FINAL REPORT

Development of Diagnostic Capability for Priority Aquatic Animal Diseases of National Significance: Spawner-Isolated Mortality Virus

Owens, L. & Cullen, B. R.

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Australian Government
Department of Agriculture, Fisheries and Forestry

JCU James Cook University

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FRDC Project No. 2001/625

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2001/625 Aquatic Animal Health Subprogram: development of diagnostic capability for priority aquatic animal diseases of national significance: spawner-isolated mortality virus

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NON TECHNICAL SUMMARY:

OUTCOMES ACHIEVED TO DATE

1. An up to date survey for SMV was carried out.

OUTPUTS

1. Detection protocols and PCR positive control was developed.
2. Sensitivity and specificity was further defined as was the type of samples suitable for testing.
3. Protocols and PCR positive control was supplied to diagnostic laboratories.
4. The re-discovery of IHHNV in Australia.

During 1993-1994, mid-crop mortality syndrome (MCMS) first appeared in farmed *Penaeus monodon* in northern Queensland, causing up to 80% mortalities in some grow-out ponds. Significant mortalities continued at least up to 1997-98. This had a significant impact on the small, but rapidly growing industry and disease was recognized as a major threat to future sustainability

In 1993, wild-caught *P. monodon* spawners which were being held as a captive population exhibited signs of lethargy, failure to feed, redness of the carapace and increased mortality. Electron microscopy revealed aggregations of virus-like particles in the cytoplasm of gut cells. The particles were hexagonally shaped suggesting icosahedral morphology, approximately 20nm in diameter, and appeared to issue from the nucleus through pores in the nuclear membrane (Fraser & Owens 1996). Selective nucleic acid digestion, chloroform treatment and bioassay experiments suggested a non-enveloped DNA virus. The virus was termed spawner-isolated mortality virus (SMV) and was suggested as being a possible parvovirus (Fraser & Owens 1996). Similar viral particles were noted in prawns from farms experiencing MCMS in the mid 1990's (Owens *et al*. 1998). Gene probes were developed for *in situ* detection. A PCR test was developed in an effort to detect the agent in prawn tissues as well as faeces, which would facilitate non-destructive sampling. These tools were

used in a survey of spawner prawns, where an overall SMV prevalence of 8.5% was noted, but ranged from 0% to 24% between prawn samples.

For optimal detection power in terms of confidence and sensitivity, the PCR test was coupled with dot blot confirmation. Although this afforded increased sensitivity and confidence in the results, it was somewhat unwieldy, and only available at one laboratory. The aims of this project were to improve the diagnostic capability for SMV by the development, application and dissemination of convenient detection methods, and to use these methods in a survey of samples from industry.

A PCR/ELISA method was developed to address the shortcomings in diagnostic capability. This method had significant advantages in terms of convenience, the components were generic and readily available commercially, and the format was amenable to high throughput applications. An increase in sensitivity of ≈10 to 100 fold was noted, and the assay did not cross-react with other viruses, crustaceans, cell lines or bacteria it was tested against. Of note, it did not cross react with infectious hypodermal and haematopoietic necrosis virus (IHHNV) strains from Australia and New Caledonia. A non-infectious positive control was developed in the form of a plasmid containing the target sequence, and was disseminated to relevant laboratories upon request.

Nucleic acid extraction methods were assessed, and the overall test was found to be suitable for prawn tissues, postlarvae and prawn faecal samples. Some form of sample quality assessment was recommended when testing batches of clinical specimens, as sample quality was variable depending on tissue type, storage and transportation regimes.

A survey consisting of a total of 876 samples of prawn tissues, postlarvae and prawn faecal samples from 5 separate facilities was carried out over the 2002/2003 period. The prevalence of SMV was low, at 2.17%, with most positives coming from specific prawn batches at one facility. Based on low prevalence and previously estimated production losses associated with the presence of SMV, the impact of SMV on the prawn culture industry was unlikely to have been significant at the time of this current survey.

KEYWORDS: spawner-isolated mortality virus, marine prawns, aquaculture, detection test, PCR, ELISA

2.0 Acknowledgments

Assistance was received from the Tropical Aquaculture Research Team at the Australian Institute of Marine Science.

Thanks to all the Queensland prawn farms and hatcheries who contributed samples to this project. To maintain confidentiality, none have been mentioned by name, but the support to this project was greatly appreciated.

3.0 Background

Prior to 1994, Australia was regarded by the prawn aquaculture industry to be largely free of costly prawn pathogens. During 1993-1994, mid-crop mortality syndrome (MCMS) first appeared in farmed *Penaeus monodon* in northern Queensland, causing up to 80% mortalities in some grow-out ponds. Significant mortalities continued at least up to 1997-98. This had a significant impact on the small, but rapidly growing industry and disease was recognized as a major threat to future sustainability. The immediate consequence was a dramatic slowing in the rate of industry growth. Significant progress has now been made in understanding 2 of the key viral pathogens, and losses due to disease have subsequently decreased. However, the industry remains vulnerable to further disease problems due to a poor knowledge of other potential pathogens and an inadequate health management capacity.

During the past 3-4 years, research has focussed on 2 viruses that appeared to have been a major cause of stock losses associated with MCMS. Aquaculture CRC project A.1.4 has investigated the yellow head related virus LOV/GAV which was first detected in Australia in 1995. An understanding of the epidemiology of LOV/GAV is now emerging. Its host range and susceptibility has been defined; its relationship to exotic yellow head virus from Thailand has been determined.

FRDC project 96/301 has investigated spawner isolated mortality virus (SMV). In 1993, wild-caught *P. monodon* spawners which were being held as a captive population exhibited signs of lethargy, failure to feed, redness of the carapace and increased mortality. Electron microscopy revealed aggregations of virus-like particles in the cytoplasm of mid-gut cells. The particles were hexagonally shaped suggesting icosahedral morphology, approximately 20nm in diameter, and appeared to issue from the nucleus through pores in the nuclear membrane (Fraser & Owens 1996). Melanin problems prevented the observation of virions from CsCl gradient preparations. Selective nucleic acid digestion, chloroform treatment and bioassay experiments suggested a non-enveloped DNA virus. The virus was termed spawner-isolated mortality virus (SMV) and it was suggested that it was a possible parvovirus (Fraser & Owens 1996).

When prawn farms were experiencing MCMS in the mid 1990's, tissue homogenates from these prawns were subjected to CsCl gradients and ultracentrifugation. One band that commonly appeared in these preparations had a buoyant density of 1.4 gml^{-1} (parvoviruses have a buoyant density of 1.39-1.43gml^{-1}). TEM of negatively stained aliquots revealed particles approximately 20-25nm in diameter. DNA was extracted from the gradient purified bands, ssDNA was complemented by using Klenow polymerase, and the resulting dsDNA was ligated to EcoR1 adapters and subsequently digested with BamH1. This was ligated into pGEM7zf(+) which had been doubly digested with EcoR1 and BamH1, and transformed into *E. coli* JM109 cells. Clones which contained large inserts were selected by electrophoresis following plasmid digestion with EcoR1 and BamH1. Inserts that contained prawn DNA were eliminated using dot-blot hybridisation against *P. monodon* DNA. Selected clones were partially sequenced and compared to the GeneBank database to determine those most likely to contain viral DNA. A probe was constructed through digoxigenin labelling methods (Owens *et al*. 1998). This probe was used for *in situ* hybridisation studies on MCMS prawns, and on experimentally infected prawns from the studies done during the initial observation of SMV in captive prawns. Positive *in situ*

staining was observed predominantly in cells of the apical end of the hepatopancreatic tubules, midgut caecae, midgut, hindgut caecae and hindgut folds. The gene probe did not react to prawn tissues known to contain monodon baculovirus, hepatopancreatic parvovirus, and Australian IHHNV (Owens *et al*. 1998).

A PCR test was developed which allowed the detection of SMV in prawn faeces. PCR testing of faeces is a non-invasive procedure which ensures no impact on subsequent spawner survival or spawning efficiency. A survey of 909 faecal samples from *P. monodon* spawners showed a correlation between SMV infection and decreased productivity. Spawners that were PCR-positive produced postlarvae with a 23% reduced survival compared to postlarvae from PCR-negative spawners (Owens *et al*. 2003). Other viruses were not assayed as part of this survey, and therefore the role of other viruses was not assessed. This decreased survival has been estimated to cost the industry A$3 million/year in lost production. Screening of spawners for SMV will be a benefit to industry in improving PL survival and may also reduce the risk of disease during grow-out.

There is no practical surveillance methods recognised by the Office International des Epizooties (OIE) for SMV and confirmatory diagnosis involves use of the electron microscope to visualise the virions of SMV. Obviously, this is clumsy, expensive, impractical and most unsatisfactory. Laboratory based PCR techniques are available only at James Cook University (JCU). There is an urgent need to get these techniques to other laboratories in an easy to use format to help the industry and to meet Australia's OIE reporting requirements.

The current SMV PCR with the dot blot confirmation test is 100% specific and sensitive with no cross reactions against anything that has been tested. It has been found to work equally well in samples from all species known to carry SMV (eg. *P. monodon* and *P. merguiensis*). It meets the standard approaches and techniques listed in the OIE Diagnostic Manual for Aquatic Animal Diseases. Completion of this project means all other aims of the program "Development of Diagnostic Capability for Priority Aquatic Animal Diseases of National Significance" for SMV will be met.

4.0 Need

Mid-crop mortality syndrome caused significant losses in prawn farms from Queensland and New South Wales. Estimated losses over the period 1994-1998 were $44 million (CRC Project A1.4). Two viruses are thought to be involved, LOV/GAV and SMV. This project focussed on detecting and controlling SMV. A survey of 909 faecal samples from spawners correlated SMV PCR-positive results with a decrease in productivity. Spawners with PCR-positive faecal samples produced postlarvae with a 23% reduced survival compared to PCR-negative spawners. A similar negative relationship between PCR-positive spawners and the survival of progeny has been shown with white spot syndrome virus (WSSV) in Asia. A robust PCR for use with faecal samples had been developed, and this project refined the technique using PCR/ELISA. A non-infectious positive control was offered and sent to testing laboratories. Screening at the hatchery provides maximal downstream effect on controlling disease in the industry. There was a need to develop time and cost-effective screening tools for SMV to reduce the mortality caused by this virus.

Furthermore, SMV has been listed by the OIE (Office International des Epizooties, 2000) as an “other significant disease”, so Australia has reporting obligations as a signatory to the regulations of the World Trade Organisation. Development of a diagnostic kit for SMV will make it easier for more laboratories to test for SMV and therefore help Australia meet these obligations.

5.0 Objectives

1. Produce a non-infectious, SMV-positive control for use by other laboratories and for inclusion in a diagnostic kit.

    This objective was successfully achieved. A positive control was developed in the form of a plasmid containing a target sequence for PCR. This was stable for transportation and storage, non-infectious and of virtually unlimited supply. Relevant laboratories were contacted and the positive control dispatched as requested.

2. Develop a kit based on ELOSA (enzyme-linked oligosorbent assay) to detect SMV infections in spawners and post-larvae.

    A PCR/ELISA configuration was successfully developed for the detection of SMV in prawn tissues, postlarvae and prawn faecal samples. The method could be applied to virtually any PCR, and the components were generic and readily available commercially. The diagnostic capability of SMV was improved.

3. Quantitatively measure the sensitivity and specificity of the ELOSA.

    Significant improvements in sensitivity (≈10-100 fold) were observed using the new method, with detection down to single-figure copies of genome evident. No inappropriate assay cross-reactivity was observed throughout the project, against any other viruses, crustacean, cell lines or bacteria tested.

4. Screen spawners and postlarvae in commercial hatcheries using the SMV ELOSA to establish prevalence and to correlate SMV detection with available production parameters.

    A total of 876 samples were tested for SMV. They consisted of tissues from grow-out prawns, tissues from spawners, whole postlarvae and prawn faecal samples over 5 separate facilities. The results of the SMV survey were included in this report.

6.0 Construction of a positive control for use in spawner-isolated mortality virus detection by PCR.

The initial phase of the project was to produce a non-infectious positive control for use in the PCR-based detection of SMV, and to assist in the further refinement of assays. The methods, sequence, provision and use of the positive control are described below.

6.1 Methods

6.1.1 Template. To test the operation of the PCR and to develop a PCR-specific positive control, a plasmid containing a previously cloned 2kb insert of SMV DNA was subjected to PCR.

6.1.2 PCR. The SMV200 PCR was designed from a previously sequenced 2kb length of SMV genome, to produce an amplified product of 207bp. This PCR had been previously used successfully on samples of prawns faeces. The primer sequences were as follows:
SMV200 forward: 5'-TAGCTATTTTTTGGTCGTCTG-3'
SMV200 reverse: 5'-GCCGCAATTTACCAGTGTTTGAAG-3'

The following components were included in standard PCR: 200µM dNTP's, 2mM $MgCl_2$, 0.5µM each primer, 1U Taq polymerase (MBI Fermentas), PCR buffer (750mM Tris-HCl pH 8.8, 200mM $(NH_4)_2SO_4$, 0.1% Tween 20) (MBI Fermentas), 1-5µl template, deionised water to a final volume of 50µl.

A touchdown PCR strategy was used to minimise the possible occurrence of spurious bands. Thermocycling consisted of an initial denaturation step of 94°C for 4 minutes, 7 cycles (94°C for 45 seconds, annealing temperature 65°C to 58°C in 1°C decreasing intervals per cycle for 50 seconds, 72°C extension for 1 minute), 30 cycles (94°C for 45 seconds, 58°C for 50 seconds, 72°C for 1 minute), final extension 72°C for 4 minutes.

6.1.3 Visualisation of PCR products. PCR products were run in TAE buffer on 1.5% agarose gels containing 0.5µg/ml ethidium bromide, and visualised under UV light.

50x TAE buffer	Working Solution
242g Tris base	40mM Tris-acetate
57.1ml glacial acetic acid	1mM EDTA
100ml of 0.5M EDTA (pH 8.0)	

(Sambrook & Russell 2001).

6.1.4 DNA quantitation. The quantity of DNA was estimated using a spectrophotometer (Shimatzu) measuring absorbance at 260nm wavelength. Calculation was based on an absorbance of 1 being equivalent to 50µg/ml of double-stranded DNA. Purity was assessed by calculating the ratio of absorbance at 260nm / absorbance at 280nm, which should be around 1.8 to 2 for pure preparations (Sambrook & Russell 2001).

