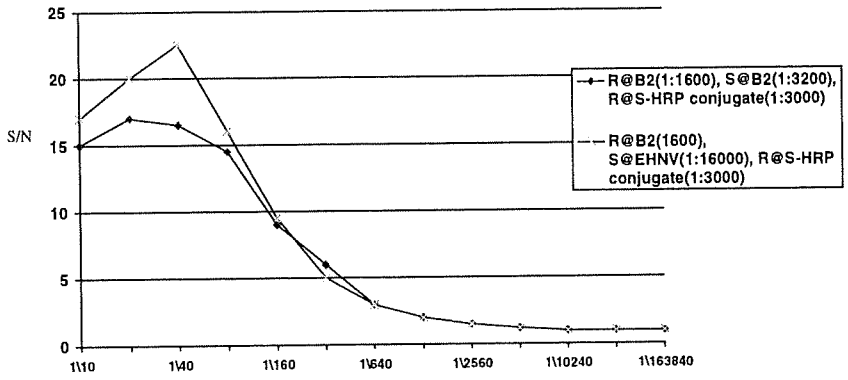
Dilution of R @ B2

Fig. 2.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1\163840
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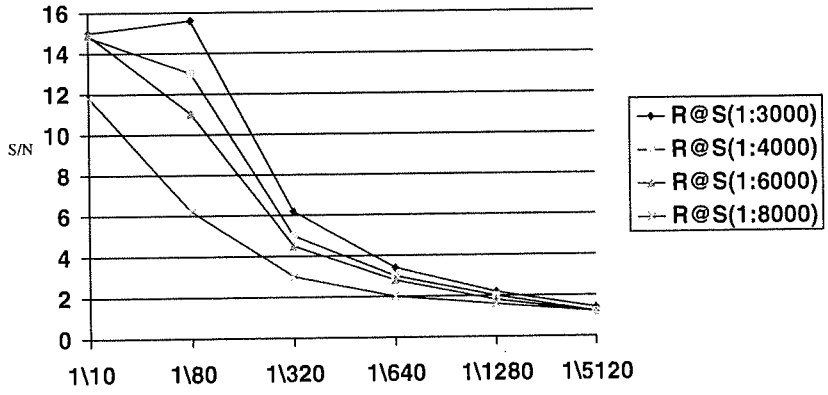
R@B2(1600), S@EHNV(1:16000), R@S-HRP conjugate(1:3000)
Dilution of EHNV antigen and control

Fig. 3.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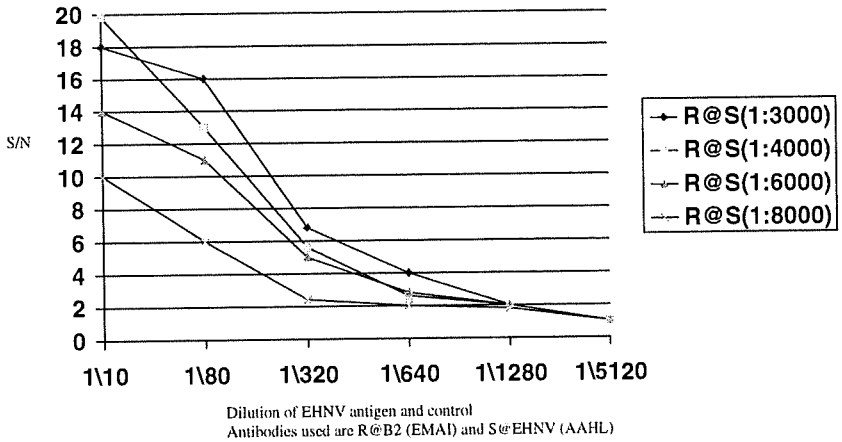
R@S(1:3000)
R@S(1:4000)
R@S(1:6000)
R@S(1:8000)
Dilution of EHNV antigen and control
Antibodies were R@B2 (EMAI) and S@B2 (EMAI).

Fig. 4.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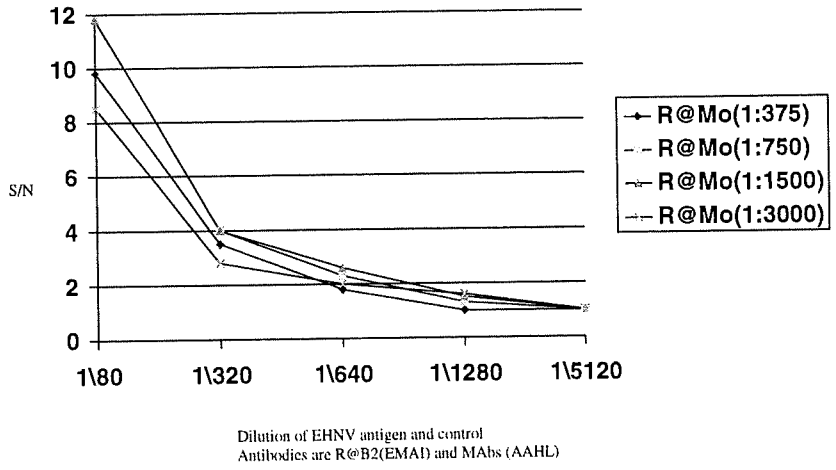
1\5120
R@S(1:3000)
R@S(1:4000)
R@S(1:6000)
R@S(1:8000)
Dilution of EHNV antigen and control
Antibodies used are R@B2 (EMAI) and S@EHNV (AAHL)

Fig. 5.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R@Mo(1:750)
R@Mo(1:1500)
R@Mo(1:3000)
Dilution of EHNV antigen and control
Antibodies are R@B2(EMAI) and MAbs (AAHL)

Fig. 6.

Table 1. *Configuration and sensitivities of six ELISA protocols.*

ELISA No.	Capture antibody	2nd antibody	Conjugate	Analytical Sensitivity * ($TCID_{50}$)
1	Rabbit-anti-EHNV (EMAI) 1:1600	Sheep-anti-EHNV (EMAI) 1:3200	Rabbit-anti-sheep-HRP 1:3000	2.53×10^2
2	Rabbit-anti-EHNV (EMAI) 1:1600	Sheep-anti-EHNV (AAHL) 1:16000	Rabbit-anti-sheep-HRP 1:3000	2.53×10^2
3	Rabbit-anti-EHNV (EMAI) 1:1600	MAb(2C1/B12) 1:32	Rabbit-anti-mouse-HRP 1:750	5.06×10^2
4	Rabbit-anti-EHNV (EMAI) 1:1600	MAb1 1:8	Rabbit-anti-mouse-HRP 1:750	5.06×10^2
5	Rabbit-anti-EHNV (EMAI) 1:1600	MAb2 1:8	Rabbit-anti-mouse-HRP 1:750	5.06×10^2
6	Rabbit-anti-EHNV (EMAI) 1:1600	MAb5 1:8	Rabbit-anti-mouse-HRP 1:750	5.06×10^2

* Defined as one dilution above the dilution of antigen at which the antigen titration curve reached the baseline.

Evaluation of the sensitivity and specificity of monoclonal antibody and polyclonal antibody based ELISA for detection of EHNV from tissue culture supernatants

(i) *Sample selection*

Uninfected fish: 100 Redfin Perch and 100 Rainbow Trout which were obtained from known EHNV-free populations were selected. Liver + kidney + spleen from individual fish were cultured on BGF cells and the cultures were all free of cytopathic effect after two passages.

Infected fish: 100 Redfin Perch and 100 Rainbow Trout from known EHNV-infected populations were selected. Liver + kidney + spleen from individual fish were cultured on BF-2 cells and all cultures displayed cytopathic effect typical of EHNV.

Culture supernatants from this work were tested in each of the 6 ELISA protocols. The culture supernatants included material from laboratory accessions from 1990-1994 (i.e. stored for different periods), supernatants stored at 4^0C, -20^0C or -80^0C and passage level of cultures ranging from first to fourth as shown in the Table-2.

Table 2 *History of samples used in ELISA protocols.*

		Number of EHNV infected supernatants	Number of EHNV free supernatants
Species	• Redfin	89	100
	• Rainbow trout	98	100
Temp of storage	4°C	70	92
	-20°C	101	108
	-80°C	16	-
Years of storage	5	-	50
	4	45	58
	3	50	69
	2	39	-
	1	63	23
Passage level	1	109	61
	2	65	89
	3	6	50
	4	7	-

(ii) *Controls used for each ELISA experiment*

Controls were included on each ELISA plate in each experiment. The controls included a series of dilutions (1:10, 1:100, 1:200, 1:500, 1:1000, and 1:10000), in maintenance medium, of the EHNV strain B86/8774 (X852). Cell culture medium (CCM) was used as a negative control. Controls were dispensed in 5 ml aliquots and stored at 4°C.

(iii) *Procedure of testing*

A Micro-rack tube system (Interpath services P/L, Caringbah, NSW) was used to dispense the culture supernatants and controls, using a multichannel pipette, into Linbro 96 well microtitre plates (Linbro/Titertek, ICN Pharmaceutical P/L, Seven Hills, NSW). Before each ELISA run, the

racks were inverted twice to ensure proper dispersion of the samples. The racks were stored at 4^0C for the duration of the trial.

Single batches of 10 x PBS, ABTS, stop solution, primary antibody, secondary antibody, and conjugates were used for all six ELISA's.

(iv) *Control of variability in ELISA between experiments*

In addition to rigorous laboratory quality control, a novel method for reducing plate to plate variation in antigen capture ELISA was evaluated. This was based on a method described for antibody-detection ELISA (Whittington 1992, *J. Immun. Methods* 148:57-64). Four controls which had OD values covering the range of response of the ELISA were selected from each plate and used to calculate a correction factor for each plate. The variability in the OD responses of the four controls before and after correction of data is shown in the table. The procedure resulted in a useful reduction in variability of the data in most cases.

Table 3 *Variability of data from different ELISAs.*

ELISA No.	Mean OD				Coefficient of variation before correction %				Coefficient of variation after correction %			
	Control				Control				Control			
	1	2	3	4	1	2	3	4	1	2	3	4
1	2.7	1.5	0.8	0.2	2.8	4.4	6.2	6.6	3.9	2.1	3.9	4.3
2	2.6	1.5	0.8	0.1	3.9	5.0	9.5	17.4	6.1	5.8	6.0	11.5
3	2.6	1.0	0.4	0.1	3.8	8.9	11.7	26.0	8.9	5.8	6.9	19.2
4	2.7	1.2	0.6	0.1	2.8	10.5	9.1	17.1	5.2	9.4	6.7	14.6
5	2.6	1.1	0.6	0.1	3.8	12.3	13.9	24.6	8.4	10.7	6.9	19.0
6	2.6	0.9	0.4	0.1	2.9	10.0	14.0	23.6	7.8	10.2	8.6	18.8

(v) *Comparison of sensitivity and specificity of six ELISA protocols*

Sensitivity was defined as the percentage of EHNV infected cell culture supernatants with an ELISA OD exceeding an arbitrarily determined positive/negative (P/N) threshold. Specificity was defined as the percentage of EHNV free cell culture supernatants with an ELISA OD less than the P/N threshold.

For each ELISA, a P/N cutoff OD to maximise sensitivity and specificity was selected by analysis of the frequency distribution of the corrected OD data. The cutoffs selected for each of the six ELISA's are given in the table. There were no significant differences in the operating characteristics of the six ELISA's. The P/N cutoffs ranged from ELISA OD 0.3-0.5 with the specificity and sensitivity ranging from 98.9%-100%.

Table 4 *Sensitivity and specificityof the different ELISA protocols.*

ELISA No.	P/N cut-off	EHNV-free			Specificity %	95% confidence limits	EHNV-infected			Sensitivity %	95% confidence limits
		+ve	-ve	Total			+ve	-ve	Total		
1	0.5	0	200	200	100	97.4-100	185	2	187	98.9	95.1-100
2	0.3	0	200	200	100	97.4-100	186	1	187	99.5	96.1-100
3	0.5	2	198	200	99	95.4-100	185	2	187	98.9	95.1-100
4	0.5	1	199	200	99.5	96.3-100	186	1	187	99.5	96.1-100
5	0.4	1	199	200	99.5	96.3-100	187	0	187	100	97.2-100
6	0.4	1	199	200	99.5	96.3-100	187	0	187	100	97.3-100

Further analysis of the data showed that species of fish, temperature and duration of storage of the supernatants, and passage level of the culture had no effect on the results.

CONCLUSION:

1. **Sensitivity and specificity of MAb-based assays are equivalent to the polyclonal assays.**
2. **Some Mabs are not suitable for routine use because they are inactivated by gamma irradiation.**

7.4 Assessment of Sensitivity and Specificity of ELISAs for Tissue Homogenates

METHODS

Selection and processing of specimens

Uninfected fish:

150 Redfin Perch (ME93/23) (average 84.4 g, 15 cm) and 150 Rainbow Trout (ME93/20) (average 210 g 25 cm) were obtained from known EHNV-free populations (Tasmania). Fish were dissected, individual organs were dispensed in sterile tubes containing 300 ml sterile PBS and stored at -20°C. Tissue homogenates were prepared in disposable plastic tubes using a fitted pestle, vortexed with glass beads, and then clarified by centrifugation as described by Whittington and Steiner (1993). All tissue homogenates were shown to be negative in virus isolation.

The liver, spleen and kidney from 120 redfin perch and 120 rainbow trout were individually homogenised, pooled (33µl of each) and stored at -80°C to be tested later. Liver, spleen, kidney, gonad and gut from an additional 30 redfin perch and 30 rainbow trout were individually homogenised and stored at 4°C for testing the next day or at -80°C for testing later. Once tested as individual samples, these homogenates were stored at -80°C. Later, the liver, kidney and spleen homogenates were thawed, pooled and 200 ml of homogenising medium was added. Thus there were a total of 150 pooled homogenates from these species.

91 golden perch fingerlings (ME93/4) (average 0.7 g weight, range 25-40 mm forklength) from Narrandera NSW were collected and stored at -80°C. The body of each fish, excluding head and tail, was homogenised as above and stored at 4°C overnight. These homogenates were processed routinely for culture in BF-2 cells and found to be free of EHNV by the absence of cytopathic effect.

Infected fish:

Homogenates of liver, kidney and spleen from 19 adult redfin perch with clinical EHN (ME91/60, ME91/50, ME91/42 and ME92/35) were prepared from tissues frozen at -20°C, processed routinely for culture in BF-2 cells and found to contain EHNV. 236 rainbow trout fingerlings (ME93/63) (average 0.37 g, 3.2 cm) were obtained from a known EHNV-infected population and stored at -80°C. They were thawed and dissected over a period of 2 months. The viscera of each fish was removed aseptically and homogenised as above. The clarified homogenates were stored at 4°C overnight and then inoculated individually into cultures of BF-2 cells. Two passes were undertaken and the cell culture supernatants were then tested using ELISA 1. 123 fish were shown to be positive for EHNV by cytopathic effect and ELISA 1. Due to the removal of 300µl of neat homogenate for virus isolation, the addition of 300µl of homogenising medium was required in order for the homogenates to be tested in all 4 ELISA's.

Procedure of testing

A micro-rack tube system (Interpath services P/L, Caringbah, NSW) was used to dispense the tissue homogenates and controls, using a multichannel pipette, into Linbro 96 well microtitre plates (Linbro/Titertek, ICN Pharmaceuticals P/L, Seven Hills, NSW). Before each ELISA run, the racks were inverted twice to ensure proper dispersion of the samples.

ELISA solutions, primary antibody and conjugates

Single batches of primary antibody R@B2, conjugate KPL (for sheep second antibodies) and conjugate R@Mo (for mouse second antibodies) were used for all ELISAs.

ELISA configurations

Four of the 6 ELISA configurations shown in the previous section were used in this trial. The configurations used are shown below:

Assay	Capture Ab/	2nd Ab/	Conj./diln
1	R@B2(EMAI) 1:1600	S@B2(EMAI) R@ 1:3200	Sh-HRP 1:3000
2	R@B2(EMAI) 1:1600	S@EHNV(AAHL) 1:16000	R@Sh-HRP 1:3000
5	R@B2(EMAI) 1:1600	MAb2 1:8	R@Mo-HRP 1:750
6	R@B2(EMAI) 1:1600	MAb5 1:8	R@Mo-HRP 1:750

Monoclonal antibody 2 was selected because it showed slightly better sensitivity and specificity compared to MAbs C1 and 1 when tested against tissue culture supernatants (refer to previous section). Monoclonal antibody 5 was selected because it was formed from a different parent clone than the other 3 monoclonals and it appears to be able to differentiate between EHNV, BIV and FV3.

Controls for ELISA

The controls were a series of dilutions, in maintenance medium, of the EHNV strain B86/8774 (X852) as described in the previous section. Four of these were selected to correct plate to plate variation (refer to previous section).

RESULTS

General analysis

Tissue homogenates from three species of fish were tested in the four versions of the EHNV ELISA test. The sample descriptions and summary ELISA data are given in Table 1.

Table 1. *Classification of samples with a summary of their ELISA results (mean ± S.D.)*

Species	Population status	Virus isolation	n	ELISA OD (mean ± S.D.)			
				ELISA 1 (S@B2)	ELISA 2 (S@EHNV)	ELISA 5 (MAb2)	ELISA 6 (MAb5)
Rainbow trout	Infected	positive	123	0.94 ± 0.78	0.95 ± 0.77	0.70 ± 0.76	0.68 ± 0.76
	Infected	negative	113	0.34 ± 0.41	0.34 ± 0.47	0.26 ± 0.42	0.30 ± 0.48
	Free	negative	150	0.26 ± 0.13	0.21 ± 0.11	0.16 ± 0.05	0.15 ± 0.06
Redfin perch	Infected	positive	19	2.39 ± 0.63	2.78 ± 0.80	2.67 ± 0.77	3.07 ± 0.97
	Free	negative	150	0.27 ± 0.15	0.20 ± 0.11	0.17 ± 0.06	0.14 ± 0.05
Golden perch	Free	negative	91	1.14 ± 0.17	0.51 ± 0.05	0.30 ± 0.09	0.24 ± 0.14
Total			646				

Sensitivity was defined as the percentage of infected fish that gave a positive result in the test. Specificity was defined as the percentage of disease free (uninfected) fish that gave a negative result in the test.

