DITAC / FRDC PROJECT

IN JAPAN

(21 October - 1 December 1994)

Research Report

Prepared by
Dr John GLAZEBROOK

APPLICATION OF DIAGNOSTIC TESTS DEVELOPED IN JAPAN TO THE DETECTION OF BARRAMUNDI PICORNA-LIKE VIRUS (BPLV) IN AUSTRALIA

A. INTRODUCTION

The history of BPLV in Australia is one in which mass mortalities have occurred repeatedly among cultured larvae almost completely without warning. There is no published data on where the virus comes from (i.e. its source or origin), or, in the case of stock being transferred interstate, the prevalence of asymptomatic carriers. Current methods of detection rely on light and electron microscope techniques only.

Because of inadequate health certification, mass mortalities have now occurred in South Australia on two occasions (March and April 1994), as well as in the north of the continent. In addition, healthy carrier fish were detected by electron microscopy at Robe in South Australia in 1993, resulting in 26,000 fish being destroyed (P. Durham, pers. comm.). There is now a serious risk of exposing native freshwater finfish in the Murray Darling River system to the virus because of the establishment of growout facilities in the region. Until recently, no bath exposure trials have been carried out on species native to Australia's largest river system. In a separate piece of work carried out for the Murray Darling Basin Commission and the Victorian Department of Conservation and Natural Resources, Macquarie Perch, Silver Perch, Murray Cod were shown to be susceptible to BPLV, and two farmed species, viz. Rainbow trout and Brown trout, seemed to be capable of harbouring the virus asymptomatically.

My reason for going to Japan was to see whether immunological and molecular tests developed for the detection of the closely related Striped Jack nervous necrosis virus (SJNNV) in marine finfish could be applied to BPLV in Australia.

The following is a summary of results obtained.

B. SAMPLES TAKEN TO JAPAN FOR ANALYSIS

1. The range of samples taken to Japan for analysis included:

- *Known positive controls* - larvae from previous mass mortality events in Qld, the Northern Territory and South Australia.
- *Known negative controls* - larvae and fingerlings from current production runs in north Queensland, with a history of no signs of disease up to 42 days.

In addition, 9 finfish species (6 native and 3 introduced) which had been bath exposed to BPLV were tested by FAT and PCR to confirm or deny previous clinical and pathological observations.

The progress of research was greatly hastened by the fact that all tests were "up and running" on my arrival.

FIXED SAMPLES TAKEN TO JAPAN FOR HISTOPATHOLOGY

Nine species of native and introduced finfish that had been bath exposed to BPLV

(i) Barramundi only (known positives) from previous mass mortality events

Northern Territory - Larvae from Stokes Hill Hatchery and Bousted Farm (February 1991).

Queensland - Larvae from mass mortality at Sea Harvest Pty Ltd, north Queensland (January 1989).

South Australia - Embedded tissues from mass mortality events in March/April 1994; courtesy Dr P. Durham.

larvae	no. 017-12A (20-30 fish).
	no. 017-A13 (20-30 fish).
fingerlings	no. 019-14 (5 fish).
	no. 019-15 (5 fish).

(Ii) From a current (Sept 1994) production run in north Queensland (known negatives)

Cairns/Bungalow sample recently received; hatchery reared; 25 d old (9 fish).

(Iii) From bath exposure trials

1	Macquarie Perch	A* challenged NT 18 Feb - dead 22 Feb.
		A** challenged Qld 18 Feb - survived 18 Mar.
		A*** challenged NT/Qld 18 Feb - survived 18 Mar.
		B control.
2	Brook Trout	A challenged NT 1 Oct. PM 21 Oct - 2 fish.
		B control
3	Rainbow Trout	A challenged NT 1 Oct. PM 21 Oct - 3 fish.
		B control - 3 fish.

4	Snapper	A challenged NT 1 Oct. PM 21 Oct - eye only. B control - not available.
5	Mulloway	A challenged NT 1 Oct. PM 21 Oct. B control.
6	Golden Perch	A challenged NT 18 Mar. PM 3 weeks later. B control.
7	Barramundi	A challenged NT 4 Mar - survived 21 Mar.

FROZEN SAMPLES TAKEN TO JAPAN FOR ELISA AND PCR

(i) Known Positives

Tissue homogenates from whole larvae (Stokes Hill hatchery and Bousted Farm). Collected Feb 1991.

Whole fish - fingerlings from Bungalow, 25 days old. Collected Sept 1994.

(ii) Bath Exposure Trials

Macquarie Perch	1 challenged NT* 18 Feb - dead 22 Feb. 2 challenged Qld** 18 Feb - dead 3 Mar. 3 control.
Brook Trout	4 challenged NT 1 Oct. PM 21 Oct. 5 control.
Rainbow Trout	6 challenged NT 1 Oct. PM 21 Oct. 7 control.
Mulloway	8 challenged NT 1 Oct. PM 21 Oct. 9 control.
Golden Perch	10 challenged NT 18 Mar. PM 3 weeks later. 11 control.
Goldfish	12 challenged Qld 18 Feb. PM 18 Mar. 13 control.
Silver Perch	14 challenged NT 18 Feb - dead 3 days later. 15 control.
Murray Cod	16 challenged Qld 18 Feb. PM 7 Mar.
Barramundi	17 challenged NT 4 Mar. PM 14 Apr.

* Fish challenged with Northern Territory strain of BPLV

** Fish challenged with Queensland strain of BPLV

RESULTS

Future sampling and sample storage in Australia *(based on advice from Japanese colleagues)*

For Diagnostic Work

Detection of asymptomatic carriers and investigation of mass mortalities:

- For FAT/ELISA collect moribund larvae (20-30/batch); store in 10% NBF at –80°C, respectively.
- For PCR use moribund or recently dead larvae (at least 10).

For Epidemiological Studies

Broodstock:

- **PCR** - biopsy sample from gonads at the start of the breeding season and immediately before spawning; use new primer set. Also sperm (0.1 g before and after spawning).
- **ELISA** - 0.1 g eggs/sperm before and after spawning; when specific rabbit anti BPLV serum becomes available.

Larvae and Fingerlings:

- **FAT** - moribund fish <9 days old, 21 days old and 42 days old. Dissect out eyes, brain and spinal cord in fingerlings.
- **PCR** - dissect out eyes and brain from fingerlings.

SUMMARY AND CONCLUSIONS

The frozen and fixed material taken to Japan from Australia was unsatisfactory in several respects:

- Homogenised larvae were collected from Stokes Hill Hatchery and Bousted Farm in February 1991 and frozen in sea water. They had been stored in liquid nitrogen and in a deep freeze (–60°C) for almost four years. In contrast, the fingerlings from Bungalow were only two months old.
- Too few fish were provided from the bath exposure trials for the results to be meaningful.
- Two controls were absent.