6.1.5 Cloning of PCR Products. PCR products were purified using the Qiagen PCR Product Purification Kit according to manufacturer's instructions. The amount of product was estimated by spectrophotometry. PCR products were cloned using the pGEM-T Easy Vector System (Promega), according to the manufacturer's instructions, and a number of

clones were selected based on blue/white colony screening. Vector putatively containing the SMV200 insert was extracted from bacterial cells using the Promega Wizard Plus SV Miniprep DNA Purification System. The extracted plasmid was tested with the SMV200 PCR to verify the presence of insert. Transformed *E. coli* JM109 cells containing the SMV insert were stored at -70°C.

6.1.6 Sequencing of cloned insert. Three clones were sequenced and compared to obtain a consensus sequence. The sequence of the insert was determined using pUC/M13 universal primers (Promega), with the CEQ Dye Terminator Cycle Sequencing Quick Start Kit (Beckman Coulter). Sequencing products were analysed with a Beckman CEQ 2000 DNA Analysis System by the Advanced Analytical Centre, James Cook University, Townsville. Chromatograms were aligned and analysed using Sequencher software (Gene Codes).

6.2 Results

The SMV200 PCR produced a product of around 200bp when examined using electrophoresis (Figure 6.1). Sequencing indicated the product was 207bp, and had the following sequence (bold/italics indicates priming sites):

TAGCTATTTTTGGTCGTCTGACATCGTAACCACTTCTTTTTTTGAAGTGTCATT
ATGTTTATGAACCATTCCTATTTGATTAAAGAATTTTTTTAATGGACTTTCAATA
GCTTCTTGTGTGCTATTCGTTGTTTTTTTTATATTAGATAGTGGTTTTTTAGCCAC
TTGTATCCTATTTTCTT***CTTCAAACACTGGTAAATTGCGGC***

Upon comparison, this sequence matched that of the relevant region of the original 2kb fragment of SMV sequence.

The *E. coli* containing the SMV insert were stored in Luria Broth (LB) with 10% glycerol at -70°C and liquid nitrogen.

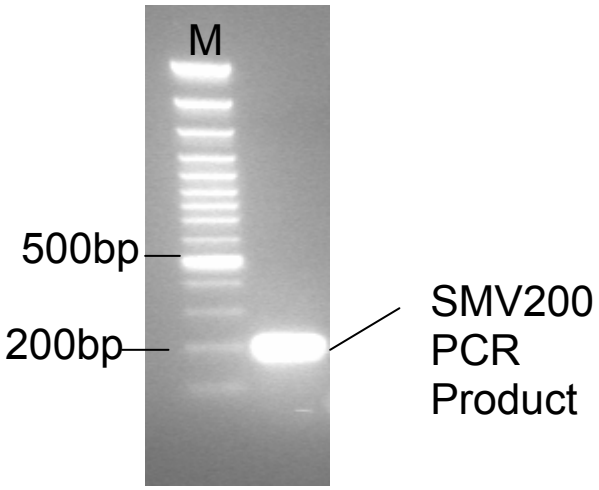

Figure 6.1: Product obtained using the SMV200 PCR. M = DNA marker (1.5% agarose gel).

6.3 Discussion

An important part of quality control in PCR based tests is the inclusion of a positive control with every batch of test samples. A sample containing the actual target virus is desirable for this purpose, but is not readily available in the absence of *in vitro* culture methods and due to inherent difficulties in obtaining purified virus in adequate quantity. Quarantine and transportation difficulties also arise in the event that multiple laboratories are participating in viral testing. A plasmid containing the target sequence for a particular PCR provides a convenient alternative. Laboratories wishing to carry out new tests are at least able to establish the successful operation of the PCR dynamics prior to the handling of clinical material. The inclusion of a plasmid sample in every batch that is tested guards against PCR setup or equipment errors, providing a greater degree of confidence in test results.

Transformed *E. coli* cells and plasmid can been stored for long periods and easily cultured when required, providing a virtually unlimited supply of material. The materials are non-infectious, negating quarantine and biohazard issues associated with the transportation of the material to other laboratories.

The positive control for the SMV200 PCR has been made available to research and diagnostic laboratories. The following have been contacted with regards to the provision of the positive control, and plasmid has been dispatched upon request.

Australian Institute of Marine Science, Townsville, Queensland.
Oonoonba Veterinary Laboratory, Queensland Department of Primary Industries, Townsville, Queensland.
Cooperative Research Centre for Aquaculture, CSIRO Livestock Industries, Brisbane, Queensland.
University of Queensland, Brisbane, Queensland.
University of Sydney, Camden, New South Wales.
New South Wales Fisheries, Wollongbar, New South Wales.
Agriculture, Fisheries and Forestry – Australia, Canberra, Australian Capital Territory.
Australian Animal Health Laboratory, CSIRO Livestock Industries, Geelong, Victoria.
Fish Health Diagnostic Services, Prospect, Tasmania.
University of Tasmania, Launceston, Tasmania.
Fisheries Department of Western Australia, Perth, Western Australia.
Berrimah Veterinary Laboratory, Department of Primary Industries and Fisheries, Berrimah, Northern Territory.
Mahidol University, Bangkok, Thailand.
University of Arizona, Arizona, USA.
Moana Technologies, Hawaii, USA.
IFREMER, Noumea, New Caledonia.

7.0 Evaluation of DNA extraction method for use on prawn tissues and postlarvae

The acquisition of high quality nucleic acid from a test sample is an inherent part of PCR. The higher the quality of nucleic acid obtained, the greater the probability of an accurate result and successful PCR amplification. As PCR-based tests are prone to corruption by poor quality nucleic acid and the presence of inhibitors, the nucleic acid extraction process on prawn samples was examined in terms of nucleic acid yield, purity, degradation and ability to PCR.

7.1 Methods

7.1.1 DNA extraction method. The High-Pure PCR Template Preparation Kit (Roche) or similar has been used in other studies investigating prawn viruses (Durand & Lightner 2002, Tang & Lightner 2002), and was evaluated for its suitability to this project. The kit was based on tissue digestion, followed by selective binding of nucleic acids to silica based spin columns, washing to remove cellular components and final elution of nucleic acid in 200µl of Tris buffer. As a standard method, samples consisted of approximately 50mg of prawn tissue or postlarvae. Frozen samples were used directly upon thawing, whereas samples in 70% ethanol were dried at room temperature for ½ - 1 hour to remove excess ethanol before processing. Samples were placed in a 1.5ml microfuge tube (Sarsdedt) containing the digestion buffer provided in the kit, and ground briefly with a plastic pestle (Astral Scientific). The extraction process was carried out as per the manufacturer's instructions for mouse tail samples. A variation was also tried, in which 50mg of prawn tissue and 1ml of PBS was added to a stomacher bag. This was homogenised with a hammer, boiled for 5 minutes and 200µl was applied to the extraction kit. Each batch of extractions included a negative control, in which an extraction was done on 200µl of sterile deionised water. These blanks were included as negative controls in all PCRs to check for reagent contamination.

7.1.2 Evaluation of DNA quality and quantity. The quantity and purity of the resultant nucleic acid samples were estimated using spectrophotometry (Section 6.1.4). The extent of DNA shearing was determined by running 10µl of the nucleic acid sample in TAE buffer on a 1% agarose gel containing 0.5µg/ml ethidium bromide, and visualised under UV light. The interpretation of the level of shearing by this method can be somewhat subjective, but was nevertheless valuable as a general indicator of sample DNA quality.

7.1.3 Ability to PCR. To determine whether the extracts were suitable for use in PCR, the nucleic acid samples were tested using decapod specific primers (143F 5'-TGC-CTT-ATC-AGC-TNT-CGA-TTG-TAG-3' and 145R 5'-TTC-AGN-TTT-GCA-ACC-ATA-CTT-CCC-3') yielding an 848bp product, based on 18S rRNA sequence of decapods (Lo *et al*. 1996). The following was included in each PCR: 200µM dNTP's, 1.5mM $MgCL_2$, 1U Taq polymerase (MBI fermentas), PCR buffer (750mM Tris-HCl pH 8.8, 200mM $(NH_4)_2SO_4$, 0.1% Tween 20) (MBI Fermentas), 1µM each primer, 1-3µl template, deionised water to 50µl final reaction volume. Thermocycling consisted of an initial denaturation at 94°C for 3 minutes, followed by 40 cycles of 94°C for 1 minute denaturation, 55°C for 1 minute annealing, 72°C for 3 minutes extension, and a final extension of 72°C for 5 minutes. The 848bp expected product was visualised on 1.2% agarose gels. Negative controls where no DNA template was added were included with each batch.

7.1.4 Postlarvae samples. All postlarvae samples had been previously stored in 70% ethanol. Samples processed included 14 extractions (samples 1-14) consisting of 30 postlarvae each, 1 extraction consisting of 50 postlarvae (sample 15), and 1 extraction of 50 postlarvae that had their eyes removed with a sterile scalpel prior to extraction (sample 16). All were tested with the decapod PCR and for DNA shearing. Samples 1-14 were evaluated with spectrophotometry.

7.1.5 Penaeus stylirostris samples. Twenty-four *Penaeus stylirostris* around 4-5g in size from New Caledonia were fixed in 10% formalin for 24 hours then transferred to 70% ethanol. A sample of intestine, gill and pleopod from one prawn were pooled to form a sample for extraction, resulting in a total of 24 extractions. These were all evaluated for DNA shearing, by spectrophotometry and with the decapod-specific PCR.

7.1.6 Penaeus merguiensis samples. Separate samples of hepatopancreas, intestine, gill and pleopod from 18 frozen adult *Penaeus merguiensis* were processed, resulting in 72 extractions. These were evaluated for DNA shearing, by spectrophotometry and with the decapod-specific PCR.

7.1.7 Prawn samples spiked with SMV200 plasmid. Separate samples from *Penaeus merguiensis* were spiked with SMV200 plasmid. These consisted of 50mg of intestine, 50mg of muscle tissue, and an additional 50mg of intestine sample processed by boiling in PBS. One of each of these samples was spiked with 118ng of SMV200 plasmid prior to being put through the extraction kit, one of each was spiked with SMV200 plasmid to a final concentration of 1.2ng/µl after being put through the extraction kit, and one of each sample was left unspiked. Each preparation was evaluated by spectrophotometry. One microlitre and 5µl of each preparation was used as template in the decapod-specific PCR, and also in the standard SMV200 PCR (Section 6.1.2). Negative controls (no template) and positive controls (SMV200 plasmid) were included in each SMV200 PCR batch.

7.2 Results

7.2.1 DNA extraction from postlarvae. The extraction method worked well for obtaining DNA from postlarvae that had been fixed in 70% ethanol. From Table 7.1, nucleic acid concentration ranged from 152.5ng/µl to 240.1ng/µl (av = 193.2ng/µl). Purity was generally good, with A_{260nm}/A_{280nm} ratios ranging from 1.51 to 1.86 (av = 1.69). All samples were positive with the decapod-specific PCR (Figure 7.1), indicating suitability for use in assays using PCR. Very little DNA shearing was evident, indicating suitable sample quality (Figure 7.2). No differences could be observed in terms of sample quality or ability to PCR between postlarvae samples with or without eyestalks removed (data not shown).

Table 7.1: Parameters indicating nucleic acid quality from postlarvae samples.

Sample Number	Decapod PCR	DNA Concentration (ng/µl)	DNA Purity A_{260nm}/A_{280nm}
1	+	183.5	1.54
2	+	177	1.78
3	+	165.5	1.74
4	+	172.5	1.78
5	+	193.5	1.76
6	+	228.5	1.62
7	+	212.5	1.51
8	+	175.5	1.70
9	+	152.5	1.52
10	+	212.5	1.65
11	+	187	1.83
12	+	179	1.83
13	+	224.5	1.57
14	+	240.1	1.86
15	+	NA	NA
16	+	NA	NA

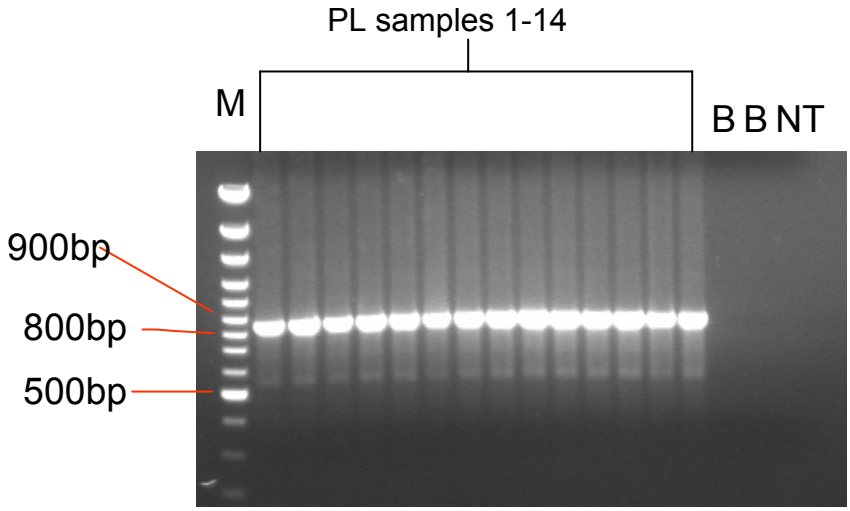

Figure 7.1: Decapod-specific PCR on postlarvae samples. M=DNA marker, PL=postlarvae, B=blank water extraction (negative control), NT=no template (negative control). 1.2% agarose gel.

