FINAL REPORT

Aquatic Animal Health Subprogram: development of diagnostic and reference reagents for epizootic haematopoietic necrosis virus of finfish

Richard Whittington and Kylie Deece

June 2004

FRDC Project No. 2003/621

Faculty of Veterinary Science
The University of Sydney

Australian Government
Fisheries Research and Development Corporation

Professor Richard Whittington and Ms Kylie Deece

Aquatic Animal Health Subprogram: development of diagnostic and reference reagents for epizootic haematopoietic necrosis virus of finfish.

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2003/621 Aquatic Animal Health Subprogram: development of diagnostic and reference reagents for epizootic haematopoietic necrosis virus of finfish

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OBJECTIVES:

1. To provide quality-controlled antibody reagents and protocols to detect EHNV
2. To provide quality controlled DNA reagents and protocols to differentiate EHNV from related viruses including BIV
3. To provide stocks of reference-strain EHNV and fish tissues containing reference-strain EHNV as controls for diagnostic testing
4. To develop and assess new storage conditions, guidelines for reconstitution and shelf life for antibody and DNA reagents

NON TECHNICAL SUMMARY:

The quantity and value of aquaculture production is likely to increase relative to wild harvest fisheries globally and as a consequence the international community is taking great interest in disease threats to finfish aquaculture. Epizootic haematopoietic necrosis (EHN) is one of the viral diseases of fish listed by the Office International des Epizooties (OIE) and occurs in parts of Australia. Because of the extreme virulence of the causative agent EHN virus (EHNV), its restricted geographic range and limited opportunities for study outside Australia, this country hosts the OIE Reference Laboratory for EHNV, based jointly at the University of Sydney Faculty of Veterinary Science and CSIRO Australian Animal Health Laboratory. In addition to providing research and diagnostic referral services to the Australian industry, the reference laboratory provides technical advice, protocols and reagents to laboratories throughout the world, thereby ensuring international diagnostic capability. This is required under international guidelines in trade in aquatic animal products, administered by the OIE. The OIE Reference Laboratory for EHNV represents an important contribution by Australia to the international community.

Following stakeholder consultations, the FRDC Aquatic Animal Health Subprogram identified EHNV reference laboratory functions as one of a number of high priority issues for funding under the Federal Government's "Building a National Approach to Animal and Plant Health" program. The OIE Reference Laboratory for EHNV has

provided reagents, protocols and diagnostic referral services to fish health laboratories in Australia and other countries for more than 10 years. Research on protocols for improved viral detection and differentiation from related viruses has been ongoing, and has been published in high quality journals. However, many of the original reagents were prepared in 1989-1992 and stocks of quality-controlled batches were almost exhausted. Furthermore, new protocols had recently been developed using modern tools of molecular biology but standardized DNA reagents were not available. The aim of this project was therefore to provide quality-controlled viral, tissue, antibody and DNA reagents and protocols to detect EHNV and to differentiate it from related viruses including BIV. A further aim was to develop and assess new storage conditions, guidelines for reconstitution and shelf life for these reagents.

As a result of this project reagents and protocols for the detection of EHNV using the latest technology in ELISA, immunohistochemistry and molecular biology have been prepared. The reagents have been evaluated at an independent laboratory and are now available to laboratories in Australia and internationally. EHNV is a very serious pathogen. Consequently the reagents have been prepared using a new approach which will facilitate easy shipment in a stable form with no biosecurity risk. This is important in the current era of bioterrorism.

Stocks of reagents produced in this project are likely to be sufficient for 15 years based on current demand.

OUTCOMES ACHIEVED TO DATE:

1. A stock of quality-controlled antibody reagents and protocols to detect EHNV
2. A stock of quality controlled DNA reagents and protocols to differentiate EHNV from related viruses including BIV
3. A stock of reference-strain EHNV and fish tissues containing reference-strain EHNV as controls for diagnostic testing
4. Protocols for preparation of reagents using new storage conditions, guidelines for reconstitution and shelf life data for antibody and DNA reagents

These outcomes have been achieved within the life of the project and are of direct benefit to fish farmers by enabling diagnostic and certification testing for EHNV and indirect benefit to the entire Australian aquaculture industry by demonstrating technical and operational competencies in fish disease diagnosis to the international community, with whom we trade.

KEYWORDS:

EHNV, diagnosis, epizootic haematopoietic necrosis virus, finfish, trade access, disease

FULL REPORT

Acknowledgements
This project was made possible through the cooperation of a number of outside agencies and scientists. During an unexpectedly difficult and delayed university laboratory refurbishment Dr Peter Kirkland, Elizabeth Macarthur Agricultural Institute kindly made facilities available which enabled us to grow and purify EHNV. Mrs Christine Hornitzsky and Mrs Madeline Saunders provided great support for Ms Kylie Deece while Dr Leslie Reddacliff assisted in the location of paraffin blocks from experiments conducted more than 10 years ago. Dr Dean Gilligan, NSW Fisheries Narrandera expertly coordinated and supported wild capture of redfin perch for the animal experiments. Dr Mark Crane, Fish Diseases Laboratory, CSIRO AAHL kindly undertook an independent evaluation of the new reagents and protocols for both ELISA and PCR in a very efficient manner, allowing this report to be completed by the due date.

Background
Australia is unlucky to be home to two of the eleven Office International des Epizooties (OIE) listed viral diseases of finfish. One of them is called epizootic haematopoietic necrosis virus (EHN). Because of the extreme virulence of the causative virus, its restricted geographic range and limited opportunities for study outside Australia, this country hosts the International Reference Laboratory for EHNV, based jointly at the University of Sydney Faculty of Veterinary Science and CSIRO Australian Animal Health Laboratory. In addition to providing research and a diagnostic referral service to the Australian industry, the reference laboratory provides technical advice, protocols and reagents to laboratories throughout the world, thereby ensuring international diagnostic capability. This is required under international guidelines in trade in aquatic animal products, administered by the Aquatic Animal Health Standards Commission of the OIE. The Reference Laboratory represents an important contribution by Australia to the international community. The expertise in Australia came about through research on EHNV funded from FRDC (FRDC92/66).

Need
Relationship to R&D plans and strategies
In the May 2000 Budget, the Federal Government announced its *Building a National Approach to Animal and Plant Health* program to maintain Australia's status as a source of high quality agricultural produce with work on aquatic animal health to be funded via AFFA through the FRDC Aquatic Animal Health Subprogram. Following stakeholder consultations, seven projects focusing on the development of improved diagnostic methods for diseases of fish, crustaceans and molluscs were funded under this Subprogram. The Subprogram recently requested advice from aquatic animal health specialists in Australia on priority suggestions for the remaining uncommitted funds. The '*Maintenance of reagent stocks for diagnosis of important diseases e.g. EHN*' was identified by this forum as one of a number of high priority issues and subsequently forwarded to the Subprogram. The Subprogram's Steering Committee and Scientific Advisory Committee supported this and other suggestions because of their national significance. This project was undertaken in response to this need.

Pragmatic need

The OIE Reference Laboratory for EHNV provides research and diagnostic referral services to fish health laboratories in Australia and other countries. Reagents have been supplied upon request for more than 10 years. Research on protocols for improved viral detection and differentiation from related viruses is ongoing, and has been published in high quality journals. These protocols are supplied, together with reference reagents, to any diagnostic laboratory upon request. As many of these original antibody reagents were prepared in 1989-1992, they were deemed likely to be near the end of their shelf life, or stocks of quality-controlled batches were almost exhausted. Furthermore, new protocols have recently been developed using modern tools of molecular biology (Marsh et al 2002 Rapid differentiation of Australian, European and American ranaviruses based on variation in major capsid protein gene sequence. Mol.Cell. Probes 16:137-151). For routine use these require development of new reagents, for example standardized DNA solutions.

This project is an operation to replenish stocks of reagents for existing tests and to create stocks for the more newly developed tests. In future we aim to meet costs by charging for supply of reagents, something that OIE Aquatic Animal Health Standards Commission has agreed may be necessary to support reference laboratory activities. Stocks of antibody reagents produced in this project are likely to be viable and sufficient for 15 years assuming freeze drying is successful and based on current levels of demand, but this will also be dependent on future demand, which is not easily predicted.

Objectives

1. To provide quality-controlled antibody reagents and protocols to detect EHNV
2. To provide quality controlled DNA reagents and protocols to differentiate EHNV from related viruses including BIV
3. To provide stocks of reference-strain EHNV and fish tissues containing reference-strain EHNV as controls for diagnostic testing
4. To develop and assess new storage conditions, guidelines for reconstitution and shelf life for antibody and DNA reagents

Methods

The methods used in this project are based on those given in the reference list at the end of this report, as well as methods developed in the project, which are described below and in the results.

Objective 1. To provide quality-controlled antibody reagents and protocols to detect EHNV

After optimisation of methods, a reference isolate of EHNV was propagated in BF2 cell monolayers in cell factories (large-scale production) to produce a large quantity of infectious virus. This was separated from cell culture components by differential and density gradient centrifugation, again after optimisation. Purity of protein was confirmed by SDS-PAGE, electron microscopy and immunoblotting using antisera prepared against the cell culture antigens. Antibodies against purified EHNV were raised in sheep, goats and rabbits. Those from sheep and goats were compared for specificity. All reagents were compared with remaining stocks of reference reagents. The reagents were titrated for antigen capture ELISA against control

antigens and working dilutions were defined for routine use. Purified rabbit antibodies were titrated for immunohistochemical detection of EHNV in tissues from infected fish.

Objective 2. To provide quality controlled DNA reagents and protocols to differentiate EHNV from related viruses including BIV

Methods for purification of DNA from cultured EHNV and BIV were compared and the DNA samples were then distinguished using our published methods which are based on PCR for the major capsid protein with restriction endonuclease analysis. Stocks of DNA were prepared and working dilutions established for routine use as control target DNA in diagnostic PCR. Validity was determined across a range of isolates of EHNV from south eastern Australia, BIV and DNA from an unrelated exotic iridovirus (Red Sea Bream Iridovirus, RSIV).

Objective 3. To provide stocks of reference-strain EHNV and fish tissues containing reference-strain EHNV as controls for diagnostic testing

Reference strains of EHNV and the closely related Australian ranavirus BIV were propagated in cell culture, quantified by $TCID_{50}$ assays, confirmed by PCR of capsid protein and aliquoted for storage and future distribution. Inactivation methods were compared to facilitate distribution of viral antigen to reduce the biosecurity risk.

Fifty juvenile 0+ redfin perch (*Perca fluviatilis)* (approximately 50 mm forklength) were caught in Blowering Dam NSW, transported to Camden and acclimated in aquaria. They were experimentally inoculated with EHNV and monitored for signs of disease, which is expected in all fish inoculated with EHNV. None of the fish succumbed to initial immersion inoculation at a water temperature of 25°C. This was repeated once at a water temperature of 20°C, and as no fish succumbed, an intraperitoneal inoculation of 8 fish was then undertaken at the same water temperature. Four fish were moribund (1) or died (3) between days 3 and 10 post inoculation and these fish had early histopathological changes consistent with EHNV infection. The reasons for the failure to induce the usual epizootic syndrome in these redfin perch was unclear but could include attenuation of the virus through laboratory handling or immunity of the fish. It was beyond the scope of this project to determine the exact reason. At this point the fish trials ceased as it was unlikely that sufficient material to meet project objectives could be obtained to meet project milestones on time and within budget and efforts were redirected to acquire samples of infected redfin liver from archives held in regional veterinary laboratories in New South Wales. Paraffin blocks were located after an intensive search for samples. Multiple sections were taken from a number of blocks and assessed for suitability as controls following immunohistochemistry. The paraffin blocks have been secured by the laboratories for future reference. A commercial kit was used to prepare immunoperoxidase stained sections using the manufacturer's instructions (Dako LSAB + Kit, Peroxidase, Universal K0679, Dako Corporation, Carpinteria USA), with modification to reveal antigens damaged by tissue fixation.

Objective 4. To develop and assess new storage conditions, guidelines for reconstitution and shelf life for antibody and DNA reagents

To simulate a variety of storage and transport scenarios, antibody reagents were stored frozen at –20°C with and without cryoprotectant and were also lyophilised and stored at 4°C. Freeze dried reagents were subjected to temperatures ranging from 4°C to 50°C for periods of up to 72 hours, reconstituted in water or buffer with cryoprotectant then stored at –20°C. The effect of temperature on control antigen was also evaluated. The activity of these preparations was compared by ELISA. The shelf life of antibody reagents was determined after lyophilisation (then stored at 4°C), dilution in cryoprotectant (then stored at –20°C) or frozen storage (-20°C).

Results/Discussion

Objective 1 To provide quality-controlled antibody reagents and protocols to detect EHNV

1A. Factory scale production of EHNV

The methods of Steiner *et al*, (1991) were used with the following changes to enable use of cell factories. After a series of optimisation experiments, a total of seven NUNC^{TM} Cell Factories (10-tray) were used to produce large quantities of EHNV. Each factory was seeded with BF-2 cells (1800mL media) and incubated at 22°C until confluent. The cells were then inoculated with EHNV (18mL) and incubated at 22°C until 100% CPE was observed. Only one factory was harvested at a time and each was given a batch number to monitor purity between factories.

1B. Purification of EHNV

Modification and optimisation of the methods of Steiner *et al* (1991) were required to allow for the increased yields of EHNV from cell factories. Cell debris was removed through centrifugation and the virus was pelleted. Three variations of the method were assessed after this step to determine a safe stopping point. The first involved resuspending the pellet in 2mL 100mM Tris-HCl and immediately loading the virus onto the sucrose gradients. For the second, the virus was resuspended as in the first, but stored overnight at 4°C, before being loaded onto sucrose gradients the following morning. The third involved a 16-hour centrifugation step (overnight) to pellet the virus, followed by resuspension and loading of the sucrose gradients.

To prevent overloading of the gradients, and to account for the 3.7 times increase in surface area (or the amount of virus produced) compared to the original method in flasks or roller bottles, a maximum volume of 270µL of the suspension was loaded onto each of ten preformed discontinuous sucrose gradients (15-60%) and centrifuged according to published methods (Appendix 3.1). Of the two bands present, only band 2 (lower band containing purified virus) was aspirated and pelleted before being resuspended in 200µL of 100mM Tris-HCl.

Batches from each method were shown to be pure by SDS-PAGE (Figure 1). Immunoblotting using antisera prepared against cell culture antigens further confirmed that the viral suspension contained only traces of cellular contaminants (Figure 2). The protein concentration of each batch of virus was determined using a Bio-Rad Bradford Protein Assay Kit. Results are shown in Table 1.

Of the three method variations assessed, methods 2 and 3 offered more flexibility, however the protein concentration of batch 4 from the third method was lower when compared to batches from methods 1 and 2. Based on these results, the purification method was updated to incorporate a safe stopping point as described for method 2 (Appendix 3.1). Method 1 was not practical, requiring 14 to 16 hours continuous working time.