C. Results of Analyses Performed in Japan

1. Histopathology

Between February and May 1994 a total of 9 finfish species (6 native and 3 introduced) were bath-exposed to BPLV for the Murray Darling Basin Commission and Victorian Department of Conservation and Natural Resources. With the exception of Mulloway and Snapper which were obtained from the Brackiswater Fisheries Research Station at Salamander Bay in New South Wales, all were temperate, freshwater species. These fish tests and controls were taken to Japan for histopathological examination.

2. Results

As with barramundi, the main pathological change observed was vacuolation (Figs 1 & 2). Fish found to be susceptible to BPLV (mortality rates 50-100%) viz Macquarie Perch, Silver Perch and Murray Cod, showed the greatest extent of vacuolation (pp 7). This change was minimal in fish with low grade infections (possible asymptomatic carriers) viz Mulloway and Brown Trout and absent in those resistant to infection, i.e. Golden Perch, Goldfish and Rainbow Trout. The grey matter was the area primarily affected.

PATHOLOGICAL CHANGES OBSERVED IN FISH BATH EXPOSED TO BPLV

SPECIES	NO EXAMINED CONTROL /TEST	EXTENT OF VACUOLATION		
		BRAIN	RETINA	SPINAL CORD
Macquarie Perch *Macquaria australasica*	10/10	+++	++	+
Silver Perch *Bidyanus bidyanus*	7/7	+++	++	++
Murray Cod *Maccullochella peeli*	7/7	++	++	-
Golden Perch *Macquaria ambigua*	7/7	-	-	-
Goldfish *Cyprinus carpi*	7/7	-	-	-
Barramundi *Lates calcarifer*	10/10	++	+	+++
Mulloway *Argyrosomus bololepidotus*	5/5	+	-	-
Snapper *Pagrus auratus*	5/5	-	-	-
Rainbow Trout *Oncorphynchus mykiss*	10/10	-	-	-
Brown Trout *Salmo trutta*	10/10	-	+	-

2. Fluorescent antibody test (FAT)

An **antigenic relationship between BPLV and SJNNV** was confirmed. The BPLV infected fish (larvae and fingerlings) had come from a recent mass mortality events in South Australia (March/April 1994). Rabbit anti SJNNV serum was used in these tests and reacted with BPLV. A strong positive reaction was recorded in the eyes, brain and spinal cord of larvae and in the brain of fingerlings.

For **asymptomatic carriers**, one out of five fingerlings obtained from Bungalow (Cairns) showed a strong positive reaction. **Note**: This was the first time BPLV had been detected by FAT in apparently healthy barramundi.

Fish from the **bath exposure trials** (conducted on behalf of the Murray Darling Basin Commission and Victorian Department of Conservation and Natural Resources) were all negative except for Brown Trout and Mulloway which showed a weak positive reaction (6-9 cells) in the retina and/or brain (Fig 8) . This result suggested that these fish were capable of acting as asymptomatic carriers of BPLV.

3. ELISA/Western Blot

The samples used in these studies were tissue homogenates of larvae collected from mass mortality events at Stokes Hill Hatchery and Bousted Farm in Feb 1991 (Barramundi 1 and 2, respectively). Recently acquired, clinically normal fingerlings from Bungalow and positive/negative striped jack controls were also included (Figs. 9 & 10).

All three barramundi samples were negative, in that their absorbance readings at 405 nm were <0.1. On the other hand, the striped jack positive control, recorded a value of 1.0 at a dilution of 1280 (Fig. 9). The former result was most likely due to a low concentration of the virus in the barramundi samples rather than the nature of the antiserum used (rabbit anti SJNNV serum).

Western Blot

The coat protein of BPLV could also not be detected by Western Blot analysis in the three barramundi samples tested (Fig. 10). In contrast, clear banding was apparent in the two striped jack samples - a purified form of the virus (sample 1) and a homogenate of infected larvae (sample 2).

These tests will be repeated when the appropriate rabbit anti BPLV serum becomes available.

4. Polymerase chain reaction (PCR)

Three different samples were used in this study: barramundi from Stokes Hill hatchery and Bousted Farm in the Northern Territory (Government and privately owned, respectively), where mass mortalities occurred in Jan/Feb 1991; and Bungalow in Cairns (Sept 1994), where the fish showed no signs of disease up to 42 days.

Electrophoresis of viral RNA extracted from diseased fishes

After ethidium bromide staining, two viral RNA bands from the Cairns/Bungalow samples were visualised in 1% agarose gel. They appeared to have the same molecular weights as SJNNV RNA1 and SJNNV RNA2 (Fig. 11). No bands were observed from the Stokes Hill and Bousted samples. This may have been due to a very low concentration of the virus in the original samples.

A paper comparing the physico-chemical properties of BPLV and SJNNV is being prepared in conjunction with Japanese colleagues.

Northern hybridisation

Only the SJNNV RNA2 reacted positively in the Northern Hybridisation assay using a SJNNV RNA2 cDNA clone as an identification probe.

Reverse transcription polymerase chain reaction using 5 primer sets designed for SJNNV

Only the T4 region of BPLV RNA2 was amplified from the Bungalow sample whereas the other two samples (Stokes Hill and Bousted) produced no amplification products from the five primer sets employed for SJNNV RNA (Fig. 12).

This suggests that BPLV and SJNNV are genetically related and that very little virus, if any, was present in the Stokes Hill and Bousted samples. However, as these samples were homogenates, tissue enzymes could have interfered with the PCR reaction.

In tests carried out by the Fish Pathology laboratory at Hiroshima University, the other four VNN agents consistently produced T2 and T4 amplification products when tested with the five primer sets.

Cloning and sequencing

The T4 region of BPLV RNA2 was sequenced (Fig. 13) and compared with the same region of the coat protein gene for the five VNN agents. It contained 421 base pairs (bp) and showed approximately 70% homology with the T4 regions of SJNNV and Tiger puffer nervous necrosis virus (TPNNV) and nearly 90% homology with red spotted grouper nervous necrosis virus (RGNNV) and Japanese flounder nervous necrosis virus T4 regions (Tables 1 & 2).

Reverse transcription polymerase chain reaction using primers designed for BPLV

With a new primer set the target region of BPLV (324 bp) was clearly amplified from the Bungalow sample (Fig. 14). However, all fish bath exposed to BPLV were negative (Table 3). This may have been due to insufficient fish being available for testing (1 only from each group) or the low infectivity of the original sample (used as an inoculum).

The new primer set is **not** specific for BPLV but could be described as a "best fit". TPNNV and RGNNV are identical in the region targeted. The primer will differentiate these viruses from SJNNV, JFNNV and BFNNV. The intensity of the band obtained from BPLV is also stronger (comparing Figs. 12 & 14).

5. *Purification of virus from infected fish and expression of coat protein gene of BPLV from recombinant E. coli*

Purification of virus from infected fish

After equilibrium centrifugation, only one band was obtained. Analysis of the nucleic acid extracted from this band revealed that no viral RNA or any other RNA was present in it. The Bungalow sample apparently contained insufficient virus for purification by this method.