The sensitivity and specificity of each ELISA was determined initially using the whole disease free population (virus isolation negative) regardless of species of fish (n=391), and the virus isolation positive samples from the infected population, regardless of species of fish (n=142).

The sensitivity and specificity at several OD cut-offs were calculated for each test (Table 2 and Figs 1a - h) 1). ELISA 1 (S@B2) had relatively poor specificity compared to ELISA 2 (S@EHNV), the other polyclonal ELISA. The specificities of these tests at a common level of sensitivity (78%) were 55.8% and 64.7% respectively, values that were significantly different (Chi square test, P=0.01). ELISA 1 had a sensitivity of only 33.8% at a specificity of 99.0%, compared with a sensitivity of 55.6% for ELISA 2 at 99.7% specificity (Chi square test, P=0.0002).

Both MAb-based tests, ELISA 5 (MAb2) and ELISA 6 (MAb5) were relatively insensitive at levels of specificity considered usual for such assays. ELISA 5 had a sensitivity of only 47.2% while ELISA

6 had a sensitivity of only 40.9% at a specificity level of 99.2% (Chi-square test, P=0.28). Both assays were characterised by a rapid decline in sensitivity with small increases in OD cut-off.

Polyclonal ELISA 2 was more sensitive (60%) than monoclonal ELISA 5 (47%) at a specificity of 99% (Chi square test P=0.03). These results suggest that polyclonal ELISA 2 should be used for routine screening of samples for EHNV, and that monoclonal ELISA 5 should be used to re-examine samples suspected to be false-positive reactors.

Table 2. *Sensitivity and specificity of each ELISA test at different OD cut-off. Tissues from rainbow trout, redfin perch and golden perch are included.*

ELISA	OD Cut-off	Sensitivity (n = 142)	Specificity (n = 391)
1. S@B2	0.20	100	
	0.30	78.2	55.8
	0.40	72.5	65.0
	0.50	63.4	70.6
	0.60	59.2	75.2
	0.70	53.5	76.5
	1.40	33.8	99.0
2. S@EHNV	0.20	100	
	0.30	78.9	64.7
	0.40	73.2	72.6
	0.50	64.1	85.2
	0.60	59.9	99.5
	0.70	55.6	99.7
5. MAb2	0.20	100	
	0.30	58.5	87.7
	0.40	52.1	98.0
	0.50	47.2	99.2
	0.60	43.0	99.7
	0.70	38.7	99.7
6. MAb5	0.20	100	
	0.30	58.5	92.3
	0.40	48.6	96.9
	0.50	43.7	98.5
	0.60	40.9	99.2
	0.70	35.9	100

Effect of fish species on ELISA results

EHNV-free golden perch had significantly greater ELISA OD than EHNV free redfin perch or rainbow trout in each ELISA (P=0.0000) (Table 1). Very high OD were obtained with the polyclonal ELISAs, especially ELISA 1. The OD for golden perch in the monoclonal ELISAs were on average about twice those of redfin perch and rainbow trout. These results suggest that golden perch are inherently cross-reactive in these tests. The cross reactive antigen has not been identified.

The sensitivity and specificity of each ELISA test was recalculated after excluding golden perch. The results are given in Table 3 and Figs1b,d,f,h. There was an improvement in specificity at any given OD cut-off and there was less difference between the performance of polyclonal and monoclonal ELISAs. For example, at a specificity of 99-100%, sensitivities were 53.5, 59.9, 52.1 and 48.6% across the assays. These differences were not significant (Chi square test of largest difference, P=0.06). The improvement in specificity enabled a lower OD cut-off to be used, thus increasing the effective sensitivity of each assay.

In infected fish, the OD of redfin perch were greater than those of rainbow trout (P=0.0000). This suggests that viral replication and antigen accumulation is more marked in redfin perch.

Table 3. *Sensitivity and specificity of each ELISA test for analysis of tissue homogenates of rainbow trout and redfin perch at different OD cut-off. Golden perch were excluded to enable estimation of cut-offs for species not affected by tissue antigen cross reactions.*

ELISA	OD Cut-off	Sensitivity (n = 142)	Specificity (n = 300)
1. S@B2	0.10	99.3	3.3
	0.20	88.0	38.7
	0.30	78.2	72.7
	0.40	72.5	84.7
	0.50	63.4	92.0
	0.60	59.2	97.3
	0.70	53.5	99.0
	0.90	47.2	100
2. S@EHNV	0.10	99.3	10.3
	0.20	89.4	60.3
	0.30	78.9	84.3
	0.40	73.2	93.3
	0.50	64.1	98.0
	0.60	59.9	99.7
	0.70	55.6	99.7
	1.15	40.85	100
5. MAb2	0.10	94.4	4.0
	0.20	74.7	80.7
	0.30	58.5	96.0
	0.40	52.1	100
6. MAb5	0.10	97.2	9.0
	0.20	76.8	87.3
	0.30	58.5	98.3
	0.40	48.6	100

Positive-negative cut-off OD for each ELISA

In order to achieve specificity of 99-100% in rainbow trout and redfin perch, positive-negative cut-off OD of 0.7, 0.6, 0.4 and 0.4 were selected for ELISA 1, ELISA 2, ELISA 5 and ELISA 6 respectively (based on the data in Table 3). Higher cut-offs would need to be used in other species of fish to guarantee a high level of specificity.

The suggested cut-offs in other species of fish such as golden perch are 1.40 (ELISA 1), 0.70 (ELISA 2) and 0.6 (ELISAs 5 and 6) (Table 2). The relation between the OD values of the control antigens and the suggested OD cut-offs for rainbow trout and redfin perch are shown in Figs 2a - d. For each ELISA, the OD cut-off is at the lower end of the dose response curve and approximates that of control antigen 3 (equivalent to a 1:500 dilution of stock antigen X852).

Virus isolation (CPE) negative fish from EHNV affected populations

Rainbow trout from infected populations were examined individually by cell culture. Virus was isolated from some fish but not others (Table 1). The ELISA OD of virus isolation negative fish from the infected populations were higher than those from uninfected rainbow trout (P=0.02 ELISA 1; P=0.001 ELISA 2; P=0.007 ELISA 5; P=0 ELISA 6), suggesting that EHNV antigen was present in the former group. The number of virus isolation (CPE) negative fish that had positive results in each ELISA is given in Table 4. Although the monoclonal antibody assays (ELISAs 5 and 6) detected a greater number of fish than did the polyclonal ELISAs (ELISAs 1 and 2), the differences were not significant (Chi-square P=0.14)

These data suggest that many of the virus isolation negative fish contained EHNV antigen. Storage of the fish/tissues for prolonged periods prior to analysis might have influenced the virus isolation results, reducing the frequency of a positive result.

Table 4. *Fish with negative virus isolation but positive ELISA results. Some rainbow trout from the infected population had negative virus isolation but positive ELISA results. These fish contained non-viable virus that was detectable only by ELISA.*

ELISA	OD Cut-off	No. positive	No. negative	Total
1. S@B2	0.7	6	107	113
2. S@EHNV	0.6	7	106	113
5. MAb2	0.4	11	102	113
6. MAb5	0.4	12	101	113

Infected fish with negative ELISA results

Some fish known to contain EHNV (i.e. virus isolation positive fish) gave negative results in one or more ELISAs. This is because cell culture is inherently more sensitive than ELISA. Many cell cultures did not develop CPE (i.e. become positive) until the second pass, indicating that only small amounts of virus were present in these individuals. About 42-47% of the ELISA negative infected fish were in this category (Table 5). In the remaining fish there was still insufficient virus to enable detection by ELISA, despite CPE appearing in the first pass.

Table 5. *Infected fish with negative ELISA results. A proportion of infected fish were not detected by ELISA. In many cases this was because the amount of virus present was low. This is illustrated by the proportion of fish in which cytopathic effect (CPE) developed only in the second passage of cell culture.*

ELISA	OD Cut-off	No. negative	% CPE +ve in PII
1. S@B2	0.7	66	47
2. S@EHNV	0.6	60	43
5. MAb2	0.4	72	42
6. MAb5	0.4	76	43

Examination of specific organs of uninfected fish for cross reactivity in each ELISA

Preliminary data from our laboratories suggested that gonad might react non-specifically in ELISA for EHNV. Exclusion of gonad from visceral samples for ELISA was suggested as a means of improving the reliability of results. Consequently, to find possible sources of cross reaction in the assays, the kidney, spleen, liver, gonad and gut of 30 rainbow trout and 30 redfin perch were tested separately in each ELISA.

Overall the tissues of redfin perch were more reactive than those of rainbow trout in ELISA 1 (P=0.0000), ELISA 2 (P=0.02) and ELISA 6 (P=0.0000) so the data for each species were examined separately (Table 6). Significant differences were found in the reactivity of organs in both species of fish. In rainbow trout, liver was highly reactive relative to other organs while gonad and gut were least reactive (P=0.0000). Similar differences were noted for each ELISA. Liver was also the most reactive organ in redfin perch, while spleen was the least reactive. The differences in redfin perch were significant in ELISA 1 (P=0.0000), ELISA 2 (P=0.0000) and ELISA 5 (P=0.007), but the differences between organs in ELISA 6 were not significant (P=0.09).

Although it is inherently cross reactive, liver remains a useful organ to include in ELISA because it is a frequent site of viral replication. The data show that similar cross reactive antigens exist in EHNV and cells of host fish.

Table 6. *Reactions of organs from uninfected fish. The organs of each fish were tested individually in each ELISA. Data are mean ± S.D.*

Species and ELISA	Organ				
	Kidney	Spleen	Liver	Gonad	Gut
• Rainbow trout					
ELISA 1	0.20 ± 0.08	0.19 ± 0.13	0.47 ± 0.16	0.09 ± 0.02	0.08 ± 0.01
ELISA 2	0.17 ± 0.07	0.16 ± 0.11	0.39 ± 0.12	0.08 ± 0.01	0.08 ± 0.01
ELISA 5	0.18 ± 0.06	0.21 ± 0.20	0.27 ± 0.08	0.10 ± 0.03	0.15 ± 0.06
ELISA 6	0.16 ± 0.06	0.15 ± 0.06	0.21 ± 0.08	0.10 ± 0.02	0.13 ± 0.05
• Redfin perch					
ELISA 1	0.27 ± 0.13	0.17 ± 0.04	0.40 ± 0.19	0.32 ± 0.18	0.24 ± 0.17
ELISA 2	0.20 ± 0.08	0.13 ± 0.03	0.33 ± 0.14	0.27 ± 0.16	0.15 ± 0.07
ELISA 5	0.15 ± 0.04	0.16 ± 0.08	0.21 ± 0.10	0.17 ± 0.07	0.14 ± 0.05
ELISA 6	0.17 ± 0.07	0.17 ± 0.06	0.21 ± 0.06	0.18 ± 0.07	0.17 ± 0.06

Precision of the ELISA

Four control antigens were included on each plate and used to normalise the data for samples included on each plate. The variability in the OD of the controls before and after application of the normalisation method is given in Table 7. The normalised OD had maximum coefficients of variation ranging from 7.5 to 10.2% in ELISAs 1, 2 and 5 but up to 25.9% in ELISA 6.

Table 7. *Precision of ELISA. The coefficient of variation of each control antigen tested on 26 plates is shown before and after application of a normalisation procedure. The OD cut-off corresponding to ≥ 99% specificity is similar to the OD value of control 3. For practical purposes the OD of this control can be regarded as the cut-off in each ELISA.*

ELISA	Data	Control			
		1	2	3	4
1. S@B2	Raw	4.4	12.0	16.6	7.6
	Normalised	8.1	5.4	9.3	7.9
	OD mean ± s.d.	2.69 ± 0.12	1.93 ± 0.23	0.64 ± 0.11	0.16 ± 0.01
2. S@EHNV	Raw	4.7	8.2	14.3	12.3
	Normalised	7.5	5.1	7.2	7.1
	OD mean ± s.d.	2.68 ± 0.13	1.98 ± 0.16	0.68 ± 0.10	0.15 ± 0.02
5. MAb2	Raw	5.1	14.8	12.8	12.5
	Normalised	7.1	10.2	5.8	9.2
	OD mean ± s.d.	2.63 ± 0.13	1.51 ± 0.22	0.45 ± 0.06	0.13 ± 0.02
6. MAb5	Raw	3.8	14.1	22.3	35.9
	Normalised	12.3	12.5	11.5	25.9
	OD mean ± s.d.	2.65 ± 0.10	1.45 ± 0.21	0.44 ± 0.10	0.13 ± 0.05

In addition to plate to plate variation, intra-assay variation for duplicate samples included on the same plate was assessed (Table 8). In general, the results for duplicates in the polyclonal assays (ELISAs 1 and 2) rarely varied by more than 15% coefficient of variation. In contrast, variation greater than this magnitude was common in both monoclonal assays (ELISAs 5 and 6).

Table 8. *Intra-assay variation in EHNV ELISAs. Tissue homogenates (n=946) were tested in duplicate wells. The data are the number of samples (%) with coefficient of variation between duplicate wells of the magnitudes shown.*

Coefficient of variation	ELISA 1	ELISA 2	ELISA 5	ELISA 6
No. duplicates > 15%	29(3%)	18(2%)	221(23%)	241(25%)
No. duplicates > 50%	-	-	71(8%)	91(10%)

Methods of reducing this variability were investigated using monoclonal ELISA 5. A range of infected and EHNV free tissue homogenates from both redfin perch and rainbow trout and infected tissue culture supernatants were used. The following variations (highlighted) to the standard protocol were introduced:

1. Immulon 96 well microtitre plates
2. PBSTO (PBS, 0.1% v/v Tween 20, 0.1% w/v ovalbumin) diluent diluent
3. PBSTS (PBS, 0.05% v/v Tween 20, 2% w/v skim milk) diluent
4. PBST (PBS, 0.1% v/v Tween 20) diluent
5. 10 washes
6. PBSTO (PBS, 0.05% v/v Tween 20, 0.1% w/v ovalbumin,pH 8.0) diluent
7. PBSTO (PBS, 0.1% v/v Tween 20, 1% w/v ovalbumin) diluent

A control plate using standard conditions (PBSTO diluent [PBS pH 7.4, 0.05% v/v Tween 20, 0.1% w/v ovalbumin]; 5 washes; Linbro 96 well microtitre plates) was included with each variation. Results are given in Table 9.

Table 9. *Effect of variations to the protocol on reproducibility between duplicate samples in ELISA 5.*

Variation	Treatment		Control	
	CV > 15%	CV > 50%	CV > 15%	CV > 50%
1	1/40(3%)	-	5/40(13%)	-
2	18/84(21%)	2/84(2%)	19/84(23%)	-
3	4/41(10%)	2/41(5%)	8/41(20%)	1/41(2%)
4	11/79(14%)	6/79(8%)	9/79(11%)	5/79(6%)
5	7/40(18%)	1/40(3%)	5/40(13%)	1/40(3%)
6	11/40(28%)	3/40(8%)	5/40(13%)	1/40(3%)
7	12/40(30%)	2/40(5%)	5/40(13%)	1/40(3%)

The poor reproducibility was not overcome by alternative blocking and washing procedures, nor by changes to buffers and diluentsand was worsened by some, but was responsive to change of microtitre plate. Immulon microtitre plates resulted in a higher rate of reproducibility than Linbro plates, but OD were lower. It was not considered practical to change to Immunolon plates because this have invalidated estimates of sensitivity and specificity and the cut-off point.

Batch to batch variation in gamma irradiated reagents

The MAbs and sheep anti-EHNV polyclonal antibody were products from AAHL that required gamma irradiation prior to export from AAHL.

To determine the degree of batch-to-batch variation in irradiated MAbs, different batches of these reagents were titrated in single wells against cell culture grown EHNV strain B86/8774 (RT-X852). The response of each MAb is illustrated in Figs 3 - 9. The key list for the monoclonal antibodies is given below. Among some monoclonal antibodies, batch-to-batch variation was quite evident. There was an 8-fold difference in activity between two batches of MAb3C1/B12 (Fig 4) and MAb5 (Fig 9). An 8-fold difference was also observed between an early batch of MAb2 and two later batches (Fig 8).