Figure 1. Indicative SDS-PAGE analysis of purified EHNV. Lanes 1 – 5, batches 1 - 5 sucrose gradient purified EHNV; Lane 6, BF2 cell negative control; lane 7, molecular size markers (kDa).

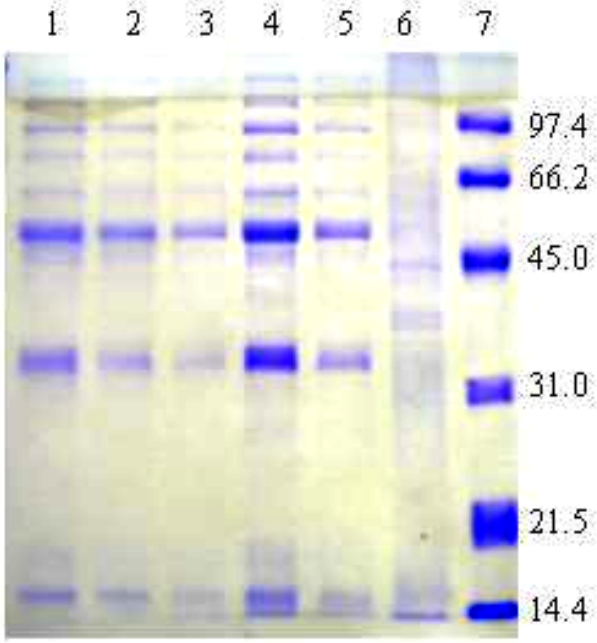


Figure 2. Indicative Western blot anaylsis of purified EHNV. Lanes 1 – 5, batches 1 - 5 sucrose gradient purified EHNV; Lane 6, BF2 cell control; lane 7, molecular size markers (kDa). The blot was stained with rabbit antiserum against BF-2 cells.

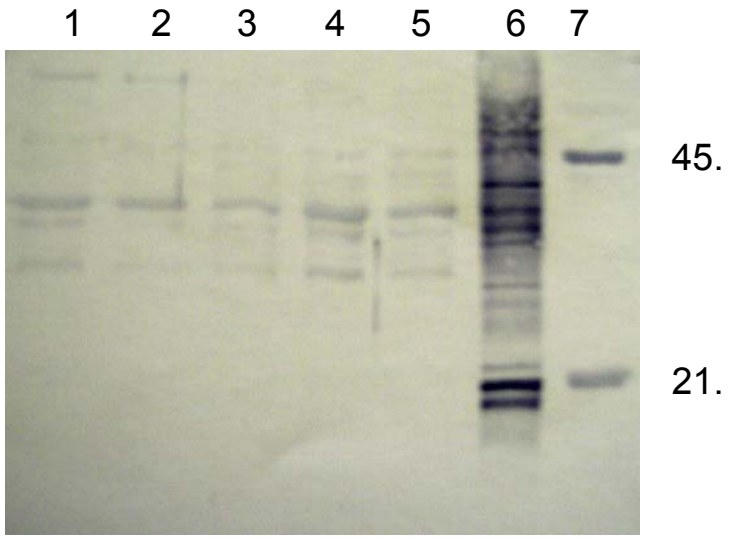
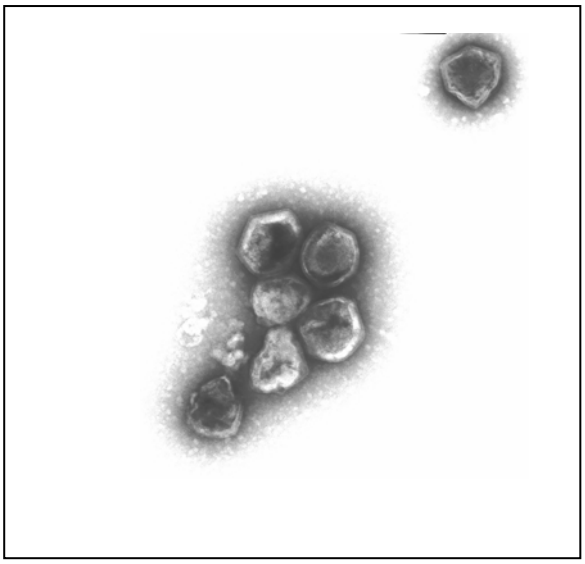


Table 1: Protein concentrations of each virus batch determined using a Bradford Assay.

Batch Number	Method	Protein (mg/mL)	Total Protein (mg)
1	1	8.6	1.72
2	1	3.6	0.72
3	3	2.0	0.4
4	1	7.0	1.4
5	2	3.6	0.72
6	2	7.0	1.4
7	2	5.6	1.12

Negative contrast electron microscopy was undertaken on a sample from Batch 7 and further confirmed the purity of the virus preparation. The sample contained mainly non-enveloped nucelocapsids and cellular debris were not apparent (Figure 3).

Figure 3. Transmission electron micrograph of EHNV purified using the optimised method



1C. Hyperimmunisation of animals

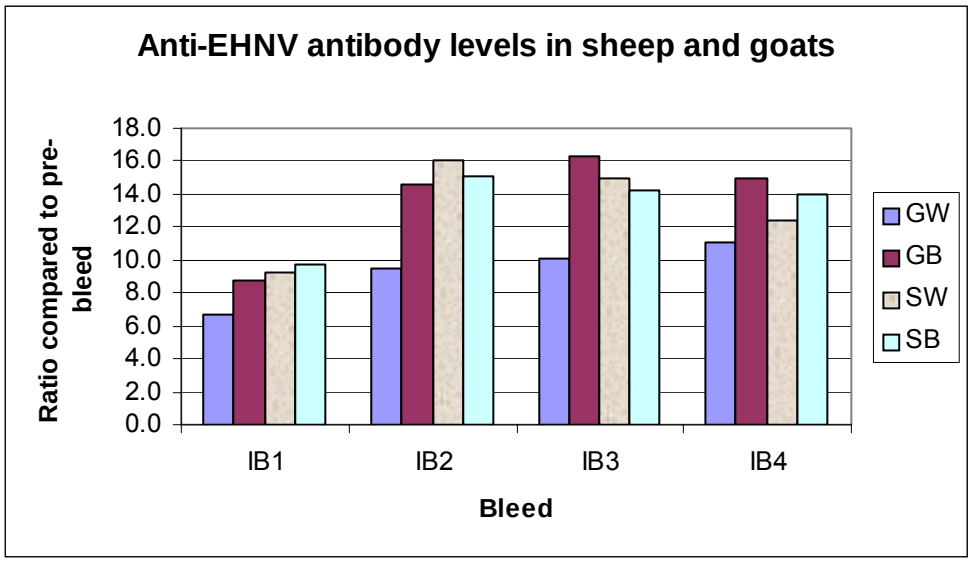
Three sheep, three goats (all less than 12 months old) and six mature New Zealand White rabbits were purchased and housed in purpose built animal house facilities. The ruminants were fed lucerne pellets, grain and hay while the rabbits were fed commercial pellets. Pre-immune blood samples were collected and screened for pre-existing antibodies against EHNV by ELISA, with negative results. These sera were stored as the pre-bleed (PB). Two sheep, 2 goats and all 6 rabbits were immunised on two occasions 5 weeks apart with purified EHNV. Blood samples were then collected at intervals and anti-EHNV titres monitored by ELISA. Serum was stored at –20°C.

Antibody production was confirmed by ELISA. When antibody titre appeared to have reached a peak level, a final bleed was collected from each animal; this was 6 weeks after the second immunisation of rabbits and 6 to 12 weeks after the second immunisation for sheep and goats (Appendix 3.2).

1D. Comparison of antibody responses between individual ruminants

Seven-to-eight-fold increases in antibody level were apparent at the first immune bled but by the third immune bleed there were 10 to 16 fold increases in antibody level compared to prebleed levels (Figure 4). One goat (GW) had a lower response compared to the three other animals at the third immune bleed, but the responses of the other animals were quite consistent.

Figure 4. Comparison of the immune response against EHNV in two sheep (SW, SB) and two goats (GW, GB) following hyperimmunisation. EHNV antigen was used in ELISA at a dilution of 1:200 while sheep and goat sera were tested at a dilution of 1:8000.



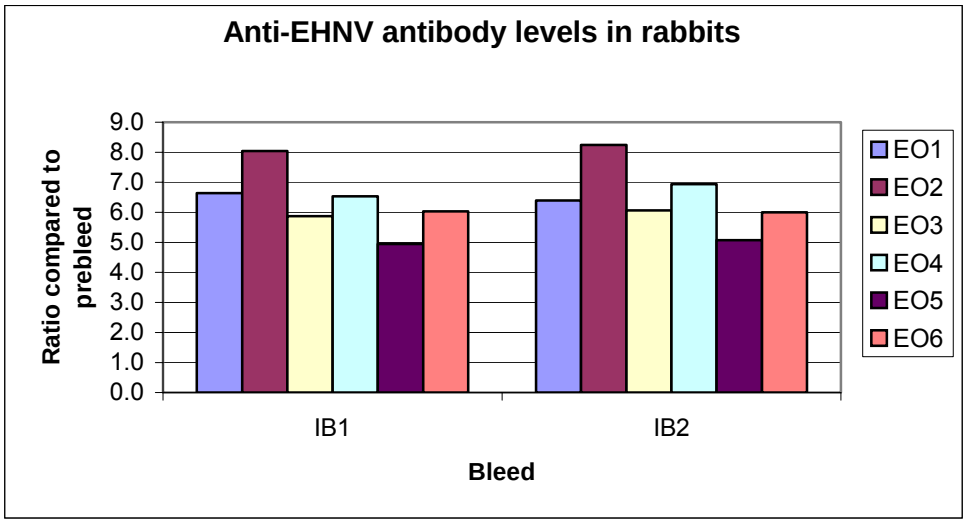
1E. Comparison of ruminant immune sera with a previous batch of antisera prepared in sheep (fishface)

When tested at a dilution of 1:8000 in an ELISA with EHNV antigen at a dilution of 1:200, the third immune bleed from the sheep and goats had OD ranging from 1.11 to 1.63, compared to 0.61 for “fishface”. Corresponding values for very low levels of antigen (1:3000) were 0.18 to 0.23 for the goats and sheep, and 0.15 for “fishface”. These data indicate that the quality of the new antisera is at least as good as, but probably better than the previous batch.

1F. Comparison of antibody responses between individual rabbits

Immune responses against EHNV were measured by ELISA and were similar between the six rabbits. There were between 5 and 8 fold increases in antibody level compared to prebleed at both the first and second immune bleeds (Figure 5).

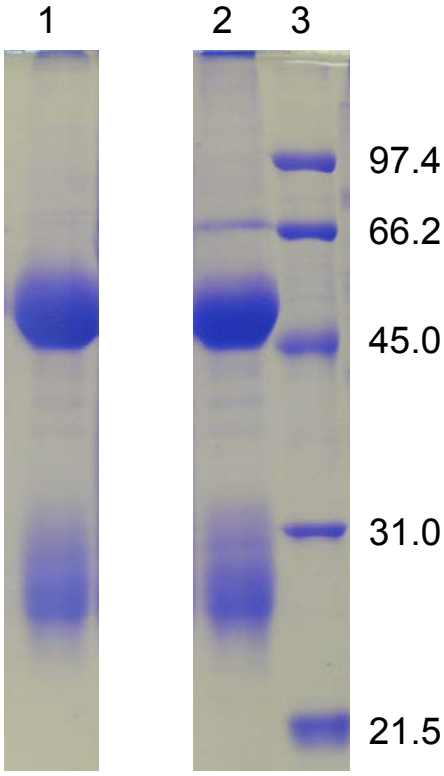
Figure 5. Anti-EHNV antibody levels in rabbits
Immune response of six rabbits (EO1 to EO6) following hyperimmunisation with EHNV. The antigen used for the ELISA was partially purified heat inactivated EHNV at a dilution of 1:200 while rabbit sera were evaluated at a dilution of 1:1600.



1G. Preparation of a pool and purification of immunoglobulin from rabbit serum

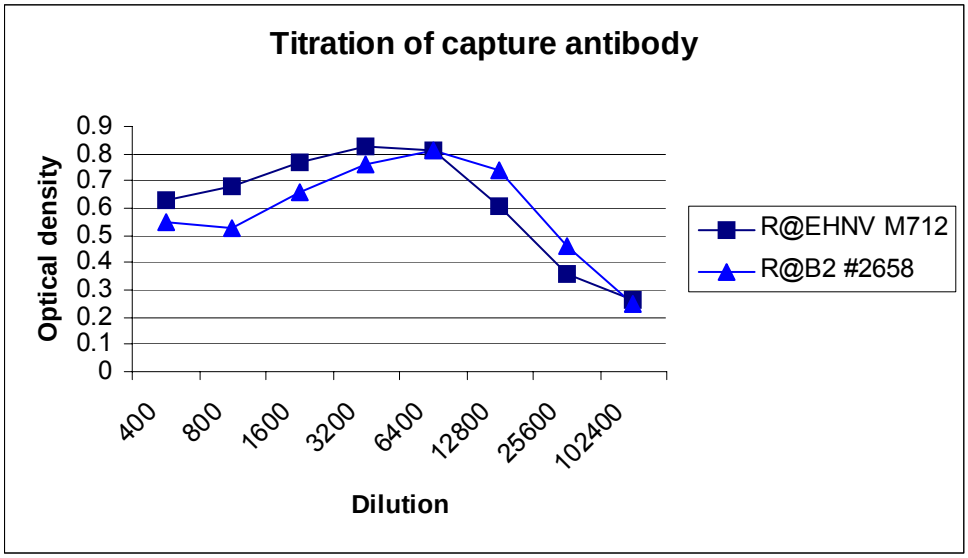
Serum was pooled from each immune bleed from all rabbits to make a total pool of approximately 530mL. Aliquots of 4mL were stored at –20°C (Lot No. M622). Affinity purification of rabbit serum IgG was performed on a 5 ml bed volume column containing protein A-sepharose (Pharmacia). Serum was thawed and centrifuged at 3000g for 10min to pellet debris. The supernatant was diluted 1:2 in starting buffer (25mM Tris-HCl, pH 7.2, 0.15M NaCl, 0.01% sodium azide), a maximum of 4mL was loaded onto the column and washed with 25mL of starting buffer at a flow rate of 60mL/hr. Immunoglobulins (Ig) were eluted with 30mL elution buffer (0.1M glycine-HCl, pH3.0, 0.15M NaCl) in 2.5mL fractions and immediately neutralised with 35µL of 2M Tris buffer. Fractions were pooled and dialysed overnight against starting buffer, and then concentrated by dialysis against carboxymethylcellulose (Aquacide II, Calbiochem) to the original volume of serum loaded (2mL). This method was repeated until a pool of 100mL of purified Ig was collected. The purity was confirmed by SDS-PAGE. Only trace amounts of serum protein contamination were revealed. These amounts were similar to those in the previous batch of this reagent which was prepared in 1990 (Figure 6). Aliquots of 1mL were freeze-dried, labeled Rabbit anti-EHNV Affinity Purified and stored at 4°C (Lot No. M708).