Expression of coat protein gene from E. coli

From the total RNAs, about 1380 bp of cDNA was amplified by PCR. It was cloned in the plasmid (pET-BP7). One major protein about 42 kDa was detected by SDS PAGE analysis and Coomassie Blue stain from the induced cells transformed by pET-BP7 (Fig. 15A). However, it was not detected in un-induced cells. This protein was almost the same size as the SJNNV coat protein, so it was considered it was the BPLV coat protein expressed from the cloned open reading frame (ORF). This expressed BPLV coat protein reacted with rabbit anti SJNNV serum (Fig. 15B). This result suggested that BPLV and SJNNV possessed the same coat protein antigens.

The ability to express the coat protein of BPLV by recombinant techniques and use this for rabbit inoculation to produce a specific anti BPLV serum, will hasten the adoption of FAT and ELISA tests in Australia. There has been a chronic shortage of infective material for BPLV research in Australia, mainly due to the tendency of farmers to dispose of dead fish as soon as they are discovered.

D. *Summary/Conclusions*

An antigenic relationship between BPLV and 5 nervous necrosis agents (NNV) that occur in cultured finfish in Japan was confirmed.

The Fluorescent Antibody Test was used successfully for the detection of BPLV in clinically affected barramundi and asymptomatic carriers of the virus (larvae and fingerlings).

For the ELISA test to work for BPLV a large amount of viral antigen as coat protein will be required, either from a recent mass mortality event or recombinant DNA technology as in Japan. This test can then be used in epidemiological studies (to determine whether the virus is vertically transmitted). Rabbit anti BPLV serum will be produced by intramuscular injections of purified virus or expressed coat protein.

For the first time, BPLV was detected in healthy carrier fish from northern Australia by PCR. The T4 region of the BPLV RNA gene was sequenced for comparison with the 5 UNN agents. A more specific primer for BPLV was produced. For this test, care should be taken in selecting only the eye or brain tissue to avoid tissue enzyme interference. Sea water contamination of the samples is undesirable.

E. *Future BPLV research in Australia*

Application will be made to the Fisheries Research and Development Corporation for financial support over the next three years to investigate the epidemiology of BPLV and carry out extensive testing of field samples collected under Australian conditions.

FAT and **PCR** will be trialed as a screening test for larvae and fingerlings from current production runs in the tropics. **PCR** will also be applied to the gonads of broodstock to determine whether BPLV is being vertically transmitted. **ELISA** will also be employed to test broodstock and their eggs or sperm. The end result will be improved methods of Health Certification for barramundi.

F. *Acknowledgements*

The results of work undertaken by Associate Professor T Nakai and three of his postgraduate students, as well as the author, are included in this report.

LIST OF FIGURES

Histopathology

Histopathology-Bath Exposure Trials (MDBC/Vic Dept CNR)

Fluorescent Antibody Test (FAT)

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Polymerase Chain Reaction (PCR)

Expression of coat protein of BPLV RNA2 from recombinant E. coli

LIST OF TABLES

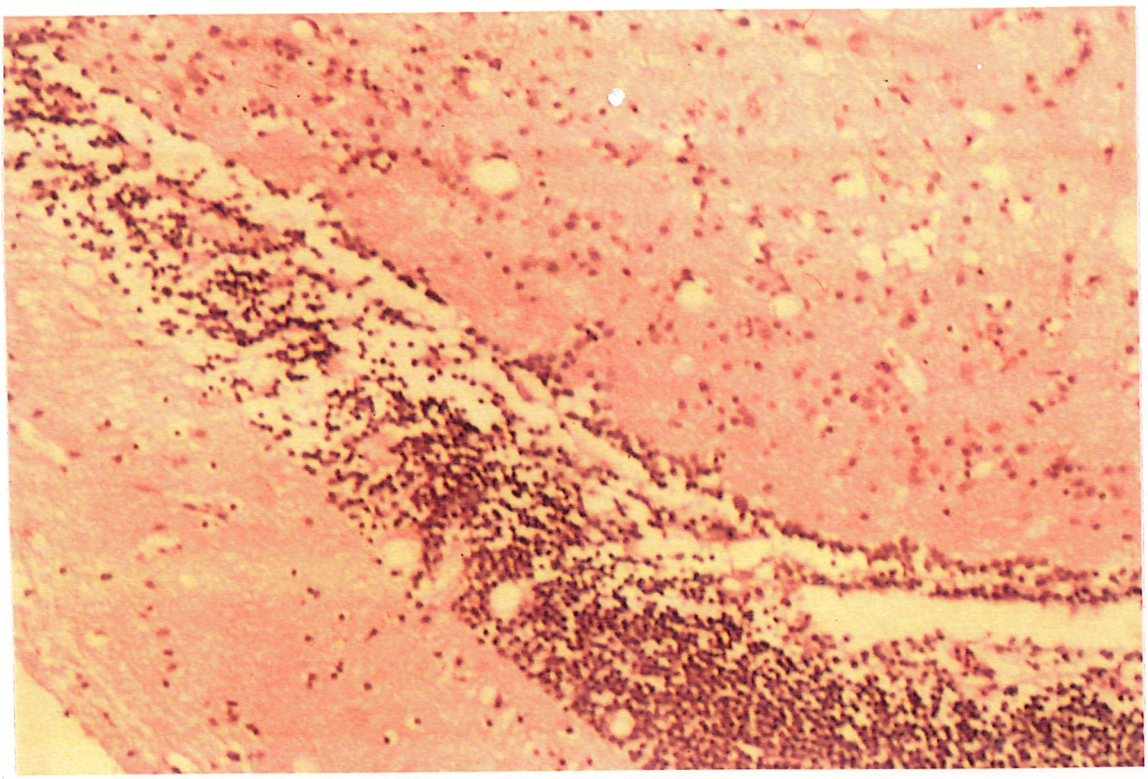


Fig. 1. Brain vacuolation in Murray Cod. x 62.5.