Key list for monoclonal antibodies:

Experiment 1 (Figures 3 & 4)

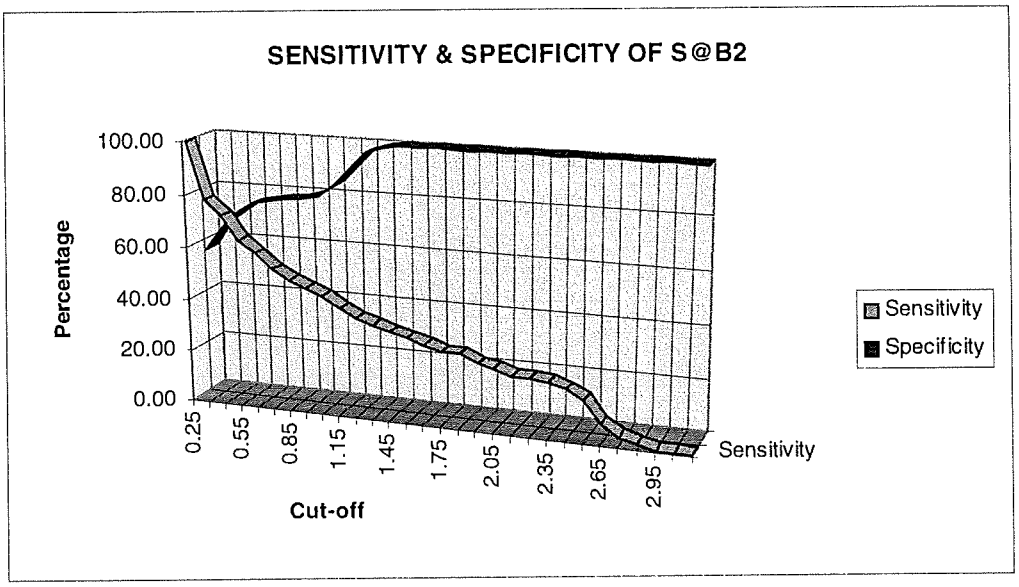
Monoclonal batch	Clone	ME accession no.
MAb5(B7)	3B1/A7	94/38
MAb5(B22)	3B1/A7	95/17
MAbC1(B1)	3C1/B12	94/25
MAbC1(B23)	3C1/B12	95/17

Experiment 2 (Figs 5, 6 & 7)

MAb11(B13)	7D12/A3	95/8
MAb11(B24)	7D12/A3	95/17
MAbC1(B1)	3C1/B12	94/25
MAbC1(B15)	3C1/B12	95/8
MAbC1(B23)	3C1/B12	95/17
MAb5(B7)	3B1/A7	94/38
MAb5(B17)	3B1/A7	95/8
MAb5(B22)	3B1/A7	95/17

Experiment 3 (Graphs 8 & 9)

MAb2(B4)	107(192)	94/38
MAb2(B26)	107(192)	95/38
MAb2(B28)	107(192)	95/45
MAb5(B22)	3B1/A7	95/17
MAb5(B27)	3B1/A7	95/38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40.00
20.00
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Percentage
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2.65
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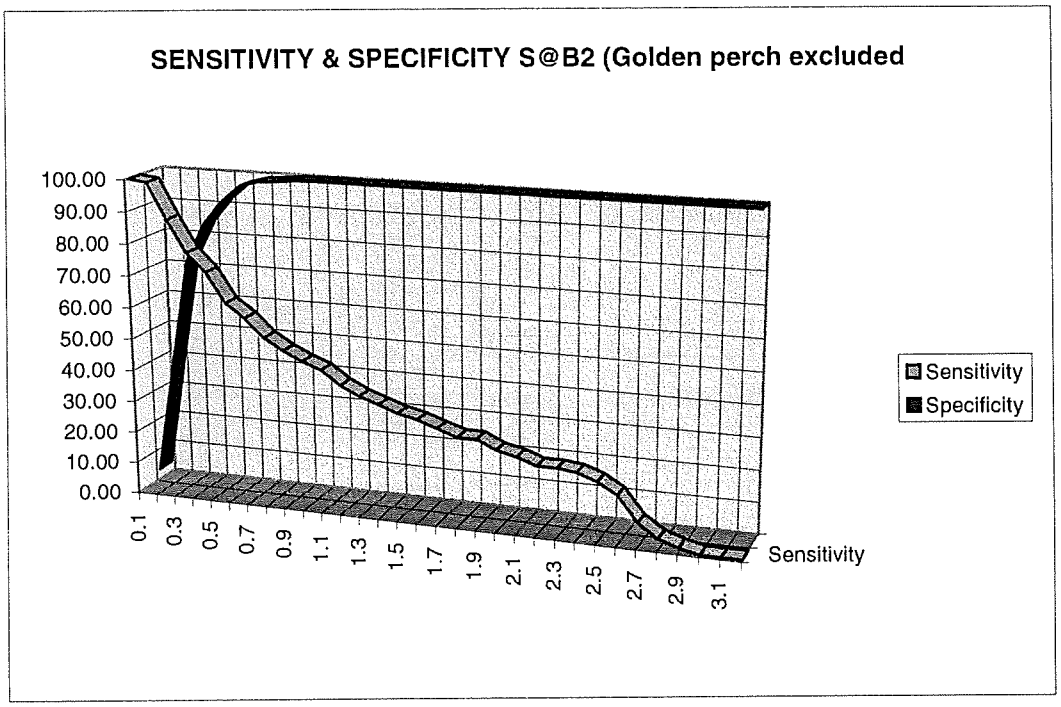
Cut-off
Sensitivity
Sensitivity
Specificity

Fig 1a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0.3
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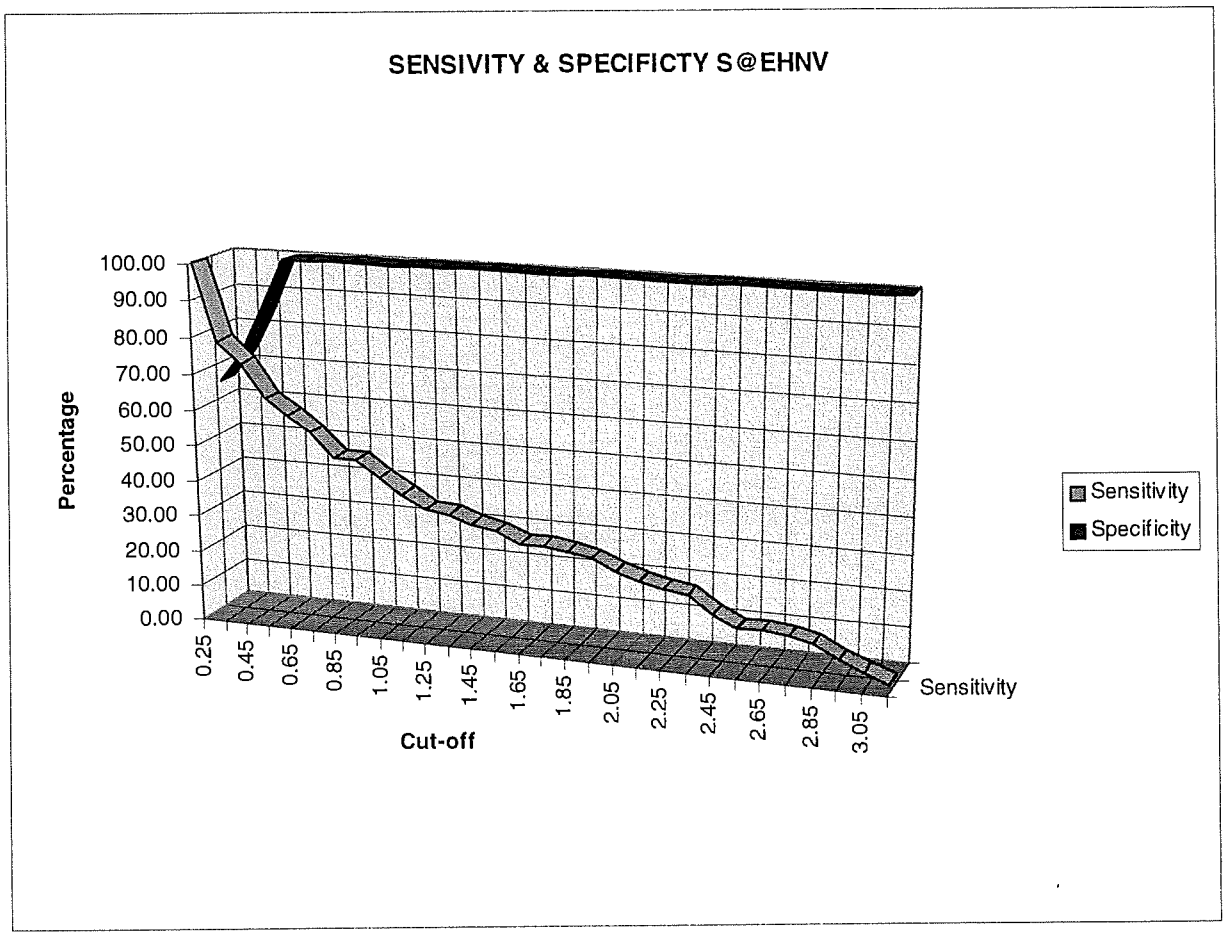
3.1
Sensitivity
Sensitivity
Specificity

Fig 1b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1.45
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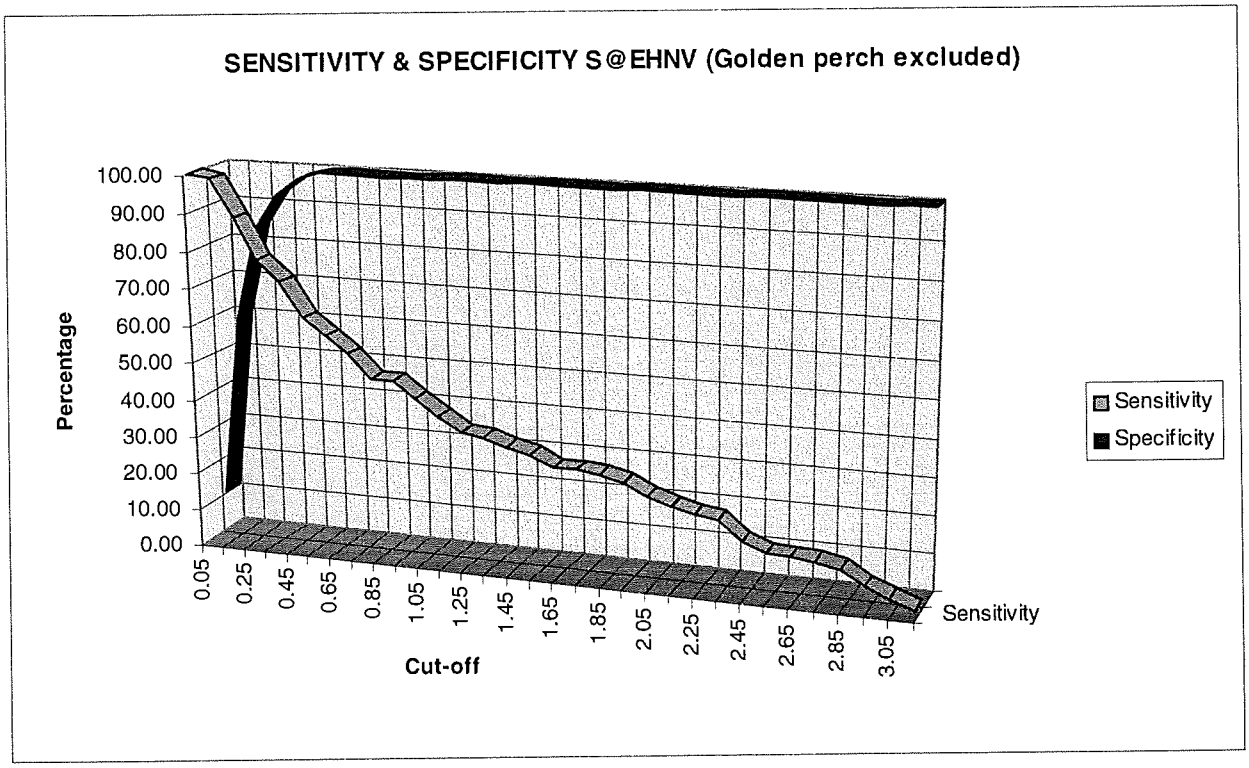
3.05
Cut-off
Sensitivity
Sensitivity
Specificity

Fig 1c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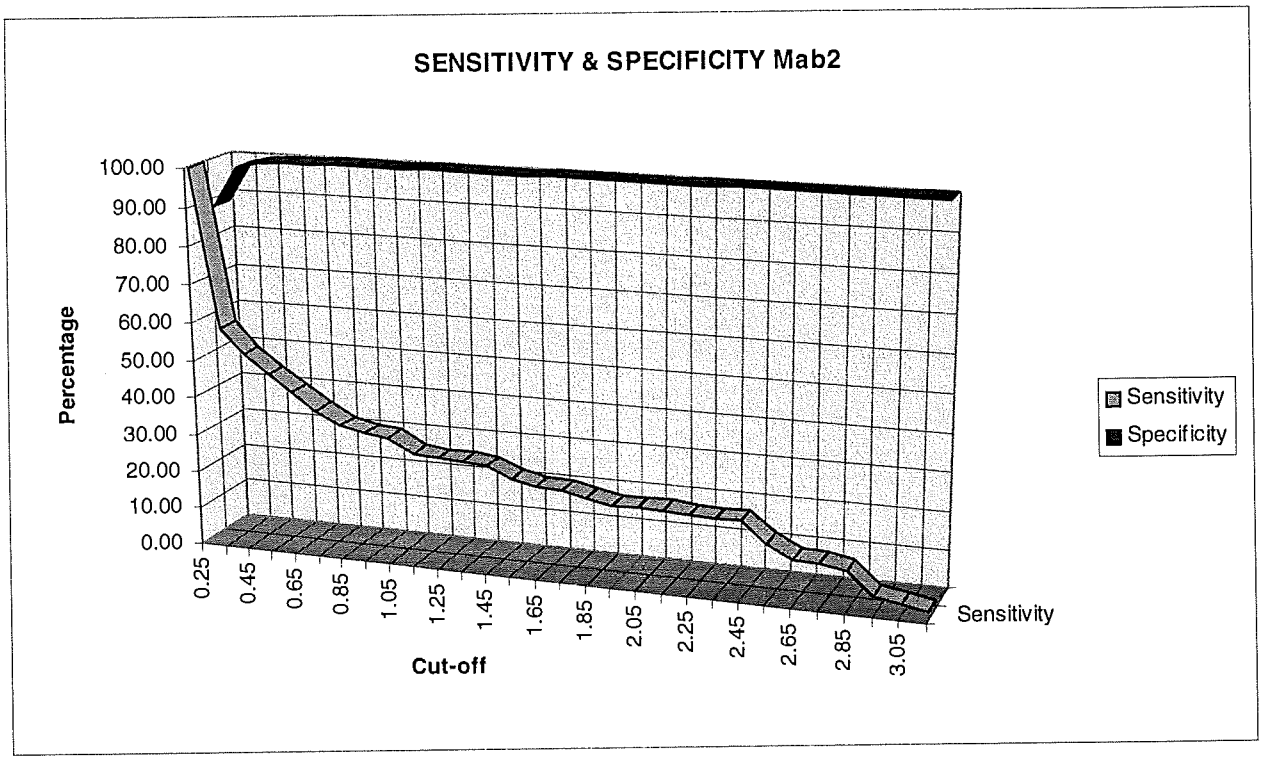
Cut-off
Sensitivity
Sensitivity
Specificity

Fig 1d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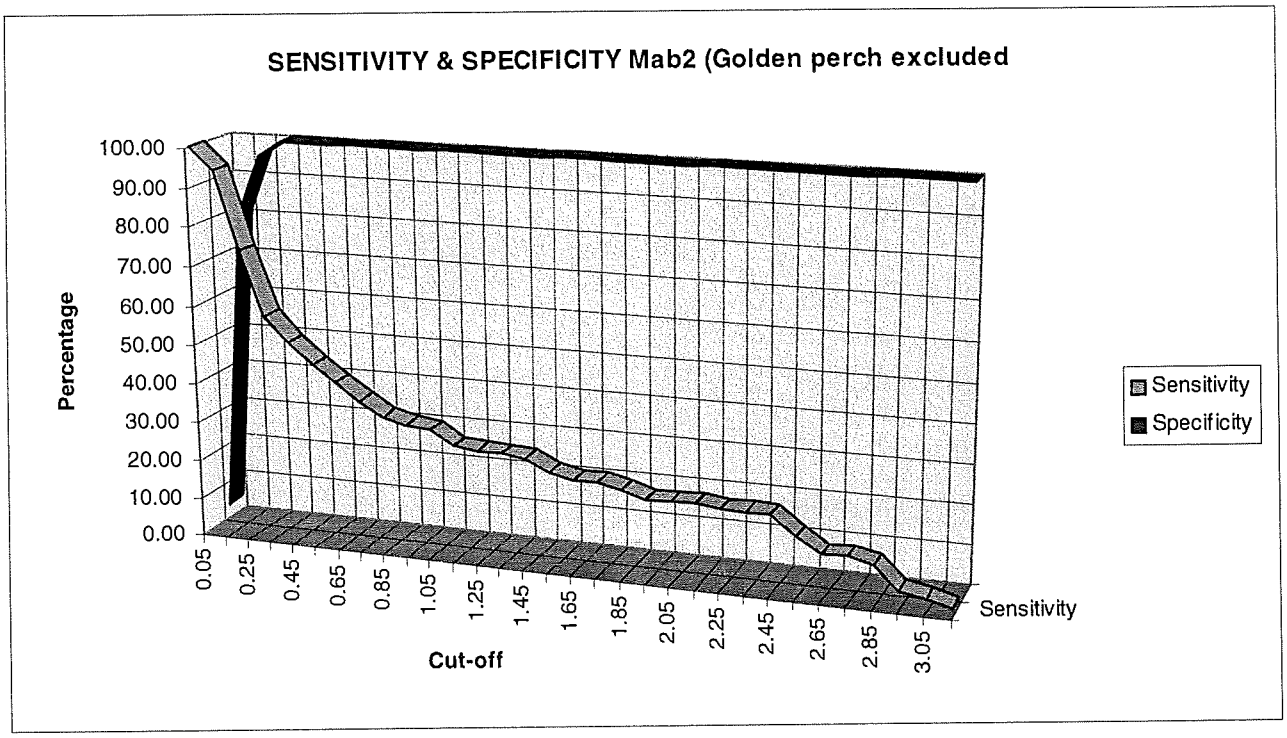
Sensitivity
Sensitivity
Specificity

Fig 1e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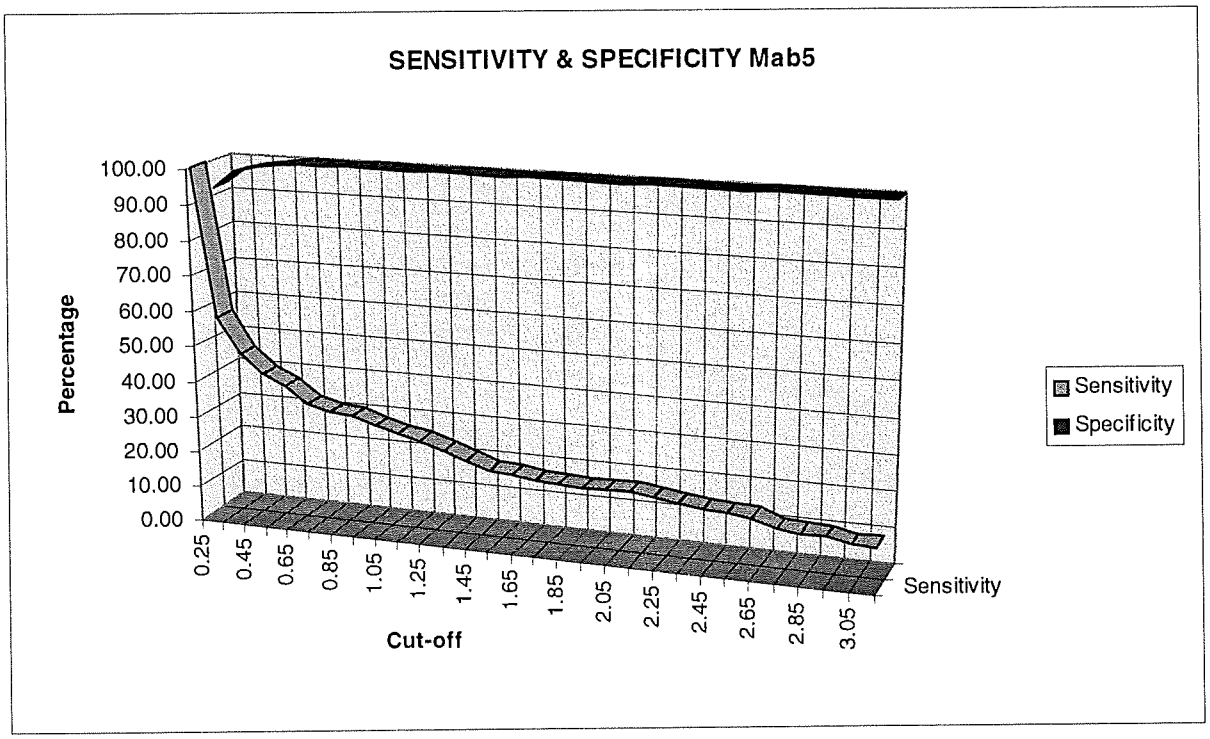
3.05
Cut-off
Sensitivity
Sensitivity
Specificity