*Figure 6. SDS-PAGE analysis of affinity purified rabbit immunoglobulins. Lane 1, the batch prepared in 2004 (Lot No. M708); lane 2, the previous batch prepared in 1990 (*RxB2*), lane 3 molecular size markers (kDa). The proteins are heavily loaded to reveal serum contaminants and for this reason the H and L chains do not run to predicted molecular size.*



A titration in ELISA of the new batch of affinity purified rabbit immunoglobulin in comparison with the previous batch confirmed that the new batch had a similar activity (Figure 7).

Figure 7. Titration of capture antibody
A comparison of the specific reactivity of an older standard reagent (R@B2 Lot No. #2658) and the new affinity purified rabbit anti-EHNV reagent (Lot No. M712 prepared as a working stock from Lot No. M708) when used as capture antibody in ELISA to detect EHNV antigen. EHNV antigen was used at a dilution of 1:200.



1H. Preparation of a pool of ruminant anti-EHNV antisera

Sera from immune bleed 3 (IB3) from both sheep were pooled and mixed well (a total of 280mL). Aliquots of 1mL (x100) were freeze-dried, labeled Sheep anti-EHNV and stored at 4°C (Lot No. M716). The remaining serum was stored in 10mL aliquots at –20°C, and labeled as above.

Preliminary titrations of sheep anti-EHNV Lot No. M726, which was prepared as a working stock from Lot No. M716, confirmed that the reagent was likely to be useful in ELISA at dilutions ranging from 1:8000 to 1:64,000, with further titrations required.

1I. Creation of a new control EHNV antigen for ELISA

Two control antigens were evaluated in this study as replacements for a gold standard antigen which has been stored at –80°C since 1996:

a. Lot No. M729, a double heat inactivated EHNV culture supernatant
b. Lot No. 86/8774-4-5-01, which is live and has to be heat inactivated immediately prior to use.

Titration data for these antigens are provided in Tables 2 and 3. The assay was performed with new batches of antibody reagents (rabbit anti-EHNV Lot No. M712 at a dilution of 1:12,800; sheep anti-EHNV Lot No. M726 at a dilution of 1:32,000; KPL conjugate Lot No. M761 at a dilution of 1:1500).

The gold standard antigen results for these titrations were as follows:
B 1.21 D 0.49 F 0.16

Table 2. Titration data for control antigen Lot No. 86/8774-4-5-01

Dilution series A	OD	Dilution series B	OD
5	**1.66**	50	1.26
10	1.74	100	0.80
20	1.64	**200**	**0.51**
40	**1.16**	400	0.29
80	0.90	800	0.17
160	0.49	1600	0.12
320	0.33	**3200**	**0.10**
640	0.19	6400	0.09

Table 3 Titration data for control antigen Lot No. M729

Dilution series A	OD	Dilution series B	OD
5	**1.89**	50	1.59
10	1.87	100	1.16
20	1.75	200	0.82
40	1.52	400	0.46
80	1.11	800	0.25
160	0.70	1600	0.15
320	**0.48**	**3200**	**0.13**
640	0.24	6400	0.10

The recommended dilutions for the above antigens as secondary gold standards are highlighted in bold in the tables. A dilution of 1:5 of each antigen is suggested to represent the highest signal in the assay.

1J. Definition of a new protocol for EHNV ELISA using appropriate dilutions for the new reagents

Titrations were undertaken with the new reagents. The recommended protocol for detection of EHNV by ELISA is provided at Appendix 3.10. The OIE Reference Laboratory will continue to improve this protocol and provide mean and standard deviation data for the control antigens.

1K. Define the working dilution of new rabbit antisera in immunohistochemistry

Sections of liver of redfin perch experimentally infected with EHNV were sectioned at 10 um and stained using rabbit anti-EHNV antibodies prepared at a range of dilutions from 1:1000 to 1:10,000. Dilutions of 1:1500 to 1:2500 appeared optimal, with good staining of focal areas of cellular degeneration and necrosis (Figures 8 and 9), and no background staining in these sections or control sections reacted with non-immune rabbit serum. The recommended protocol for immunostaining is provided in Appendix 3.11.

Figure 8.
Photomicrograph of redfin liver (MN92/995) with a large focus of cellular degeneration associated with replication of EHNV. The fish was infected experimentally. Immunoperoxidase stain, haematoxylin counterstain. The rabbit anti-EHNV reagent was used at a dilution of 1:2500. Left, low power magnification; right, high power magnification. The darker areas contain EHNV antigen.

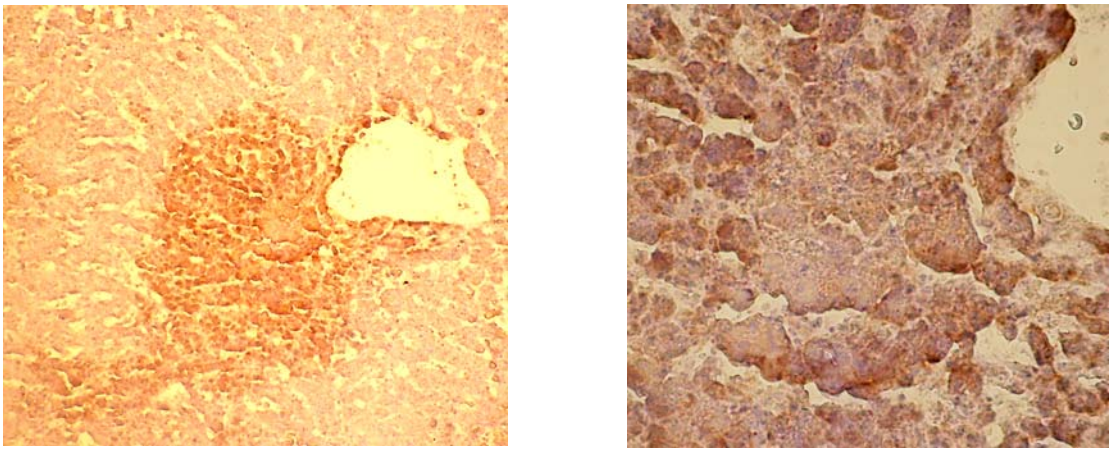
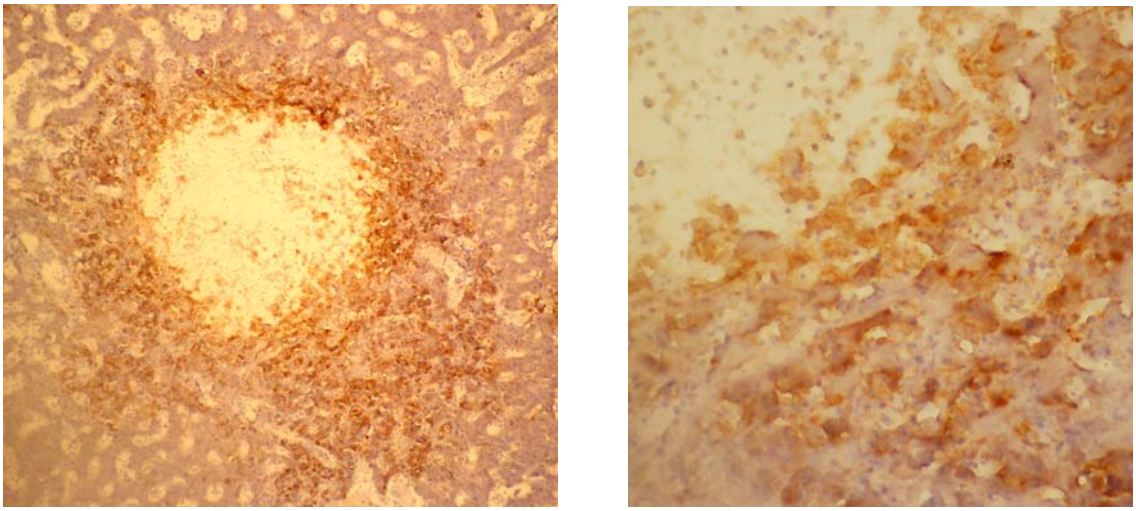


Figure 9.
Photomicrograph of redfin liver (MN91/4267) with a more advanced lesion than is present in Figure 8. There is large focus of liquefactive necrosis associated with replication of EHNV. The fish was infected experimentally. Immunoperoxidase stain, haematoxylin counterstain. The rabbit anti-EHNV reagent was used at a dilution of 1:1500. Left, low power magnification; right, high power magnification. The darker areas contain EHNV antigen.



Objective 2 To provide quality controlled DNA reagents and protocols to differentiate EHNV from related viruses including BIV

2A. Comparison of DNA extraction techniques

To prepare the EHNV and BIV DNA controls for PCR, five nucleic acid extraction techniques were assessed. These comprised three commercially available kits using membrane/filter tubes, an organic solvent extraction method and boiling (105°C for 20min) to release nucleic acids. Stock EHNV (Lot No. M577; $10^{7.8}$ $TCID_{50}$/mL) and BIV (Lot No. M594; $10^{7.4}$ $TCID_{50}$/mL) tissue culture supernatants (TCSN) stored at –20°C were used. Each extract was serially diluted in TE buffer (10mM Tris-HCL, 1mM EDTA, pH8.0) from 10^{0} to 10^{-7} and stored at –20°C before being tested in four EHNV PCR assays (Table 4). Amplification reactions in a final volume of 50µl (including 5µl DNA sample) contained 250ng of each working primer, 200µM of each of the nucleotides dATP, dTTP, dGTP and dCTP, 10 x PCR buffer (66.6mM Tris-HCl, 16.6mM $(NH_4)_2SO_4$, 2.5mM $MgCl_2$, 1.65 mg/mL bovine serum albumin, 10mM beta-mercaptoethanol) and 2U Taq polymerase. Two negative controls were included, one comprising PCR cocktail only and the second containing 5µl TE buffer.

Table 4. EHNV PCR assays and primer sequences

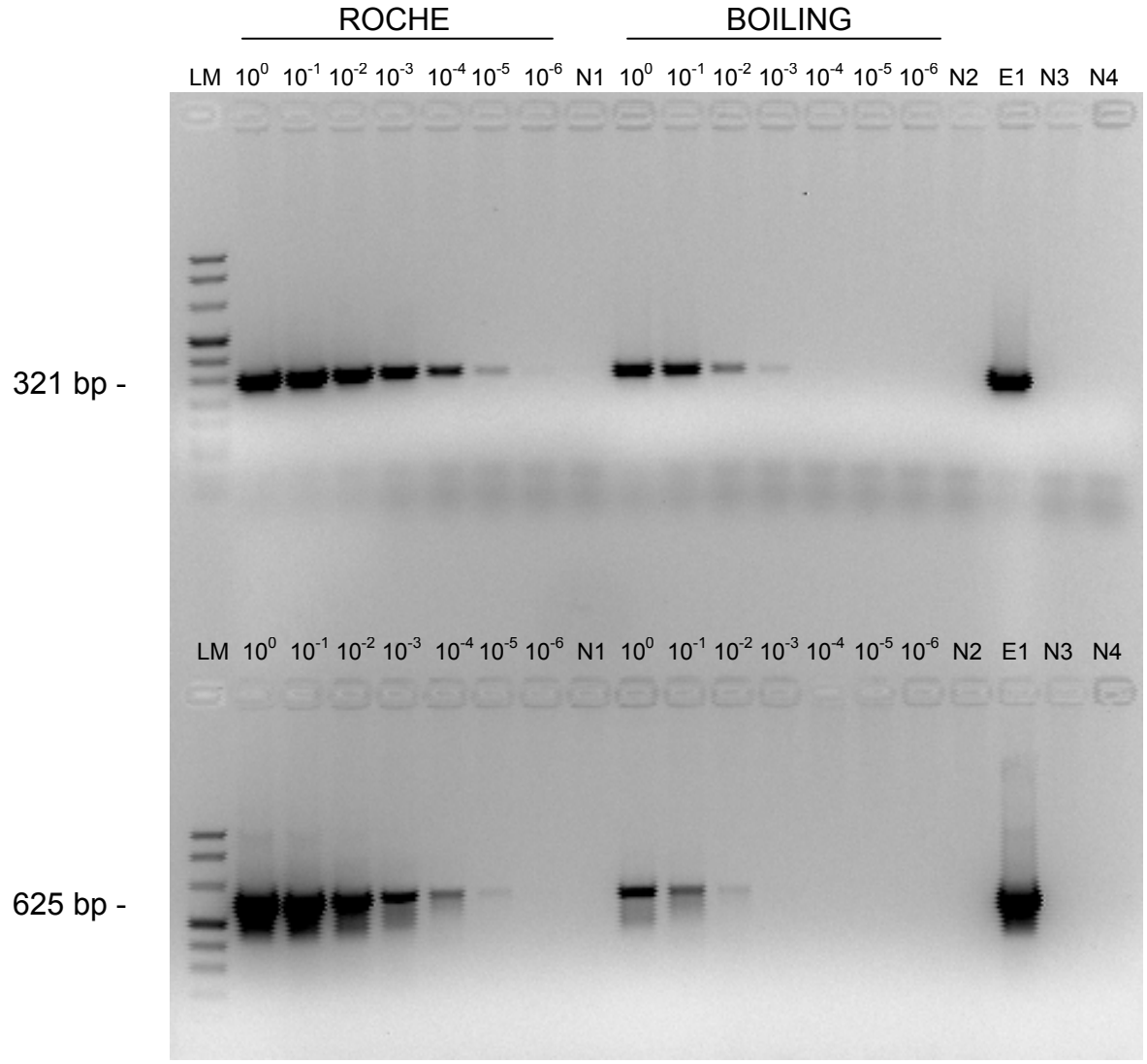
PCR assay (Name)	Primer	Sequence	Product Size	Gene Location
1 (MCP-1)	M151	AAC CCG GCT TTC GGG CAG CA	321bp	MCP 266 - 586
	M152	CGG GGC GGG GTT GAT GAG AT		
2 (MCP-2)	M153	ATG ACC GTC GCC CTC ATC AC	625bp	MCP 842 - 1466
	M154	CCA TCG AGC CGT TCA TGA TG		
3	MCP-5	CGC AGT CAA GGC CTT GAT GT	586bp	MCP 900 - 1484
	MCP-6^R	AAA GAC CCG TTT TGC AGC AAA C		
4	P505	GAT CCA CAC GGC CTG ACA CCG	200bp	Unknown open reading frame
	P506	GAT CCG AAA GAC AGC GGT CGA		

PCR Assays 1 & 2: Marsh *et al*. (2002) Mol. Cell. Probes 16:137-151
PCR Assays 3 & 4: Hyatt *et al*. (2000) Arch. Virol. 145:301-331

Reaction conditions for PCR assays 1 and 2 included an initial denaturation cycle at 94°C for 3min, followed by 35 cycles of 94°C for 30s, 50°C for 30s and 72°C for 1min, and a final extension of 72°C for 5min. PCR assays 3 and 4 had cycle times of 94°C for 1min, 50°C for 2min and 72°C for 2min for 25 cycles followed by an extension at 72°C for 15min. Products were run on 2% (w/v) agarose gels stained with ethidium bromide and the results from each extraction method were analysed for sensitivity across all four PCR assays for both EHNV and BIV.