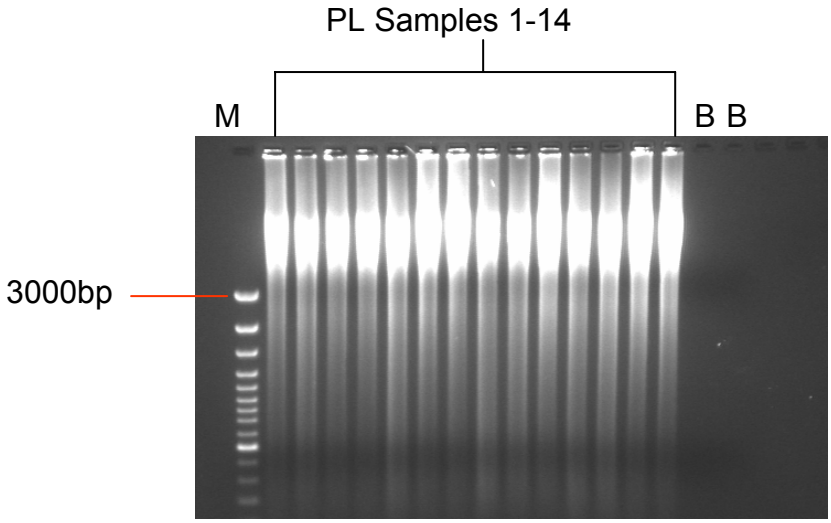

Figure 7.2: Nucleic acid extractions on postlarvae samples run on a 1% agarose gel. M=DNA marker, PL=postlarvae, B=blank water extraction (negative control).

7.2.2 DNA extraction from Penaeus stylirostris samples. Results from the *Penaeus stylirostris* samples indicated the extraction technique was suitable for use on prawn tissues which had been fixed in formalin and transferred to 70% ethanol (Table 7.2). Nucleic acid concentration ranged from 100.4ng/µl to 157.5ng/µl (av = 133.8ng/µl). Purity indicators were generally favourable, with A_{260nm}/A_{280nm} ratios ranging from 1.29 to 2.01 (av = 1.77). Twenty out of the 24 samples (83.3%) were positive for the decapod PCR (Figure 7.3), indicating suitability for PCR based tests. Some shearing was noted when samples were run on agarose gels (Figure 7.4). As these samples consisted of a number of pooled tissue types, it was uncertain whether the presence of a particular tissue was responsible.

Table 7.2: Parameters indicating nucleic acid quality from *Penaeus stylirostris* samples.

Sample Number	Decapod PCR	DNA Concentration (ng/µl)	DNA Purity A_{260nm}/A_{280nm}
1	+	120.7	1.89
2	+	142.7	1.86
3	-	107.8	1.98
4	+	130.5	1.81
5	+	142.7	1.91
6	+	137.9	1.81
7	-	100.4	1.93
8	+	117.1	2.01
9	+	137.9	1.89
10	+	117.1	2.00
11	+	130.5	1.89
12	-	108.9	1.94
13	+	137.6	1.80
14	+	148.7	1.29
15	+	142.4	1.57
16	-	148.7	1.66
17	+	148.7	1.52
18	+	157.5	1.48
19	+	142.4	1.78
20	+	142.4	1.83
21	+	148.7	1.49
22	+	116.8	1.93
23	+	148.7	1.46
24	+	133.6	1.85

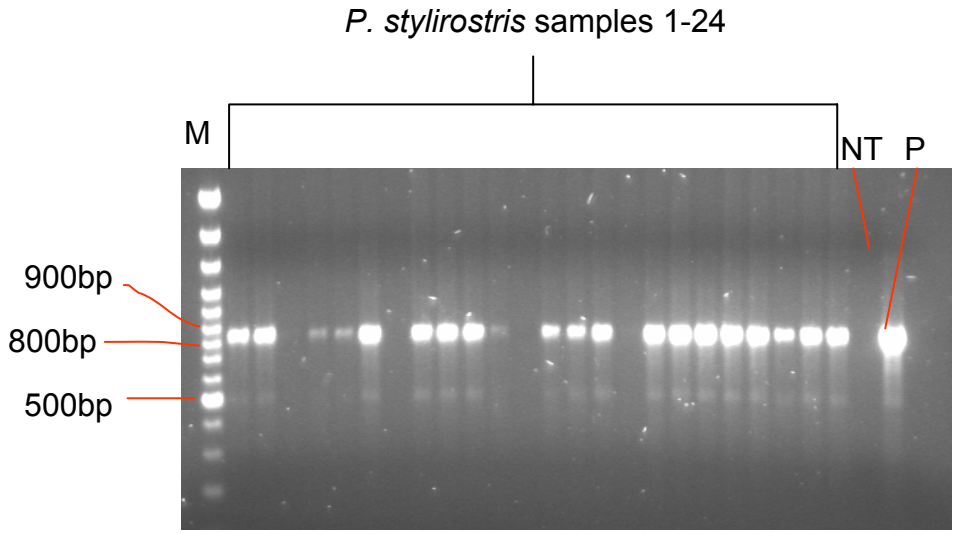

Figure 7.3: Decapod-specific PCR on *P. stylirostris* samples. M=DNA marker, NT=no template (negative control), P=positive control prawn sample. 1.5% agarose gel.

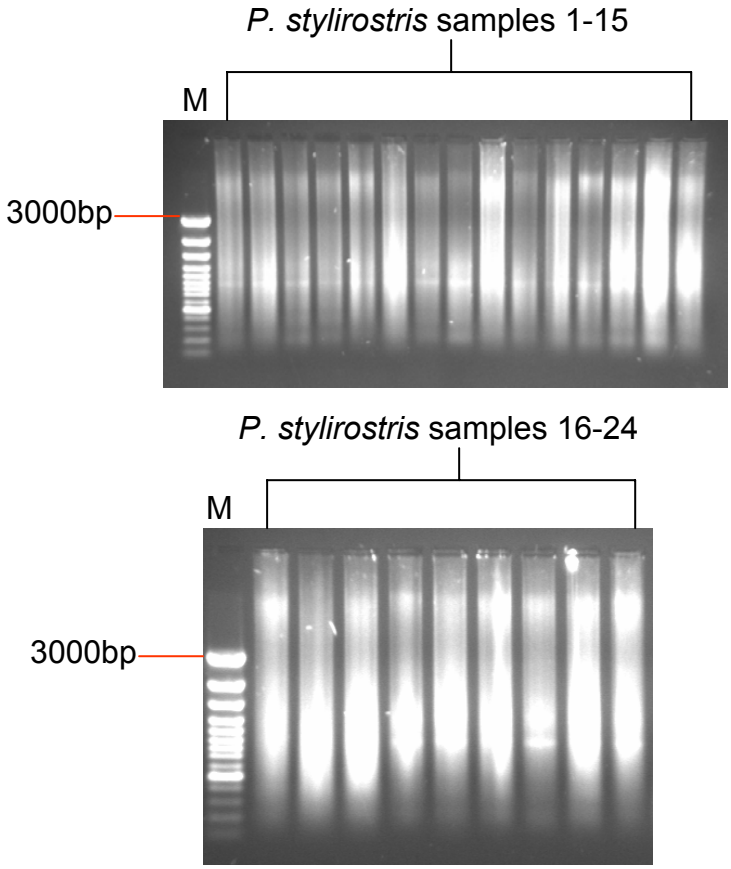

Figure 7.4: Nucleic acid extractions on *P. stylirostris* samples run on a 1% agarose gel. M=DNA marker.

7.2.3 DNA extraction from Penaeus merguiensis samples. Indicators of DNA quality were variable for the different frozen prawn tissue samples. Seventy out of the 72 samples tested (97.2%) were positive using the decapod PCR (Figure 7.5), indicating suitability for use in PCR based assays (Table 7.3). DNA concentration ranged from 25.9ng/µl to 149.7ng/µl (av = 78.8ng/µl) for hepatopancreas, 30.3ng/µl to 128.4ng/µl (av = 88.3ng/µl) for gill, 9ng/µl to 157.6ng/µl (av = 86.0ng/µl) for intestine, and 49.7ng/µl to 143.1ng/µl (av = 113.3ng/µl) for pleopod. The A_{260nm}/A_{280nm} indicator of purity ranged from 1.21 to 2.04 (av = 1.82) for hepatopancreas, 1.67 to 1.92 (av = 1.82) for gill, 1.27 to 1.97 (av = 1.82) for intestine, and 1.58 to 1.97 (av = 1.85) for pleopod. Although there was variability between prawns, the degree of DNA shearing was generally dependent upon tissue type. Generally, pleopod tissues showed very little shearing, indicating high quality DNA. Moderate levels of shearing were evident on most gill samples, with intestine faring slightly worse. Hepatopancreas samples generally exhibited the highest level of shearing.

Table 7.3: Parameters indicating nucleic acid quality from *Penaeus merguiensis* samples.

Prawn Number	Tissue	Decapod PCR	DNA Concentration (ng/µl)	DNA Purity A_{260nm}/A_{280nm}
1	Hepatopancreas	+	143.1	2.02
	Gill	+	128	1.75
	Intestine	+	95.3	1.95
	Pleopod	+	112	1.86
2	Hepatopancreas	+	148.9	1.96
	Gill	+	63.8	1.79
	Intestine	+	65.5	1.92
	Pleopod	+	143.1	1.85
3	Hepatopancreas	+	86.5	2.03
	Gill	+	92.6	1.86
	Intestine	+	42.1	1.87
	Pleopod	+	125.4	1.79
4	Hepatopancreas	+	26.5	1.59
	Gill	+	103.6	1.87
	Intestine	+	116.2	1.93
	Pleopod	+	127.6	1.92
5	Hepatopancreas	+	51.3	2.04
	Gill	+	30.3	1.88
	Intestine	+	9	1.27
	Pleopod	+	65.5	1.95
6	Hepatopancreas	+	52.3	1.81
	Gill	+	121.1	1.77
	Intestine	+	91	1.81
	Pleopod	+	123.5	1.84
7	Hepatopancreas	+	45.6	1.81
	Gill	+	74.3	1.87
	Intestine	+	86.9	1.97
	Pleopod	+	130.9	1.86
8	Hepatopancreas	+	42.7	1.76
	Gill	+	127.6	1.88
	Intestine	+	157.6	1.56
	Pleopod	+	117	1.87
9	Hepatopancreas	+	70.5	1.85
	Gill	+	128.4	1.79
	Intestine	+	119.2	1.80
	Pleopod	+	127.6	1.88
10	Hepatopancreas	+	80.1	1.99
	Gill	+	83.5	1.83
	Intestine	+	49.1	1.76
	Pleopod	+	95.5	1.91
11	Hepatopancreas	+	94.8	1.90
	Gill	+	127.6	1.89
	Intestine	+	66.8	1.72
	Pleopod	+	125	1.87

12	Hepatopancreas	+	38.2	1.91
	Gill	+	101.6	1.85
	Intestine	+	77.9	1.89
	Pleopod	+	113.9	1.84
13	Hepatopancreas	+	134.7	1.88
	Gill	+	46.6	1.78
	Intestine	+	86.15	1.93
	Pleopod	+	77.1	1.97
14	Hepatopancreas	+	28	1.77
	Gill	+	74.3	1.79
	Intestine	+	94.5	1.85
	Pleopod	+	49.7	1.91
15	Hepatopancreas	+	25.9	1.74
	Gill	+	90.4	1.92
	Intestine	+	109.7	1.91
	Pleopod	+	107.5	1.94
16	Hepatopancreas	+	149.7	1.62
	Gill	+	68.1	1.82
	Intestine	+	119.6	1.90
	Pleopod	-	138.6	1.74
17	Hepatopancreas	+	56.3	1.94
	Gill	+	103.9	1.82
	Intestine	+	99.7	1.90
	Pleopod	+	134	1.58
18	Hepatopancreas	+	142.8	1.21
	Gill	+	23.5	1.67
	Intestine	+	61.5	1.87
	Pleopod	-	125.2	1.76

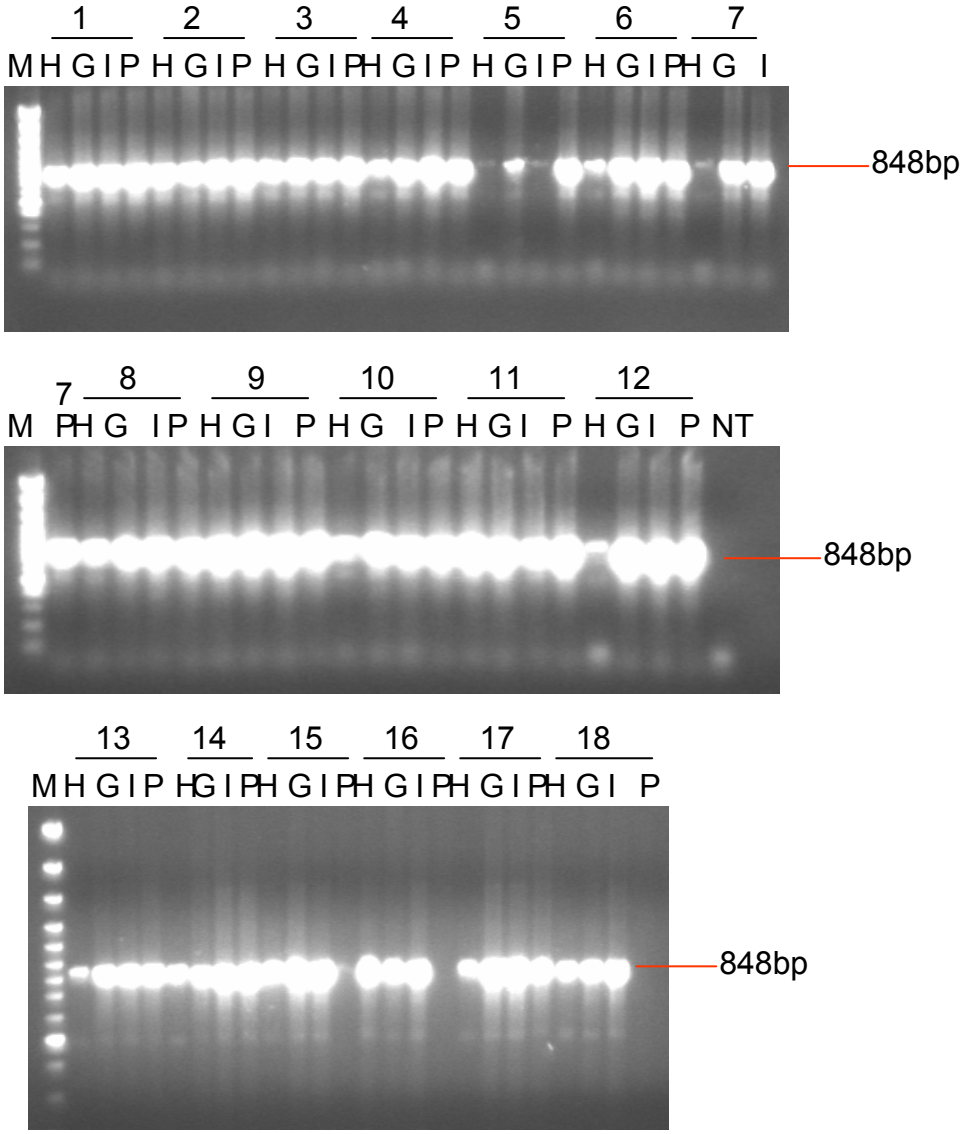

Figure 7.5: Decapod-specific PCR on *P. merguiensis* tissue samples. H=hepatopancreas, G=gill, I=intestine, P=pleopod, M=DNA marker, NT=no template (negative control), numbers designate individual prawns. 1.5% agarose gel.