Fig 1f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0.45
0.65
0.85
1.05
1.25
1.45
1.65
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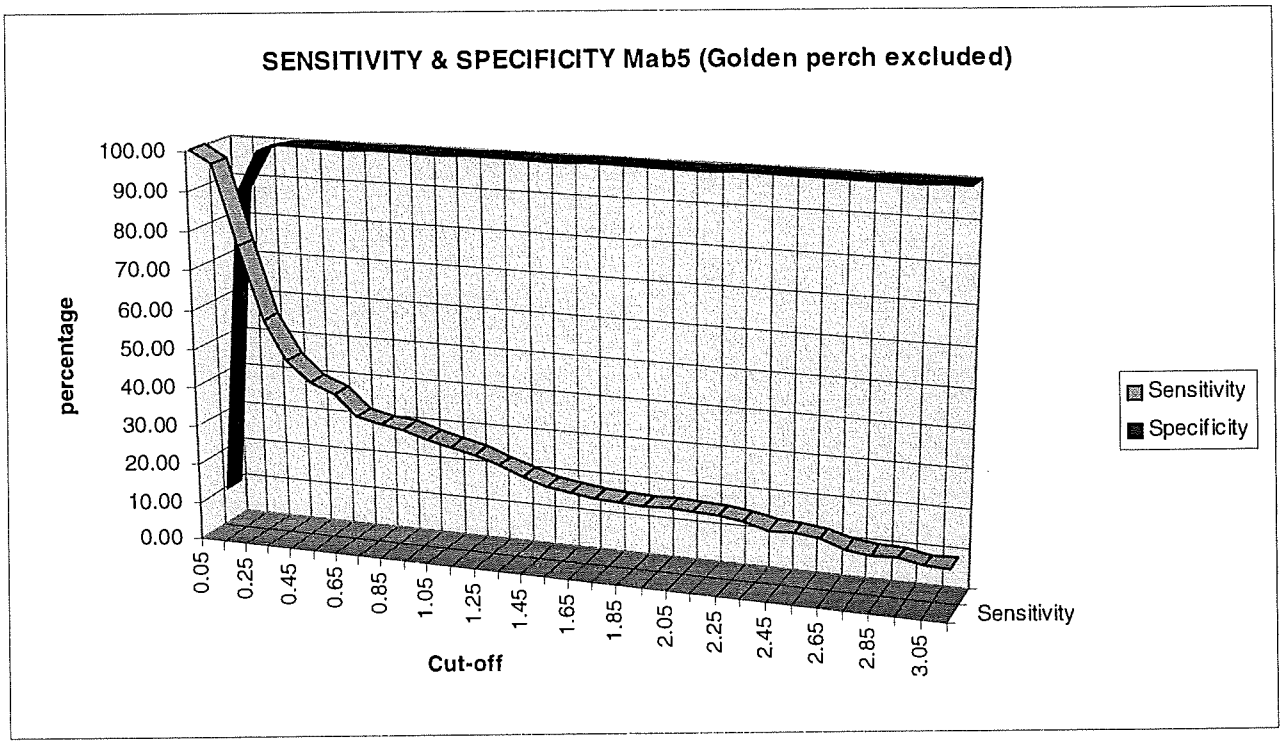
3.05
Cut-off
Sensitivity
Sensitivity
Specificity

Fig 1g

Fig 1h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80.00
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60.00
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40.00
30.00
20.00
10.00
0.00
0.05
0.25
0.45
0.65
0.85
1.05
1.25
1.45
1.65
1.85
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3.05
Cut-off
Sensitivity
Sensitivity
Specificity

Fig 2a **S@B2 control data**

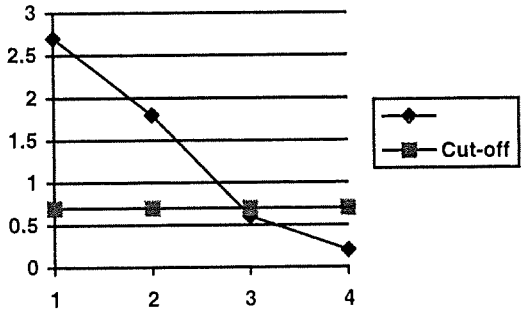


Fig 2b **S@EHNV control data**

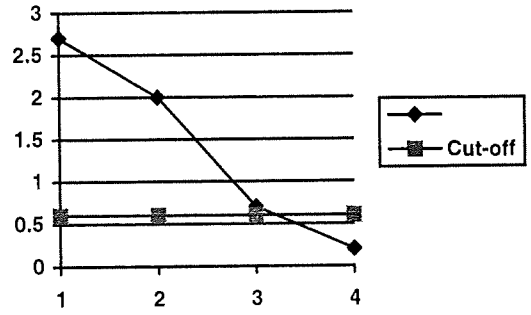


Fig 2c **Mab2 control data**

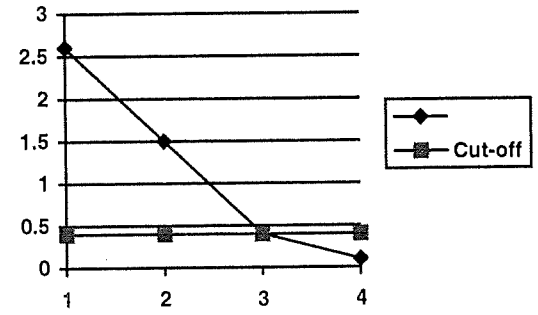
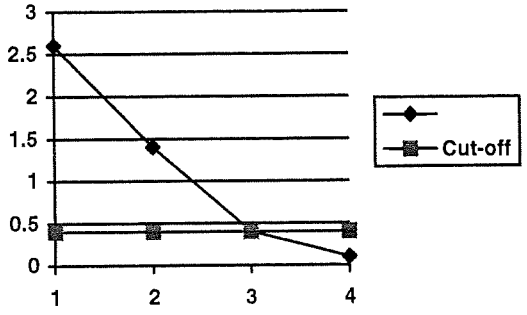


Fig 2d **Mab5 control data**



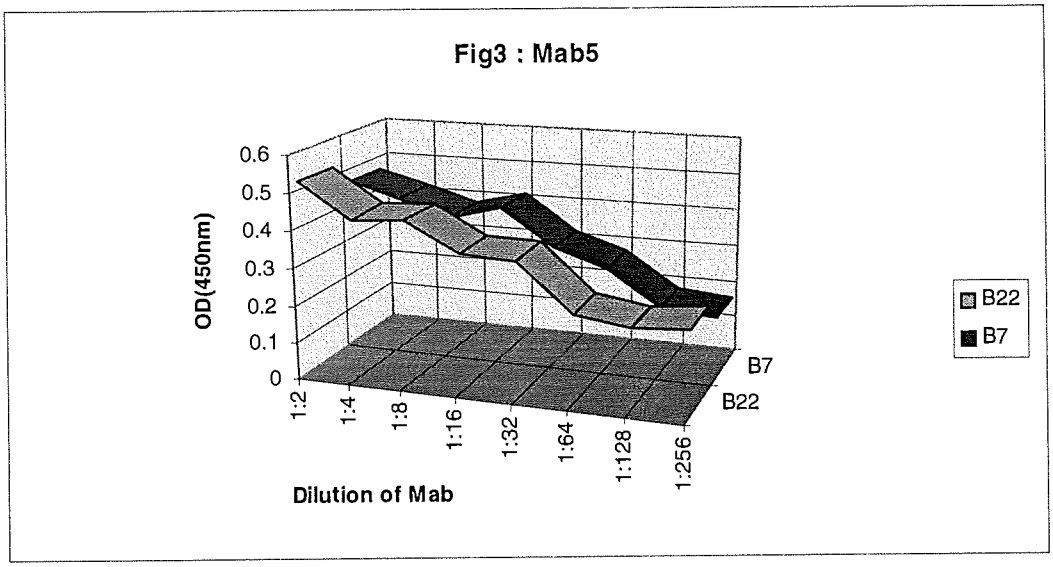


Fig3 : Mab5
OD(450nm)
0.6
0.5
0.4
0.3
0.2
0.1
0
1:2
1:4
1:8
1:16
1:32
1:64
1:128
1:256
Dilution of Mab
B7
B22
B22
B7

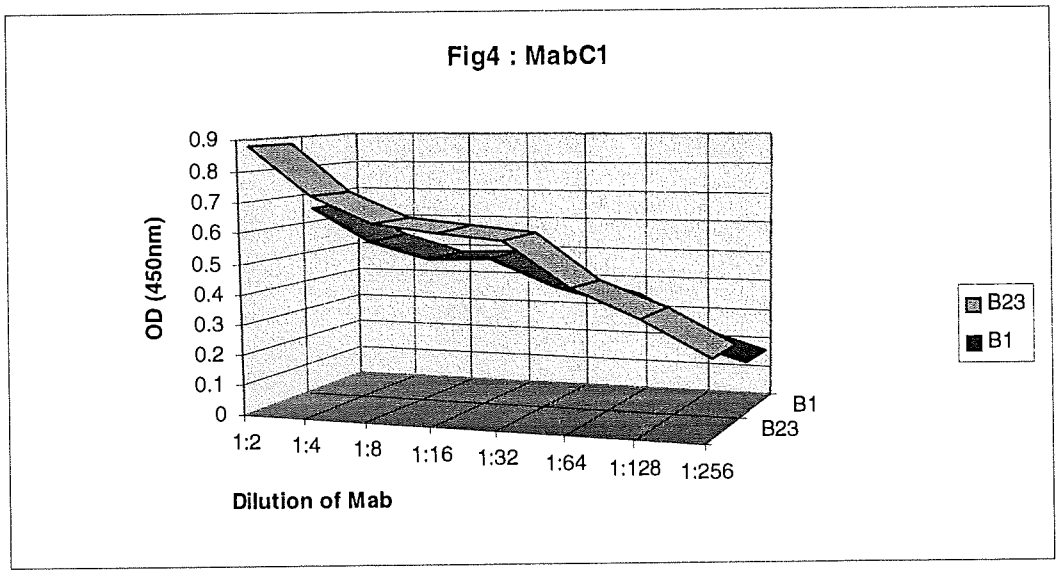


Fig4 : MabC1
OD (450nm)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1:2
1:4
1:8
1:16
1:32
1:64
1:128
1:256
Dilution of Mab
B1
B23
B23
B1

Fig5 : MAb11

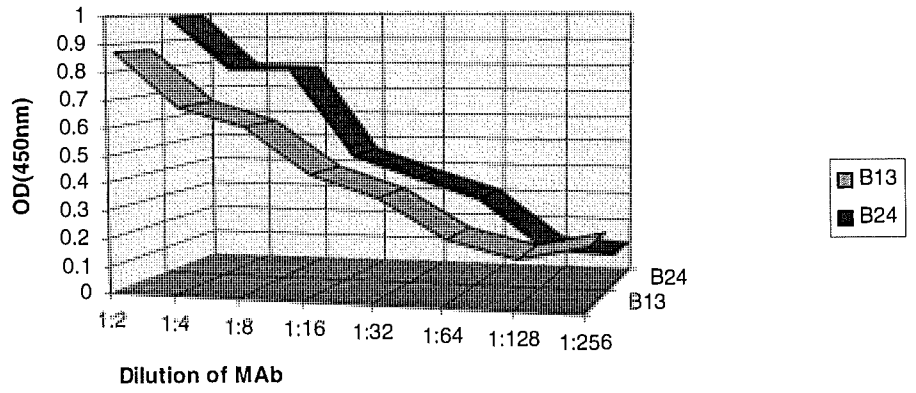


Fig. 6 MAbC1

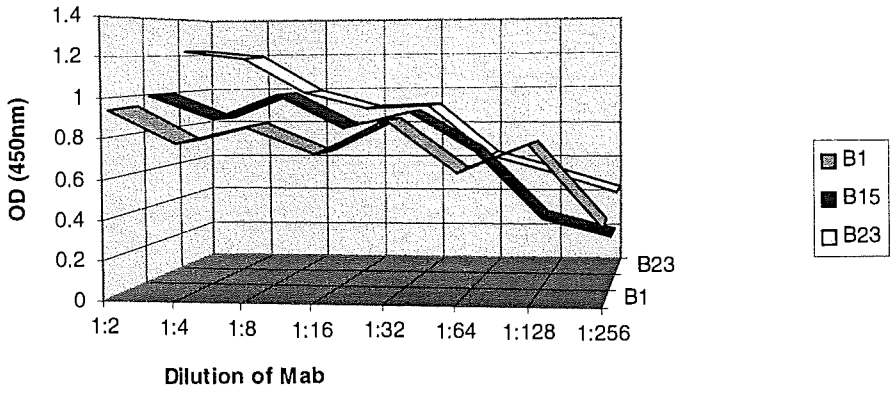


Fig7 : MAb5

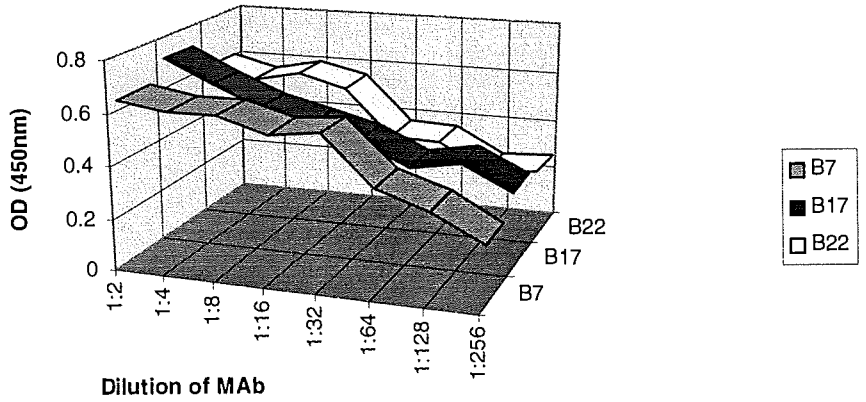


Fig8 : MAb2

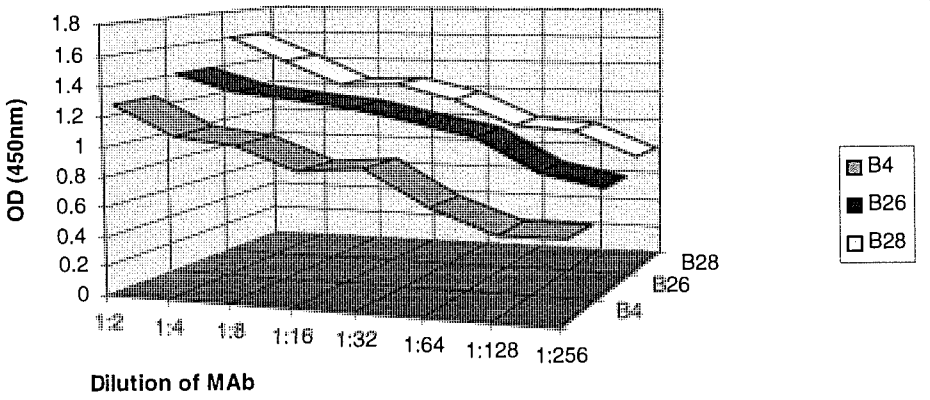


Fig9 : MAb5

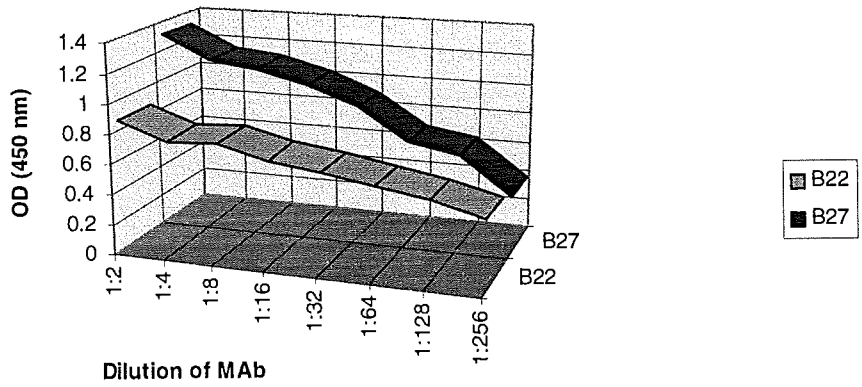
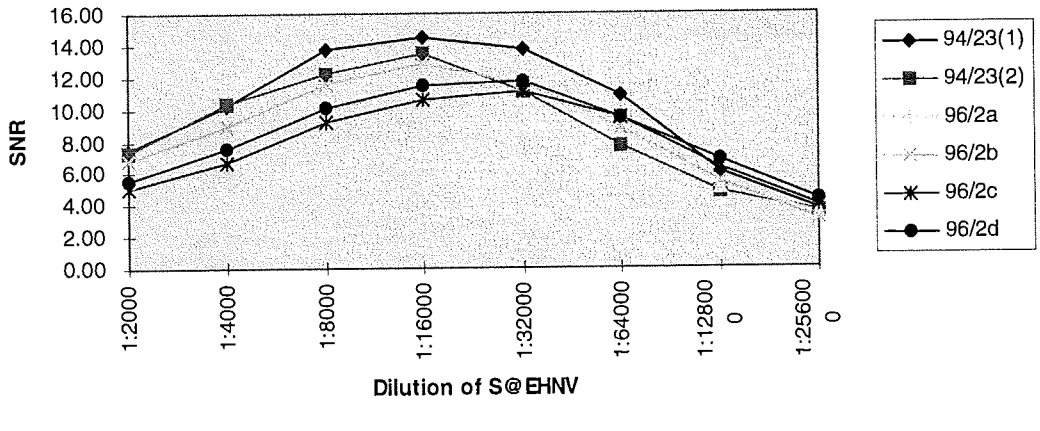