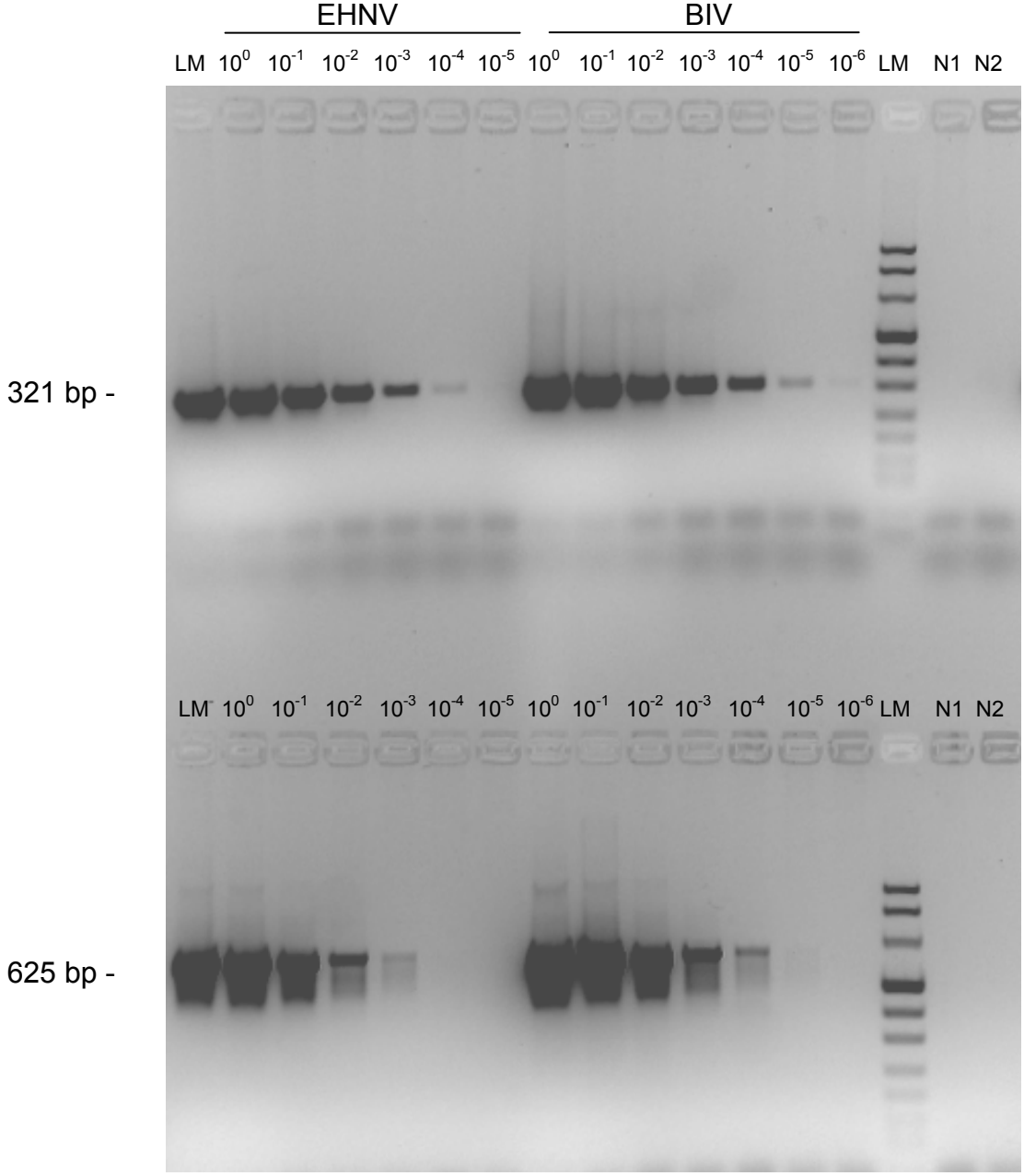
The Roche High Pure Viral Nucleic Acid Kit produced DNA samples that reacted in all four PCR assays at a higher dilution than extracts prepared using the other methods (Appendix 3.3). Experiments using BIV showed the same increase in sensitivity with the Roche method compared to boiling (Figure 10).

Figure 10.
Results of BIV DNA extraction using the Roche kit and boiling. Amplified product from PCR Assay 1 is shown in the top lanes, PCR Assay 2 in the bottom lanes. LM = lane marker, N1 and N2 = extraction negatives (TE buffer), N3 = PCR negative (TE buffer), N4 = PCR mix control, E1 = EHNV control (Roche extract).



Further experiments were performed to test the option of freeze-drying DNA extract, live virus and heat killed virus samples for use as DNA controls for PCR. Freeze-dried virus was reconstituted and extracted through boiling or with a Roche Kit before being tested in PCR assay 1. Freeze-dried EHNV and BIV DNA was reconstituted and tested in all four PCR assays. Results are summarised in Appendix 3.4 and shown in Figure 11. Freeze-drying of DNA extracted with the Roche method had no effect on the level of sensitivity. The whole viral preparations also functioned as suitable controls but were less convenient than the freeze dried DNA extract, which required no additional treatment after reconstitution.

Figure 11.
Amplified product from EHNV and BIV Freeze-dried DNA tested in PCR Assay 1 (shown on the top lanes) and PCR Assay 2 (bottom lanes). LM = lane marker, N1 = PCR negative (TE buffer), N2 = PCR mix control.



2B. Large scale preparation of EHNV and BIV DNA samples as PCR controls

The ROCHE High Pure Viral Nucleic Acid Kit was chosen to extract DNA which would be used as a PCR control. Bulk extractions were performed for each virus on separate days to ensure that no cross contamination occurred. As before, stock EHNV (Lot No. M577) and BIV (Lot No. M594) TCSN stored at –20°C were used. DNA was extracted according to the manufacturer's instructions. Briefly, the viral suspension (neat TCSN) is incubated with Proteinase K, detergent and chaotropic salt to lyse the virus. The released nucleic acids bind selectively to glass fibre fleece in a filter centrifuge tube and remain bound during a series of wash and spin steps to remove contaminants such as cellular components. Nucleic acids are then eluted from the glass fleece with a low salt solution and collected in a centrifuge tube for storage at –20°C. Extracts were pooled for each virus, mixed thoroughly then freeze-dried in 50µL aliquots and stored at 4°C (100 x BIV PCR Control Lot No. M 709; 200 x EHNV PCR Control Lot No. M710).

2C. Evaluation of Control DNA in PCR

One vial each of EHNV and BIV PCR Control DNA was reconstituted in 500µL TE Buffer (10^{-1}) and allowed to stand for 5min at room temperature, before the contents were transferred to a screw cap vial. Each control was serially diluted 10-fold in TE buffer from 10^{-1} to 10^{-7} and stored at –20°C before being tested in the four EHNV PCR assays. Results are shown in Table 5.

Table 5: EHNV and BIV PCR Control results

Final dilution relative to original DNA sample	**PCR Assay 1**		**PCR Assay 2**		**PCR Assay 3**		**PCR Assay 4**	
	EHNV	BIV	EHNV	BIV	EHNV	BIV	EHNV	BIV
10^{-1}	+	+	+	+	+	+	+	+
10^{-2}	+	+	+	+	+	+	+	+
10^{-3}	+	+	T	T	T	T	T	T
10^{-4}	T	T	-	-	-	-	-	-
10^{-5}	-	-	-	-	-	-	-	-
10^{-6}	-	-	-	-	-	-	-	-
10^{-7}	-	-	-	-	-	-	-	-
TE Control	-	-	-	-	-	-	-	-

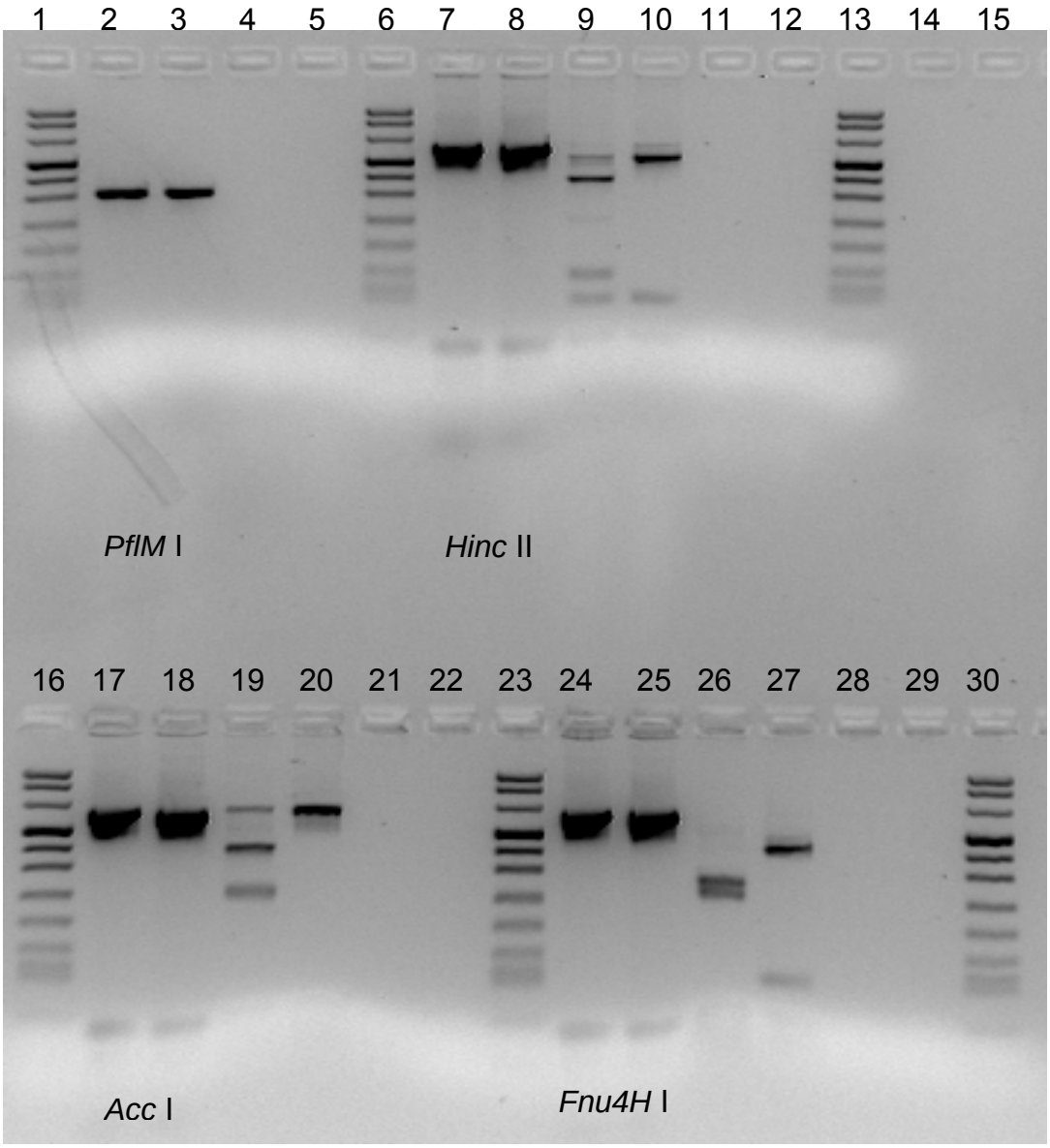
A dilution of 10^{-2} was chosen as a working control as it was the highest dilution that produced a strong band across all four PCR assays. This working control was then freeze/thawed at least four times and tested each time in PCR. It consistently produced a strong band suggesting it is robust and appropriate for use as a control. Compared to the initial experiment a 10-fold decrease in apparent DNA concentration or analytical sensitivity occurred for both EHNV and BIV controls across all four assays. This is likely to be due to the extractions being performed at factory scale before pooling, or to the change in freeze-drying vials (from heat sealed tubes to stoppered ampoules).

EHNV and BIV PCR product from PCR assay 1 and 2 was subjected to Restriction Enzyme Analysis (REA) with the enzymes described in Table 6 below (taken from Marsh *et al*, 2002). All endonucleases were used according to manufacturers' instructions. REA reactions were prepared by adding 5µL of PCR product, 2U of the appropriate restriction endonuclease, 1.6µL of buffer (supplied with restriction endonuclease), 1.6µL of 100µg/mL bovine serum albumin (for *PflM* I and *Hinc* II) and made up to a final volume of 16µL with sterile purified water. Restriction digests were incubated for 2-4h at the recommended temperatures and assessed by agarose gel electrophoresis in 3% gels. The resulting patterns confirmed the identity of each virus (Figure 12). The recommended protocol for rapid identification of EHNV and differentiation from related viruses using PCR-REA is provided in Appendix 3.12.

Table 6: REA Patterns for EHNV and BIV

PCR Assay	Restriction Enzyme	Predicted Band Sizes (bp)	Virus
1 (321bp)	*PflM* I	321	EHNV, BIV
2 (625bp)	*Hinc* II	100, 138, 387	EHNV
		100, 525	BIV
	Acc I	238, 387	EHNV
		625	BIV
	Fnu4H I	33, 38, 44, 239, 271	EHNV
		3, 33, 38, 44, 108, 399	BIV

Figure 12. Differentiation of EHNV and BIV. PCR product from EHNV and BIV freeze-dried DNA PCR control was digested with the enzymes shown. For the PflM I digest, the samples are in the following order EHNV, BIV, N1 and N2. For all other enzyme digests, the samples are EHNV-uncut, BIV-uncut, EHNV, BIV, N1 and N2. Lanes 1, 6, 13, 16, 23 and 30 contain the molecular size marker. Lanes 4,11,21 and 28 are negative controls (N1) containing REA cocktail and MQW only. Lanes 5,12,22 and 29 are negative controls (N2) containing REA cocktail only.



The freeze dried EHNV and BIV DNA PCR controls were also screened with two PCR assays (major capsid protein gene and the ATPase gene) for an unrelated Iridovirus (red sea bream iridovirus) (RSIV) with negative results.

PCR assays 1 and 2 were tested with a range of EHNV and BIV isolates in the collection at the University of Sydney (Table 7). All isolates gave positive results in both assays.