7.2.4 DNA extraction from prawn samples spiked with SMV200 plasmid. All samples were positive using the decapod-specific PCR, indicating their suitability for PCR based assays (Table 7.4, Figure 7.6). All the extracts which were spiked with SMV200 plasmid were positive using the SMV200 PCR (Figure 7.7), with the non-spiked samples remaining negative for that test. Formation of primer dimers in negative samples noted in this gel (Figure 7.7) was rectified in subsequent PCRs by reducing the concentration of primers. Using the boiling method for the intestine samples resulted in less yield, probably primarily due to the PBS dilution factor. The purity was also slightly less for samples treated in this way.

Table 7.4: Parameters indicating nucleic acid quality from prawn samples spiked with SMV200 plasmid.

Plasmid Treatment	Tissue	Decapod PCR		SMV200 PCR		DNA Concentration (ng/µl)	DNA Purity A_{260nm}/A_{280nm}
		1µl	5µl	1µl	5µl		
Spiked Before Extraction	Muscle	+	+	+	+	27.9	1.72
	Intestine	+	+	+	+	54	1.93
	Boiled Intestine	+	+	+	+	9.1	1.4
Spiked After Extraction	Muscle	+	+	+	+	21.6	1.82
	Intestine	+	+	+	+	26	1.78
	Boiled Intestine	+	+	+	+	8.6	1.69
Non-Spiked	Muscle	+	+	-	-	26.7	1.78
	Intestine	+	+	-	-	68	1.89
	Boiled Intestine	+	+	-	-	3.7	1.11

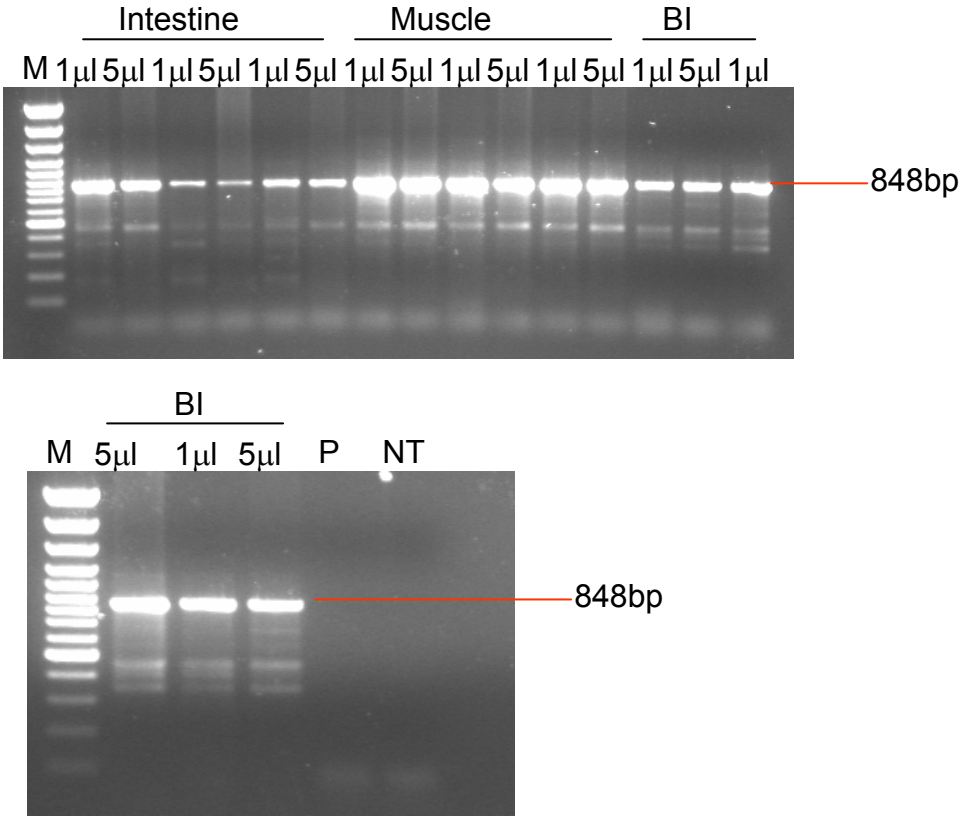

Figure 7.6: Decapod-specific PCR on plasmid-spiked prawn tissue samples. BI=boiled intestine, M=DNA marker, P=SMV200 plasmid (negative control), NT=no template (negative control), 1µl or 5µl designates volume of nucleic acid preparation used as PCR template. 1.5% agarose gel.

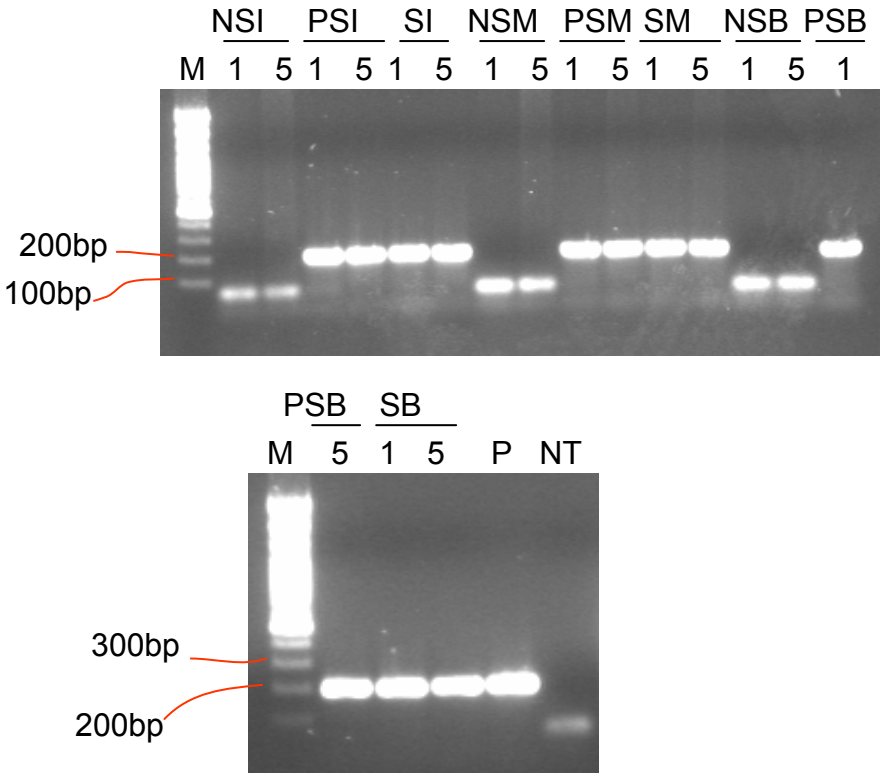

Figure 7.7: SMV200 PCR on plasmid-spiked prawn tissue samples. One or 5 designates µl volume of nucleic acid preparation used as PCR template, NSI=non-spiked intestine (intestine sample not spiked with plasmid), PSI=post-spiked intestine (intestine sample spike with plasmid after extraction), SI=spiked intestine (intestine sample spiked with plasmid before extraction), NSM=non-spiked muscle (muscle sample not spiked with plasmid), PSM=post-spiked muscle (muscle sample spiked with plasmid after extraction), SM=spiked muscle (muscle sample spiked with plasmid before extraction), NSB=non-spiked boiled intestine (boiled intestine sample not spiked with plasmid), PSB=post-spiked boiled intestine (boiled intestine spiked with plasmid after extraction), SB=spiked boiled intestine (boiled intestine spiked with plasmid before extraction), M=DNA marker, P=SMV200 plasmid (positive control), NT=no template (negative control). The <100bp product was primer dimer, this was subsequently reduced by lowering the amount of primer added to the PCR.

7.3 Discussion

The overall results indicated that the High-Pure PCR Template Preparation Kit (Roche) was suitable for extracting DNA from prawn tissues of various types and fixation treatments. The yields were generally good, the purity was high in most instances, and the DNA was able to be amplified by PCR. Successful amplification of plasmid-spiked prawn samples indicated that the SMV200 PCR was not adversely affected by the presence of prawn DNA in the test sample.

Postlarvae fixed in 70% ethanol gave particularly good results, and indicated that such samples were well suited to interrogation by PCR. As prawn eyes contain high levels of melanin, a known PCR inhibitor, there was some initial concern that the eyes of postlarvae would have to be removed prior to processing. However, no differences could be noted between postlarvae samples with or without eyes, in terms of DNA quality or ability to PCR. This indicated that the

extraction method successfully excluded melanin, and negated the labour intensive step of removing eyestalks.

Fixing prawn samples in formalin is sometimes required prior to transportation in order to avoid the potential translocation of pathogens. The results presented here indicated that prawn samples fixed briefly (24hrs) in formalin before being transferred to 70% ethanol would be suitable for testing by PCR. Samples fixed in formalin for longer times would need to be treated with caution, and individual assessment in terms of nucleic acid quality using methods described above would be required to indicate their suitability for testing.

Prawn samples are commonly submitted frozen, as this is often the most convenient form of sample storage and transportation. No gains were apparent from boiling the intestine tissues prior to extraction, so this step was deemed unnecessary for tissue samples. These results indicated that frozen prawn samples were suitable for use with the extraction method, but some caution was warranted. DNA quality indicators showed differences depending on the type of tissue. Hepatopancreas samples fared worst, followed by intestine and gill, with pleopod samples indicating higher quality nucleic acid. This was not surprising, as enzymatically active digestive tissues such as hepatopancreas and intestine are likely to cause more rapid sample degradation than the more benign tissues such as gill and pleopod. This iterated the importance of assessing the potential testing suitability of samples based on tissue type as well as host species, and the desirability of carrying out suitable checks prior to the application of a particular pathogen detection / diagnostic system. It is also desirable to choose potential tissues for testing based on increasing the chances of obtaining high quality templates, but this is not always possible due to the tissue tropism typical of many pathogens. Samples intended to be submitted frozen should be frozen immediately on site, and care taken to ensure they remain so during transportation. This is especially important if the target tissue is susceptible to rapid degradation, such as the hepatopancreas.

8.0 PCR/ELISA Protocol for the detection of spawner-isolated mortality virus (SMV)

For ease of presentation and reference, the methodology for the SMV PCR/ELISA is described here in full. This also serves as a quick reference guide to those wishing to use the method for testing, or to apply the methods to another PCR. Throughout the remainder of this document, unless otherwise stated, the term "SMV PCR/ELISA" refers to the method outlined below.

8.1 Methods

The detection of spawner-isolated mortality virus (SMV) in crustacean tissues consisted of a PCR with the addition of dig-11-dUTP, yielding a product of 207bp. Products were hybridised to a biotinylated probe and visualisation took place in a streptavidin-coated microtire plate. Products could also be visualised on an agarose gel, although products appear around 230-250bp due to the incorporated digoxigenin.

8.1.1 DNA Extraction. Extraction of nucleic acid was as discussed in Section 7.0. The method consisted of taking a 50mg sample, grinding briefly with a pestle, and then using the High Pure PCR Template Preparation Kit (Roche) according to the manufacturer's instructions.

8.1.2 SMV200 PCR Protocol. The PCR to amplify a region of SMV genome yielding a 207bp product was run using standard PCR methods, with the addition of digoxigenin-11-2'-deoxy-uridine-5'-triphosphate (Roche) in the PCR mix. The following components were included in the PCR: 200µM dNTP's, 10µM DIG-11-dUTP (Roche), 2mM $MgCl_2$, 0.5µM each primer, 1U Taq polymerase (MBI Fermentas), PCR buffer (750mM Tris-HCl pH 8.8, 200mM $(NH_4)_2SO_4$, 0.1% Tween 20) (MBI Fermentas), 1-5µl template, deionised water to 50µl final volume. Below is a suggested setup for PCR:

Per Tube (50µl)	
dH_2O	to 50µl
10 x PCR buffer with $(NH_4)_2SO_4$	5µl
dNTP's (2mM stock)	5µl
dig-11-dUTP (1nmol/µl stock)	0.5µl
primer SMV200 forward (100µM stock)	0.25µl
primer SMV200 reverse (100µM stock)	0.25µl
1U Taq (1U/µl stock)	1µl
$MgCl_2$ (25mM stock)	4µl
Template	1-5µl

A touchdown PCR strategy was used to minimise the possible occurrence of spurious bands. This involved progressively lowering the annealing temperature at each cycle from 65°C to 58°C, and then running 30 cycles at 58°C annealing temperature. The following program was the outline for the thermocycler:

Initial denaturation step of 94°C for 4 minutes, 7 cycles (94°C for 45 seconds, annealing temperature 65°C to 58°C in 1°C decreasing intervals per cycle for 50 seconds, 72°C extension for 1 minute), 30 cycles (94°C for 45 seconds, 58°C for 50 seconds, 72°C for 1 minute), final extension 72°C for 4 minutes.

Primer sequences were as follows: SMV200 forward (5'-TAG-CTA-TTT-TTT-GGT-CGT-CTG-3'), SMV200 reverse (5'-GCC-GCA-ATT-TAC-CAG-TGT-TTG-AAG-3').

8.1.3 Hybridisation. After PCR, 10µl of the PCR product was added to 90µl of hybridisation buffer containing 1.3nM biotinylated probe, in a thin walled PCR tube. The probe was biotin labelled at the 5' end (Sigma) and consisted of the following sequence: 5'-AACAACGAATAGCACACAAGAAGC-3'. The tubes were then placed in a thermocycler for a 5 minute denaturation step at 94°C, followed by a 5 minute annealing step at 62°C, then returned to ambient temperature.