Fig10 : Signal to noise ratio (SNR)



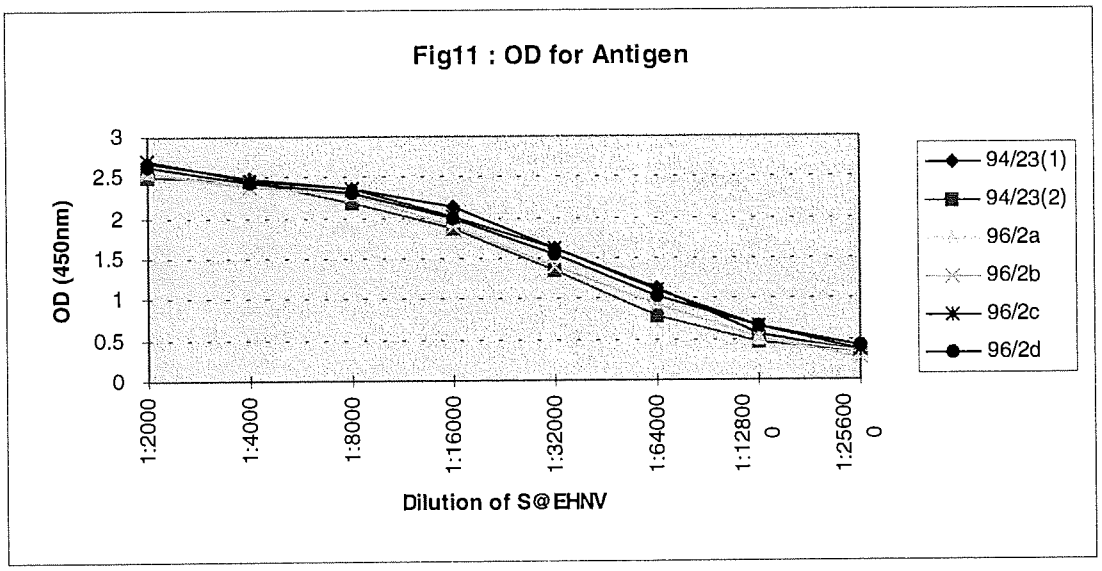


Fig11 : OD for Antigen
OD (450nm)
3
2.5
2
1.5
1
0.5
0
1:2000
1:4000
1:8000
1:16000
1:32000
1:64000
1:12800
1:25600
Dilution of S@EHNV
94/23(1)
94/23(2)
96/2a
96/2b
96/2c
96/2d

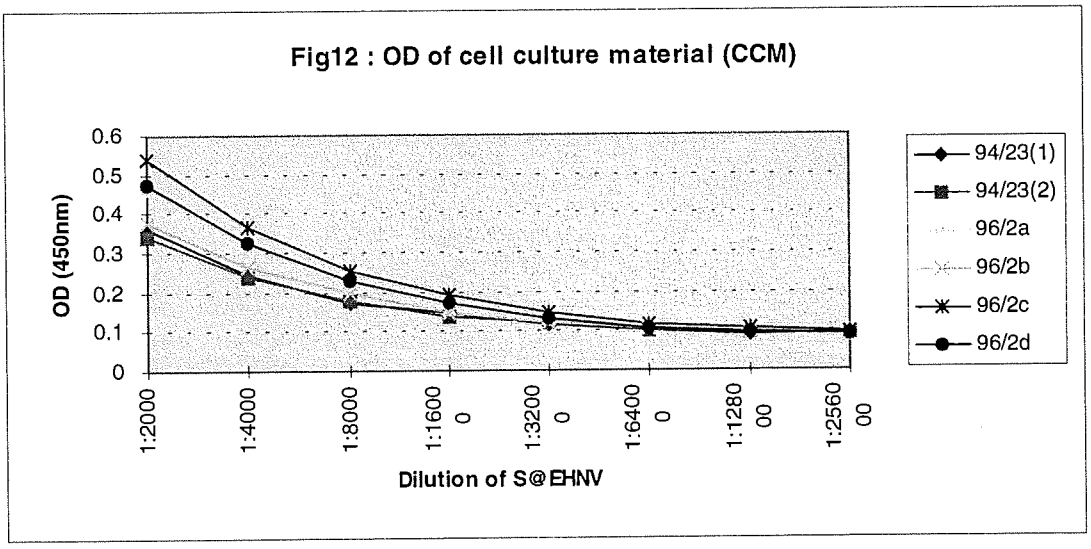


Fig12 : OD of cell culture material (CCM)
OD (450nm)
0.6
0.5
0.4
0.3
0.2
0.1
0
1:2000
1:4000
1:8000
1:16000
1:32000
1:64000
1:12800
1:25600
Dilution of S@EHNV
94/23(1)
94/23(2)
96/2a
96/2b
96/2c
96/2d

Fig13 : NON-IRADIATED (INF) vs RADIATED (NI) VIRUS [a = 1:10]

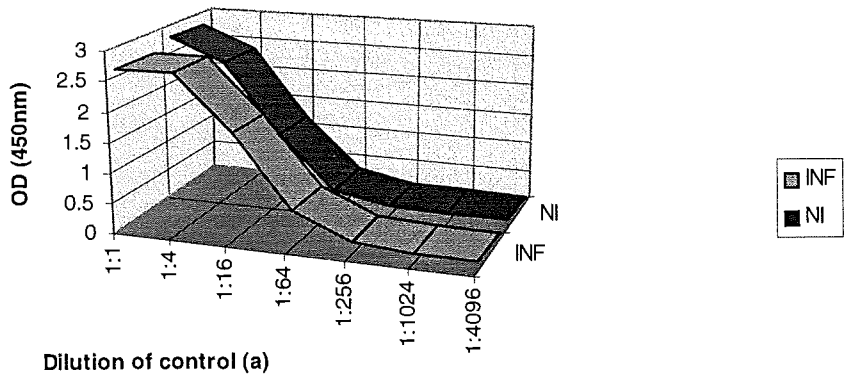


Fig14 : NON-IRRADIATED (INF) vs RADIATED (NI) VIRUS (b = 1:100)

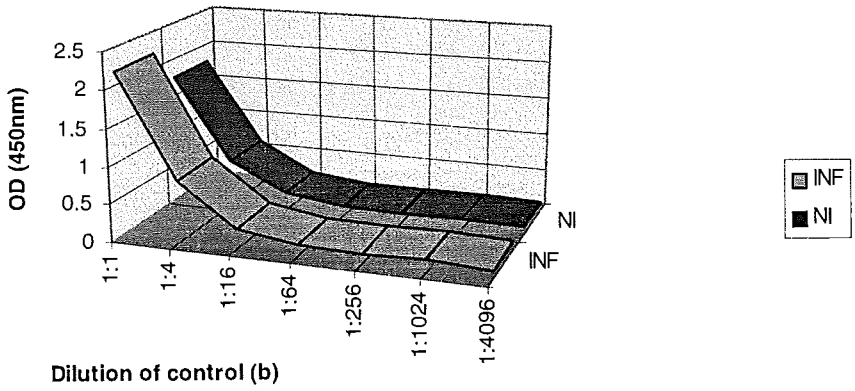


Fig15: NON-IRRADIATED (INF) vs RADIATED (NI) VIRUS [C = 1:200]

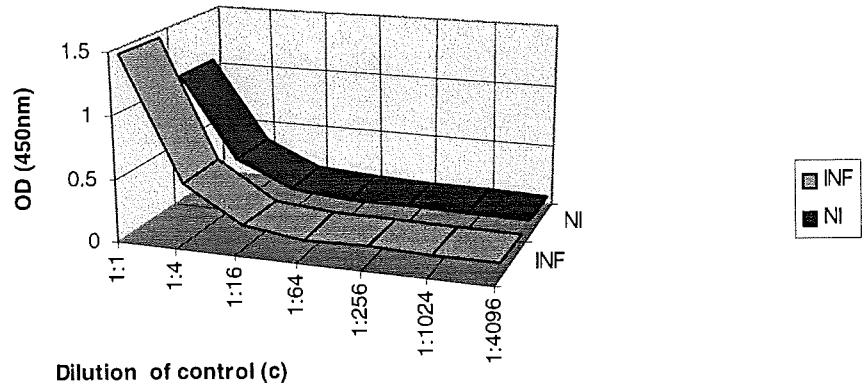


Fig16 : NON-IRRADIATED (INF) vs RADIATED (NI) VIRUS [d = 1:500]

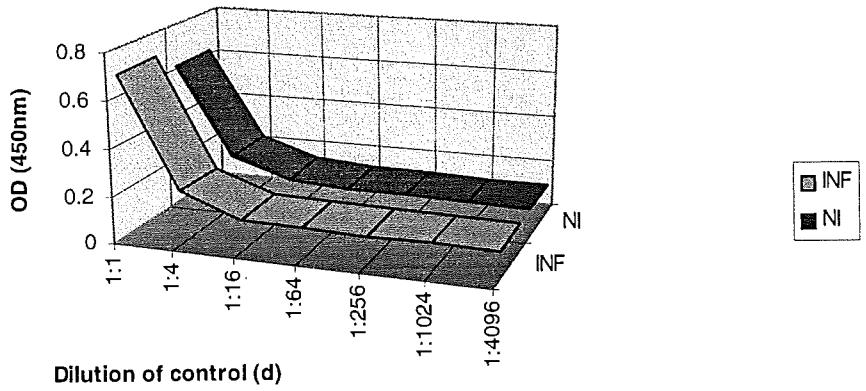


Fig17 : NON-IRRADIATED (INF) vs RADIATED (NI) VIRUS [E = 1:1000]

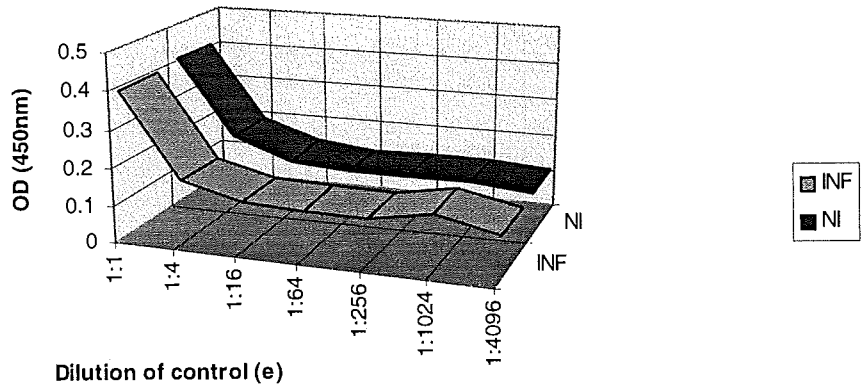
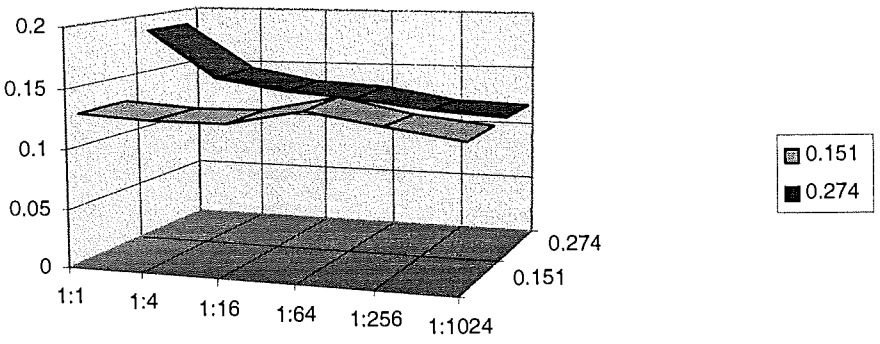


Fig18 : NON-IRRADIATED (INF) vs RADIATED (NI) VIRUS [F = 1:10000]



A large batch of sheep anti-EHNV (AAHL) was irradiated in 1996 (ME96/2). It consisted of 32 x 50µl volumes of undiluted serum and 64 x 200µl volumes of a 1:10 dilution (in TSGM) in a square cryobox. Two of the undiluted samples from different locations in the cryobox were diluted 1:10 in TSGM at EMAI (ME 96/2a,2b). Two of the 1:10 dilutions that were situated in different positions in the cryobox (ME96/2c-edge, 2d-centre) were also selected. For comparison, an older batch of this reagent, received as undiluted serum at EMAI in 1994 and diluted 1:10 at the time of receipt [ME94/23(1)], as well as a freshly made 1:10 dilution of the undiluted serum [ME94/23(2)] were used. All reagents were stored at -20°C at EMAI. Each batch was then titrated against antigen (X852, stock no.1, 17/11/95) and cell culture medium (sonicated BGF cells) at a dilution of 1:100. The results are given in Figures 10,11,12.

The optimal dilution for all batches of reagent was 1:16,000. However, a lower signal to noise ratio was observed with the batch supplied in 1996 (ME96/2) compared with the batch supplied in 1994 (ME94/23). The samples that were diluted in TSGM at AAHL (96/2c,2d) showed the lowest signal to noise ratio, particularly the sample taken from the edge of the box (96/2c). **It is therefore recommended that only undiluted stocks of this reagent be irradiated and transported**. It is uncertain whether irradiation was the only variable as there may have been differences in transport and handling of the different batches from AAHL. Differences in storage of the reagent between EMAI and AAHL might also have accounted for the differences.

Effect of gamma irradiation and transport on control antigens

It is desirable to use an inactivated EHNV control antigen where the test is to be conducted in a geographic region free of EHNV. The activity of live and irradiated control antigens was therefore compared. Control antigens consisting of a series of dilutions of X852 were prepared in duplicate at EMAI. The dilutions were (a) 1:10, (b) 1:100, (c) 1:200, (d) 1:500, (e) 1:1000, (f) 1:10000. Non-irradiated controls were kept at EMAI at -80^{0}C and moved to 4^{0}C on the day before testing. Duplicates were sent to AAHL on wet ice to be gamma irradiated (60kGy). These were then returned to EMAI on wet ice, stored at -20^{0}C for a week, then moved to 4^{0}C on the day before testing. Each of the irradiated and non-irradiated controls was diluted and tested ihn ELISA 2. The results are given in Figs 13 - 18. Irradiated controls (a)-(d) had a slightly lower activity than non-irradiated controls, while irradiated controls (e) and (f) had a slightly higher activity. Similar results were obtained in a titration of irradiated and non-irradiated parent control X852 (86/877) (Fig 18), suggesting that the effects of irradiation are independent of antigen concentration. It should be noted that irradiation alone may not be responsible for the reduction in antigenic activity. Other factors that may have contributed are the temperature at which the controls were stored and/or the conditions during transportation. From a practical viewpoint, irradiation of control antigen does not have a significant effect, and the OD of control (d) remains close to the positive-negative cut-off point.

CONCLUSIONS:

1. ELISA 2 is recommended for routine use. The suggested OD cut-off for use of this test in rainbow trout and redfin perch is 0.6, providing specificity of 99-100% and sensitivity of 60%. The OD value of control antigen 3 approximates the cut-off. The cut-off should be increased to 0.7 to maintain specificity if the test is used on species other than rainbow trout or redfin perch.

2. ELISA 5 should be used if greater specificity is required, where species other than rainbow trout and redfin perch are being tested in the absence of cell culture, or where the results of ELISA 2 need to be confirmed. A cut-off of 0.4, corresponding to the OD of control antigen 3, should be used when testing tissues from rainbow trout or redfin perch. This provides specificity of >99% and sensitivity of 52%. A cut-off of 0.6 should be used in species other than rainbow trout and redfin perch, providing specificity of >99% and sensitivity of 43%.

3. The precision of ELISA 2 and ELISA 5 is better than 10% coefficient of variation across plates and days.

4. Liver, kidney and spleen should be included in homogenates to be tested by ELISA. In the case of small fish, whole fish or all viscera can be included.

7.5 Use Of Inactivated EHNV In Antigen Capture ELISAs

METHODS & RESULTS

Inactivated vs Infectious EHNV

A stock of EHNV was prepared, this stock was isolate B86/8774. The stock was divided into two batches, one for gamma irradiation (60 kGy) [refer below] and the other kept as a control (infectious virus) . Each batch was then divided into three lots, the first of stored at +4°C, the second at -20°C and the third at -80°C. All samples were tested in the EHNV antigen-capture (Hyatt *et al* 1991). The dose of effective irradiation was determined by plaque reduction assay (Table 1), 30 kGy were found to be sufficient.

TABLE 1 *Dose irradiation versus infectivity*

LEVEL OF IRRADIATION (kGy)	PLAQUE FORMING UNITS (PFU/ml)	OBSERVATIONS
0	1.5×10^6	Infection apparent
10	5.7×10^6	Infection apparent
30	0	No obvious infection
50	0	No obvious infection
70	0	No obvious infection

The ELISA results from assays incorporating gamma irradiated and infectious viruses are shown in Table 2. The data show that over the first nine months the level of reactivity (inactivated virus and antibody interactions) drops significantly. During this period of testing a single batch of antibodies were used; i.e. the same tubes were used and stored at +4°C. The protocol of the test was then changed such that stocks of antibodies were aliquoted into small volumes and stored. All subsequent testing was performed using fresh aliquotes of -80°C antibodies.