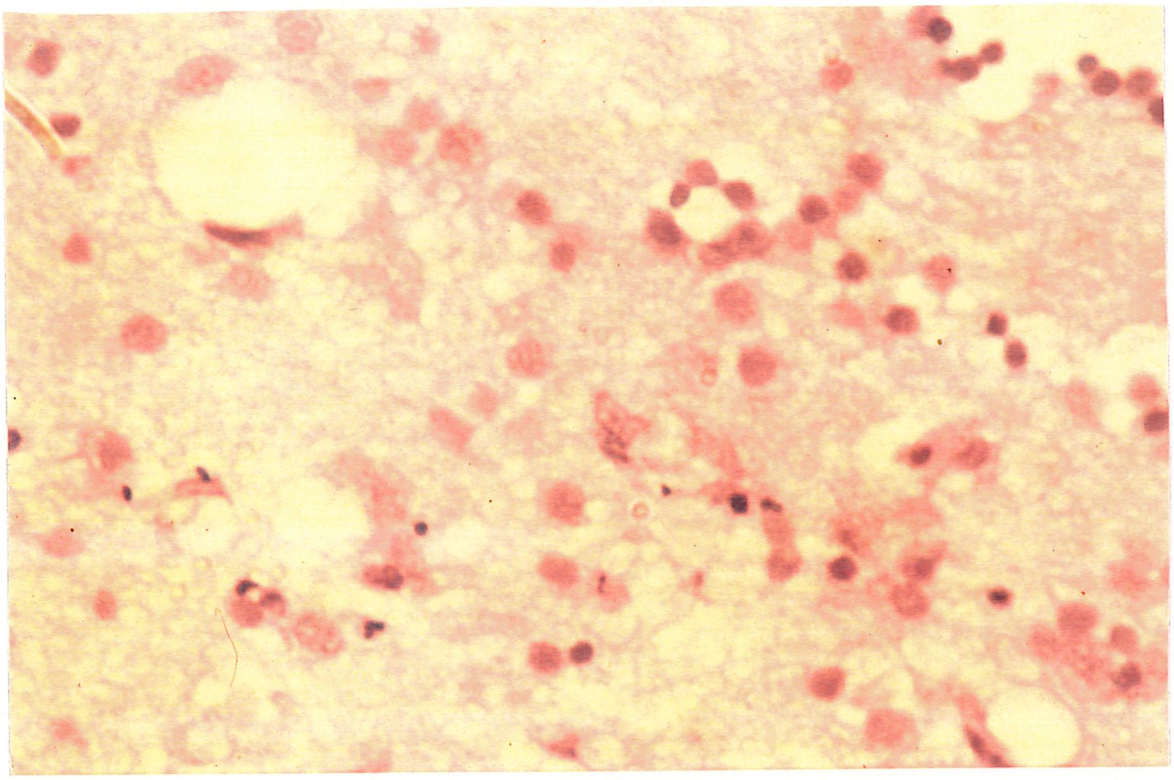


Fig.2. Brain vacuolation in Murray Cod. x 250.

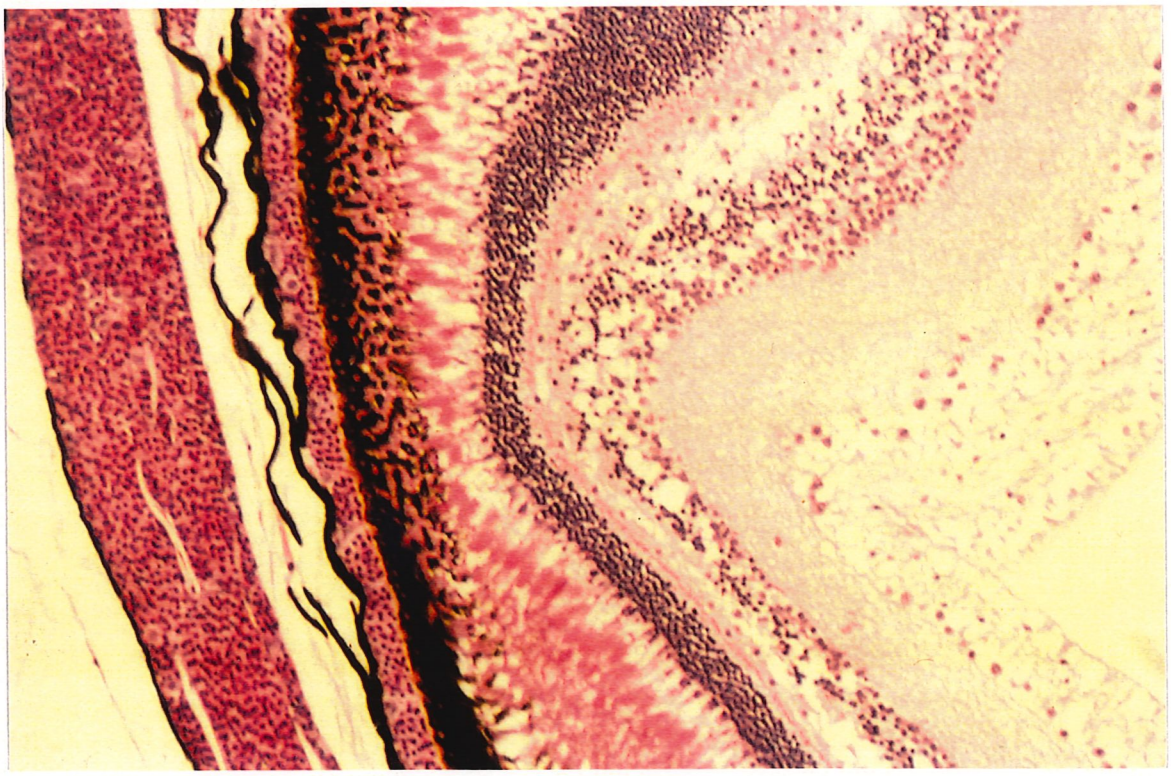


Fig. 3. Retinal vacuolation in Silver perch (*Bidyanus bidyanus*). x 62.5.