8.1.4 Detection. After hybridisation, the mix (100µl) was transferred to a well of a streptavidin coated microtitre plate (Thermo Labsystems) and incubated at room temperature for 1 hour. Contents of the plate were then flicked out and wells washed 3 times with Tris buffered saline containing Tween 20 (TBST) using a large bore wash bottle. The inverted plate was tapped on paper towel to remove excess liquid.

As a blocking step, 200µl of post-coating buffer (TropBio) was added to each well and incubated for 1 hour at room temperature. This was then flicked out and the wells washed 3 times with TBST. The inverted plate was tapped on paper towel to remove excess liquid.

To each well, 100µl of a 1:1000 dilution of anti-digoxigenin-POD, Fab fragments (Roche) in ELISA diluent (TropBio) was added and incubated at room temperature for 30 minutes. Contents of the wells were flicked out and washed 3 times with TBST. The inverted plate was tapped on paper towel to remove excess liquid.

To each well, 100µl of ABTS single step solution (KPL Laboratories) was added and incubated for 30 minutes at room temperature.

Positive samples were indicated by the development of green colour in the wells. The plate could be read by eye, or absorbance measured at 409nm. Alternatively, dual absorbance readings could be used, with absorbance at 492nm subtracted from absorbance at 414nm. The dual wavelength system was most commonly used during validation and clinical testing. Absorbance above 0.5 was nominally designated as positive, and below 0.2 as negative. However, absorbance levels for positive samples were often above 1, with negative values often below 0.1.

8.1.5 Reagents and buffers.

<u>TBST</u>
100mM Tris, pH 7.5
150mM NaCl
Autoclave
Add 0.1% v/v Tween 20

<u>Hybridisation buffer</u>
4 x SSC
20mM EDTA
20mM HEPS
Autoclave
Add 0.15% Tween 20

4 x SSC
0.6M NaCl
0.06M Tri-Sodium Citrate
Autoclave

Streptavidin-coated microtitre plates: Biobind assembly strip, streptavidin coated, ThermoLabsystems, catalogue # 95029263

Digoxigenin –11-dUTP, 1nmol/µl, Roche, catalogue # 1558706

Anti-digoxigenin-POD, Fab fragments, Roche, catalogue # 1207733

8.2 Discussion

After some initial trials, the above method was formulated and applied. The method and components were chosen based on availability, ie., all the components were of a generic nature and readily available from commercial scientific suppliers. The method and components were of a non-test specific nature, in that the method could be readily applied to any current PCR. The only differences between the tests would be the PCR itself, and the design of the particular internal probe. The 96-well format ensures ease of use and time efficiency for high sample throughput applications, and as the plates can be used in strips of 8 wells, are also suitable for processing small numbers of samples without unnecessarily discarding consumables. Sensitivity and specificity issues are discussed in subsequent sections of this report. Unless otherwise stated, this was the method used in validation and clinical testing, and is referred to as the SMV PCR/ELISA throughout the text.

9.0 Sensitivity and Specificity of the SMV PCR/ELISA

The sensitivity of viral detection methods is a key issue in screening prawns. Knowledge of the detection limit of an assay gives an indication as to the level of viral load in an animal that is likely to be detected; and the relative risk of prawns with low level infections escaping detection can be appreciated. The sensitivity of the SMV assay is investigated and described in this section. Potential cross-reactivity with other organisms can lead to false-positive tests, so the assay was used to test a range of samples, in order to interrogate its specificity.

9.1 Methods

9.1.1 Sensitivity of the standard SMV PCR using pure plasmid. SMV200 plasmid positive control was prepared by growing *E. coli* JM109 cells containing the SMV200 insert in Luria Broth (LB) containing 100µg/ml ampicillin overnight at 37°C. Plasmid was harvested using the Wizard Plus SV Miniprep DNA Purification System (Promega), and the amount of plasmid estimated by spectrophotometry (Section 6.1.4). A plasmid preparation containing 10.65ng/µl DNA was 10-fold serial diluted in deionised water to 10^{-9} dilution. One microlitre of each dilution was used as template for the standard SMV200 PCR (without dig-11-dUTP), "no template" negative controls were included. PCR products were visualised on 1.5% agarose gels.

9.1.2 Sensitivity of the SMV PCR/ELISA using pure plasmid. SMV200 plasmid and serial dilutions were prepared as above, down to 10^{-12} dilution. The neat plasmid preparation had an initial concentration of 18.5ng/µl. One microlitre of each dilution was used as template for the SMV200 PCR, in which dig-11-dUTP was included. These samples were analysed by agarose gel electrophoresis, and with detection by the ELISA protocol (Section 8.0). Controls included:
-tube with no template, dig-11-dUTP included
-tube with no template, dig-11-dUTP excluded
-tube with 1µl of 10^{-2} dilution of plasmid (0.185ng), dig-11-dUTP excluded

9.1.3 Sensitivity of the SMV PCR/ELISA using plasmid-spiked prawn samples. A nucleic acid extraction was performed on a 50mg sample of pooled intestine, gill and pleopod tissues from a *Penaeus stylirostris* originating from New Caledonia, using the High Pure PCR Template Preparation Kit (Roche). The sample had tested negative for SMV, positive for the decapod-specific PCR, and had a nucleic acid concentration of 120.7ng/µl (A_{260nm}/A_{280nm} = 1.89) by spectrophotometry. Nine microlitres (1086.3ng) of this prawn extract was placed into each of 12 microfuge tubes. To the first tube, 1µl of a 20.25ng/µl SMV200 plasmid preparation was added, this was then 10-fold serial diluted through the remaining tubes. One microlitre of each of these dilutions was used as template for the SMV PCR/ELISA protocol. Non-spiked prawn extract and no template controls were included. Results of the PCR were visualised using agarose gels and ELISA.

9.1.3 SMV200 PCR/ELISA using various concentrations of prawn nucleic acid. To determine whether the amount of prawn genomic material affected the SMV PCR/ELISA, for example, through PCR inhibition, a constant amount of SMV200 plasmid was added to samples of various concentrations of prawn genomic material.

A sample of 30 *Penaeus monodon* postlarvae was put through the High Pure PCR Template preparation Kit (Roche) using the method described in Section 7.0. The extract

had a nucleic acid concentration of 177ng/µl, an A_{260nm}/A_{280nm} ratio of 1.78, displayed minimal shearing, tested positive for the decapod-specific PCR and negative for the SMV PCR/ELISA. The following samples were prepared and used as template for the SMV PCR/ELISA:
Sample 1: 88.5ng prawn nucleic acid, 13.6pg SMV200 plasmid
Sample 2: 88.5ng prawn nucleic acid, 1.36pg SMV200 plasmid
Sample 3: 177ng prawn nucleic acid, 13.6pg SMV200 plasmid
Sample 4: 177ng prawn nucleic acid, 1.36pg SMV200 plasmid
Sample 5: 354ng prawn nucleic acid, 13.6pg SMV200 plasmid
Sample 6: 354ng prawn nucleic acid, 1.36pg SMV200 plasmid
Sample 7: 531ng prawn nucleic acid, 13.6pg SMV200 plasmid
Sample 8: 531ng prawn nucleic acid, 1.36pg SMV200 plasmid
Sample 9: 708ng prawn nucleic acid, 13.6pg SMV200 plasmid
Sample 10: 708ng prawn nucleic acid, 1.36pg SMV200 plasmid
Sample 11: 885ng prawn nucleic acid, 13.6pg SMV200 plasmid
Sample 12: 885ng prawn nucleic acid, 1.36pg SMV200 plasmid

A "no template" (negative control) tube and a tube containing 13.6pg of straight SMV200 plasmid (positive control) were included in the PCR, and a well in which only hybridisation buffer and probe was added as the test sample (negative control) was included in the ELISA.

9.1.4 Specificity of the SMV PCR/ELISA. The SMV PCR/ELISA was tested against a variety of samples readily available at Microbiology and Immunology, James Cook University, in order to investigate the specificity of the assay (Table 9.4). Nucleic acid was extracted using the High Pure PCR Template Preparation Kit (Roche) using the manufacturer's protocol most suitable for the sample type. The nucleic acid extracts were used as template for the SMV PCR/ELISA, with negative controls (no template) and positive controls (SMV200 plasmid) included in every batch.

9.2 Results

9.2.1 Sensitivity of the standard SMV PCR using pure plasmid. Pure plasmid was readily detectable down to 1.065fg (equivalent to ≈300 copies) of plasmid DNA containing the SMV200 insert (Figure 9.1). A faint band was present at 0.1065fg (≈30 copies), but was not easily discernable.

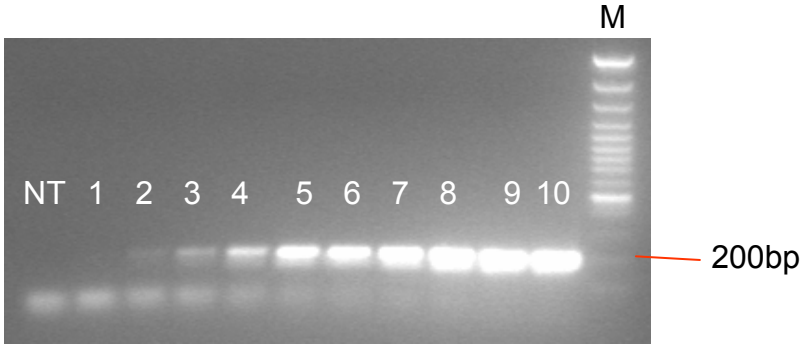

Figure 9.1: SMV200 PCR on serial dilutions of SMV200 plasmid. 1=10^{-9} dilution (0.0165fg, 3 copies), 2=10^{-8} dilution (0.1065fg, 30 copies), 3=10^{-7} dilution (1.065fg, 300 copies), 4=10^{-6} dilution (10.65fg, 3 000 copies), 5=10^{-5} dilution (0.1065pg, 30 000 copies, 6=10^{-4} dilution (1.065pg, 300 000 copies), 7=10^{-3} dilution (10.65pg, 3 000 000 copies), 8=10^{-2} dilution (0.1065ng, 30 000 000 copies), 9=10^{-1} dilution (1.065ng, 300 000 000 copies), 10=neat plasmid (10.65ng, 3 000 000 000 copies), NT=no template (negative control), M=DNA marker. 1.5% agarose gel.

9.2.2 Sensitivity of the SMV PCR/ELISA using pure plasmid. When the test plasmid dilutions were visualised using agarose gel electrophoresis, 1.85fg (≈500 copies) was detectable (Figure 9.2). A faint band was present at 0.185fg (≈50 copies), but it was less discernable at this level. The products appeared slightly larger than the expected 207bp product due to the incorporation of dig-11-dUTP.

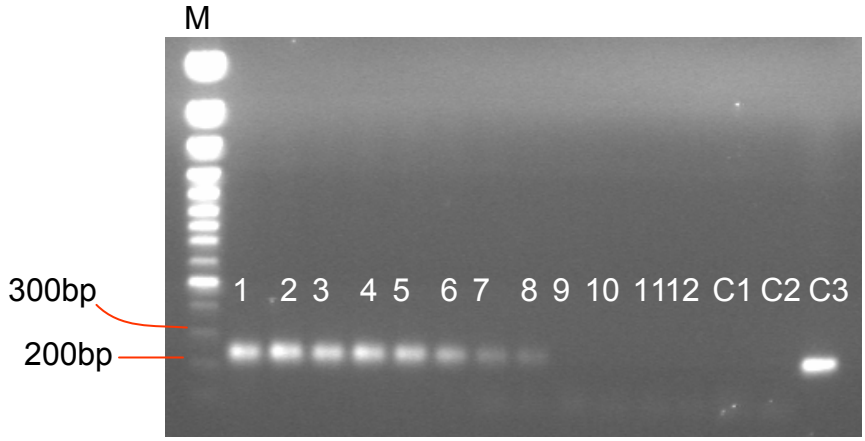

Figure 9.2: SMV200 PCR (dig-11-dUTP included) on serial dilutions of SMV200 plasmid. Products appear larger than the expected 207bp due to the incorporation of dig-11-dUTP. 1=10^{-1} dilution (1.85ng, 500 000 000 copies), 2=10^{-2} dilution (0.185ng, 50 000 000 copies), 3=10^{-3} dilution (18.5pg, 5 000 000 copies), 4=10^{-4} dilution (1.85pg, 500 000 copies), 5=10^{-5} dilution (0.185pg, 50 000 copies), 6=10^{-6} dilution (18.5fg, 5 000 copies), 7=10^{-7} dilution (1.85fg, 500 copies), 8=10^{-8} dilution (0.185fg, 50 copies), 9=10^{-9} dilution (0.0185fg, 5 copies), 10=10^{-10} dilution (0.00185fg, 0.5 copies), 11=10^{-11} dilution, 12=10^{-12} dilution, C1=control 1 (no template, dig-11-dUTP included), C2=control 2 (no template, no dig-11-dUTP), C3=control 3 (1µl of 10^{-2} plasmid dilution, no dig-11-dUTP), M=DNA marker. 1% agarose gel.

When visualised using the SMV200 ELISA protocol, the products were easily detectable down to 0.0185fg, or approximately 5 copies (Figure 9.3). Positives had absorbances over 1.5, whereas negatives had absorbances less than 0.05 (Table 9.1).