Table 7. PCR results obtained for EHNV and BIV isolates tested with PCR assay 1 and 2

Sample Accession No.	Virus Isolate	Species	PCR results	
			Assay 1	Assay 2
93/0035	BIV	Frog	+	+
93/0049	EHNV	Rainbow Trout	+	+
93/0050	EHNV	Redfin Perch	+	+
93/0051	EHNV	Rainbow Trout	+	+
93/0052	EHNV	Redfin Perch	+	+
93/0090	EHNV	Redfin Perch	+	+
93/0091	EHNV	Rainbow Trout	+	+
96/2476	EHNV	Rainbow Trout	+	+
96/0296	EHNV	Rainbow Trout	+	+

Objective 3 To provide stocks of reference-strain EHNV and fish tissues containing reference-strain EHNV as controls for diagnostic testing

3A. Preparation of batches of EHNV and BIV in BF2 cells

Heat inactivation methods were evaluated. Langdon (1989) has previously reported that EHNV is inactivated by heating at 40°C for 24 hrs or 60°C for 15 min. EHNV TCSN was placed in either 1.5 ml screw capped vials, 175cm^2 tissue culture flasks or 50 ml centrifuge tubes then exposed to temperatures ranging from 55° to 70°C for between 10 mins and 2 hrs using either a heating block or hybridisation as appropriate for each type of vessel (Appendix 3.5). Viral inactivation was unreliable when EHNV TCSN was placed in 1.5 ml vials in a heating block, probably because droplets inside the lid were not always exposed to an appropriate temperature for long enough as the lid protruded slightly from the block. In one experiment EHNV TCSN placed in a 175cm^2 flask survived heating at 65°C for 15 mins in a hybridisation oven. It was possible that virus survived in dried droplets on the wall of this large vessel. For routine work it is recommended that EHNV TCSN be inactivated by placing in a small sealed tube (1.5 – 50 ml) with minimal air space and heating at 65°C for 15 min in an oven or similar device where the entire tube is exposed to heat.

EHNV strain X852 (derived from 86/8774 by culture in 1988) was recovered from storage at –80°C, grown in BF2 cells at 22°C, aliquoted in 1 ml volumes, titrated using a limiting dilution method ($TCID_{50}$ $10^{7.3}$/ml), titrated in ELISA (see objective 1 for the results) and then:

a. Double heat inactivated, 1 ml x 183 vials and stored at –20°C in preparation for freeze drying (Lot No. M729).
b. Stored at –20°C, 1 ml x 33 vials (Lot No. M769)
c. Stored at –80°C, 1 ml x 33 vials (Lot No. M769)

BIV strain ME93/35 (derived from an isolated supplied by James Cook University in 1993) was recovered from storage at –80°C, grown in BF2 cells at 22°C, aliquoted in 1 ml volumes, titrated using a limiting dilution method ($TCID_{50}$ $10^{7.4}$/ml) and then:

a. Stored at –20°C, 1 ml x 50 vials (Lot No. M594)
b. Stored at –80°C, 1 ml x 25 vials (Lot No. M594)

3B. Formalin-fixed paraffin embedded tissue controls for immunohistochemistry

As outlined in the methods redfin perch were relatively refractory to infection and paraffin blocks from previous experimental studies of EHNV infection in redfin perch were sourced from regional veterinary laboratories and secured. Multiple sections were cut from each block, floated onto Super Frost Plus G/Edge glass slides and stored at room temperature.

Objective 4 To develop and assess new storage conditions, guidelines for reconstitution and shelf life for antibody and DNA reagents

4A. Determination of the shelf life of rabbit and sheep antisera stored in various ways

Within the 18 month time frame of this project it was clearly not possible to determine the shelf life of new reagents that became available towards the end of the period. Consequently their likely shelf life was determined by inference from near-identical reagents produced in the past. To define the minimum shelf-life of the new batches of antibodies, existing sample vials of rabbit and sheep anti-EHNV antibodies, which had been stored under various conditions since production in the late 1980's and early 1990's, were diluted and/or reconstituted and the activity of each preparation was compared by ELISA. The results are shown in Appendix 3.6.

There was no decrease in activity of these reagents over a number of years. From these results, the minimum shelf-life for affinity purified rabbit anti-EHNV antibodies stored either freeze dried at 4°C, frozen at –20°C or diluted 1:10 in TSGM and kept at –20°C is at least 4 years. Sheep anti-EHNV antisera will remain active for at least 15 years when stored frozen at –20°C or when diluted 1:10 in TSGM and kept at –20°C, and for at least 4 years when freeze dried and stored at 4°C. Note that these figures are minimal, and are limited by the period of observation. The oldest purified rabbit immunoglobulins available were 4 years old, and their real shelf life is predicted to be similar to the sheep antibodies.

4B. Lyophilisation and QC testing of new anti-EHNV antibody reagents

Affinity purified rabbit anti-EHNV was freeze-dried in 100 x 1mL aliquots (Lot No. M708) while sheep anti-EHNV antiserum pool was freeze-dried in 100 x 1mL aliquots (Lot No. M716). Both freeze dried ELISA reagents were reconstituted in sterile purified water and stored at –20°C in a screw-cap vial. Both were diluted 1 in 10 in the cryoprotectant TSGM and stored at –20°C. These reagents were tested by ELISA after reconstitution and no decrease in activity was shown.

4C. Tolerance of ELISA reagents to temperature changes

To simulate a variety of transport scenarios and determine the resistance to temperature changes, all ELISA reagents (antibody and antigen) were subjected to a series of temperature cycles (22°C to 60°C) and exposure times (24hrs to 72hrs).

Sheep and rabbit antibodies, previously stored at –20°C in the cryoprotectant TSGM, were exposed to temperatures of 22°C, 40°C or 50°C for 72hrs. Freeze-dried preparations of sheep anti-EHNV and affinity purified rabbit anti-EHNV antibodies were placed at 40°C or 50°C for 72hrs, reconstituted in sterile purified

water, diluted with cryoprotectant and stored at –20°C. The activity of each preparation was then checked by ELISA. The results are summarised in Appendix 3.7. There was no apparent reduction in the activity of either the rabbit or the sheep anti-EHNV reagents when subjected to these temperature regimes, indicating that the reagents can be shipped with confidence using standard packaging. The freeze dried reagents are suitable for shipment in the postal system.

EHNV antigens (live and heat inactivated) taken from storage at –20°C or –80°C were exposed to temperatures of 22°C, 37°C or 60°C for 24hrs and 72hrs. Similarly, freeze-dried antigens (both live and heat inactivated) were subjected to the above conditions (excluding 22°C) and then reconstituted in sterile purified water. The viability of antigens was checked by culture in BF-2 cells while antigenicity was checked by ELISA. The results are summarised in Appendix 3.8. There was no apparent change in antigenicity when live EHNV antigen taken from storage at either –20°C or –80°C was then held at 22°C, 37°C or 60°C for up to 72 hours. There was a slight decrease in antigenicity of heat killed virus subjected to the same treatments. Both heat killed lyophilised and live lyophilised EHNV antigens lost some antigenicity when held in glass vials at 37°C or 60°C for 72 hours prior to reconstitution and testing, but remained highly antigenic if held at these temperatures for only 24 hours. Note that freeze dried live EHNV was not inactivated by heating at 60°C. The results suggest that heat inactivated EHNV antigen needs to be protected from further heating during transport.

Supplement to Objectives 1 to 4. Quality control assessment of ELISA and PCR tests at another laboratory

One of the milestones for this project was to have the quality control of the ELISA and PCR reagents confirmed at another laboratory. Dr Mark Crane at the Australian Animal Health Laboratory agreed to undertake the check testing. Reagents were supplied in June 2004 (Table 8) together with draft protocols.

Dr Crane provided a comprehensive report (Appendix 3.9) and stated that the tests yielded expected results and that the protocol was concise and easy to follow. Several suggestions were made for improvement of the protocol and these were incorporated into the final protocol.

Table 8. Reagents supplied to AAHL for check testing

Reagent	Amount	Lot No.	Storage
Rabbit anti-EHNV Affinity Purified Freeze-dried	1mL	M708	4° C
Sheep anti-EHNV Freeze-dried	1mL	M716	4° C
KPL Conjugate 1:10	100µl	3208	-20° C
Antigen EHNV Heat Inactivated	1mL	M729	-20° C
Dynatech Immulon ELISA plates	2		ambient
EHNV Purified DNA PCR Control Freeze-dried	0.5mL	M710	4° C
BIV Purified DNA PCR Control Freeze-dried	0.5mL	M709	4° C
Primer M151	100µl	P90	-20° C
Primer M152	100µl	P91	-20° C
Primer M153	100µl	P92	-20° C
Primer M154	100µl	P93	-20° C

Benefits and adoption

International trade

Exports. EHNV is regarded by OIE as a highly significant pathogen of finfish and as such Australia must ensure that fish and fish products exported to countries without EHNV (all other countries) do not pose a risk. Risk analysis based on sound scientific data, and in some cases, laboratory testing of batches of product is undertaken to show that EHNV is not present. The availability of laboratory reagents and test protocols is essential to underpin international trade. Annual reporting of the activities of the OIE Reference Laboratory, including the preparation of the latest reagents and standardised protocols provides overseas trading partners with the confidence that Australia is highly technically competent. There is national benefit.

Imports. Australia is free of European Sheatfish Virus and European Catfish Virus. These viruses are very closely related to EHNV but may have different host preferences. These viruses are highly virulent for catfish species. It is important that we have local capability to distinguish these viruses from EHNV so that a rapid response can be mounted should they be detected here. Furthermore, supply of reagents to identify these viruses in overseas laboratories enables overseas authorities to manage their industries so that product exported to Australia does not pose a risk. Annual reporting of the activities of the OIE Reference Laboratory, including the preparation of the latest reagents and standardised protocols is important in trade negotiations when Australia has concerns about the risk posed to domestic industries through animal disease introduction. Australia can demonstrate with confidence that it is highly technically competent and aware of its own disease status. Again there is national benefit.

Domestic trade

Within Australia there is uneven distribution of EHNV so that states may require testing prior to entry of finfish or fish products. For example Western Australia conducts routine testing of salmonids on WA farms and requires entry testing of salmonids from the eastern states.

The research outcomes from this project, specifically new reagents and standardised protocols will be adopted by aquatic animal health laboratories nationally.

Further Development

The OIE Reference Laboratory for EHNV will continue to conduct quality control checks of the new reagents and work to update and improve tests on an ongoing basis. There may be opportunities to develop kits for detection of EHNV.

Planned outcomes

Describe how the project's outputs will contribute to the planned outcomes identified in the application. The planned outcomes for this project were to provide:

1. A stock of quality-controlled antibody reagents and protocols to detect EHNV
2. A stock of quality controlled DNA reagents and protocols to differentiate EHNV from related viruses including BIV
3. A stock of reference-strain EHNV and fish tissues containing reference-strain EHNV as controls for diagnostic testing
4. Protocols for preparation of reagents using new storage conditions, guidelines for reconstitution and shelf life data for antibody and DNA reagents

These outcomes have been achieved within the life of the project and are of direct benefit to fish farmers by enabling diagnostic and certification testing for EHNV and indirect benefit to the entire Australian aquaculture industry by demonstrating technical and operational competencies in fish disease diagnosis to the international community, with whom we trade.

Conclusion

Reagents and protocols for the detection of EHNV using the latest technology in ELISA, immunohistochemistry and molecular biology have been prepared. The reagents and protocols have been evaluated at an independent laboratory and are now available to laboratories in Australia and internationally. EHNV is a very serious pathogen. Consequently the reagents have been prepared using a new approach which will facilitate easy shipment in a stable form with no biosecurity risk. This is important in the current era of bioterrorism. Stability and shelf life of the reagents can also now be specified.

The planned outcomes of the project were a stock of quality-controlled antibody reagents and protocols to detect EHNV, a stock of quality controlled DNA reagents and protocols to differentiate EHNV from related viruses including BIV, a stock of reference-strain EHNV and fish tissues containing reference-strain EHNV as controls for diagnostic testing and protocols for preparation of reagents using new storage conditions, guidelines for reconstitution and shelf life data for antibody and DNA reagents. These planned outcomes have been achieved within the life of the project and are of direct benefit to fish farmers by enabling diagnostic and certification testing for EHNV and indirect benefit to the entire Australian aquaculture industry by demonstrating technical and operational competencies in fish disease diagnosis to the international community, with whom we trade.

The outputs of the project, being new reagents available to other laboratories on cost recovery basis and use of these reagents to diagnose and certify Australian fish stocks with respect to infection with EHNV and to maintain international market access for Australian fish products, require communication with a target audience being primarily the wider scientific community and the aquatic animal health laboratory service. Therefore, publication of the techniques and description of the quality of the reagents produced is required. This is so that they can be used elsewhere with confidence and also to enable laboratories in Australia to carry out testing using the reagents so that trade in fish can continue. The communication methods will include publication of protocols in technical manuals including those available electronically through OIE. Relationships with end users already exist and will be promoted through the OIE Fish Diseases Commission. The activities of the

OIE Reference Laboratory for EHNV are reported annually and published internationally by OIE in the Annual Report of OIE Reference Laboratories and the OIE Manual of Diagnostic Tests for Aquatic Animals. After completion of the project, one scientific paper will be developed to summarise the methods available for detection of EHNV.

References

Gould AR, Hyatt AD, Hengstberger SH, Whittington RJ and Coupar BEH (1995). A polymerase chain reaction (PCR) to detect epizootic haematopoietic necrosis virus and Bohle iridovirus. Diseases of Aquatic Organisms 22: 211-215.

Hyatt AD, Gould AR, Zupanovic Z, Cunningham AA, Hengstberger S, Whittington RJ, Kattenbelt J and Coupar BEH (2000). Comparative studies of piscine and amphibian iridoviruses. Archives of Virology 145: 301- 331.

Langdon JS (1989). Experimental transmission and pathogenicity of epizootic haematopoietic necrosis virus (EHNV) in redfin perch, *Perca fluviatilis* L., and 11 other teleosts. Journal of Fish Diseases 12: 295-310.

Marsh IB, Whittington RJ, O'Rourke B, Hyatt AD and Chisholm O (2002). Rapid differentiation of Australian, European and American ranaviruses based on variation in major capsid protein sequence. Molecular and Cellular Probes 16: 137-151.

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 (1992). Evaluation of a simple method for improving the precision of an ELISA detecting antibody in serum. Journal of Immunological Methods 148: 57-64.

Whittington RJ and Reddacliff GL (1995). Influence of environmental temperature on experimental infection of redfin perch (*Perca fluviatilis*) and rainbow trout (*Oncorhynchus mykiss*) with epizootic haematopoietic necrosis virus, an Australian iridovirus. Australian Veterinary Journal 72: 421-424.

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.

Appendix 1

Intellectual Property

Modified methods for growth and purification of EHNV were developed together with new and validated methods for preparation and storage of reagents. These were improvements to existing art but do not warrant patent protection. The combination of reagents and know-how could be combined into diagnostic test kits if a manufacturer could be identified and licensed to do so.

Appendix 2

Staff

The staff who were engaged on or associated with the project include:

Professor Richard Whittington

Ms Kylie Deece, Technical Officer

Mrs Anna Waldron, Laboratory Manager

Ms Natalie Schiller, Technical Officer

Mr Craig Kristo, Senior Technical Officer

Mr Matt Van Dyke, Technical Officer

Mr Jiri Tasler, Technical Officer

Dr Robert Dixon, Sub Dean Animal Welfare

Mr Tho Nguyen, MVSc student

APPENDIX 3.1

Recommended EHNV Purification Method

Note: g forces in this method are based on average radius of rotor.