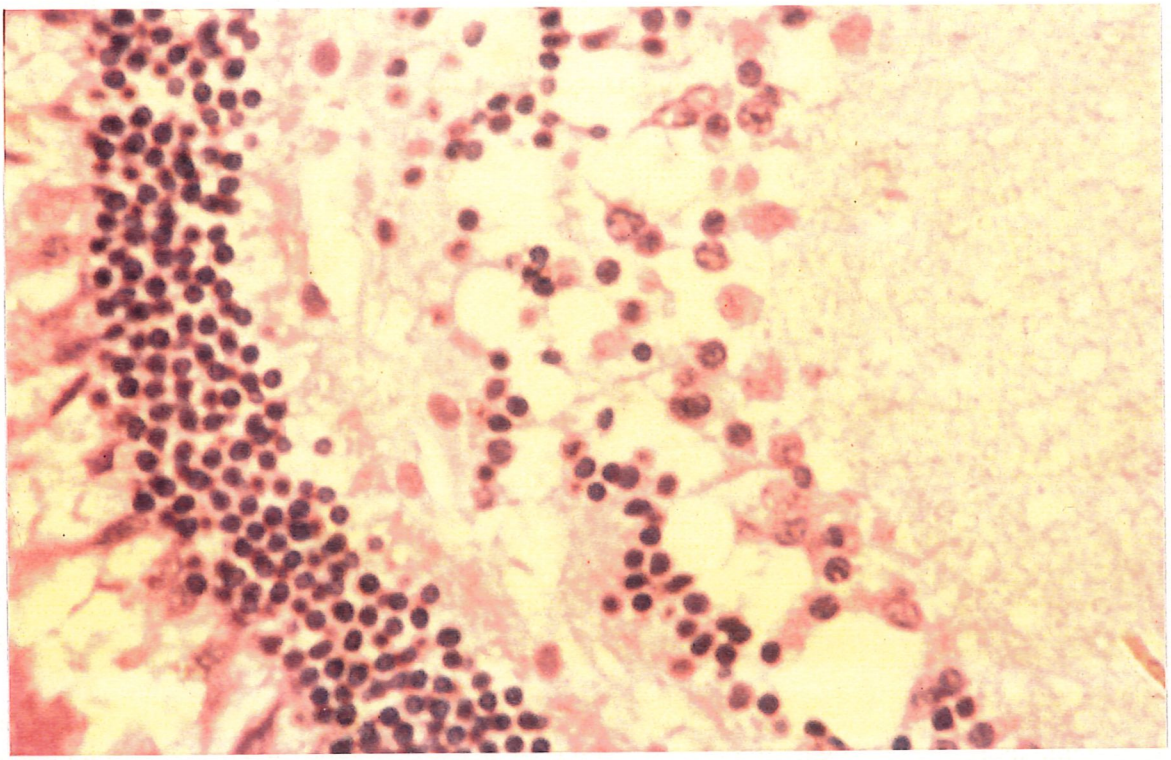


Fig. 4. Retinal vacuolation in Silver perch (*Bidyanus bidyanus*). x 250.

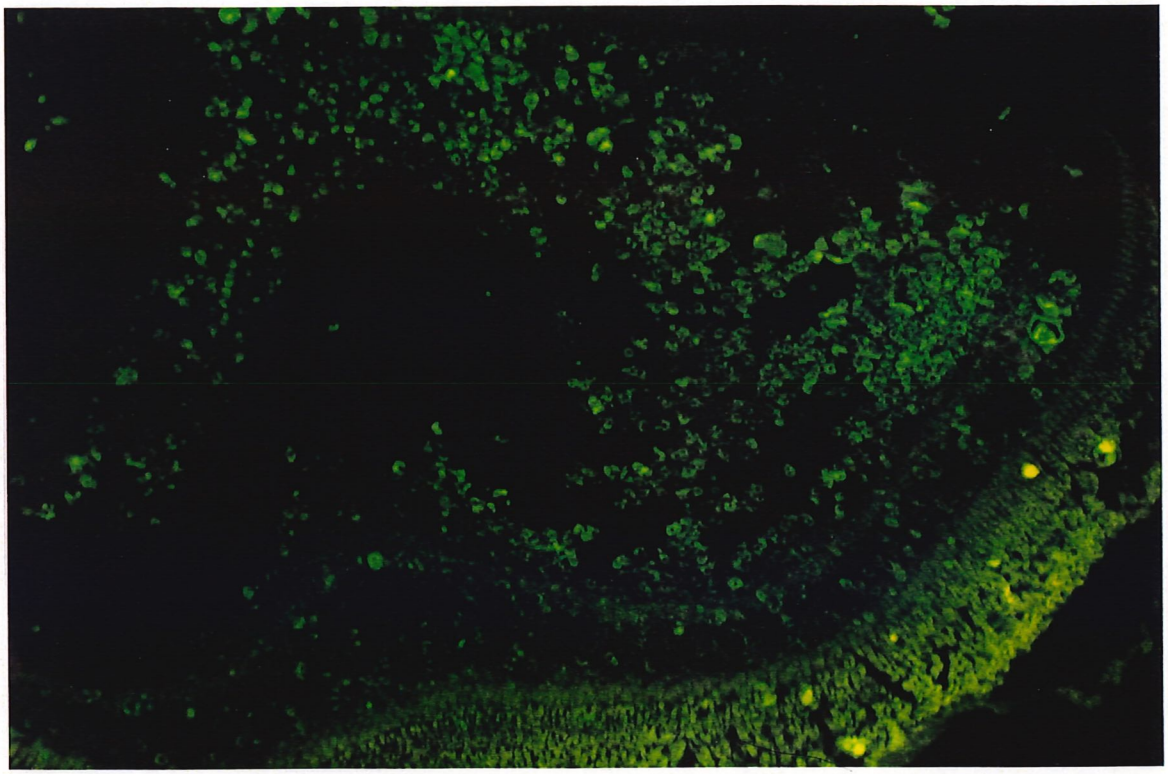


Fig. 5. Fluorescent Antibody Test (FAT). Positive reaction in 25 d old fingerlings from Cairns/Bungalow. x 62.5.

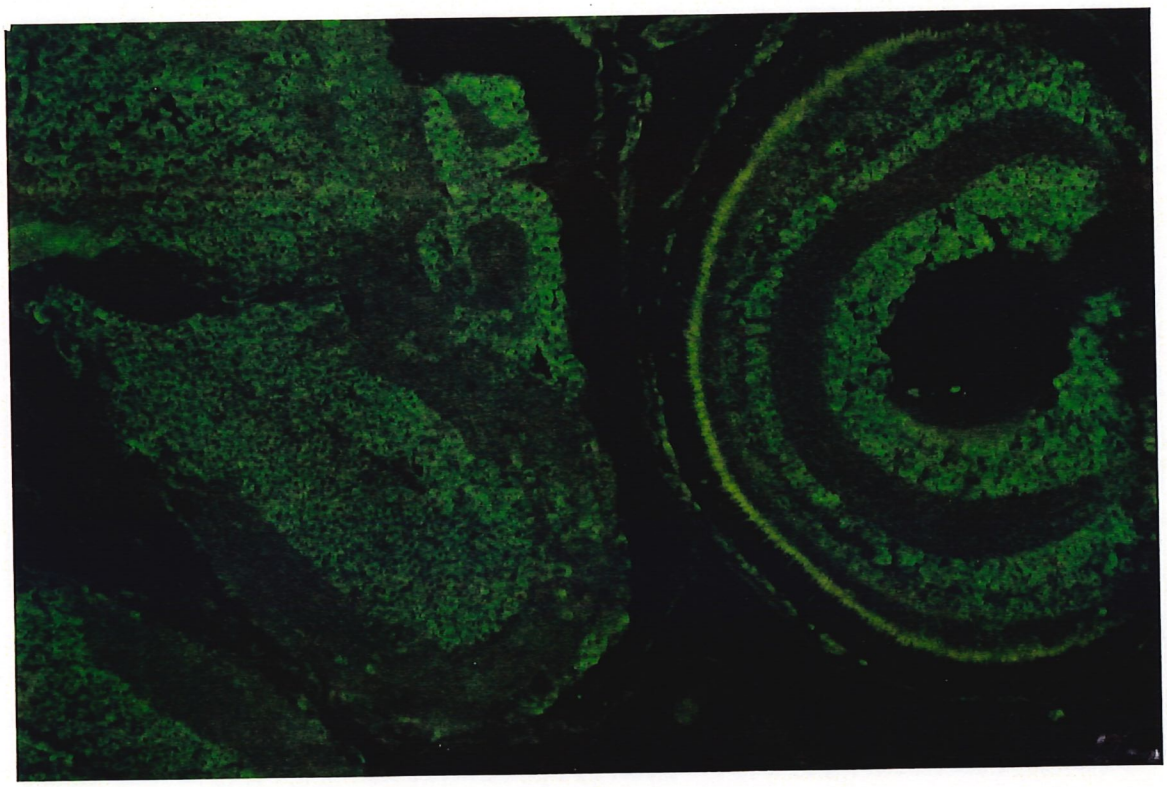


Fig. 6. FAT. Eye/brain of larval barramundi from West Beach Aquaculture. x 62.5.