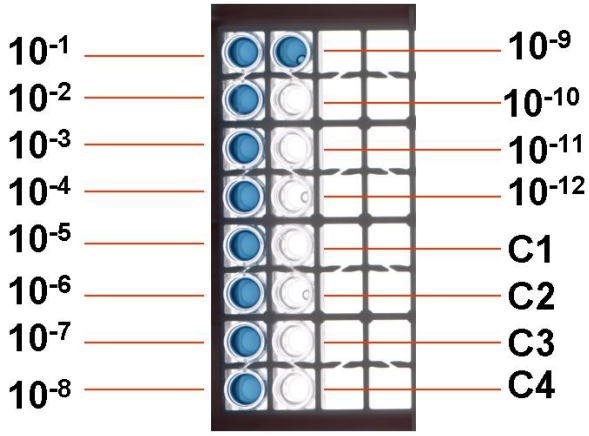

Figure 9.3: SMV200 PCR/ELISA on serial dilutions of SMV200 plasmid. 10^{-1} dilution (1.85ng, 500 000 000 copies), 10^{-2} dilution (0.185ng, 50 000 000 copies), 10^{-3} dilution (18.5pg, 5 000 000 copies), 10^{-4} dilution (1.85pg, 500 000 copies), 10^{-5} dilution (0.185pg, 50 000 copies), 10^{-6} dilution (18.5fg, 5 000 copies), 10^{-7} dilution (1.85fg, 500 copies), 10^{-8} dilution (0.185fg, 50 copies), 10^{-9} dilution (0.0185fg, 5 copies), 10^{-10} dilution (0.00185fg, 0.5 copies), 10^{-11} dilution, 10^{-12} dilution, C1=control 1 (no template, dig-11-dUTP included), C2=control 2 (no template, no dig-11-dUTP), C3=control 3 (1µl of 10^{-2} plasmid dilution, no dig-11-dUTP), C4=control 4 (hybridisation buffer and probe only added to the ELISA plate).

Table 9.1: Absorbance readings corresponding to the plate depicted in Figure 9.3.

	1	**2**
A	2.120	1.748
B	2.157	0.019
C	2.146	0.018
D	1.948	0.029
E	1.938	0.019
F	1.887	0.021
G	1.729	0.047
H	1.565	0.018

9.2.3 Sensitivity of the SMV PCR/ELISA using plasmid-spiked prawn samples. When the test plasmid dilutions were visualised using agarose gel electrophoresis, 20.25fg (≈ 6 000 copies) was detectable (Figure 9.4). A faint band was present at 2.025fg (≈ 600 copies), but was less discernable at this level. The products appeared slightly larger than the expected 207bp product due to the incorporation of dig-11-dUTP.

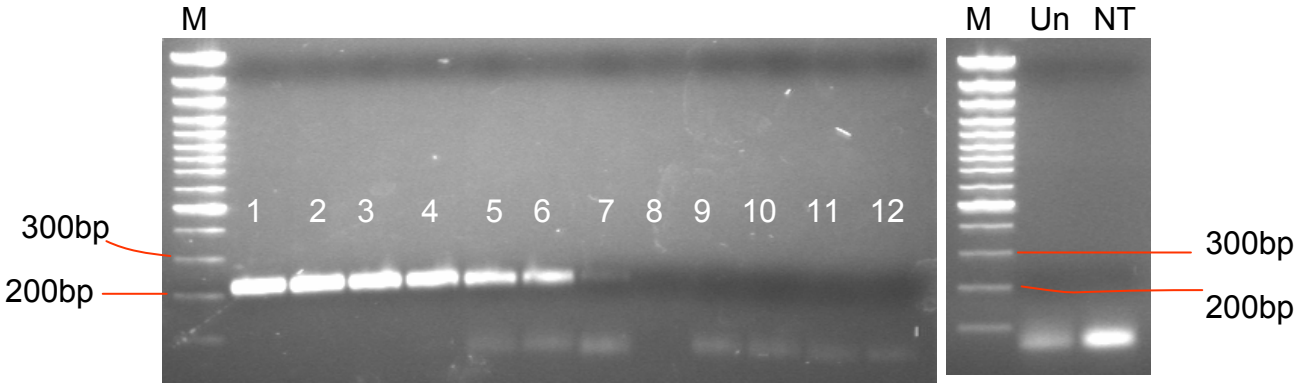

Figure 9.4: SMV200 PCR (dig-11-dUTP included) on prawn extract spiked with serial dilutions of plasmid. 1=10^{-1} dilution (2.025ng, 600 000 000 copies), 2=10^{-2} dilution (0.2025ng, 60 000 000 copies), 3=10^{-3} dilution (20.25pg, 6 000 000 copies), 4=10^{-4} dilution (2.025pg, 600 000 copies), 5=10^{-5} dilution (0.2025pg, 60 000 copies), 6=10^{-6} dilution (20.25fg, 6 000 copies), 7=10^{-7} dilution (2.025fg, 600 copies), 8=10^{-8} dilution (0.2025fg, 60 copies), 9=10^{-9} dilution (0.02025fg, 6 copies), 10=10^{-10} dilution (0.002025fg, 0.6 copies), 11=10^{-11} dilution, 12=10^{-12} dilution, Un=un-spiked prawn extract, NT=no template (negative control), M=DNA marker. 1.5% agarose gel.

When visualised using the SMV200 ELISA protocol, the products were detectable down to 0.02025fg, or approximately 6 copies, although the absorbance reading was only slightly above 0.5 at this level (Table 9.2).

Table 9.2: Absorbance readings for SMV PCR/ELISA on plasmid-spiked prawn extracts.

	1	**2**
A	2.107 (10^{-1})	0.516 (10^{-9})
B	1.952 (10^{-2})	0.029 (10^{-10})
C	1.905 (10^{-3})	0.026 (10^{-11})
D	1.756 (10^{-4})	0.028 (10^{-12})
E	1.687 (10^{-5})	0.026 (Un)
F	1.502 (10^{-6})	0.022 (NT)
G	1.384 (10^{-7})	0.021 (Blank)
H	1.023 (10^{-8})	0.025 (Blank)

10^{-1} dilution (2.025ng, 600 000 000 copies), 10^{-2} dilution (0.2025ng, 60 000 000 copies), 10^{-3} dilution (20.25pg, 6 000 000 copies), 10^{-4} dilution (2.025pg, 600 000 copies), 10^{-5} dilution (0.2025pg, 60 000 copies), 10^{-6} dilution (20.25fg, 6 000 copies), 10^{-7} dilution (2.025fg, 600 copies), 10^{-8} dilution (0.2025fg, 60 copies), 10^{-9} dilution (0.02025fg, 6 copies), 10^{-10} dilution (0.002025fg, 0.6 copies), 10^{-11} dilution, 10^{-12} dilution, Un=un-spiked prawn extract, NT=no template (negative control), Blank= hybridisation buffer and probe only added to the ELISA plate.

9.2.4 SMV200 PCR/ELISA using various concentrations of prawn nucleic acid. All the samples spiked with SMV200 plasmid were positive for the SMV PCR/ELISA; absorbance readings for the ELISA are presented in Table 9.3. This indicated that the assay worked successfully in samples which ranged from 88.5ng to 885ng of total prawn nucleic acid. This covered the range of total DNA which would typically be expected in prawn tissue samples using the Roche extraction method. This indicated that there was no observable difference in the performance of the assay (no inhibition) over DNA ranges which would normally be expected in test samples, and demonstrated that the amount of sample template added to the PCR was not a critical parameter for effective assay performance.

Table 9.3: Absorbance readings for the SMV PCR/ELISA on test samples containing various amounts of prawn nucleic acid

Sample	**Prawn Nucleic Acid (ng)**	**SMV200 Plasmid (pg)**	**Absorbance**
1	88.5	13.6	1.687
2	88.5	1.36	1.533
3	177	13.6	1.556
4	177	1.36	1.471
5	354	13.6	1.651
6	354	1.36	1.490
7	531	13.6	1.519
8	531	1.36	1.345
9	708	13.6	1.317
10	708	1.36	1.273
11	885	13.6	1.301
12	885	1.36	1.219
NT	-	-	0.019
PC	-	13.6	1.806
Hyb	-	-	0.015

NT= no template (negative control), PC= SMV200 plasmid only (positive control), Hyb= hybridisation buffer and probe only added to ELISA plate (negative control).

9.2.5 Specificity of SMV PCR/ELISA. All samples of various crustaceans, viruses, cell cultures and bacteria tested negative for the SMV PCR/ELISA (Table 9.4). Although the material chosen was largely due to availability, there was no indication of inappropriate assay cross-reactivity.

Table 9.4: Absorbance readings for the SMV PCR/ELISA on test samples of various crustaceans, viruses, cell cultures and bacteria

Sample and Source	Absorbance
Penaeus monodon, northern Queensland	0.064
Penaeus merguiensis, northern Queensland	0.026
Penaeus stylirostris, New Caledonia	0.042
Cherax quadricarinatus, northern Queensland	0.019
Infectious hypodermal and haematopoietic necrosis virus (*Parvoviridae*), New Caledonia, *Penaeus stylirostris* pleopod samples that tested positive for IHHNV, 15 samples	0.042 0.037 0.034 0.042 0.048 0.045 0.040 0.048 0.046 0.047 0.035 0.046 0.040 0.039 0.041
Infectious hypodermal and haematopoietic necrosis virus (*Parvoviridae*), Australia, *Penaeus monodon* pleopod samples that tested positive for IHHNV, 8 samples	0.064 0.064 0.059 0.079 0.085 0.100 0.068 0.078
Canine parvovirus (*Parvoviridae*), cultured in feline kidney (FK) cells	0.025
Bohle iridovirus (*Iridoviridae*), cultured in bluegill fry (BF2) cells	0.024
Mareck's disease serotype 1 (*Herpesviridae*), vaccine	0.024
Bovine herpesvirus 1 (*Herpesviridae*), cultured in Madden Darby Bovine Kidney (MDBK) cells	0.022
Dengue virus serotype 2 (*Flaviviridae*), cultured in Madden Darby Bovine Kidney (MDBK) cells	0.023
Dengue virus serotype 3 (*Flaviviridae*) cultured in Madden Darby Bovine Kidney (MDBK) cells	0.022
Kokobera virus (*Flaviviridae*), cultured in Madden Darby Bovine Kidney (MDBK) cells	0.029
Kunjin virus (*Flaviviridae*), cultured in Madden Darby Bovine Kidney (MDBK) cells	0.023
Murray Valley encephalitis virus (*Flaviviridae*), cultured in Madden Darby Bovine Kidney (MDBK) cells	0.025
Bovine viral diarrhoea virus (*Flaviviridae*), cultured in Madden Darby Bovine Kidney (MDBK) cells	0.023

Bovine enterovirus (*Picornaviridae*), cultured in Madden Darby Bovine Kidney (MDBK) cells	0.021
Bovine parainfluenza-3 virus (*Paramyxoviridae*), cultured in Madden Darby Bovine Kidney (MDBK) cells	0.033
Newcastle disease virus (*Paramyxoviridae*), cultured in chicken embryos	0.035
Avian adenovirus (*Adenoviridae*), cultured in chicken primary embryonic liver (PEL) cells	0.038
Chicken primary embryonic liver (PEL) cells	0.031
Bluegill fry (BF2) cells	0.040
Vibrio harveyi strain 20	0.019
Vibrio harveyi strain 642 (includes *Vibrio harveyi* myovirus-like bacteriophage)	0.016
Vibrio harveyi strain 645	0.016
Vibrio harveyi strain 47-6661 (includes *Vibrio harveyi* podovirus-like bacteriophage)	0.017
Vibrio harveyi strain ISO7	0.018
Vibrio cholera JCU09051	0.016
Vibrio cholera JCU09049	0.017
Vibrio parahemolyticus	0.016
Vibrio parahemolyticus JCU09075	0.016
Vibrio alginolyticus	0.017
Vibrio alginolyticus JCU09041	0.015
Vibrio mimicus	0.013
Pseudomonas nautica	0.016
Pseudalteromonas atlantica	0.017
Pseudalteromonas citrea	0.016
Pseudalteromonas rubra	0.017
NT	0.024
NT	0.025
NT	0.029
NT	0.020
NT	0.017
NT	0.018
PC	1.929
PC	2.440
PC	2.447
PC	2.466
PC	1.701
PC	2.203
Hyb	0.023
Hyb	0.024
Hyb	0.023
Hyb	0.020
Hyb	0.017
Hyb	0.016
Hyb	0.009
Hyb	0.016

Hyb	0.015
Hyb	0.016
Hyb	0.015
Hyb	0.015

NT= no template (negative control), PC= SMV200 plasmid only (positive control), Hyb= hybridisation buffer and probe only added to ELISA plate (negative control).

9.3 Discussion

The PCR/ELISA protocol generally resulted in increases in sensitivity of 10 to 100 fold over standard gel methods when used in the detection of SMV genome. Indeed, it was indicated that the assay could detect single figure copy numbers of target, which is approaching the theoretical limit of PCR. Using plasmid would be considered ideal conditions for PCR, compared to the use of whole virus particles/genome. Nevertheless, direct comparisons have indicated significant increases in sensitivity using the PCR/ELISA. This increases the probability of detecting low level infections or carrier states, and reduces the potential of infected animals slipping through a screening program.

It is useful to extrapolate the sensitivity to the viral load required in prawn tissues; when using a 50mg tissue sample with the spin column extraction method, and using 3µl of extract as template in the PCR/ELISA with a detection limit of 5 copies. On average, the prawn would need to contain around 6666 viral particles per gram of prawn tissue in order for the infection to be detected. It should be noted that the theoretical detection limit for PCR, ie, detection of one genome copy, would still require, on average, 1333 viral particles per gram of prawn tissue in order to be detected. The detection limit of all tests should always be kept in mind, as a negative result does not indicate “virus free”, but rather indicates “below detectable level”. This detectable level is an inherent measure of the sensitivity of a particular assay.

There has been no indication that prawn DNA adversely affected the SMV PCR, as indicated by its operation over wide ranges of sample nucleic acid concentrations. Some PCR’s can be sensitive to the amount of total DNA added as template, and therefore test samples require quantitation prior to application to the reaction. The results indicated that the amount of total DNA added was not a critical factor in this case, and therefore careful quantitation would not be obligatory over typically expected ranges.

The samples used for the specificity trial were chosen largely due to availability and convenience, in that they were readily available at the laboratory at James Cook University. No cross reactivity was noted with any sample of viral, crustacean, cell line or bacterial origin that was included in the trial. However, potential cross-reactivity with any particular genome of interest would need to be tested accordingly. Of note, the SMV test did not cross react with canine parvovirus, or prawn samples containing 2 different strains of infectious hypodermal and haematopoietic necrosis virus (Australian strain and New Caledonian strain).