1. One NUNC factory was seeded with BF-2 cells, incubated and inoculated as for preparation of stock cultures (Inoculum = 18ml, Vol MEM = 1800ml/factory).

2. The lysed cells and virus were harvested at 12,000g for 30 min at 4°C (8,400rpm Beckman J2-HS centrifuge with JA-10 rotor).

3. The supernatant (SN1) was stored at 4°C while the pellet was resuspended in a small volume (1-5 ml) of SN1 and subjected to three rapid freeze/thaw cycles using liquid nitrogen and a 37°C water bath, followed by ultrasonication for three 1-minute cycles at 4°C.

4. The suspension was then centrifuged at 6,500g for 15 min at 4°C (7,400rpm Beckman J2-HS Centrifuge with JA-20 rotor).

5. This supernatant (SN2) was pooled with SN1 and stored overnight at 4°C, while the pellet was discarded. **Safe stop overnight.**

6. Virus was pelleted from the pooled SN by centrifugation at 10,000 g for 8hrs* at 4°C (7,600rpm Beckman J2-HS Centrifuge with KA-10 rotor), resuspended in 2 ml total volume of 100mM Tris-HCl pH 8.6 and stored overnight at 4°C. **Safe stop overnight.** *To overcome clumping which occurs if a shorter high speed spin is used to pellet the virus (eg 45,000 g for 45 min).

7. A volume of 270µl of the suspension was loaded onto ten preformed discontinuous gradients of 15-60% sucrose (in 100mM Tris-HCl, pH 8.6, 2.5ml of each sucrose conc, approx 1.6ml needed to fill tube ie 270µl sample + 1330µl Tris-HCl), and centrifuged at 150,000g for 45 min at 4°C. Note: 45 min is sufficient for the virus to equilibrate, there was no change in the location of the resulting bands after 2 hr spin. Slow deceleration also has no effect (34,000rpm Beckman Optima™L-90K with SW 41 Ti rotor).

8. Two bands are usually obtained. Band 1 = upper fuzzy band (faint) just below middle of the tube (38% sucrose). Band 2 = lower discrete band (50% sucrose) which contained purified virus. Bands were harvested separately by aspiration with a 21 G needle and 2 ml syringe, diluted in 100mM Tris-HCl, pH 8.6, until the concentration of sucrose was about 10%, and then pelleted through a 5ml cushion of 20% sucrose at 80,000 g for 1hr at 4°C (25,000rpm Beckman Optima™L-90K with SW 41 Ti rotor).

9. Pellets were resuspended in 200 ul of 100mM Tris-HCl, pH 8.6, and stored at 4°C in sterile 2.0ml screw cap tubes.

APPENDIX 3.2

Animal Inoculation and Blood Collection Details

Pre-bleed Date

- 17/12/2003 Six rabbits, three sheep and three goats; SVC# 03/032

Inoculation Dates

- 24/12/2003 Virus batch 1 was administered to six rabbits, two sheep and two goats; approximately 170µg per 2mL dose.
- 28/01/2003 Virus batches 3 and 4 were administered to six rabbits, two sheep and two goats; 170-180µg per 2mL dose.

Immune Bleed Dates

- IB1 13/02/2004 Rabbits, Sheep and Goats; SVC# 04/009 – #04/011
- IB2 25/02/2004 Sheep, Goats and Rabbits; SVC# 04/013 - #04/015
- IB3 10/03/2004 Sheep and Goats; SVC# 04/047 and #04/048; Rabbits euthanased
- IB4 21/04/2004 Goats and Sheep; SVC# 04/031 and #04/030

APPENDIX 3.3

Comparison of DNA Extraction Methods Using EHNV and BIV TCSN Across Four PCR Assays

	Extraction 1				Extraction 2				Extraction 3				Extraction 4				Extraction 5			
	QIAGEN QIAamp® DNA Mini Kit				**ROCHE** High Pure Viral Nucleic Acid Kit				**BOILING** 105°C for 20 min				**PROMEGA** Wizard® SV Genomic DNA Purification System				**Phenol/ Chloroform** Eppendorf Phase Lock Gel			
Volume TCSN used:	200µl				200µl				200µl				200µl				200µl			
Reaction No.	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
Neat EHNV	+	+	+	+	+	+	+	+	+	T	T	+	+	+	+	+	+	+	+	+
10^{-1}	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10^{-2}	+	+	+	+	+	+	+	+	+	T	T	+	+	-	+	+	+	T	+	+
10^{-3}	+	T	+	+	+	+	+	+	T	-	T	T	T	-	T	T	T	-	T	T
10^{-4}	T	-	+	T	+	-	+	+	-	-	T	T	-	-	-	-	-	-	-	-
10^{-5}	-	-	T	-	T	-	T	T	-	-	-	-	-	-	-	-	-	-	-	-
10^{-6}	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TE control	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Neat BIV					+	+			+	+										
10^{-1}					+	+			+	T										
10^{-2}					+	+			T	T										
10^{-3}					+	+			T	-										
10^{-4}					+	T			-	-										
10^{-5}					T	T			-	-										
10^{-6}					-	-			-	-										
TE control					-	-			-	-										

T = Trace result
Shading indicates technique was not tested

Reactions 1 and 2 were conducted exactly as described in the methods.
Reactions 3 and 4 were conducted using the primers described in Table 4, the buffers described in Section 2A and cycling conditions described by Hyatt *et al* (2000).

APPENDIX 3.4

Comparison of DNA Extraction Methods Using Freeze Dried EHNV and BIV

	Extraction 6				Extraction 7				Extraction 8				Extraction 9				Extraction 10					
	F.D. Live Virus Reconstituted with 200µl MQW				F.D. Heat Inact. Virus Reconstituted with 200µl MQW				F.D. Live Virus Reconstituted with 200µl MQW				F.D. Heat Inact. Virus Reconstituted with 200µl MQW				F.D. ROCHE Extract Reconstituted with 50µl MQW					
Extraction Method	BOILING 105°C for 20 min				BOILING 105°C for 20 min				ROCHE High Pure Viral Nucleic Acid Kit				ROCHE High Pure Viral Nucleic Acid Kit				NIL Used directly in PCR					
Reaction No.	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3		4	
Neat EHNV	+				+				+				+	+			+	+	+	+	+	+
10^{-1}	+				+				+				+	+			+	+	+	+	+	+
10^{-2}	+				+				+				+	+			+	+	+	+	+	+
10^{-3}	T				T				+				+	+			+	+	+	+	T	+
10^{-4}	?				-				T				T	T			+	T	T	+	-	+
10^{-5}	-				-				?				?	-			T	-	-	T	-	T
10^{-6}	-				-				-				-	-			-	-	-	-	-	-
TE control	-				-				-				-	-			-	-	-	-	-	-
Neat BIV	+				+				+				+	+			+	+	+	+	+	+
10^{-1}	+				+				+				+	+			+	+	+	+	+	+
10^{-2}	+				+				+				+	+			+	+	+	+	+	+
10^{-3}	T				T				+				+	+			+	+	+	+	T	+
10^{-4}	-				-				T				T	T			+	T	T	+	-	+
10^{-5}	-				-				?				?	-			T	-	-	T	-	T
10^{-6}	-				-				-				-	-			-	-	-	-	-	-
TE control	-				-				-				-	-			-	-	-	-	-	-

T = Trace result
Shading indicates not tested

PCR assays were performed under the conditions described for the MCP-1 and MCP-2 PCR assays (Marsh et al, 2002).

Reactions 1 and 2 were conducted exactly as described in the methods.
Reactions 3 and 4 (left column) were conducted using the primers described in Table 4, the buffers described in Section 2A and cycling conditions described by Hyatt *et al* (2000).

Reactions 3 and 4 (right column) for extraction 10 were conducted with the cycling conditions described by Marsh *et al* (2002), which improved analytical sensitivity.

APPENDIX 3.5

EHNV Heat Inactivation Trials

Comparison of temperature/time and volume/surface area using a heating block and a hybridisation oven.

		Heating block				Hybridisation oven			
Temp	**Time**	29/01[1]	02/02[1]	10/02[1]	12/02[1]	24/05[1]	28/05[2]	01/06[2]	18/06[3]
55°C	15min				+				
60°C	10min				-	-			
	15min	-			-	-			
	2hrs				+				
65°C	10min	-			+	-			
	15min	+	-	-	-	-	-	+	-
70°C	10min					-			
	15min	-			-	-			

Shading indicates technique was not tested.

[1] Trial sample was 1mL of EHNV TCSN in a 1.5mL tube.
[2] Trial sample was 100mL EHNV TCSN in a 175cm^2 TC flask.
[3] Trial sample was 50mL EHNV TCSN in a 50mL centrifuge tube.

APPENDIX 3.6

Comparison of antibody storage conditions

SAMPLE	STORAGE	ANTIGEN DILUTION*			
		1:5	1:40	1:200	1:3000
Rabbit anti-EHNV 1:6400	Freeze-dried (02/2000 4°C) from R@B2 #2657 (10/1999)	2.28	2.20	0.68	0.16
	FD trial (vial reconstituted 11/1999 –20°C 1:10) from R@B2 #2657 (10/1999)	2.54	2.17	0.72	0.18
	R@B2 #2657 (10/1999) stored neat at –20°C	2.63	2.0	0.81	0.18
	Diluted R@B2 1:10 (10/1999-20°C) from R@B2 #2657 (10/1999)	2.56	2.09	0.78	0.16
Sheep anti-EHNV (Fishface) 1:16000	Freeze-dried (02/2000 4°C) from FF 96/2 (09/1989)	2.64	2.25	1.29	0.29
	FD trial (vial reconstituted 11/1999 –20°C 1:10) from FF 96/2 (09/1989)	2.71	2.26	1.11	0.25
	FF 96/2 (09/1989) stored neat at –20°C	2.42	2.09	1.03	0.23
	Diluted FF 1:10 (11/1999 –20°C) from FF 96/2 (09/1989)	2.48	2.0	0.72	0.17

*data are ELISA OD at each antigen dilution

APPENDIX 3.7

Effect of temperature changes on rabbit and sheep antibody used in ELISA

Sample	Storage Conditions				Antigen Dilution
	0hrs	72hrs			
	Control	22°C	40°C	50°C	
Rabbit anti-EHNV in cryoprotectant from –20°C	1.42	1.57	1.50	1.53	1:40
	0.59	0.71	0.63	0.67	1:200
Sheep anti-EHNV in cryoprotectant from –20°C	As above	1.35	1.46	1.45	1:40
		0.60	0.63	0.71	1:200
Freeze-dried Rabbit anti-EHNV	As above		1.28	1.44	1:40
			0.60	0.60	1:200
Freeze-dried Sheep anti-EHNV	As above		1.43	1.45	1:40
			0.66	0.86	1:200

Shading indicates condition not tested

APPENDIX 3.8

Effect of temperature changes on EHNV ANTIGEN used in ELISA and Cell Culture

EHNV Antigen	Storage Conditions							Evaluation
	Control	22°C		37°C		60°C		
	0hrs	24hrs	72hrs	24hrs	72hrs	24hrs	72hrs	
Live -20°C	2.53	2.39	2.67	2.77	2.34	2.58	2.53	ELISA 1:40
	1.92	1.79	1.90	1.71	1.62	1.77	1.96	ELISA 1:200
	+				+			Cell Culture
Live -80°C	2.68	2.50	2.25	2.55	2.59	2.63	2.59	ELISA 1:40
	1.85	1.63	1.87	1.83	1.80	1.81	1.76	ELISA 1:200
	+				+			Cell Culture
Heat Inactivated -20°C	2.78	2.56	2.50	2.57	2.83	2.39	2.33	ELISA 1:40
	1.63	1.60	1.49	1.50	1.57	1.57	1.47	ELISA 1:200
	-							Cell Culture
Heat Inactivated -80°C	2.69	2.49	2.65	2.61	2.53	2.61	2.41	ELISA 1:40
	1.93	1.67	1.95	2.00	1.84	1.48	1.43	ELISA 1:200
	-							Cell Culture
Freeze-dried Live	2.44			2.31	2.06	2.41	1.17	ELISA 1:40
	1.58			1.34	1.53	1.30	0.39	ELISA 1:200
	+			+	+	+	+	Cell Culture
Freeze-dried Heat Inactivated	2.74			2.45	2.37	2.41	2.44	ELISA 1:40
	1.70			1.54	1.04	1.37	0.89	ELISA 1:200
	-							Cell Culture

Shading indicates condition not tested

APPENDIX 3.9

Report From Dr Mark Crane, Fish Diseases Laboratory
Australian Animal Health Laboratory Geelong
25th June 2004

FRDC PROJECT NUMBER: 2003/621

PROJECT TITLE: Aquatic animal health subprogram: development of diagnostic and reference reagents for epizootic haematopoietic necrosis virus of finfish

PRINCIPAL INVESTIGATOR: Prof. Richard Whittington, U. Sydney

Background

AAHL was nominated as a second diagnostic laboratory to undertake evaluation of reagents developed as part of FRDC Project No. 2003/621: Aquatic animal health subprogram: development of diagnostic and reference reagents for epizootic haematopoietic necrosis virus of finfish.

EHNV PCR and REA

Methods

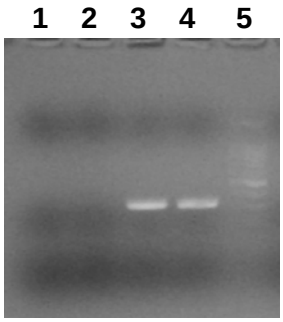
MCP-1 and MCP-2 PCRs were performed on EHNV and BIV DNA provided by Prof. Richard Whittington, University of Sydney.

In brief, in Isolation Room 2 in the South Suite, each DNA was reconstituted in 500µL of TE. A 1/10 dilution was made of each DNA sample and 2µL used for PCR analysis. Using Qiagen HotStar Taq and the primers provided, both PCRs were performed under the prescribed conditions in the diagnostic PCR suite at AAHL.

Results

i. MCP-1 PCR

EHNV and BIV were both detected in the MCP-1 PCR, yielding a 321bp product (see figure 1). Restriction enzyme analysis was **not** completed on the PCR products from MCP-1 PCR since the described enzyme (PflM I) does not distinguish ENHV and BIV.

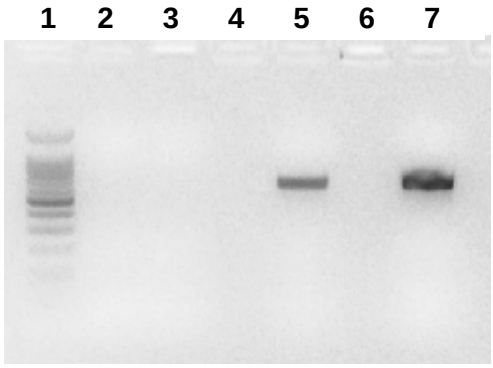


Legend
Lane 1: negative control
Lane 2: TE negative control
Lane 3: BIV DNA
Lane 4 EHNV DNA
Lane 5: 100bp Marker

FIGURE 1. PHOTOGRAPH OF A GEL DEPICTING THE AMPLICONS YIELDED BY MCP-1 PCR

ii. MCP-2 PCR

EHNV and BIV were both detected in the MCP-2 PCR, yielding a 625bp product (see figure 2). Products from the MCP-2 PCR were excised from a 2% gel and purified using the Qiagen Gel Extraction Kit. Purified PCR product was eluted in 40µL.