TABLE 2 *Intensities of Antigen Capture ELISA Reactions (Optical Densities) Using Non-Infectious (gamma irradiated) and Infectious Virus that were Stored at Different Temperatures.*

DAYS	NON-INFECTIOUS VIRUS *Freeze dried antigen* (Optical density at 450 nm)			INFECTIOUS VIRUS *Stored as solution* (Optical density at 450nm)		
	(+4°C)	(-20°C)	(-80°C)	(+4°C)	(-20°C)	(-80°C)
1	1.81	2.13	1.81	1.68	2.13	2.00
33	1.83	1.93	2.12	2.06	2.06	2.11
64	1.05	1.25	1.27	1.20	1.37	1.51
96	0.54	0.84	0.90	0.71	0.73	0.96
180	0.49	0.58	0.63	0.56	0.45	0.54
216	0.66	0.76	0.82	0.76	0.72	0.80
248	0.68	0.80	0.90	0.82	0.76	0.89
283	1.36	1.31	1.29	1.18	1.13	1.36
307	0.66	1.22	1.12	1.001	0.90	1.14
339	1.54	1.60	1.64	1.63	1.42	1.55
370	1.76	1.95	2.03	1.93	1.93	2.23
398	1.18	1.47	1.53	1.47	1.20	1.44
430	1.60	1.87	1.88	2.019	1.90	2.04
444	1.51	1.69	1.61	1.69	1.56	1.82
462	1.37	1.47	1.37	1.59	1.47	1.58
477	1.89	2.0	1.90	2.29	1.83	1.95
486	1.45	1.69	1.51	1.84	1.34	1.61
500	1.36	1.65	1.61	1.56	1.48	1.71
515	1.81	1.88	1.82	2.24	1.70	2.63
528	1.34	1.94	1.86	1.93	1.81	2.18
542	1.45	1.54	1.43	1.92	1.82	1.47
1012	1.42	1.59	1.47	1.54	1.45	1.64
1124	1.37	1.84	1.40	1.55	1.32	1.65

TABLE 3 *Variation In Intensities Of Antigen Capture ELISA Reactions (optical densities) Using Non-Infectious (gamma irradiated) and Infectious EHNV.*

(Viruses were stored at different temperatures. The means (averages) are shown for two time periods the first (1-307 days) where two common vials of antibodies were used and the second (399-500 days) where fresh aliquotes of antibodies were used).

TIME PERIOD (Days)	NON-INFECTIOUS VIRUS *Freeze dried antigen* (Mean±standard deviation)			INFECTIOUS VIRUS *Stored as a solution* (Mean±standard deviation)		
	(+4°C)	(-20°C)	(-80°C)	(+4°C)	(-20°C)	(-80°C)
1- 307	1.01±0.53	1.2±0.53	1.21±0.49	1.11±0.49	1.14±0.60	1.26±0.54
399 - 500	1.51±0.21	1.71±0.19	1.68±0.17	1.78±0.26	1.57±0.26	1.72±0.26

Examination of Tables 2 & 3 at the various time points and temperatures reveal:

(1) Continued use of a antibody stored at 4°C will result in a progressive reduction in optical densities as the antibody deteriorates.

(2) Use of fresh antibody with stored antigens (EHNV) results in consistent ELISA readings. This is also seen from the data in Table 2 reveals the variation in results from using a common pool of antibody (days 1 - 307) to using a fresh (previously un-opened) aliquote (days 399 - 500).

(3) Gamma irradiation/freeze drying of EHNV does not lead to a reduction in its antigenic activity. The results do indicate that storage (+4°C) of inactivated, freeze dried virus is not the optimal storage regime as it leads to slightly suppressed ELISA values.

Infectivity of stored "non-treated" virus

During this study the titre of the non-gamma irradiated virus was assessed. Table 4 shows the decline in infectious virus over a three year period at different storage temperatures. The original titre was 6 x 10^7 PFU/ml. These results are important if infectious virus stocks are to be stored for extended periods. The results indicate that infectious virus should be kept at -80°C and not stored in the fridge (+4°C) or in the freezers (-20°C). The same may be valid for clinical tissues (destined for the isolation of possible viruses).

TABLE 4 *Effect On The Titre (Infectivity) Of Non-Irradiated Stocks Of EHNV of Storage for 3 Years at Different Temperatures.*

TEMPERATURE	TITRE (Plaque forming units(PFU)/ml)
-80°C	$3x10^7$
-20°C	$9.3x10^4$
+4°C	$8.8x10^4$

Longevity of stored antibodies

Although this was not detailed in the original research plan the longevity of antibodies stored under different temperatures was considered to be an important area of investigation. Upon the eventual distribution of the ELISA kit, the storage conditions of both antibodies and inactivated antigens will have to be documented.

The polyclonal antisera R@B2 (EMAI) and S@B2 (EMAI) were stored routinely at -20°C. No loss of activity was observed over a 5 year period using control antigens also stored at -20°C. Affinity purified antibodies derived from R@B2 (EMAI) were stable at -20°C for at least 2 years.

Storage conditions of other antibodies were also assessed. The antibodies investigated were *"AAHL sheep anti-EHNV"* and *"AAHL rabbit anti-EHNV";* it is assumed that the results from these two polyclonal antibodies will be indicative for similar antibodies. The results reveal that after 112 days there has been no significant deterioration of antibody reactivity. The antigen used in these experiments was derived from the supernatant of freshly infected (EHNV) CHSE tissue culture cells. Whilst data for the storage of MAbs was not performed it was also found that polyclonal antibodies diluted 1:10 in 25mM Tris HCL pH 7.2, 0.15M NaCl, 50% (v/v) glycerol were stable for at least 2 years at -20°C. As these remain in the liquid state this is the preferred means of storage of working stocks of antibodies for use in the detection of EHNV.

CONCLUSIONS

1 Inactivated EHNV can be stored for at least three years at 4°C, -20°C and -80°C.

2 Antibodies should be aliquoted and stored at -20°C and used fresh to achieve consistent results.

3 Live virus should be stored at -80°C if infectivity is to be optimally conserved.

7.6 Minimum Sampling Sizes Required for Disease-Free Certification of Commercial Fish stock.

The optimisation, standardisation and evaluation of the antigen capture ELISA in this study has enabled rational assessment of strategies to detect EHNV. Sample sizes need to be defined for two purposes:

1. Diagnosis of EHNV. Diagnosis of EHNV is straightforward and requires only submission of samples for diagnosis in the manner usual for fish diseases, i.e. submission of at least 12 affected fish. In our experience high levels of EHNV or EHNV antigen will be present in at least 25% of affected fish during an outbreak and a sample of 12 fish would enable a diagnosis to be reached by ELISA or virus isolation.

2. Certification for freedom from EHNV. Certification of freedom from EHNV requires a series of assumptions and the application of statistical sampling theory. The assumptions used here are:

   i) That infection is present at a rate of 2% in the population

   ii) The population consists of more than 10,000 individuals

   iii) The test (antigen capture ELISA) has 100% specificity and 60% sensitivity

   iv) An error rate, i.e. failure to detect infection when it is in fact present, of either 5% or 1% is acceptable

   v) The sample is collected randomly

These assumptions are consistent with accepted international practice for disease certification.

The sample sizes required are given below:

Error rate	Sample size for ELISA
5%	249
1%	382

Smaller samples may be used if several tests are used in series to increase the sensitivity of detection of EHNV. Combined use of EHNV antigen capture ELISA and virus isolation would result in a sensitivity of perhaps 90%. In this case appropriate sample sizes would be:

Error rate	Sample size for ELISA plus virus isolation
5%	165
1%	254

7.7 DEVELOPMENT OF A POLYMERASE CHAIN REACTION (PCR) TO DETECT and DIFFERENTIATE EHNV and BOHLE IRIDOVIRUS (BIV).

METHODS

Virus isolates and cells

Isolates of EHNV (Table 1) and tissues from experimentally infected redfin perch and rainbow trout (Table 2) were used in this study. The isolates of EHNV were selected from a reference collection to represent a range of geographical areas across Victoria and New South Wales (NSW) and the two host species from which the virus has been isolated. Other viruses included two isolates of BIV (Hengstberger *et al.* 1993), BIV(1) representing the original isolate (Speare and Smith, 1992) and BIV(2) from laboratory BIV infected barramundi (*Lates calcarifer*) (Moody and Owens, 1994); FLDV (Pearce *et al.*, 1990) and DPEV. FV3, goldfish virus, sheatfish virus and catfish virus (refer to introduction) were not used in this study as they are exotic to Australia and were not available at the time this study was undertaken because of importation restrictions.

All EHNV and BIV isolates, (samples 1 - 12) were passaged in bluegill fry (BF-2) cells (ATCC CCL 91) as described by Hengstberger *et al.* (1993). Samples 13 and 14 did not grow in cell culture and the samples therefore consisted of erythrocytes from a diamond python and nodular lesions from a barramundi respectively. In these latter samples, the presence of virus within these samples were confirmed by negative contrast electron microscopy and the examination of the relevant ultrathin sections by transmission electron microscopy (data not shown).

Samples of ENNV-infected redfin perch and rainbow trout tissues were obtained from experimentally infected animals. Rainbow trout were infected by intraperitoneal injection with strain 86/8774 (Table 2) and died 3 - 4 days later. Redfin perch were infected by bath inoculation with the same strain of EHNV and died 8 - 10 days later. Tissue samples from kidney, liver and spleen were collected and pooled for anaylses. EHNV was detected by virus isolation in the tissues from 2 of the 3 rainbow trout and all three redfin as well as by ELISA in the tissues of all six fish. Un-infected redfin perch and rainbow trout tissues were obtained from animals collected from areas where epizootics of EHNV infection have never been reported. These samples were prepared for examination and analysed by EHNV antigen-capture ELISA in addition to being passaged through BF-2 cells (Whittington *et al.* 1994); all such samples were negative by virus isolation and ELISA antigen-capture ELISA.

Preparation of samples for PCR

Samples comprised tissue culture supernatants (samples 1 - 12) and clinical tissues (sample 13 - 18), (Tables 1 and 2). As EHNV and BIV are associated with the insoluble cytoskeletal matrix of

the host cells (Eaton *et al.*, 1991) all samples were prepared by the methods described by Eaton *et al.* (1991), Hyatt *et al.* (1991), Hengstberger *et al.* (1993) or by Whittington and Steiner (1993). All such samples were diluted to 15% (w/v) in RSB (10mM Tris HCL (pH 7.4), 10mM NaCl, 1.5mM $MgCl_2$). Prior to each homogenisation the equipment was autoclaved, washed in 0.1M HCl followed with 0.1M NaOH and phosphate buffered saline (PBS). This procedure effectively inactivates any residual nucleic acid which may cause a false signal. Aliquots of the wash PBS were analysed to assess the preparative procedure for the presence of contaminating nucleic acids. Tissue culture supernatants and tissue homogenates, including erythrocytes, were diluted in H_2O (1:50), boiled for 5 min, vortexed and then stored on wet ice.

Tissue culture supernatants from EHNV-infected cell cultures were also used to assess the sensitivity of the test. In these analyses the supernatants, which had a virus titre of 10^7 plaque forming units per ml, were serially diluted and the sample prepared for PCR as described above.

PCR primers and amplification of viral genomes

The genome of EHNV is approximately 125±10kb and BIV 109±12kb (Hengstberger *et al.*, 1993). To date neither genome has been sequenced and thus sequences of the genome which are unique to either virus remain unknown.

DNA was extracted from purified EHNV and cloned into M13 bacteriophage vectors (Viera and Messing, 1982) and inserts sequenced using Sequenase (USB) according to the manufacturer's instructions. DNA sequences of several inserts were scanned for the presence of an open reading frame which served as the template for the synthesis of DNA deoxyoligonucleotides using an Applied Biosystems DNA synthesiser. Two primers P505 (5'-GATCCACACGGCCTGACACCG) and P506 (5'-GATCCGAAAGACAGCAGCGGTCGA) were designed to amplify sequences of approximately 300bp from trout EHNV using the polymerase chain reaction (PCR) (Saiki *et al.*, 1988.) using 30 cycles and parameters of 94°C for 1 min, 60°C for 2 min and 72°C for 2 min and reaction conditions as described for Taq DNA polymerase from Perkin Elmer-Cetus. DNA products were visualised using ethidium bromide staining of 2% agarose gels after electrophoresis in Tris-acetate-EDTA (TAE) buffer.
Hybridisation analyses and Southern blots were as described in Hengstberger *et al.* (1993) The PCR probe was prepared by excising the EHNV DNA amplified after PCR and electrophoresis in a 2% agarose-TAE gel. This was purified using "Geneclean" (Biolabs 101) and 5'-termini phosphorylated using T4 polynucleotide kinase and γ-^{32}P-ATP. Prior to hybridisation the labelled probe was denatured by heating to 100°C for 5 min. and immediately placed on ice.

RESULTS AND DISCUSSION

The potential of the PCR test to detect the presence of EHNV is shown in Fig. 1 (A and B). The genomic DNA of several Australian iridoviruses were tested for their ability to generate a specific DNA fragment after PCR using synthetic DNA primers. It was observed that only EHNV (isolated from redfin perch and rainbow trout) and the closely related BIV (Hengstberger *et al.*, 1993) genomes served as templates during the test. Neither fish lymphocystis virus (FLDV) or a recently discovered iridovirus from a Diamond python (DPEV) could be amplified (Table 3 and Fig.1B) thus indicaticating that the test is specific for EHNV and BIV. Alternatively, it could be argued that tissues containing FLDV and DPEV contain substances which may inhibit the PCR test.

However, ELISA tests and immunoelectron microscopical analyses have shown that polyclonal antibodies against EHNV fail to recognise these antigens (data not shown) thus inferring that the viruses are significantly different from EHNV and BIV. In addition. positive controls consisting of FLDV or DPEV (as above) but containing EHNV DNA produced positive PCR products thus indicating that the original negative results were representative and not due to inhibition of the PCR reaction by exogenous material. Sequence of the PCR products showed that the sequences are essentially identical and therefore the primers are recognising the cognate region of each of the viral genomes; FLDV and DPEV do not possess the equivalent genomic regions (unpublished).

Samples of EHNV isolated from fish originating from different regions of Victoria and N.S.W. were also analysed for their ability to generate a positive signal using the PCR test. All were found to be positive (Table 3). This indicated that within the limits of the PCR test, there appeared to be no great genomic variability among the EHNV isolates such that some could not be amplified. The sensitivity of the PCR test was analysed by performing PCR reactions on serial dilutions of a titred virus stock. Consistent positive signals were seen when at least 1 to 10 infectious virus particles were present in the initial sample material finally incorporated into the test sample (not shown).

Six samples from both uninfected rainbow trout and redfin perch were examined by PCR with negative results. Virus isolation and antigen capture ELISA (Hyatt *et al.*, 1991; Whittington and Steiner, 1993) also showed these samples to be negative and thus in light of their history (i.e. collected from geographical areas known to be EHNV free) could be defined as EHNV-negative material. Analyses of DNA from experimentally infected redfin perch and rainbow trout showed the presence of EHNV positive PCR products (Fig. 2).

Southern blot analysis of NcoI restricted EHNV and BIV genomic DNA using a labelled PCR probe derived from EHNV (Fig. 1C) showed that this probe could also be used in a simple diagnostic test to differentiate between these viruses despite their close homology (approximately 98%) at the nucleotide level (manuscript in preparation).

The results presented in this paper illustrate that the PCR test amplified target DNA from EHNV and the closely related BIV. The addition of these test to the panel of diagnostic protocols so far described for the detection of EHNV and BIV, namely antigen capture ELISA, fluorescence and electron microscopy (Hyatt *et al.*, 1991; Steiner *et al.*, 1991; Whittington and Steiner, 1993), will enhance the armouridium of diagnostic tests and facilitate future epidemiological, pathogenicity and carrier studies.

TABLE 1: Isolates of Australian iridoviruses used in this study. Redfin perch (RF), rainbow trout (RT), tissue culture supernatant TCS).

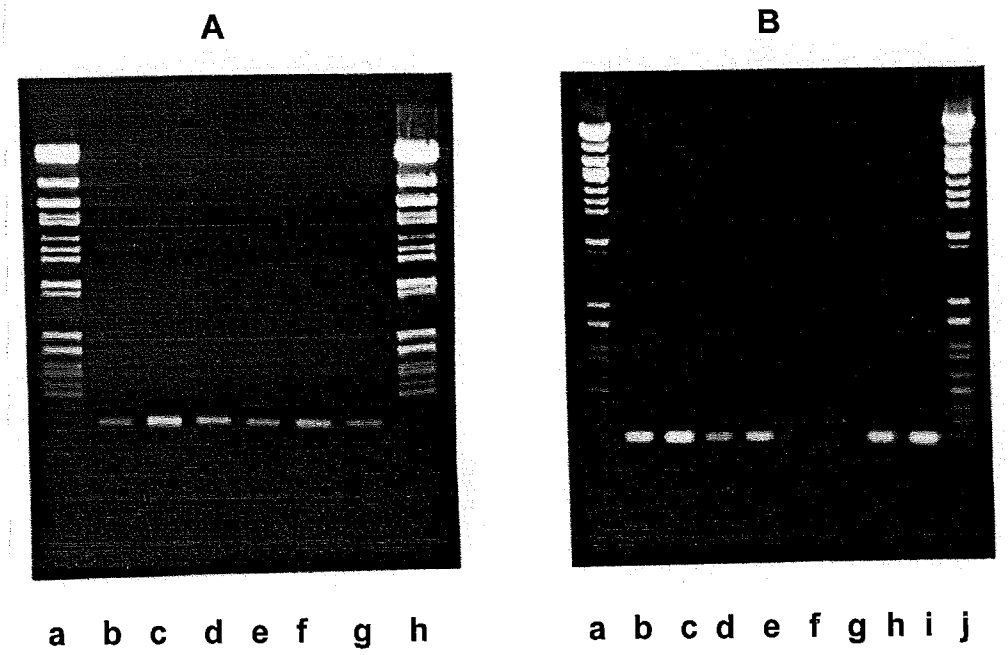
VIRUS	ISOLATE	SAMPLE NUMBER	HOST SPECIES	SAMPLE	LOCATION
EHNV	9009041231	1	RP	TCS	Lake Winnekie, Victoria
	9009031621	2	RT	TCS	Mt Dandenong, Victoria
	9009031622	3	RP	TCS	Greenhill Lake, Ararat, Victoria.
	A91:0069 9103210069	4	RP	TCS	Lake Mokoan, Victoria
	ME: 91/78	5	RP	TCS	Lake Burley Griffin
	ME: 90/24	6	RP	TCS	Blowering dam, NSW
	B87:8169	7	RP	TCS	Lake Nillahcootie Victoria
	B86:8774	8	RT	TCS	Adaminaby, NSW
	A94:0045	9	RT	TCS	Snowy Mts, NSW
	A91:024	10	RT	TCS	Goulburn, Victoria
BIV(1)	-	11	Frog	TCS	Queensland
BIV(2)	-	12	Barramundi	TCS	Queensland
FLDV	89:0248	13	Barramundi	Surface nodules	Northern Territory
DPEV	940074	14	Diamond python	Erythrocytes	Melbourne, Victoria

TABLE 2: EHNV-infected fish used in this study.