10.0 Testing of Clinical Samples for Spawner-isolated Mortality Virus

Prawn samples were tested from a number of different facilities in a attempt to obtain an insight into the prevalence of SMV. To preserve anonymity, the samples taken from Australia were referred to as originating from "marine prawns", ie, individual species have not been indicated. Facilities at where sampling took place are referred to as "facility A, B, etc". This follows the convention of the QDPI Report to Farmers, Aquaculture Production Survey. The SMV PCR/ELISA was utilised for the majority of test samples, except for samples which were processed prior to the development of the assay. In these cases, the standard SMV200 PCR and gel electrophoresis was used. Samples consisted predominantly of frozen prawns (spawners and grow-out), postlarvae preserved in 70% ethanol and prawn faecal samples.

10.1 Methods

10.1.1 Frozen prawn tissues. Where possible, prawns were frozen on site immediately upon collection, and transported on ice to the laboratory at James Cook University. Tissues of interest (predominantly gut and hepatopancreas) were aseptically dissected out of the prawn carcass. A 50mg tissue sample was processed for DNA extraction with the High Pure PCR Template Preparation Kit (Roche) using the method described in Section 7.0. Typically, 1-5µl of the extraction was used as template for the SMV PCR/ELISA as described in Section 8.0.

10.1.2 Postlarvae fixed in 70% ethanol. Postlarvae were collected on site and placed immediately into 70% ethanol for storage and transportation to the laboratory at James Cook University. For one extraction, 30 postlarvae were aseptically transferred to a sterile Petri dish, and allowed to dry at room temperature for ½ - 1 hour to remove excess ethanol. Postlarvae were then processed using the Roche kit as described in Section 7.0. Typically 1-5µl of the extraction was used as template for the SMV PCR/ELISA as described in Section 8.0. Fixed juvenile or adult prawn tissues were processed in a similar manner following dissection.

10.1.3 Prawn faecal samples. Prawn faecal samples were collected on site using a sterile plastic pipette, and transferred to a 1.5ml microfuge tube containing 500µl of Tris buffer (10mM Tris, pH 8.5). Tubes were placed in boiling water for 2 minutes, then stored at -20°C until processing. All transportation was done under ice. When ready for processing, the faecal samples were centrifuged at 16 000 x *g* for 5 minutes to pellet the sample, and the supernatant discarded. The digestion buffer supplied with the Roche kit was added (200µl), and the sample briefly ground with a plastic pestle (Astral Scientific). The sample was processed through the kit according to the manufacturer's protocol, using a 1 hour digestion step at 55°C. Typically, 5µl of the extraction was used as template for the SMV PCR/ELISA described in Section 8.0.

10.1.4 Controls. A blank DNA extraction was performed on 200µl of sterile deionised water along with every batch of extractions, to test for contamination of kit components. An additional negative control where no template was added was run with every PCR/ELISA batch, as was a positive control consisting of the SMV200 plasmid as template. Wells in which only hybridisation buffer and probe were added without sample (negative controls) were included in each ELISA run. To assess sample DNA quality after nucleic acid extraction, the decapod-specific PCR described in Section 7.1.3 was also carried out on clinical samples. In many cases this was done on every sample, but was omitted where the DNA quality of a sample batch was determined to be acceptable via the testing of representative samples.

10.1.5 Confirmation by sequencing. To confirm that SMV positive test signals were due to amplification of the expected target sequence, 3 positive samples from facility D were sequenced. The positive samples were run again using the standard SMV200 PCR (without the addition of dig-11-dUTP). The PCR products were purified with the QIAquick PCR Purification Kit (QIAGEN) and cloned using the pGEM T-easy Vector system (Promega) according to the manufacturers' protocols. The recombinant plasmid was purified using the Wizard Plus SV miniprep system (Promega) and analyzed through PCR with M13 primers to confirm the presence of insert. Positive clones were sequenced using M13 universal primers (Promega) and a DYEnamic ET Dye Terminator Kit (Amersham Biosciences). Reaction products were analysed with a MegaBACE Sequence Analyser (Amersham BioSciences) at the Advanced Analytical Centre at James Cook University. Chromatograms were compared, aligned and analysed using Sequencher software (Genecodes Corporation).

10.1.6 Test for plasmid contamination. As a precaution, 10 SMV-positive samples from facility D were tested using a PCR with M13 primers. This PCR was used to screen plasmids prior to sequencing, and positive amplification indicates the presence of plasmid. The 10 SMV-positive samples were subjected to this PCR, which would indicate whether the SMV-positive signals were due to contamination with the SMV200 positive control.

10.2 Results

10.2.1 Controls. All positive and negative controls performed as expected. Extractions performed on the blank deionised waters were all negative, which indicated no contamination of kit components during the nucleic acid extraction steps. The "no template" PCR negative controls, and ELISA wells in which only hybridisation buffer and probe were added were always negative, and the SMV200 plasmid positive controls showed a strong positive signal in every run.

10.2.2 Facility A. A total of 122 samples were processed, from marine prawns collected during the period 14/06/02 – 13/10/03 (Table 10.1, Appendix 3). These included 57 prawns from grow-out (57 gut samples, 10 pleopod samples, 37 hepatopancreas samples) which had been frozen, and a total of 840 postlarvae (30 postlarvae pooled for one sample) that had been fixed in 70% ethanol. Of the 122 samples, 74 were tested with the decapod PCR, of which all were positive (30 gut samples, 10 combined gut and pleopod samples, 14 postlarvae samples), indicating that sample collection and processing was operating acceptably. Due to sample processing prior to the development of the SMV PCR/ELISA, 20 samples (10 gut samples, 10 combined gut and pleopod samples) were tested using the standard SMV PCR and visualisation on agarose gels. The rest were tested using the SMV PCR/ELISA. All samples tested were negative for the SMV detection assays.

10.2.3 Facility B. A total of 80 samples were processed from marine prawns collected on the 03/02/03 (Table 10.2, Appendix 3). These included 20 prawns from grow-out (20 hepatopancreas samples, 20 gill samples, 20 gut samples, 20 pleopod samples). These prawns had not been frozen on site prior to transportation on ice, but were stored at -20°C upon arrival at the laboratory at James Cook University. All samples were tested with the decapod-specific PCR, of which 57 (71.25%) were positive (35% of hepatopancreas samples, 50% of gut samples, 100% of gill samples, 100% of pleopod samples). It was evident that some sample and DNA degradation had occurred in the susceptible hepatopancreas and gut tissues, so these samples would be considered compromised. All samples were tested with the SMV PCR/ELISA. One hepatopancreas sample tested positive for SMV.

10.2.4 Facility C. A total of 70 faecal samples were collected from individually housed marine prawn spawners in the period 21/10/03 – 31/10/03 (Table 10.3, Appendix 3.). All samples were tested with the decapod-specific PCR, of which 34 (48.57%) were positive. As only very small amounts of prawn nucleic acid would be expected to be present in these samples, this indicated the collection and extraction processes were taking place in an acceptable manner. All samples were tested with the SMV PCR/ELISA, and all samples proved negative for this assay.

10.2.5 Facility D. Two lines of sampling were undertaken at this facility. In the first, a total of 117 marine prawn samples were collected over the period 04/09/02 – 19/05/03 (Table 10.4, Appendix 3). This included 43 frozen prawns (43 gut samples, 38 hepatopancreas samples, 18 gill samples, 18 pleopod samples). All samples were tested with the decapod-specific PCR, of which 109 (93.16%) were positive (88.89% of pleopod samples, 90.70% of gut samples, 94.74% of hepatopancreas samples, 100% of gill samples). All samples were tested using the SMV PCR/ELISA. Overall, 12 samples (10.26%) were positive for the assay. This included 5 gut samples, 4 hepatopancreas samples, 2 gill samples and one pleopod sample. These positive samples were all from a single batch of 18 spawners, and equated to 9 (50%) of the prawns in this batch testing positive for SMV.

The second sampling regime involved the processing of 448 gut samples from individual prawns during 2003. Satisfactory function of the extraction process was established at the start of the sampling effort, so the decapod-specific PCR was not routinely carried out on these samples. Of these, 6 (1.34%) were positive by SMV PCR/ELISA.

10.2.6 New Caledonian P. stylirostris. A total of 39 *P. stylirostris* samples were processed (Table 10.5, Appendix 3). These included 24 samples (combined gut, gill, pleopod samples) from grow-out prawns which had been fixed in 10% formalin for 24 hrs then transferred to 70% ethanol. Due to sample processing prior to the development of the SMV PCR/ELISA, these were tested using the standard SMV PCR and visualisation on agarose gels. Fifteen pleopod samples (frozen samples) from prawns purchased at a seafood vendor in Canberra in 2002 were tested with the SMV PCR/ELISA. All samples were tested with the decapod PCR, of which 35 (89.74%) were positive (83.33% of the formalin-fixed samples, 100% of the frozen samples), indicating sample collection and processing were operating satisfactorily. All samples were negative for the SMV detection assays.

10.2.7 Sequencing. The sequence obtained from the 3 positive samples matched the sequence of the SMV plasmid (Figure 10.1), confirming that the SMV PCR/ELISA was targeting the expected sequence in clinical samples.

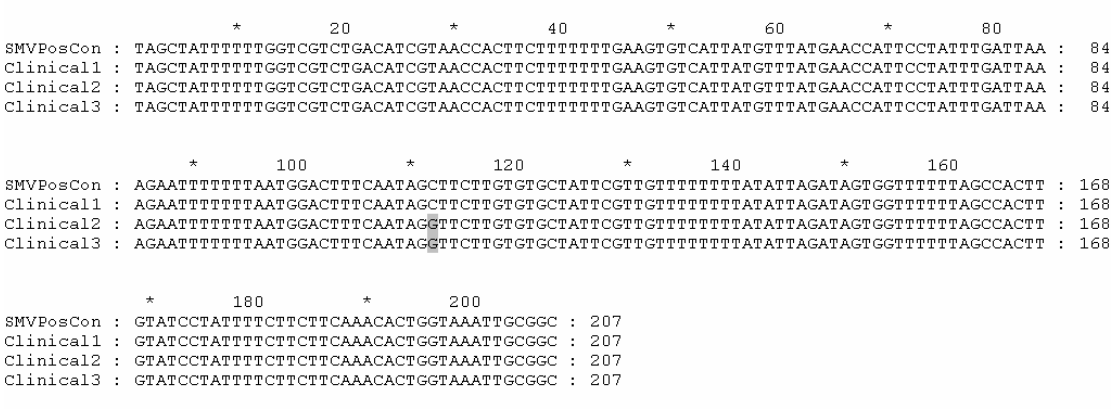

Figure 10.1: Sequence obtained from SMV-positive clinical samples (Clinical 1, 2, 3) compared with sequence from SMV200 positive control plasmid (SMV Pos Con).

10.2.8 Test for plasmid contamination. All 10 SMV-positive clinical samples were negative for the M13 plasmid screening PCR, which indicated that the SMV-positive signal was not due to contamination with SMV200 positive control.

10.3 Discussion

The occurrence of SMV-positive penaeid samples from the industry in the 2002/2003 time frame was rare. In a total of 876 individual samples processed, 19 (2.17%) were positive for SMV. The samples processed were derived from 607 individual prawns, 840 postlarvae and 70 prawn faecal samples, originating from 5 different facilities. Eighteen of the positives were from a single facility (facility D), with 1 positive from another (facility B). The samples from facility B showed some evidence of degradation, so the prevalence in these samples may have been underrepresented, but to what degree cannot be speculated.

In a survey of 909 broodstock spawners in 1998/1999, overall prevalence was 8.5% (Owens *et al*. 2003). These spawners consisted of 9 groups, with variable prevalence between groups ranging from 0% to 24%. There was some evidence of lower survival of progeny from spawners which tested positive for SMV (Owens *et al*. 2003). Although follow up survival data was not available for the more recent survey, the low prevalence indicated that the potential impact of SMV on the industry production (at least in the facilities tested) was low. SMV did not appear to be widespread throughout cultured prawns, with positives predominantly found in one facility, and only in a limited number of batch animals. If a lowering of survival of progeny from these animals occurred at the same level as that indicated in the 1998/1999 survey, the overall impact on production would not have been great.

These results indicated a low overall prevalence of SMV in cultured marine prawns during the 2002/2003 sampling period. Whether this was largely a function of the particular facilities tested, ie, if they were indicative of the whole industry, or whether the prevalences are likely to be stable over time is currently unknown. Surveys over 2 different periods (1998/1999 and 2002/2003) both indicated fairly low overall prevalences (8.5% and 2.17% of samples respectively).

11.0 Benefits and Adoption

The main beneficiaries will be diagnostic laboratories that service the prawn industries. The method developed was accurate, sensitive and specific. However, in the PCR/ELISA format costs were approximately $10.00 per sample; this includes the DNA extraction. Wage costs added approximately another $10.00 per sample, giving a cost structure of around $20.00 per sample for this technology.

Adoption has been patchy but committed where it has occurred. One farm had its entire broodstock screened for SMV in 2003 with a view to be totally free in the future. Another hatchery has requested training in the use of the SMV PCR/ELISA for increased understanding and possible implementation.

12.0 Further Development

In terms of the detection assay, the greatest cost was due to the addition of dig-11-dUTP. The omission of this component would lead to the most significant advantages in terms of assay cost. The use of other formats, such as dual probe systems could be investigated, to establish whether a cheaper test could be accommodated without losses in convenience, sensitivity or specificity. The assay method could be applied to the detection of other genomes. The modification and use of this type of assay in the detection of other pathogens could be attempted, particularly in cases where an increase in sensitivity was desired.

The biology of SMV is still relatively unknown, but it is presumed to be a parvo-like virus. Further investigations such as attempts at purification and/or obtaining additional sequence information pertaining to the currently used nucleic acid signature may be useful in elucidating the nature of SMV in prawns and prawn farms.

The data at present tended to suggest that SMV may not be having an overly significant impact on industry production, so further work on SMV may be of questionable value to industry. The recent confirmation of IHHNV (a prawn parvovirus) in Australian cultured prawns is of unknown significance. The possibility of IHHNV being widespread in the Australian industry is an avenue of potential focus and resource allocation.

13.0 Planned Outcomes

1. The kit will allow data to start being collected on SMV to meet our OIE reporting requirements.

SMV has been listed by OIE (Office International des Epizooties). The development of improved diagnostic capability in terms of sensitivity and convenience should afford robust sampling and reporting opportunities. This improved capability has been achieved.