Legend
Lane 1: 100bp marker
Lane 2: negative control
Lane 3: TE negative control
Lane 4: Blank
Lane 5: BIV DNA
Lane 6: Blank
Lane 7: EHNV DNA

FIGURE 2: PHOTOGRAPH OF A GEL DEPICTING THE AMPLICONS YIELDED BY MCP-2 PCR

iii. REA

Both purified PCR products were digested with Acc I and Hinc II. Ten microlitres of purified PCR product was digested with each enzyme for 2 hours at 37ºC before being inactivated at 65ºC. Both digested and undigested products were run on a 3% gel (figure 3).

When digested with Acc I, EHNV MCP-2 PCR product yielded bands of 387 and 238bp (Figure 3 lane 5). The BIV MCP-2 PCR product was not cut by Acc I (figure 3 lane 6).

When digested with Hinc I, the EHNV MCP-2 PCR product yielded bands of 387, 138 and 100bp (lane 8). When the BIV MCP-2 PCR product was digested with Hinc I, the resulting bands were approximately 500bp and a 100bp (lane 9).

Conclusions

Tests yielded expected results.

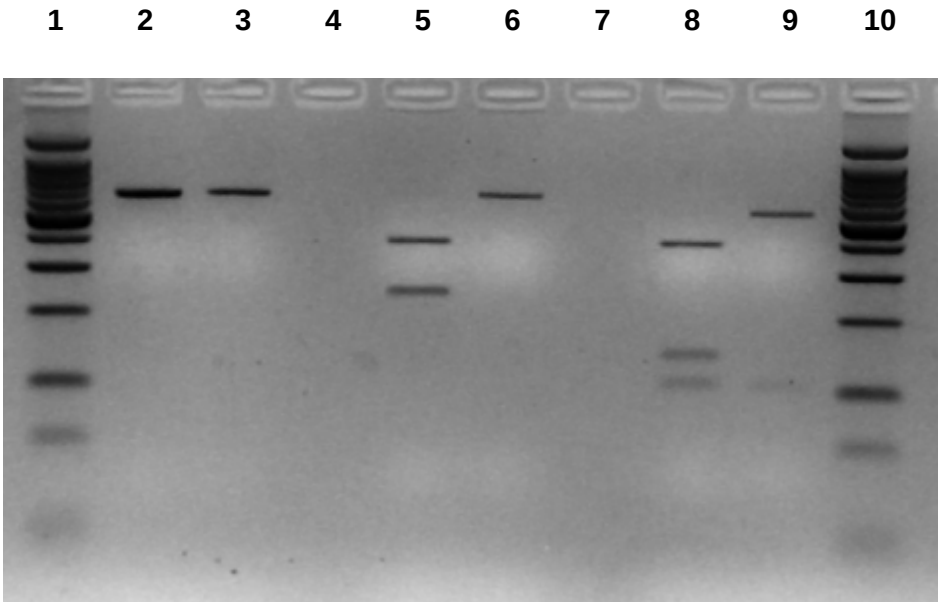


Figure 3: Restriction enzyme analysis of MCP-2 PCR Products

Legend

Lane 1 100bp Marker
Lane 2 EHNV Undigested
Lane 3 BIV Undigested
Lane 5 EHNV digested with Acc I
Lane 6 BIV digested with Acc I
Lane 8 EHNV digested with Hinc I
Lane 9 BIV digested with Hinc I
Lane 10 100bp Marker
Lane 4/7 Blank

EHNV ELISA

Methods

EHNV ELISA was performed as per protocol supplied by Prof. R. Whittington. The ELISA was performed by two of AAHL's staff members:

- Sandy Crameri, staff member of OIE Reference Laboratory for EHNV (Dr. Alex Hyatt, co-nominated expert, OIE Reference Laboratory for EHNV)
- John Young, staff member of AAHL Fish Diseases Laboratory

Current reagents held in stock at AAHL were compared with those supplied by University of Sydney, using both (i) standard dilutions used at AAHL and (ii) those dilutions requested by University of Sydney.

Results

Results are provided in the table below.

Points of note on this ELISA were:

1. The ELISA, and therefore the reagents, worked;
2. The new reagents did not give the same peak reading, but had a reading above background at the low positive dilution.
3. No attempt was made to measure non-specific binding by including another antigen.
4. The titration series were set up in quadruplicate instead of duplicate as we had plenty of reagents and plate space.
5. Two diluent control columns were set up for blanking as the diluent used in the two methods used different proteins. There is no difference between the blank columns so the data included is only blanked using the new reagent.

Operators found the protocol to be concise and easy to follow. Points for improvement are:

1. This method should include the methods for making up the reagents such as TSGM, borate coating buffer, ABTS solution or ABTS stop solution. The references do not have a method for TSGM so AAHL's TSGM was used on the assumption that it was the same as the reagent used in the current method.
2. The conjugate and substrate are commercial products, so it might be useful to list a recommended international supplier. This comment would not apply if the ELISA is supplied as a kit, with volumes supplied so a certain number of plates can be tested.
3. While use of equipment such as a plate shaker and an incubator may be deliberately avoided so the test can be conducted in less well-equipped laboratories, the introduction of a plate shaker so the incubation times can be reduced is recommended.

Table 1. ELISA tests conducted with the new reagents by two operators at AAHL

Operator A					
Dilution	Replicate 1	Replicate 2	Replicate 3	Replicate 4	**Mean A**
1:20	1.49	1.27	1.31	0.6	**1.17**
1:100	1.24	0.81	0.95	0.63	**0.91**
1:600	0.24	0.22	0.13	0.14	**0.18**
1:8000	0	0	0	0	**0.00**
Operator B					
Dilution	Replicate 1	Replicate 2	Replicate 3	Replicate 4	**Mean B**
1:20	1.21	0.76	0.65	0.79	**0.85**
1:100	0.83	0.4	0.49	0.58	**0.58**
1:600	0.36	0.13	0.14	0.12	**0.19**
1:8000	0.02	0.01	0.01	0	**0.01**

APPENDIX 3.10

Recommended Method
Detection Of EHNV Using ELISA

Principle of the test

EHNV particles are captured from the sample by an affinity purified rabbit antibody which is coated to the plate. EHNV is detected by a second antibody and a peroxidase-labelled conjugate using the chromogen ABTS. The enzyme is inactivated after 20 minutes and the resulting optical density (OD) is compared with standards.

Operating characteristics

The protocol is based on published procedures. When performed as described in this protocol, the operating characteristics of the test are as given in Table 1. The precision of the assay is <10% CV measured as variation in the OD of the controls between plates and over time when the recommended normalisation procedure is followed.

Table 1. EHNV ELISA operating characteristics

Sample	Positive-negative cut-off**	Sensitivity %	Specificity %
Tissues of fish*	OD 0.5	60	>99
Tissue culture supernatants with cytopathic effect (BF2 cells)	OD 0.3	>99	>99

* *redfin perch and rainbow trout only. Higher background OD occurs with golden perch. There are no data for other species.*

** *these cut-offs are determined by the OIE Reference Laboratory for EHNV and will vary with the batch of control antigen. Values above are for batch 86/8774-4-5-01.*

Test components and preparation of Reagents

1. Flat bottom microtitre plates (Linbro Cat # 76:381:04, ICN Flow; or Dynatech Immunolon 1 Cat # 011-010-3350; or equivalent) are required.

2. Affinity purified rabbit anti-EHNV capture antibody and sheep anti-EHNV second antibody reagents are supplied in freeze-dried form. Reconstitute using 1mL of purified water and allow the vial to stand at room temperature for 2 mins. Mix the vial very gently. These reagents are stable when stored at –20°C for at least 5 years. For routine use in ELISA it is recommended that working stocks of both antibodies be prepared as a 1:10 dilution in TSGM. These are stable at –20°C for at least 5 years and do not solidify at this temperature.

3. The conjugate (commercial reagent, KPL #14-23-06 0.5 mg) is supplied as a freeze dried powder. This reagent has displayed remarkable consistency in

activity between different lots over a period of 15 years. The product should be reconstituted in sterile 50% glycerol water, dispensed in 150µl aliquots and stored at –20°C as undiluted stock. A working stock is prepared by adding 900 µl of TSGM to 100µl 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.

4. EHNV heat inactivated antigen control is supplied as freeze dried powder. Reconstitute in 1 ml sterile water and store in small aliquots at –20°C. Prepare dilutions using PBSTG on the same day the test is performed. Control EHNV antigen dilutions (A, B, D and F) cover the range of the signal response of the assay and enabling a normalisation procedure to be undertaken.

Equipment

An automatic plate washer is recommended although plates can be washed by hand. The assay is sensitive to plate washing conditions. If OD of the controls is unexpectedly low, and the conjugate and other reagents are within date, the plate washer should be adjusted so that washing pressure during filling of wells and aspiration of wells is minimised.

An automatic plate reader is recommended although plates can be read by eye.

Precision calibrated pipettes (eg Gilson) should be used to prepare dilutions of all reagents and to load reagents into microtitre plate wells.

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 worksheet

Date:_____

Samples:_____

1. Coat a 96 well ELISA plate (100 ul/well) with affinity purified rabbit-anti-EHNV diluted 1:12,800 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 PBSTG diluent (PBS, 0.05% v/v Tween 20, 0.1% w/v gelatin)
up to 50 ml is required per plate:

10 x PBS	10 ml	Batch #_____
MQW	90 ml	
Tween 20	50 ul	
Gelatin	0.1 g	

Warm the solution in a microwave oven or water bath to dissolve the gelatin, then cool to room temperature.

4. Prepare blocking solution: 1% w/v gelatin in PBSTG
Require per plate 0.1 g gelatin in 11 ml PBSTG
____ g _____ ml

5. Block remaining binding sites using blocking solution (100 ul/well). Incubate at room temperature (RT) for 30 min.

6. Wash 5 times as above.

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

Incubate for 90 min at RT.

8. 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).

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

10. Add second antibody Sheep-anti-EHNV at 1:32,000 in PBSTG (100 ul/well).

Batch#_____ volume 1:10 stock _____ ul PBSTG volume ____ml

Incubate for 90 min at RT.

11. Wash plate 5 x on plate washer.

12. Add KPL conjugate KPL at 1:1500 in PBSTG (100 ul/well).

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

Incubate for 90 min at RT.

13. Wash plate 5 x on plate washer.

14. Add ABTS substrate (22 ml ABTS + 10 ul 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.

15. 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 of the duplicates: samples with CV >15% should be retested if the mean OD lies near the cut-off of 0.6.

Control antigens
The control antigens are dilutions of a heat killed cell culture supernatant of EHNV 86/8774. The controls are expected to give the following OD, although there will be some variation from lab to lab and ±10% variation should therefore be allowed:

Heat inactivated control antigen as supplied:

Control	Dilution in PBS*	OD*
A	1:5	>2.0
B	1:40	1.90
D	1:200	0.68
F	1:3000	0.16

**These dilutions and OD values are determined by the OIE Reference Laboratory for EHNV and will vary with the batch of control antigen. The values above are for batch 86/8774-4-5-01.*

The positive-negative cut-off for clarified tissue homogenate samples from redfin perch and rainbow trout in this ELISA is approximated by the OD value of control D on each plate.

Normalisation of data and decision limit quality control
If it is desired to normalise data from plate to plate and over time, or to undertake decision limit quality control, the following procedure can be followed.

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 (PCF) 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 which approximates a CV of 10%. 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 antigen capture ELISA. Decision-limit quality control using alternate QC variables may also be appropriate.

Buffers and other reagents

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

---

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

<u>Citrate phosphate buffer</u>

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 H_2O_2 per 22ml aliquot.

ABTS Cat no. A1888
Sigma Aldrich Pty Ltd
Sydney
Australia
Tel: 612 9841 6555
Fax: 612 9841 0500

www.sigmaaldrich.com

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.

KPL Conjugate #14-23-06

Reagent Supplier:
Bio-Mediq DPC Australia
PO Box 106
DONCASTER VIC 3108
Australia
Tel: 613 9840 2767
Fax: 613 9840 2767

Visit www.kpl.com for links to worldwide network distributors.

APPENDIX 3.11

Recommended Method
Detection of EHNV In Tissue Sections Using Immunoperoxidase Staining

The following protocol is intended for the qualitative demonstration of EHNV antigens in formalin–fixed paraffin-embedded tissue sections where antigens may have become cross-linked. It includes a protease digestion step which may be omitted if unfixed samples are examined.

A commercial kit (DAKO® LSAB K0679) with peroxidase-labelled streptavidin and a mixture of biotinylated anti-rabbit/anti-mouse/anti-goat immunoglobulins as link antibodies is used for staining. Other commercially supplied reagents are also used. For convenience these are also supplied by DAKO.

The primary affinity purified rabbit anti-EHNV antibody (Lot No. M708) is supplied freeze-dried by the OIE Reference Laboratory. Reconstitute using 1mL of purified water and allow the vial to stand at room temperature for 2 mins. Mix the vial very gently. This reagent is stable when stored at –20°C for at least 5 years. For routine use it is recommended that a working stock be prepared as a 1:10 dilution in TSGM. This is stable at –20°C for at least 5 years and does not solidify at this temperature.

1. Cut 5µm sections and mount on SuperFrost® Plus G/Edge slides (Menzel-Glaser, HD Scientific Cat. No. HD 041300 72P3).

2. Mark around the section with a diamond pencil to limit the spread of reagents.

3. De-paraffinise the section using the following reagents:
    a. Pre-heat slides in a 60°C incubator for 30 minutes.
    b. Place slides in a xylene bath and incubate for 5 minutes. Repeat once.
    c. Tap off excess liquid and place slides in absolute ethanol for 3 minutes. Repeat once.
    d. Tap off excess liquid and place slides in 95% ethanol for 3 minutes. Repeat once.
    e. Tap off excess liquid and place slides in distilled or deionised water for 30 seconds.

4. Expose antigens using a protease treatment. Flood slide with Proteinase K and incubate for 20 minutes (ready-to-use solution, DakoCytomation Cat. No. S3020).

5. Rinse slide by immersing 3 times in water. Place in a PBSTw bath for 5 minutes (PBS pH 7.2, 0.05% v/v Tween 20). Tap off the excess wash solution and carefully wipe around the section.