All samples represent pooled tissues (kidney, liver and spleen); redfin perch (RP), rainbow trout (RT).

VIRUS	HOST SPECIES	SAMPLE NUMBER
EHNV (B86:8774)	RP	15
EHNV (B86:8774)	RT	16
Uninfected	RP	17
Uninfected	RT	18

Fig 1. DNA products from the polymerase chain reaction (PCR) amplification using primers P505 and P506 were revealed by electophoresis through a 2% agarose gel and staining with ethidiun bromide. (A) Lanes are (a, h) λ Ava II DNA markers and (b-g) samples 1 to 6 (refer to Table 1). (B) Lanes (a, j) λ Ava II DNA markers, (b) sample 7, (c) sample 8, (d) BIV (1), (e) BIV(2), (f) DPEV, (g), FLDV, (h) sample 9 and (i) sample 10 (refer to Table 1). (C) Hybridisation of a ^{32}P-labelled PCR fragment to NcoI restricted DNA from $EHNV_{RF}$, $EHNV_{RT}$ and BIV. The positions of λ HindIII DNA size markers are indicated in kb.



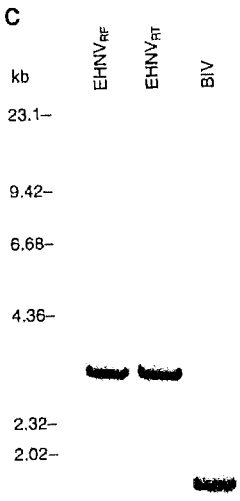
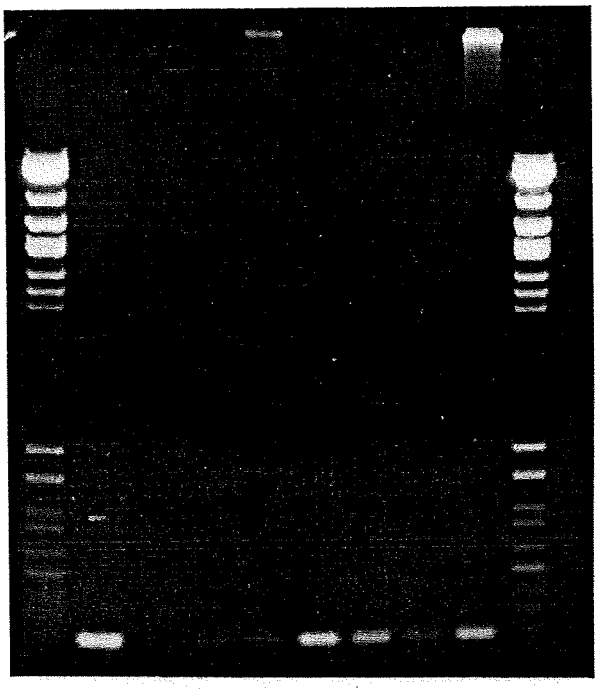


Fig 2. DNA products from the polymerase chain reaction (PCR) amplification of primers P505 and P506 are shown by ethidium bromide staining of a 2% agarose gel. Lanes are (a,j) λ Ava II DNA markers, (b) positive EHNV control (tissue culture supernatant from EHNV-infected cells), (c) negative control (uninfected redfin perch), (d-f) sample 15 (DNA products from three different experimentally infected redfin perch), (g-i) sample 16 (DNA products from three different experimentally infected rainbow trout).



CONCLUSIONS

1. The polymerase chain reaction (PCR) was used to amplify a segment of DNA of the dsDNA genome of epizootic haematopoietic necrosis virus (EHNV).

2. No PCR products were obtained from diamond python erythrocytic iridovirus (DPEV) or fish lymphocystis disease virus (FLDV) DNA.

3. EHNV isolates from redfin perch *(Perca fluvuatilis* L), rainbow trout *(Oncorhynchus mykiss* (Walbaum)) and BIV isolates from the ornate burrowing frog *(Limnodynastes ornatus* (Gray)) and barramundi *(Lates calcarifer)* generated PCR products of 235bp.

4. The tests were used to amplify DNA extracted from EHNV-infected cell cultures and infected tissues from redfin perch, rainbow trout and barramundi.

5. Hybridisation of ^{32}P-labelled EHNV PCR amplified DNA to Southern blots of NcoI restriction endonuclease digested EHNV and BIV DNA, enabled the differentiation of EHNV and BIV isolates.

6. The PCR assay described in this paper provides a method to detect/differentiate EHNV and BIV and is a valuable addition to the current EHNV diagnostic tests.

7.8 EXAMINATION OF DIFFERENT ISOLATES OF EHNV FROM WITHIN VICTORIA AND NEW SOUTH WALES

INTRODUCTION

For the past decade it has not been clear whether wild redfin perch and farmed rainbow trout were infected with similar isolates of EHNV. That is, could a strain/isolate of EHNV which caused large scale mortalities of redfin also cause fatalities in rainbow trout. The question has profound significance for the trout industry where farms source their water from river and dams and where redfin perch are, in general, common. The second question concerning field isolates of EHNV involves the number of different isolates of EHNV which exist. If more than one "type" exists then it is possible that the different types might relate to different degrees of virulence. The objective of this section of the project was to collect a diverse range of EHNV isolates. The isolates were selected based upon their geographical origin and the host species. These isolates were then analysed to provide data which could address the above questions.

METHODS

Isolates identified for study are listed in Table 1. The general geographical location and affected species are indicated.

DNA purification and Analyses

Purified EHNV (2 x 150cm^2 flasks) from affected redfin perch and rainbow trout were adjusted to 0.1 M NaCl, 5mM EDTA, 26% (w/v) sucrose and 1% SDS and mixed by rolling at 4°C for 30 min. Proteinase K was added to a final concentration of 100ug /ml and ribonuclease A to 40ug/ml were added and the incubation continued at 37°C for 18 h. Each solution was extracted twice with equal volume of phenol:chloroform. Sodium acetate was added to 0.2M followed by 2.5 volumes of ice cold ethanol. Precipitated DNA was spooled out, dried briefly under vacuum and resuspended with 10 mM Tris-HCl pH 7.5, to a concentration of 0.5 to 1.0 ug/ml. Three different restriction endonucleases were used to digisest the genome and agarose gel electrophoresis and field inversion gel electrophoresis for examing the digests.

The genome of EHNV is approximately 125±10kb and BIV 109±12kb (Hengstberger *et al.*, 1993). Earlier in the project primers were designed to the open reading frame. These primers together with those obtained later in the study were used to compare specific genome sequences of the different isolates. The later primers were to a 600 bp region of the major capsid protein of Frog virus-3 the type species of the genus *Ranavirus* within the family Iridoviridae. It is accepted that the capsid protein is a conserved protein (or contains conserved regions) within all viruses of this genus.

DNA products were visualised using ethidium bromide staining of 2% agarose gels after electrophoresis in Tris-acetate-EDTA (TAE) buffer.

EHNV ISOLATES FROM VICTORIA & NEW SOUTH WALES			
Lane & Reference Number	Species	Location	Year
1 (B87:8169)	Redfin	Lake Nillacootie	1987
2 (9009041231)	Redfin	Lake Winnekie	1990
3 (9009031621)	Redfin	Dandenong	1990
4 (9009031622)	Redfin	Ararat	1990
5 (A91006991033210069)	Redfin	Lake Makoan	1991
6 (ME 91/78	Redfin	Lake Burley Griffin, Canberra	1991
7 (ME 90/24)	Redfin	Blowering Dam, NSW	1990
8 (9009191131)	Trout	Snowy Mts, NSW	1990
9 (ME 92/14)	Trout	Snowy Mts, NSW	1992
10 (A91024910319124)	Trout	Tumut	1991
11 (940045)	Trout	Goulburn Trout Farm	1994
12 (B86:8774)	Trout	Adaminaby Trout Farm	1986

RESULTS and DISCUSSION

Analyses of the PCR products showed homologous sequences (fig.2) between all EHNV isolates. However when the entire genome was analysed by restriction enzyme digestion it was obvious that differences existed between the different isolates. There was one main group which contained isolates from both redfin perch and rainbow trout. One such isolate (#7) from Blowering Dam and collected at a time of high mortalities was identicle to isolate #8 and #9 which were collected from dead and moribund trout from a farm down stream. The identicle profiles of the isolates indicate that it was the same isolate which caused disease and death in both fish populations. It is therefore obvious that if trout farms source their water from bodies known to contain EHNV and/or EHNV-infected redfin then the possibility exists of introducing into the farms pathogenic EHNV. Whilst this has been suspected, based on epidemiological data (Whittington *et al.* 1994) this is the first scientific (molecular epidemiological) data which shows how commercial trout farms can become infected with EHNV from the surrounding water bodies.

The identification of distinct genome profiles indicates that different EHNV isolates exist. Whether the obsvered differences indicate serologically different forms of the virus or whether they represent variations within the one population is not known.

Fig 1. *Restriction endonuclease digest using the enzyme KpnI . Interpret lanes 1A and 12A and 1 and 12 respectively. M1 refer to 23.1, 9.42, 6.68, 4.36, 2.32 and 2.03 kb markers. M2 refers to high molecular weight markers 48.5, 38.4, 33.5, 29.9, 24.8, 22.6, 19.4, 17.1, 15.0, 12.2, 10.1, 8.6 and 8.3 kb. Note Isolate # 8 not shown in the gel below; however it has the same profile as lanes 8 and 9.*

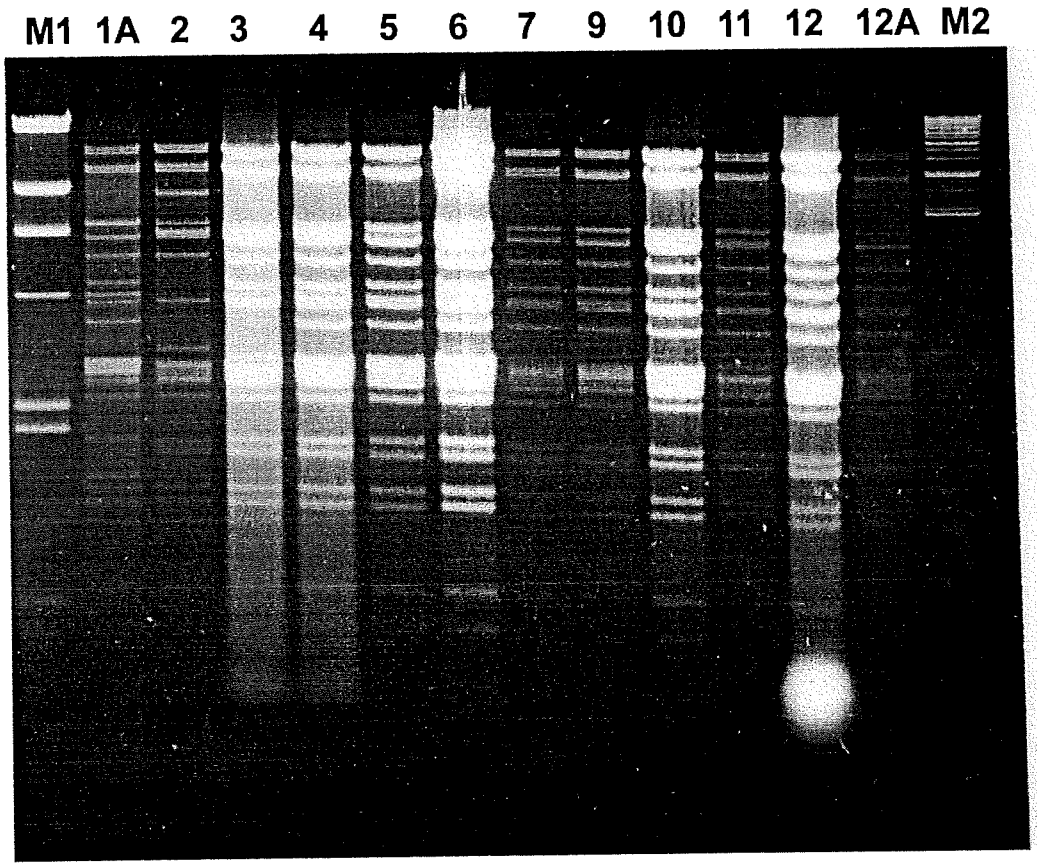


Fig 2. Sequence of EHNV coat protein. Sequence of 600bp in bold.

```
   1  ATGTCTCCTG TAACCGGTTC AGGTATCACA AGTGGTTTCA TCGACTTGGC CACTTATGAC
        M  S  P  V  T  G  S  G  I  T  S  G  F  I  D  L  A  T  Y  D

  61  AATCTCGAGA GAGCAATGTA CGGGGGCTCG GACGCCACCA CGTACTTTGT CAAGGAGCAC
        N  L  E  R  A  M  Y  G  G  S  D  A  T  T  Y  F  V  K  E  H

 121  TACCCCGTGG GGTGGTTCAC CAAGCTGCCG TCTCTGGCCG CCAAGATGTC GGGCAACCCG
        Y  P  V  G  W  F  T  K  L  P  S  L  A  A  K  M  S  G  N  P

 181  GCTTTCGGGC AGCAGTTTTC GGTCGGCGTT CCCAGGTCGG GGGATTACAT CCTCAACGCC
        A  F  G  Q  Q  F  S  V  G  V  P  R  S  G  D  Y  I  L  N  A

 241  TGGTTGGTGC TCAAGACCCC CGAGGTCAAG CTCCTGGCTG CAAACCAGCT GGGAGACAAC
        W  L  V  L  K  T  P  E  V  K  L  L  A  A  N  Q  L  G  D  N

 301  GGCACAATCA GGTGGACAAA GAACCCCATG CACAACATTG TGGAGAACGT CAACCTCTCA
        G  T  I  R  W  T  K  N  P  M  H  N  I  V  E  N  V  N  L  S

 361  TTCAACGACA TCAGCGCCCA GTCCTTTAAC ACGGCATACC TGGACGCCTG GAGCGAGTAC
        F  N  D  I  S  A  Q  S  F  N  T  A  Y  L  D  A  W  S  E  Y

 421  ACCATGCCAG AGGCCAAGCG CATAGGCTAC TATAACATGA TAGGCAACAC CAGCGATCTC
        T  M  P  E  A  K  R  I  G  Y  Y  N  M  I  G  N  T  S  D  L

 481  ATCAACCCCG CCCCGGCCAC AGGCCAGAAC GGAGCCAGGG TCCTCCCGGC CAAGAACCTG
        I  N  P  A  P  A  T  G  Q  N  G  A  R  V  L  P  A  K  N  L

 541  GTTCTTCCCC TCCCATTCTT CTTCTCCAGA GACAGCGGCC TGGCCCTGCC AGTCGTCTCC
        V  L  P  L  P  F  F  F  S  R  D  S  G  L  A  L  P  V  V  S

 601  CTCCCCTACA ACGAGATCAG GATAACAGTC AAGCTGAGGG CCATCCAGGA CCTCCTGATC
        L  P  Y  N  E  I  R  I  T  V  K  L  R  A  I  Q  D  L  L  I

 661  CTCCAGCACA ACACCACAGG GGCAATCAGC CCCATCGTGG CCGCCGACCT CGAGGGAGGT
        L  Q  H  N  T  T  G  A  I  S  P  I  V  A  A  D  L  E  G  G

 721  CTCCCCGACA CCGTCGAGGC CAACGTCTAC ATGACCGTCG CCCTCATCAC CGGGGACGAG
        L  P  D  T  V  E  A  N  V  Y  M  T  V  A  L  I  T  G  D  E

 781  AGGCAGGCCA TGAGCAGCAC AGTCAGGGAC ATGGTCGTGG AGCAGGTGCA GGCCGCCCCA
        R  Q  A  M  S  S  T  V  R  D  M  V  V  E  Q  V  Q  A  A  P

 841  GTCCACATGG TCAACCCCAG GAACGCGGCC ACCTTCCACA CCGACATGCG GTTCTCACAC
        V  H  M  V  N  P  R  N  A  A  T  F  H  T  D  M  R  F  S  H

 901  GCAGTCAAGG CCCTGATGTT TATGGTGCAG AACGTCACAC ACCCTTCCGT CGGCTCCAAT
        A  V  K  A  L  M  F  M  V  Q  N  V  T  H  P  S  V  G  S  N

 961  TACACCTGCG CCACTCCCGT CGTGGGAGTC GACAACACGG TCCTGGAGCC AGCCCTGGCG
        Y  T  C  A  T  P  V  V  G  V  D  N  T  V  L  E  P  A  L  A
```

```
1021  GTGGATCCCG TCAAGAGCGC CACGCTGGTG TACGAAAACA CCACAAGGCT CCCCGACCTG
        V  D  P  V  K  S  A  T  L  V  Y  E  N  T  T  R  L  P  D  L

1081  GGAGTCGAGT ACTACTCGCT GGTGCAGCCC TGGTACTATG CCACCTCCAT CCCAGTCAGC
        G  V  E  Y  Y  S  L  V  Q  P  W  Y  Y  A  T  S  I  P  V  S

1141  ACCGGGCACC ACCTCTACTC TTATGCCCTC AGCCTGCAGG ACCCCCACCC ATCCGGATCC
        T  G  H  H  L  Y  S  Y  A  L  S  L  Q  D  P  H  P  S  G  S

1201  ACCAATTACG GCAGACTGAC CAACGCCAGC CTTAACGTCA CCCTGTCCGC TGAGGCCACC
        T  N  Y  G  R  L  T  N  A  S  L  N  V  T  L  S  A  E  A  T

1261  ACGGCTGCCG CAGGAGGCGG AGGCGACAAC TCTGGGTACA CCACCGCCCA AAAGTACGCC
        T  A  A  A  G  G  G  G  D  N  S  G  Y  T  T  A  Q  K  Y  A

1321  CTCATCGTTC TGGCCATCAA CCACAACATT ATCCGCATCA TGAACGGCTC GATGGGATTC
        L  I  V  L  A  I  N  H  N  I  I  R  I  M  N  G  S  M  G  F

1381  CCAATCTTGT AAAGAGTATT TTTCAGCGCA AAGTCTTTTC CGTCATGGGT CCTCCATGAT
        P  I  L  -

1441  GGAAATAAAA CATGAAGTGT CCGTTTGCTG CAAAACGGGT CTTT
```

CONCLUSIONS

1. **A single isolate of EHNV (identified as having a specific restriction enzyme digestion profile) can infect both redfin perch and rainbow trout.**

2. **Various EHNV isolates (identified by specific restriction enzyme digestion profiles) exist within the Victorian and New South Wales environment.**

8.0 NATIONAL EHNV ANTIGEN CAPTURE ELISA PROTOCOL

and

OTHER USEFUL DIAGNOSTIC PROTOCOLS

8.1 NATIONAL

Antigen Capture ELISA for Detection of EHNV

The following protocol was developed at EMAI and AAHL with funding from FRDC. It is an adaptation of published procedures that have been optimised. The test has the following operating characteristics:

Sensitivity*	59.9%
Specificity*	99.5% (confirmed rainbow trout, redfin perch, golden perch)
Precision	7.2% coefficient of variation with recommended normalisation procedure
*Positive negative cut-off	ELISA OD 0.6, approximated by the signal from control antigen 3 (true normalised value 0.68)

Essential components of the assay

1. Linbro microtitre plates (Cat # 76:381:04, ICN Flow)

2. Rabbit anti-EHNV antiserum as affinity purified IgG (R@B2, EMAI reagent), used at 1:1600 final dilution

3. Sheep anti-EHNV antiserum (AAHL reagent), used at 1:16,000 final dilution

4. Rabbit anti-sheep-HRP conjugate (commercial reagent, KPL #14-23-06 0.5 mg from BIOMEDICS, Melbourne), used at 1:3000 final dilution

5. Control EHNV antigens (a, b, d and f) which cover the range of the signal response of the assay, enabling a normalisation procedure to be undertaken. These antigens are either live EHNV or gamma irradiated inactivated EHNV, according to laboratory preference.