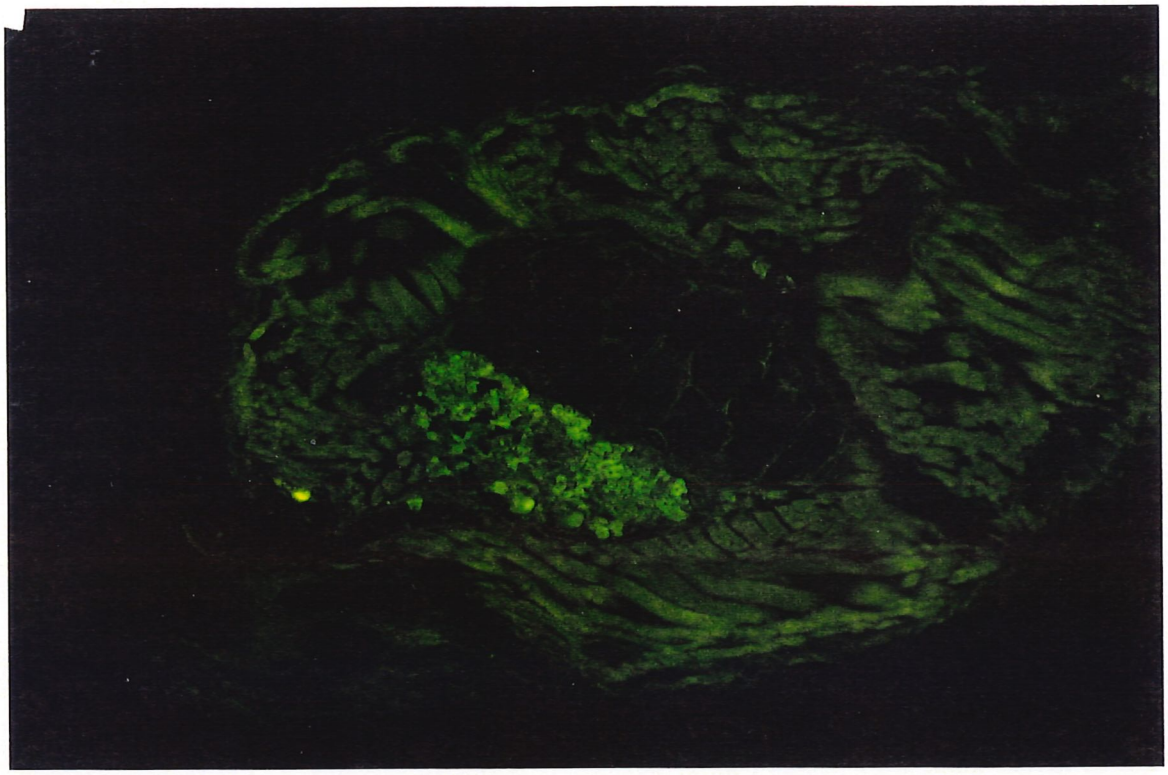


Fig 7 - Spinal cord of another larva from the same batch as Fig 6. Virus is present in the spinal cord and surrounding musculature x 62.5

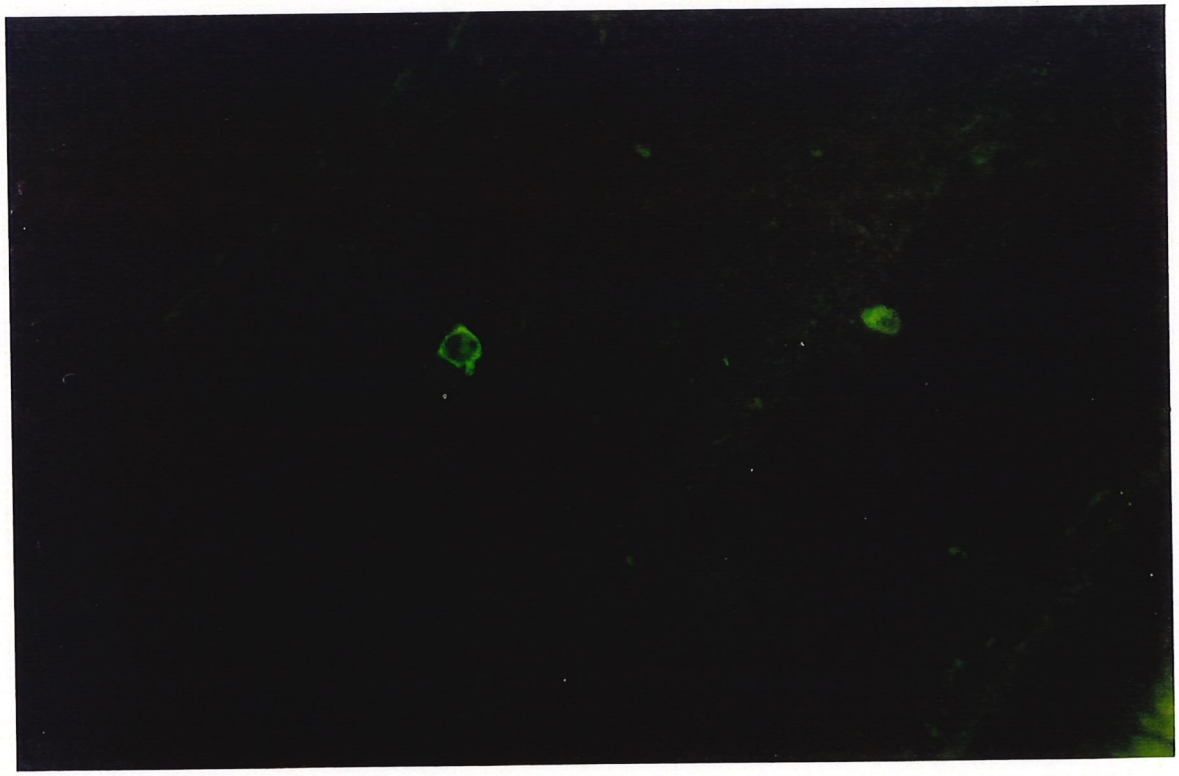
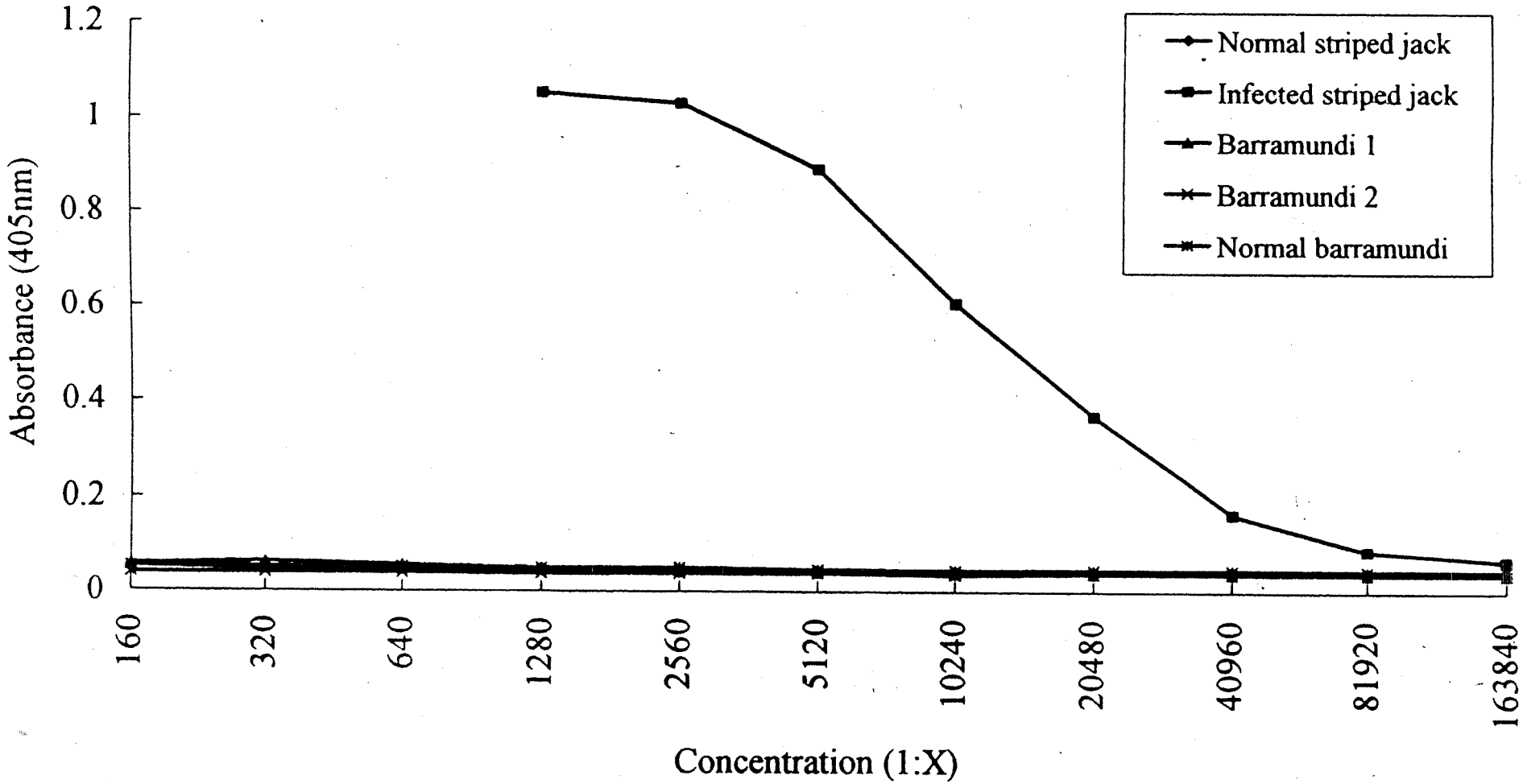
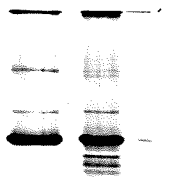