6. Perform the immunostaining reaction using the Universal DAKO LSAB®+ Kit, Peroxidase (DakoCytomation Cat No. K0679).

7. Ensuring the tissue section is completely covered, add the following reagents to the slide. Avoid drying out.
    a. 3% Hydrogen Peroxide – cover section and incubate for 5 minutes. Rinse gently with PBSTw and place in a fresh wash bath.
    b. Primary Antibody (affinity purified rabbit anti-EHNV 1:1500 Lot No. M708) and Negative Control Reagent (non-immune rabbit serum at a dilution of 1:1500, for example rabbit pre bleed SVC #03/032) on a second slide. Cover the section and incubate for 15 minutes. Rinse slides as in step a.
    c. Link – cover the section and incubate for 15 minutes. Rinse slides as in step a.
    d. Streptavidin Peroxidase – cover the section and incubate for 15 minutes. Rinse slides as in step a.
    e. Substrate-Chromogen Solution – cover the section and incubate for 5 minutes. Rinse slides gently with distilled water.
    f. Counterstain by placing slides in a bath of DAKO® Mayer's Haematoxylin for 1 min (Lillie's Modification, Cat. No. S3309). Rinse gently with distilled water. Immerse 10 times into a water bath. Place in distilled or deionised water for 2 minutes.

8. Mount and coverslip samples with an aqueous based mounting medium (DAKO® Faramount Aqueous Mounting Medium Cat. No. S3025).

Reagent supplier:

Dako Cytomation California Inc.
6392 via Real
Carpinteria, CA 93013 USA
Tel 805 566 6655
Fax 805 566 6688

Dako Cytomation Pty Ltd
Unit 4, 13 Lord Street
BOTANY NSW 2019
AUSTRALIA
Tel: 1800 653 103
Fax: 612 9316 4773

Visit www.dakocytomation.com for links to other countries.

Phosphate Buffered Saline

NaCl	8.00g
KCl	0.20g
Na_2HPO_4	1.15g
KH_2PO_4	0.20g

Combine ingredients and dissolve in 900mL of purified water, pH to 7.2 using HCl or NaOH and make up to 1000mL. Autoclave and store at RT.

APPENDIX 3.12

Recommended Method
Rapid Identification of EHNV and Differentiation From Related Viruses Using PCR-REA

Introduction
The following PCR and restriction endonuclease analysis (REA) methods allow rapid differentiation of Epizootic haematopoietic necrosis virus (EHNV), Bohle iridovirus (BIV) and Wamena virus (WV) from each other and from European catfish virus (ECV), sheatfish virus (ESV), Frog Virus 3 (FV3) and Gutapo virus (GV). Based on sequence analysis of the major capsid protein (MCP) gene, two PCR assays (MCP-1 and MCP-2) containing restriction endonuclease (RE) recognition sites can be used to differentiate between these viruses. Note that ECV and ESV cannot be distinguished from each other using these or any other methods.

PCR assay MCP-1
Amplified product from reaction MCP-1 digested with *PflM* I enables differentiation of Australian iridoviruses (EHNV and BIV) from non-Australian iridoviruses (WV, Irian Jaya; FV3/GV, Americas; and ESV/ECV, Europe).

PCR assay MCP-2
Amplified product from reaction MCP-2 digested with *Hinc* II, *Acc* I and *Fnu4H* I (individually) enables differentiation of EHNV and BIV (Australia) from each other and from WV (Irian Jaya), FV3/GV (Americas) and ESV/ECV (Europe).

Reference
Marsh IB, Whittington RJ, O'Rourke B, Hyatt AD and Chisholm O (2002). Rapid differentiation of Australian, European and American ranaviruses based on variation in major capsid protein gene sequence. Molecular and Cellular Probes 16: 137-151.

Preparation of Reagents
The EHNV Purified DNA and BIV Purified DNA PCR control reagents are supplied in freeze-dried form. Reconstitute using 0.5mL of TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0) and allow the vial to stand at room temperature for 2 mins. Mix the vial very gently.

For routine use as a PCR Control it is recommended that working stocks be prepared as a 1:10 dilution in TE buffer (pH 8.0). Aliquots of 250µl should be stored at –20°C. Each aliquot is sufficient for 50 reactions (5µl added to cocktail) and has a minimum shelf life of 6 months from date of diluting.

Primers M151 and M152 (MCP-1 PCR, 321bp), M153 and M154 (MCP-2 PCR, 625bp) are supplied in working strength and should be stored at –20°C. Primers can also be ordered from commercial suppliers. For primer sequences, refer to Table 3.

PCR Cocktail

Amplification reactions in a final volume of 50µl (including 5µl DNA sample) contain 2.5µl of each working primer, 200µM of each of the nucleotides dATP, dTTP, dGTP and dCTP, 10 x PCR buffer (66.6mM Tris-HCl, 16.6mM $(NH_4)_2SO_4$, 2.5mM $MgCl_2$, 1.65 mg/mL bovine serum albumin, 10mM beta-mercaptoethanol) and 2U Taq polymerase. Two negative controls are included, one comprising PCR cocktail only and the second containing 5µl TE buffer. Instructions on preparation of 10 x PCR buffer are included in Table 4. A PCR worksheet is also included.

PCR Conditions

Both the MCP-1 and MCP-2 reactions have the following profile: 1 cycle of denaturation at 94°C for 3min, followed by 35 cycles of denaturation at 94°C for 30s, annealing at 50°C for 30s and extension at 72°C for 1min; a final extension of 72°C for 5min, and; cooled to 4°C.

PCR results are assessed by electrophoresis in 2% agarose gels stained with ethidium bromide. Examples of results from a dilution series of control DNA in the MCP-1 and MCP-2 PCR assays are provided in Figure 1. EHNV PCR Control DNA (1:10 working stock) should give a result similar in intensity to the 10^{-3} band in both cases.

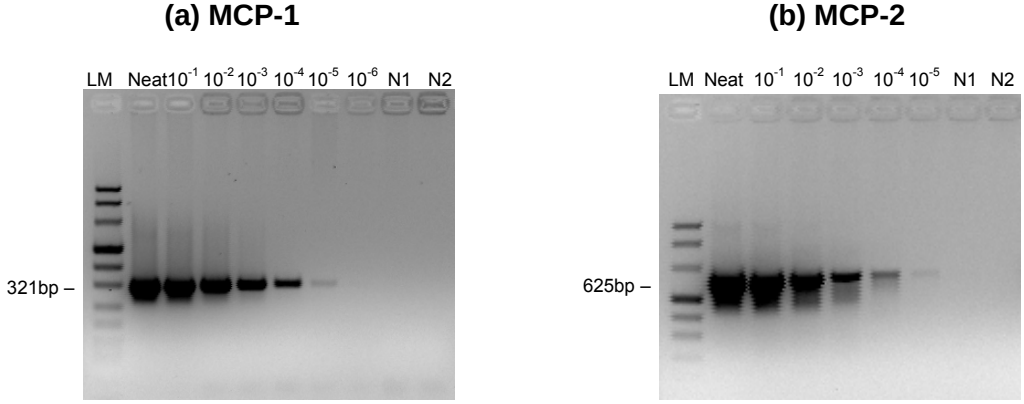


Figure 1. Product amplified from EHNV PCR Control DNA in a 10 fold dilution series in assays MCP-1 and MCP-2

Restriction Endonuclease Analysis

PCR amplicons are subjected to REA with the enzymes described in the following table (Marsh *et al*, 2002). All endonucleases should be used according to manufacturers' instructions. REA reactions are prepared by adding 4-1 µL of PCR product, 2U of the appropriate restriction endonuclease, 1.6µL of buffer (supplied with each restriction endonuclease), 1.6µL of 100µg/mL bovine serum albumin (for *PflM* I and *Hinc* II) and made up to a final volume of 16µL with sterile purified water. Restriction digests are incubated for 2-4h at the recommended temperatures and assessed by agarose gel electrophoresis in 3% gels. The predicted REA fragment patterns are given in Table 1 and illustrated in Figure 2.

Table 1. Restriction endonuclease analysis of ranavirus MCP amplicons

PCR Assay	Restriction enzyme	Predicted band sizes after restriction (bp)	Pattern applies to	REA pattern (Figure 2)
MCP-1 (321bp)	*PflM* I	321	EHNV, BIV	A
		131, 190	FV3, WV	B
MCP-2 (625bp)	*Hinc* II	100, 138, 387	EHNV	C
		100, 525	BIV, FV3	D
		100, 240, 285	WV	E
	Acc I	238, 387	EHNV	F
		625	BIV, ESV, ECV, WV	G
		164, 461	FV3, GV	H
	Fnu4H I	33, 38, 44, 239, 271	EHNV	I
		3, 33, 38, 44, 108, 399	BIV	J
		3, 38, 44, 108, 432	FV3, GV	K
		3, 9, 38, 44, 108, 151, 272	ESV, ECV	L
		3, 44, 71, 108, 399	WV	M

Differentiation of the ranaviruses

The ranaviruses are differentiated by matching the patterns observed for each restriction endonuclease as shown in Table 2.

Table 2. PCR-REA profiles for differentiation of ranaviruses

Virus	Digest Pattern				Profile
	MCP-1		MCP-2		
	*Pfl*M I	*Hinc* II	*Acc* I	*Fnu*4H I	
EHNV	A	C	F	I	1
BIV	A	D	G	J	2
FV3	B	D	H	K	3
GV	B	D	H	K	3
ESV	B	D	G	L	4
ECV	B	D	G	L	4
WV	B	E	G	M	5

Figure 2. Gel electrophoresis of ranavirus major capsid protein PCR products after restriction with the enzymes shown. The samples are from lanes 1-7: EHNV, BIV, FV3, ESV, ECV, WV and GV. Lane 8 is the molecular size marker.

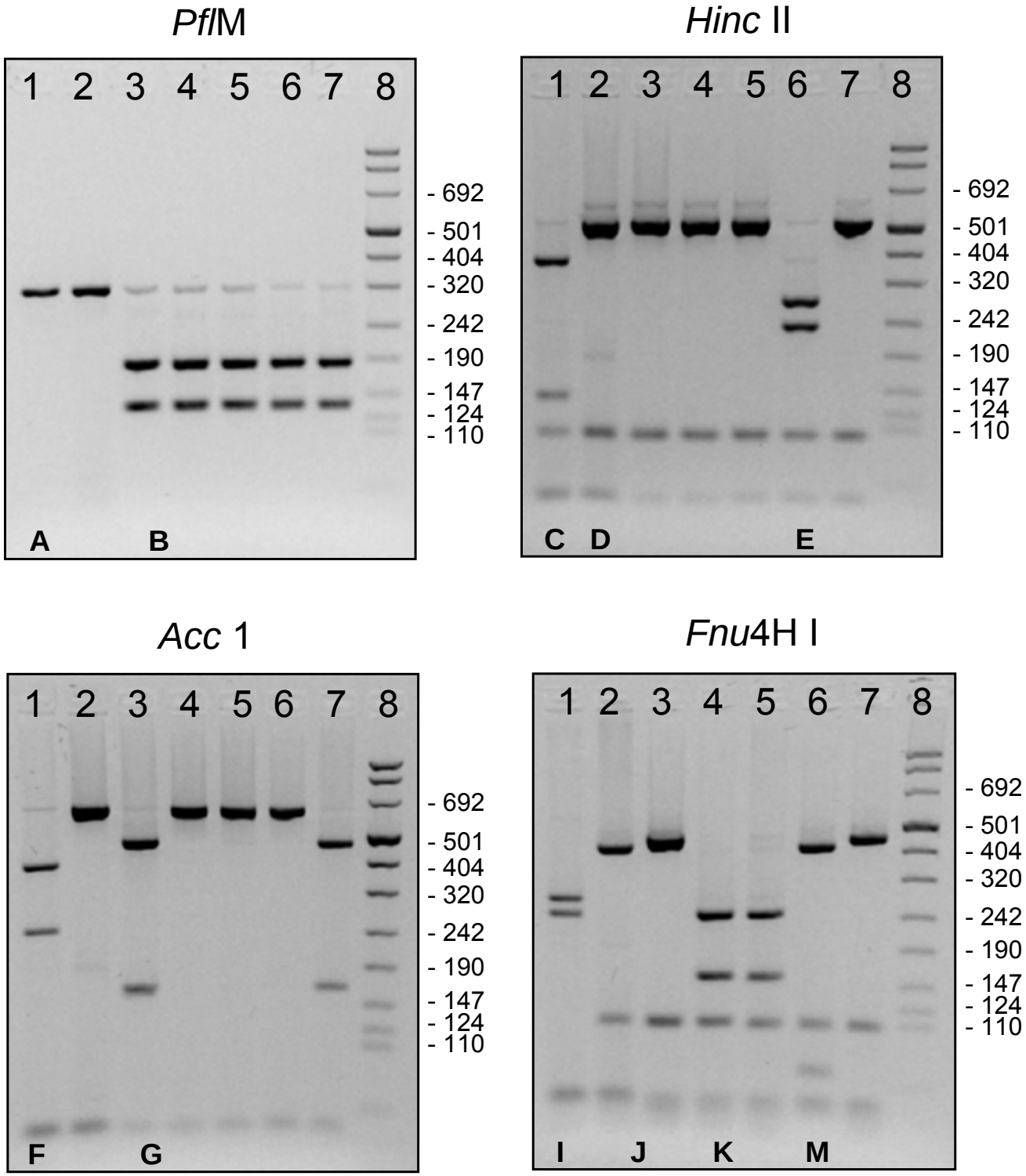


Table 3. MCP-1 and MCP-2 Primer Sequences

PCR assay	Primer	Sequence	Product Size	Gene Location
MCP-1	M151	AAC CCG GCT TTC GGG CAG CA	321bp	266 - 586
	M152	CGG GGC GGG GTT GAT GAG AT		
MCP-2	M153	ATG ACC GTC GCC CTC ATC AC	625bp	842 - 1466
	M154	CCA TCG AGC CGT TCA TGA TG		

Table 4. 10 x PCR Buffer Preparation

Ingredients	Amount	Final Concentration in 50µL PCR mix
Tris	4.050g	66.6mM
Ammonium Sulphate	1.100g	16.6mM
BSA (Albumin Bovine Fraction V Fatty Acid Free)	0.825g	1.65mg/mL
Magnesium Chloride	1.25mL	2.5mM
TE buffer (sterile)	50mL	

Aliquot into 500µL volumes and store at –20°C. For working solution, add 3.5µL of beta-mercaptoethanol per 500µl 10 x buffer. Any remaining buffer should be discarded after preparing the PCR cocktail.

PCR worksheet

PCR Experiment No._____
Date_____

Aim:_____

_____

_____

_____

PCR Reaction No._____ Name:_____
Method Version:_____

PCR Cocktail

Reagents	x 1 (µl)	x _____ (µl)
MQW #_____	24.6	
1mM dNTP #_____	10.0	
Forward Primer ID: _____ #_____	2.5	
Reverse Primer ID: _____ #_____	2.5	
10x PCR buffer (+ 3.5µl βME) #_____	5.0	
Taq 5U/µl #_____	0.4	
Final Volume		

Programme No. EHNV

Stages	Temperature (□C)	Time	Cycles
Initial denaturation	94	3 min MHS*	1
Denaturation	94	30 sec	30
Annealing	50	30 sec	
Extension	72	1 min	
Store	4	1 min	1 (hold)

* Modified Hot Start - After the thermal cycler has reached 94□C (approximately 1 minute) place the tubes in the thermal cycler.

Australian Government

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