Stability of reagents

The anti-EHNV capture antibody and second antibody are stable for at least 2 years when stored at -20°C as a 1:10 dilution in TSGM. The undiluted stock reagents which may be supplied are stable at -20°C for at least 4 years.

KPL conjugate is supplied by the manufacturer as a freeze dried powder which should be stored at 4°C. This reagent has displayed remarkable consistency in activity between different lots over a period of 5 years. The product should be reconstituted (to 1ml) in sterile 50% glycerol water, dispensed in 150µl aliquots and stored at -20°C as undiluted stock. A working stock is prepared by adding 1350 µl of TSGM to 150µl of undiluted stock. The working stock is also stored at -20°C and is stable for at least 1 year. New batches of this conjugate should be titrated against an older batch using standard protocols.

References:

Hyatt AD, Eaton BT, Hengstberger S and Russel G (1991). Epizootic haematopoietic necrosis virus: detection by ELISA, immunohistochemistry and immunoelectron microscopy. Journal of Fish Diseases 14: 605-617.

Steiner KA, Whittington RJ, Petersen RK, Hornitzky C and Garnett H (1991). Purification of epizootic haematopoietic necrosis virus and its detection using ELISA. Journal of Virological Methods 33: 199-209.

Whittington RJ and Steiner KA (1993). Epizootic haematopoietic necrosis virus (EHNV): improved ELISA for detection in fish tissues and cell cultures and an efficient method for release of antigen from tissues. Journal of Virological Methods 43: 205-220.

PROTOCOL AND WORK SHEET

Date:_____

Samples:_____

1. Coat a Linbro 96 well microtitre plate (100 ul/well) with affinity purified rabbit-anti-EHNV (R*B2) diluted 1:1600 in borate coating buffer. Incubate overnight at 4°C.

BCB batch# Capture antibody batch#_____
volume: ___ml volume 1:10 stock: ___ul

2. Wash plate five times with milli-Q purified water plus 0.05% Tween 20.

3. Prepare PBSTO diluent (PBS, 0.05% v/v Tween 20, 0.1% w/v ovalbumin-Sigma) up to 50 ml is required per plate:

10 x PBS	10 ml Batch#___
MQW	90 ml
Tween 20	50 ul
Ovalbumin	0.1 g

4. Block remaining binding sites with 1% Ovalbumin Sigma grade IIIA-5378) in PBSTO (100 ul/well).
Require per plate 0.1 g ovalbumin in 11 ml PBSTO
___ g _____ ml

Incubate at room temperature (RT) for 30 min.

5. Wash 5 times as above.

6. Work in Class II biological safety cabinet. Add tissue homogenate samples, culture supernatant samples and control antigens at 100 ul/well. The controls should be added to the lower right hand corner of the plate. All samples and controls are added to duplicate wells.

Incubate for 90 min at RT.

7. Wash plate by hand to avoid contamination of the plate washer. Work in class II cabinet. Flick contents of wells off into a waste canister or tray containing medol or 70% ethanol. Rinse plate twice with wash buffer from a wash bottle, discarding waste to the canister. (If desired, wells can be emptied using a multichannel pipette).

8. Wash plate 5 times on the plate washer as above.

9. Add second antibody Sheep-anti-EHNV at 1:16,000 in PBSTO (100 ul/well).

Batch#_____ volume 1:10 stock _____ ul PBSTO volume ___ml

Incubate for 90 min at RT.

10. Wash plate 5 x on plate washer.

11. Add KPL conjugate (KPL#14-23-06, BIOMEDIC, Doncaster, Vic) KPL at 1:3000 in PBSTO (100 µl/well).

Batch#_____ volume_____ µl 1:10 stock PBSTO volume ___ml

Incubate for 90 min at RT.

12. Wash plate 5 x on plate washer.

13. Add ABTS substrate (22 ml ABTS + 10 ul (30%)H_2O_2) (100 ul/well) and place the plate on a plate shaker. Time this step from the moment substrate is added to the first wells of plate 1.

Incubate for 20 min.

14. Immediately add ABTS stop solution (50 ul/well), shake plate briefly and read OD at 405 nm. Calculate mean ELISA OD of duplicate wells. Calculate the coefficient of variation [(SD/Mean) x 100]of the duplicates: samples with CV >15% should be retested if the mean OD lies near the cut-off of 0.6.

Control antigens and normalisation of data

The control antigens are dilutions of a cell culture supernatant of EHNV 86/8774 (EMAI X852) and are expected to give the following OD, although there will be some variation from lab to lab:

Control	Dilution culture supernatant	OD
A	1:10	2.70
B	1:100	1.90
D	1:500	0.68
F	1:10000	0.16

The positive-negative cut-off for the ELISA is approximated by the OD value of control D on each plate. For critical work to normalise data, i.e. where there is need to minimise the effects of plate to plate and day to day variation in ELISA OD due to random variation, the following procedure is recommended:

Run control antigens in ELISA on at least 5 occasions over a period of 3 weeks (total 20 separate ELISA plates). Calculate the mean OD for each control antigen. Then for each plate subsequently used, calculate a plate correction factor as follows:

PCF =[(mean OD control A/actual OD) + (mean OD control B/actual OD) + (mean OD control D/actual OD) + (mean OD control F/actual OD)]/4.

Multiply the actual mean OD of each sample by the PCF for that plate and report these values.

PCF is allowed to vary between 0.8 and 1.2. Values outside this range suggest that a plate needs to be retested. Plots of PCF over time provide a ready means for monitoring the stability of reagents, procedural variations and operator errors. This QC method has been validated for the antigen capture ELISA. Decision-limit quality control using alternate QC varibles may also be appropriate.

Borate coating buffer

Boric acid	6.18g
Disodium tetraborate ($Na_2B_4O_7.10H_2O$	9.54g
NaCl	4.38g
MQ water to	1L

Autoclave

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10 x Phosphate buffered saline

NaCl	80.00 g
KCl	2.00 g
Na_2HPO_4	11.50 g
KH_2PO_4	2.00 g

MQ water to 900ml; pH to 7.2 with HCl or NaOH; make up to 1L

Autoclave

For working strength dilute 1:10 and recheck pH.

For storage of powder in jars make up 2 x above quantity of powder; store; to make up add 1.8 L MQW, pH, make up to 2L.

ABTS

Citrate phosphate buffer

Citric acid	21.00 g
Na_2HPO_4	14.00 g

MQ water to 800 ml; adjust pH to 4.2; make up to 1L

ABTS		0.55 g
Citrate phosphate buffer	to	1L

Dispense in 22 ml aliquots and freeze.

Immediately prior to use add 10 ul (30%) H_2O_2 per 22ml aliquot.

ABTS stop solution (0.01% NaN_3 in 0.1 M citric acid)

Citric acid	10.5 g
MQW to	500 ml

Add 50 mg sodium azide or 1 ml of 5% solution.

TSGM

50 mL 10X Tris - Saline pH 7.4
250 mL Glycerol

Make up to 500 mL with Milli-Q Water
Autoclave
Add 1 mL 10% Merthiolate

Store in dark bottle at 4°C.

10X TRIS - SALINE (250 mM Tris, 1.5 M NaCl)

15.14 g Tris - HCl
43.83g NaCl
500 mL Milli-Q Water
pH 7.4

8.2 PROTOCOL FOR IMMUNO-PEROXIDASE AND FLOURESCENCE ON FORMALIN FIXED SECTIONS.

A. *Immuno-peroxidase* *Method 1*

1. Label individual slides
2. Melt wax at 60^0C, 10min.
3. Wash (x3) for 1min.
4. Rinse in 100% ethanol (tech grade) (x2, 1 min).
5. Rinse in 70% ethanol (tech grade) (1min).
6. Rinse in running tap water (1min).
7. Rinse in distilled water.
8. Trypsin digestion. Incubate slides in a coplin jar containing 0.1% trypsin in 0.1% $CaCl_2$, pH 7.8 (0.2g trypsin in 200ml 0.1% $CaCl_2$ with 1.25ml 0.1N NaOH). Warm to 37^0C before use. Incubate for 20min at 37^0C in a water bath.
9. Rinse in distilled water with agitation (x2).
10. Wash in PBSA (x1).
11. Incubate in primary antibody at desired dilution (AAHL rabbit "flopsy" 1:500 and EMAI rabbit at 1:3000) in 2% skim milk (sm) in PBS (0.4g sm in 20ml PBS) 45min, 37^0C. 200ul per slide.
12. Rinse in PBS (x3).
13. Incubate in biotinylated donkey anti-rabbit (Amersham) 1:100 in 2% sm in PBS (45min, 37^0C).

14. Rinse in PBS (x3).

15. Block endogenous peroxidase. Place slide in a coplin jar containing3% H_2O_2 in absloute methanol for 20min (RT) (20 ml of 30% H_2O_2 + 180ml methanol).

16. Wash in PBS (x3).

17. Add 2mg AEC (3-amino-9-ethylcarbazole) powder to 200 ul dimethylformamide (DMF) in an eppendorf tube). Add AEC mixture and 5ul of 30% H_2O_2 to 10ml acetate buffer. Add solution to slide and check every 5min. Stop reaction at approximately 10 tp 15 min.

18. Stop solution by adding PBS.

19. Counterstain with Mayer's haemalum (haematoxin), 90sec.

20. Rinse in running tap water.

21. Rinse in Scott's tap water until the section goes blue.

22. Rinse in running tap water.

23. Mount in Geltoi mounting medium.

A. *Immuno-peroxidase* *Method 2*

1. Cut duplicate sections and mount on poly-L-lysine coated slides

2. Remove excess tissue not required for staining, diamond pencil to limit spread.

3. Pre-heat slides in a 60°C incubator until moist. (10 min)

4. depararrafinise (rehydrating) - freshly made up solutions and start at water. (3 min each)

5. Trypsinise (0.1% trypsin/0.1% $CaCl_2$) [20 min. RT], water (3 dips), PBSTw (5 min.)

6. Block H_2O_2 (#1) [5 min, RT], PBSTw (2 x 5 min).

7. Block in goat serum (#2) (5 min. RT). Leave wet with serum - tap off the excess).

8. Primary antibody/primary control (#3), (30 min., 37°C).
   R@B2 (1:10) or control of choice, diluted in PBSTw (1:2500)
   Rinse in PBSTw (3 x 5 min).

9. Link antibody (#4) - biotinylated (30 min., 37°C).
   PBSTw (3x 3 min).

10. Peroxidase - labelled streptavidin (#5) (10 min., RT).
    (prepared immediately before)
    PBSTw (3 x 5 min).

11. Substrate #6 (10 mi., RT).
    (prepared immediately before)
    Distilled water (2 x 5 min).

12. Counterstain - Mayer's haematoxylin (2 min)
    Wash in distilled water.

13. Mount in Dako "glycergel" while still wet.
    (Pre-heat glycergel in 60°C for 30 min., pre-heat coverslips on a hot plate - 2 drops of glycerol per slide.

(PBSTw - require total volume of 4 litres of PBS/2ml TWEEN)

- **Kit (Dako LSB K680, Dako Coporation, California, USA)**
- **Known negative and positive controls should be included for each analysis.**

B. ***Immuno-fluorescence***

1. Label individual slides
2. Melt wax at 60^0C, 10min.
3. Wash (x3) for 1min.
4. Rinse in 100% ethanol (tech grade) (x2, 1 min).
5. Rinse in 70% ethanol (tech grade) (1min).
6. Rinse in running tap water (1min).
7. Rinse in distilled water.
8. Trypsin digestion. Incubate slides in a coplin jar containing 0.1% trypsin in 0.1% $CaCl_2$, pH 7.8 (0.2g trypsin in 200ml 0.1% $CaCl_2$ with 1.25ml 0.1N NaOH). Warm to 37^0C before use. Incubate for 20min at 37^0C in a water bath.
9. Rinse in distilled water with agitation (x2).
10. Wash in PBSA (x1).
11. Incubate in primary antibody at desired dilution (AAHL rabbit "flopsy" 1:500 and EMAI rabbit at 1:3000) in 2% skim milk (sm) in PBS (0.4g sm in 20ml PBS) 45min, 37^0C. 200ul per slide.
12. Rinse in PBS (x3).
13. Incubate in biotinylated donkey anti-rabbit (Amersham) 1:100 in 2% sm in PBS (45min, 37^0C).
14. Rinse in PBS (x3).
15. Add (Amersham) streptavidin-FITC (1:100 in 2% sm in PBS, 45min at 37^0C.
16. Wash in PBS (x3).
17. Add 400ul Evan's blue in 10ml PBS, 15 min 37^oC.
18. Rinse in distilled water (x3).

19. Mount in Geltoi mounting medium.

8.3 PROTOCOL FOR IMMUNO-NEGATIVE CONTRAST ELECTRON MICROSCOPY

It should be noted that in the absence of antibody tests the examination of fish tissues such as kidney, liver and spleen in addition to infected cell cultures can reveal the presence of iridoviruses. These viruses are icosahedral (approx 170 nm in diameter), form para crystalline arrays and are located within prominant virus inclusion bodies. Viruses are also observed budding from the plasma membranes of cells which contain a rarified, marjinated and often multi-lobed nucleus. This type of information is obtained by the examination of ultra-thin sections.

(A) Negative contrast immunoelectron microscopy (NCIEM).

Tissue homogenates of liver, kidey or spleen or tissue culture supernatants and cell homogenates can be examined by NCIEM.

The polyclonal and monoclonal antibodies are directed to the internal and/or major capsid protein(s). The antibodies can be used successfully in the labelling sections of glutaraldehyde fixed specimens. However for particulate samples the following protocol is recommended as the cell-surface derived envelope covers/masks the target epitipoes(s).

1. Adsord virus to carbon coated, parlodion filmed 400 mesh copper grids.
2. Fix ina mixture of 1%NP40 and 0.5% glutaraldehyde in culture medium (2 min).
3. Wash in buffer (eg 0.1M cacodylate or PBS, 3 x 5 min).
4. Wash in 1% cold water fish gelatin (CWFG) (10 min).
5. Incubate in primary antibody (can be blocked in normal goat if protein A-gold is not used). Note: Should titrate the antibodies to determine the optimum dilution. As a rough guide Mabs should be used neat and polyclonal antibodies at about 1:500. All antibodies should be diluted in CWFG. 15min to 60min at R.T.
6. Wash in PBS 6 x 3 min.
7. Label in protein A gold (dilution depends on source) or gold-labelled rabbit anti mouse (60 min) in CWFG.
8. Wash in 6 x 3min PBS.
9. Post fix in 2.5% glutaraldehyde (in PBS), 5 min.
10. Wash in water (3 x 5 min)
11. Stain in 2% PTA (pH 6.8).

(B) Post-labelling immunoelectron microscopy

The following general procedure can be used for immuno-gold labelling of sections.

1. Cut ultra-thin sections and place onto nickle grids which have been rinsed in 20% acetic acid followed by 90% ethanol and rinsed in water.

2. Place section on puddles of distilled water or PBS.

3. Block sections in normal goat (1:20) in PBS. Note: if protein A-gold is used the omit this step.

4. Incubate in the primary antibody eg R@EHNV (AAHL) (1:50) in 1% cold water fish gelatin (Sigma). [1h, RT].

5. Wash (x6) in 1% cold water fish gelatin

6. Incubate in secondary gold-labelled antibody for 1h at RT. Antibody should be diluted in [1%BSA, 0.1% TritonX, 0.1% Tween in PBS]. Note: check the class of antibodies being used and select appropriate conjugate (ie IgG1, 2a ; IgM etc..).

7. Wash (x6) in PBS.

8. Wash (x3) in distilled water.

9. Post-fix in 2.5% glutaraldehyge (aq) (5 min).

10. Wash in distilled water (x3)

11. Dry and stain.

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