Fig 8 - Retina of brown trout bath exposed to BPLV x 62.5. Note high degree of immunofluorescence in the cytoplasm of two cells.

Fig. 9. ELISA performed on Barramundi samples from Australia





1 2 3 4 5

Fig. 10. Western Blot detection of BPLV using rabbit anti SJNNV serum

1. Striped jack nervous necrosis virus (SJNNV) - purified form.
2. Infected striped jack.
3. Barramundi 1 (Stokes Hill Hatchery).
4. Barramundi 2 (Bousted Farm).
5. Clinically normal barramundi (25-day-old) ex Cairns, Bungalow.

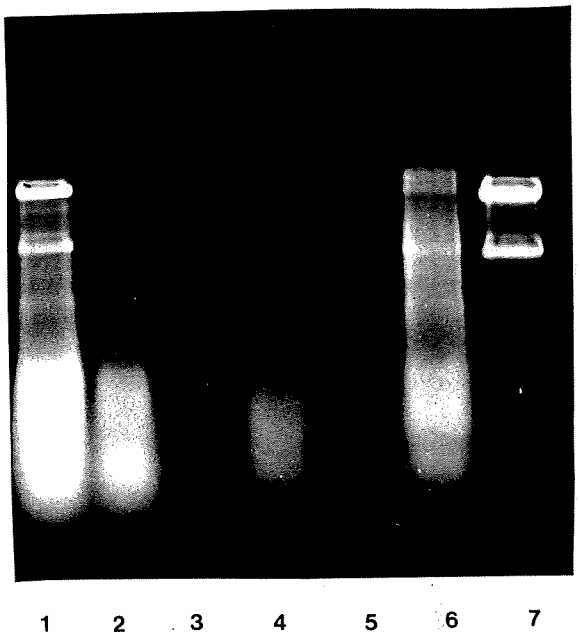
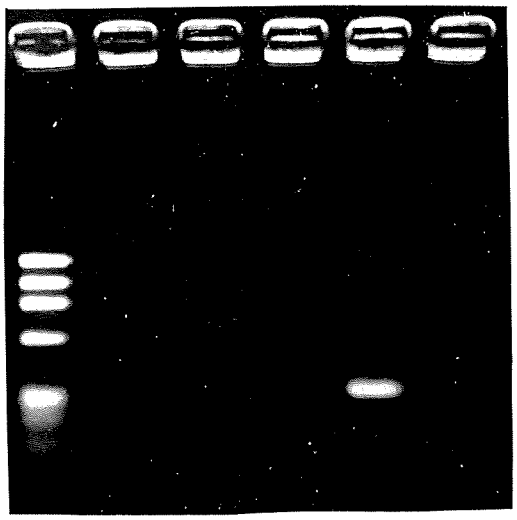


Fig. 11. Electrophoresis of viral RNA extracted from diseased fishes

1. Striped jack.
2. & 3. Barramundi from Stokes Hill, NT
4. & 5. Barramundi from Bousted Farm, NT
6. Barramundi from Bungalow, Cairns, Qld.
7. Purified SJNNV.



T4 region
of BPLV

Fig. 12. RT-PCR products using different pairs of SJNNV RNA2 primers on BPLV.

1 : CGTGTCAGTC ATGTGTCGCT GGAGTGTTCG GCTGAGCGTT CCGTCTCTTG AAACACCTGA

F2 primer *forward primer*

61 : GGAGACGACC GCTCCCATCC TGACACAAGG TCCCCTGTAC AACGATGCCC TCGCTGCCAC

121: CGACTTCAAG TCCATCCTCC TAGGGTCCAC GCAGTTGGAC ATTGCTCCTG AAGGGGCAAT

181: CTTTCAATTG GATCGACCAT TATCCATTGA TTACAGCCTA GGGACTGGTG ATGTTGACCG

241: TGCCGTCTAC TGGCACCTCA AGAAGTTCGC TGGCACATCT GCTACACCTG CAGGCTGGTT

301: TCGCTGGGGC ATCTGGGACA ACTTCAACAA AACGTTCACA GATGGCGTGA CTTACTACTC

reverse primer

361: TGACGCGCAG CCTCGTCAAA TTCTCTTGCC CGTTGGCACC GTCTTCACCC GTGTTGACTC

R3 primer

421: G

Fig. 13. BPLV T4 sequences and primer position to detect BPLV.

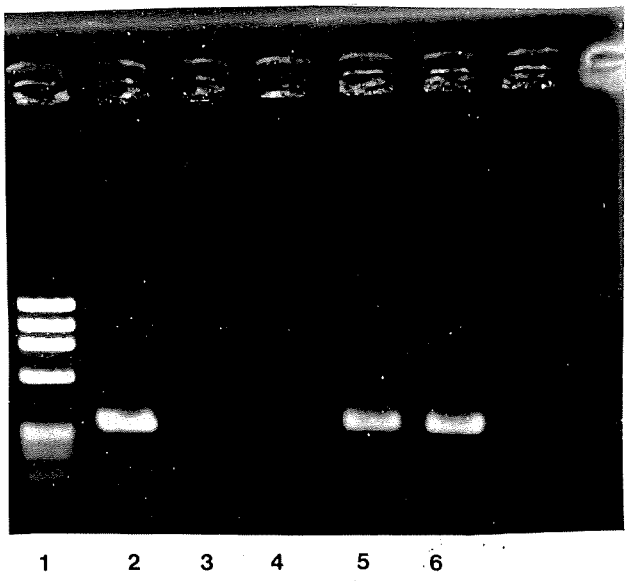
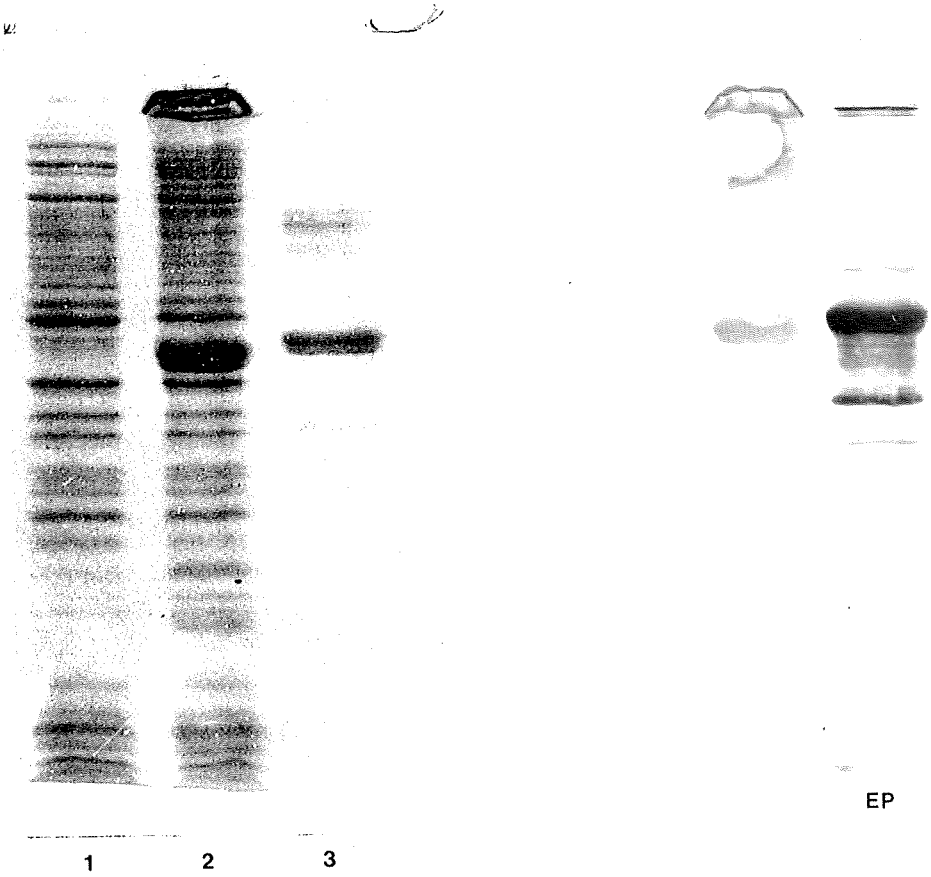


Fig. 14. RT-PCR products using one set of primers (designed for BPLV) and different sources of Barramundi.

1. Ladder.
2. RNA extracted from Barramundi in Bungalow.
3. Stokes Hill.
4. Bousted Farm
5. & 6. Bungalow.
7. Control



Figs. 15A & 15B. SDS-polyacrylamide gel electrophoresis (A) and Western blotting (B) analysis of expression protein from BPLV.

E. coli BL21 (DE3) transformed with pET-BP7, uninduced cells (Lane 1) and cells induced for 3 hours with 1 mM IPTG (Lane 2). SJNNV coat protein from 1.5 µg of purified virions (Lane 3). Western blotting analyses were performed with anti-SJNNV rabbit serum.

Table 1. Primer sequences, map position in BPLV T4 region (421bp), length, Tm, GC and orientation

primer sequences (position in BPLV T4 region)	length (bp)	Tm (°C)	GC (%)	primer orientation
5' - CGT CTC TTG AAA CAC CTG AG -3' (42-61)	20	60	50.00	sense
5' - CGT CAG AGT AGT A AG TCA CG -3' (365-346)	20	60	50.00	antisense

Table 2. Homology percentage among BPLV and 5 NNV sequences

NNV	SJ	TP	RG	JF	BF
BPLV	70	72	86	88	77

SJ Striped Jack
TP Tiger Puffer
RG Red Spotted Grouper
JF Japanese Flounder
BF Barfin Flounder

Table 3. Results of PCR on the Australian fish samples

NO	Fish species		1st	2nd	Note
1	Macquarie perch	Northern Territory	-	-	
2		Queensland	-	-	
3		control	-	-	
4	Brook trout	test	-	-	
5		control	-	-	
6	Rainbow trout	test	-	-	
7		control	-	-	
8	Mulloway	test	-	-	
9		control	-	-	
10	Golden perch	test	-	-	
11		control	-	-	
12	Gold fish	test	-	-	
13		control	-	-	
14	Silver perch	test	-	-	
15		control	-	-	
16	Murray cod	test	-	-	
17	Barramundi	test	-	-	
18		control	-	-	

*1st: using SJNNV primer
2nd: using NEW primer