2. Hatcheries can screen spawners/PLs before sale to grow-out farms. This provides quality assurance and ensures diseases are kept under control.

The methods developed and described were suitable for the testing of prawn tissues, postlarvae and faecal samples. Due to its format, it was suitable for high throughput applications; in that many samples could be run simultaneously in an efficient manner. These aspects afford hatcheries the potential to indulge in the non-destructive sampling of many spawners/family lines/postlarvae prior to transfer to grow-out phase. The extraction method considerations and PCR/ELISA detection method were applicable to the detection of other pathogens, not just SMV. This gives the opportunity to select certain broodstock / genetic lines / postlarvae batches / etc, in efforts of disease control, quality assurance or maintenance of biosecure facilities.

3. Making the detection of PCR products user-friendly allows it to be used more easily in areas remote from specialist laboratories allowing quicker, real time on-farm and diagnostic laboratory results.

The methods assessed and described, from the kit based extraction methods to final product detection, were chosen with ease of use and convenience in mind. Formats were chosen with consideration of the potential for processing large numbers of samples. The components were generic and readily available from well established commercial suppliers. This would guard against potential access problems for consumables. As an amplification process was required to ensure sensitivity, the crux of the test in terms of equipment and expertise is the running of the PCR. Therefore, facilities interested in using the test would require basic PCR capability as the minimum requirement. Electrophoresis and gel documentation capability would not be required for the test. A non-infectious, easily stored and transportable positive control was produced, and is available to interested laboratories. These aspects were intended to ensure as many laboratories as possible had the potential to carry out the procedure.

14.0 Conclusion

A significant amount of data was generated throughout this project on aspects of the handling and processing of prawn samples for examination using molecular biology techniques. Careful assessment of nucleic acid extraction methods should be considered an integral part of establishing new detection methods for prawn pathogens, and in the validation of individual samples in routine diagnostics. The sampling, storage, fixation, transportation and processing of prawn samples can have significant affects on the outcome of a specific nucleic acid detection test. Using spectrophotometry, electrophoresis and host-specific PCR to assess nucleic acid yield, purity, shearing and ability to amplify, differences were observed in relation to sample collection, storage, transportation and tissue type. Extraction using silica-based spin columns proved satisfactory for prawn material, in terms of yield and inhibitor exclusion. Significant differences were noted based on the type of tissues sampled, with enzymatically active tissues such as hepatopancreas and gut being more susceptible to degradation than gills and pleopods. Postlarvae fixed in 70% ethanol proved to be robust samples. This indicated that pre-PCR treatments should be assessed for each particular tissue or sample type, as they may not necessarily be equivalent. In routine diagnostics, the establishment of sample validity along with the specific pathogen testing is of obvious value. In cases where large batches of similarly derived samples are undergoing testing, the interrogation of representative batch samples to establish nucleic acid quality is probably all that is required, but for samples of variable or unknown pre-PCR treatment, the value of some form of quality assessment on every sample could be considered. Appropriate education of farm/industry personnel on the effects of sample collection and handling on molecular biology based tests can be beneficial.

A PCR/ELISA method was successfully developed for the detection of SMV. This methodology demonstrated significant improvements in sensitivity (≈10-100 fold) over standard PCR and gel detection, and was significantly more convenient than the previously employed dot-blot confirmation test. No inappropriate cross-reactivity with other genomes was observed throughout the project. The 96-well format was suited for multichannel pipettes and high throughput applications, but could also be used for small numbers of tests, as plates could be divided into 8-well strips. The components of the test were of a generic nature and readily available commercially. The methods and consumables could be applied to virtually any PCR, the only differences being in the primers and hybridisation probe. An extra level of confidence is gained through the use of a hybridisation probe, in that amplification must occur, and the amplicons must contain the specific probe sequence in order for a positive result to be registered. The PCR products are also available to be subjected to electrophoresis if desired. A non-infectious positive control was produced, and made available to those laboratories interested in testing for SMV. Overall, the diagnostic capability of SMV was improved, and the methods developed are available for current use, or modification and application to other tests should this be desired.

The occurrence of SMV in cultured prawns was rare. In a survey consisting of 876 samples processed from 5 different facilities, 2.17% were positive for SMV, with most of these occurring in one particular facility. This compared to an overall prevalence of 8.5% of samples during a 1998/1999 survey (Owens *et al*. 2003). Although there was some evidence that progeny from SMV-positive spawners had lower survival (Owens *et al*. 2003), the estimates of production loss from the 1998/1999 survey and the level of current SMV occurrence indicated that the 2002/2003 impact of SMV upon the industry would not have been particularly significant. Whether this was representative of the whole industry, or is likely to be stable over time is currently unknown.

Although no significant inroads were made as to the actual biology of SMV (a presumed parvo-like virus), it could be noted that the nucleic acid detection test for SMV did not cross react with IHHNV. This was this case when tested against an IHHNV strain found in Australia, which appeared most closely related to IHHNV found in Madagascar, and testing against a strain found in New Caledonia, which appeared more closely related to IHHNV found in the USA (Krabsetsve *et al*. submitted).

15.0 References

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Appendix 1: Intellectual Property

The brief for "Development of Diagnostic Capability for Priority Aquatic Animal Diseases of National Significance" states that all technology developed will be freely available to diagnostic laboratories at the completion of the project. Therefore, the intellectual property developed in this project will be in the public domain.

Appendix 2: Staff

Principal Investigator:	A/Prof Leigh Owens, Microbiology and Immunology, James Cook University
Research Officer:	Dr Bradford Cullen, Microbiology and Immunology, James Cook University

Appendix 3: Sampling of Penaeid Material for Detection of SMV

Table 10.1: Individual results of SMV testing for sampling at facility A

Sample	Collection Date	Weight (g)	Decapod PCR	Absorbance
Marine prawn, grow-out, gut sample	14/06/02	ND	+	PCR -ve
Marine prawn, grow-out, gut sample	14/06/02	12.1	+	PCR -ve
Marine prawn, grow-out, gut sample	14/06/02	6.8	+	PCR -ve
Marine prawn, grow-out, gut sample	14/06/02	9.1	+	PCR -ve
Marine prawn, grow-out, gut sample	14/06/02	9.6	+	PCR –ve
Marine prawn, grow-out, gut sample	14/06/02	4.5	+	PCR –ve
Marine prawn, grow-out, gut sample	14/06/02	6.3	+	PCR –ve
Marine prawn, grow-out, gut sample	14/06/02	7.2	+	PCR –ve
Marine prawn, grow-out, gut sample	14/06/02	5.2	+	PCR –ve
Marine prawn, grow-out, gut sample	14/06/02	4.1	+	PCR –ve
Marine prawn, grow-out, gut sample & pleopod sample combined	14/06/02	ND	+	PCR –ve
Marine prawn, grow-out, gut sample & pleopod sample combined	14/06/02	ND	+	PCR –ve
Marine prawn, grow-out, gut sample & pleopod sample combined	14/06/02	ND	+	PCR –ve
Marine prawn, grow-out, gut sample & pleopod sample combined	14/06/02	ND	+	PCR –ve
Marine prawn, grow-out, gut sample & pleopod sample combined	14/06/02	ND	+	PCR –ve
Marine prawn, grow-out, gut sample & pleopod sample combined	14/06/02	ND	+	PCR –ve
Marine prawn, grow-out, gut sample & pleopod sample combined	14/06/02	ND	+	PCR –ve
Marine prawn, grow-out, gut sample & pleopod sample combined	14/06/02	ND	+	PCR –ve
Marine prawn, grow-out, gut sample & pleopod sample combined	14/06/02	ND	+	PCR –ve
Marine prawn, grow-out, gut sample & pleopod sample combined	14/06/02	ND	+	PCR –ve
Marine prawn, grow-out, gut sample hepatopancreas sample	14/04/03	20.0	+ +	0.041 0.038
Marine prawn, grow-out, gut sample hepatopancreas sample	14/04/03	21.7	+ +	0.041 0.039
Marine prawn, grow-out, gut sample hepatopancreas sample	14/04/03	22.1	+ +	0.016 0.042
Marine prawn, grow-out, gut sample hepatopancreas sample	14/04/03	29.3	+ +	0.041 0.051
Marine prawn, grow-out, gut sample hepatopancreas sample	14/04/03	19.1	+ +	0.004 0.042
Marine prawn, grow-out, gut sample hepatopancreas sample	14/04/03	14.0	+ +	0.045 0.034

Marine prawn, grow-out, gut sample hepatopancreas sample	14/04/03	22.3	+ +	0.037 0.014
Marine prawn, grow-out, gut sample hepatopancreas sample	14/04/03	18.3	+ +	0.040 0.033
Marine prawn, grow-out, gut sample hepatopancreas sample	14/04/03	21.0	+ +	0.038 0.032
Marine prawn, grow-out, gut sample hepatopancreas sample	14/04/03	23.2	+ +	0.044 0.027
Marine prawn, grow-out, gut sample hepatopancreas sample	14/04/03	24.9	+ +	0.035 0.028
Marine prawn, grow-out, gut sample hepatopancreas sample	14/04/03	22.5	+ +	0.041 0.026
Marine prawn, grow-out, gut sample hepatopancreas sample	14/04/03	20.2	+ +	0.048 0.036
Marine prawn, grow-out, gut sample hepatopancreas sample	14/04/03	13.8	+ +	0.038 0.019
Marine prawn, grow-out, gut sample hepatopancreas sample	14/04/03	22.3	+ +	0.036 0.042
Marine prawn, grow-out, gut sample hepatopancreas sample	14/04/03	12.4	+ +	0.045 0.036
Marine prawn, grow-out, gut sample hepatopancreas sample	14/04/03	20.9	+ +	0.038 0.031
Marine prawn, grow-out, gut sample hepatopancreas sample	14/04/03	12.5	+ +	0.042 0.003
Marine prawn, grow-out, gut sample hepatopancreas sample	14/04/03	8.1	+ +	0.034 0.008
Marine prawn, grow-out, gut sample hepatopancreas sample	14/04/03	6.8	+ +	0.033 0.022
Marine prawn, grow-out, gut sample hepatopancreas sample	14/04/03	20.3	ND ND	0.027 0.027
Marine prawn, grow-out, gut sample hepatopancreas sample	14/04/03	17.0	ND ND	0.031 0.029
Marine prawn, grow-out, gut sample hepatopancreas sample	14/04/03	13.0	ND ND	0.029 0.031
Marine prawn, grow-out, gut sample hepatopancreas sample	14/04/03	14.8	ND ND	0.021 0.024
Marine prawn, grow-out, gut sample hepatopancreas sample	14/04/03	26.5	ND ND	0.032 0.028
Marine prawn, grow-out, gut sample hepatopancreas sample	14/04/03	17.9	ND ND	0.013 0.031
Marine prawn, grow-out, gut sample hepatopancreas sample	14/04/03	31.6	ND ND	0.031 0.024
Marine prawn, grow-out, gut sample hepatopancreas sample	14/04/03	34.2	ND ND	0.028 0.027
Marine prawn, grow-out, gut sample hepatopancreas sample	14/04/03	21.9	ND ND	0.032 0.036
Marine prawn, grow-out, gut sample hepatopancreas sample	14/04/03	21.9	ND ND	0.031 0.028

Marine prawn, grow-out, gut sample hepatopancreas sample	14/04/03	20.3	ND ND	0.037 0.017
Marine prawn, grow-out, gut sample hepatopancreas sample	14/04/03	22.0	ND ND	0.034 0.024
Marine prawn, grow-out, gut sample hepatopancreas sample	14/04/03	24.5	ND ND	0.035 0.025
Marine prawn, grow-out, gut sample hepatopancreas sample	14/04/03	23.4	ND ND	0.031 0.031
Marine prawn, grow-out, gut sample hepatopancreas sample	14/04/03	22.1	ND ND	0.032 0.038
Marine prawn, grow-out, gut sample hepatopancreas sample	14/04/03	25.9	ND ND	0.032 0.035
Marine prawn, grow-out, gut sample hepatopancreas sample	14/04/03	17.1	ND ND	0.034 0.035
Marine prawn, postlarvae	25/06/03	ND	+	0.028
Marine prawn, postlarvae	25/06/03	ND	+	0.029
Marine prawn, postlarvae	25/06/03	ND	+	0.021
Marine prawn, postlarvae	25/06/03	ND	+	0.022
Marine prawn, postlarvae	25/06/03	ND	+	0.030
Marine prawn, postlarvae	25/06/03	ND	+	0.022
Marine prawn, postlarvae	25/06/03	ND	+	0.022
Marine prawn, postlarvae	25/06/03	ND	+	0.039
Marine prawn, postlarvae	25/06/03	ND	+	0.037
Marine prawn, postlarvae	25/06/03	ND	+	0.022
Marine prawn, postlarvae	25/06/03	ND	+	0.030
Marine prawn, postlarvae	25/06/03	ND	+	0.035
Marine prawn, postlarvae	25/06/03	ND	+	0.021
Marine prawn, postlarvae	25/06/03	ND	+	0.019
Marine prawn, postlarvae	13/10/03	ND	ND	0.046
Marine prawn, postlarvae	13/10/03	ND	ND	0.060
Marine prawn, postlarvae	13/10/03	ND	ND	0.062
Marine prawn, postlarvae	13/10/03	ND	ND	0.055
Marine prawn, postlarvae	13/10/03	ND	ND	0.057
Marine prawn, postlarvae	13/10/03	ND	ND	0.045
Marine prawn, postlarvae	13/10/03	ND	ND	0.037
Marine prawn, postlarvae	13/10/03	ND	ND	0.042
Marine prawn, postlarvae	13/10/03	ND	ND	0.053
Marine prawn, postlarvae	13/10/03	ND	ND	0.045
Marine prawn, postlarvae	13/10/03	ND	ND	0.050
Marine prawn, postlarvae	13/10/03	ND	ND	0.050
Marine prawn, postlarvae	13/10/03	ND	ND	0.040
Marine prawn, postlarvae	13/10/03	ND	ND	0.036

ND=not done

Table 10.2: Individual results of SMV testing for sampling at facility B

Sample	Collection Date	Weight (g)	Decapod PCR	Absorbance
Marine prawn, grow-out	03/02/03	11.4		
hepatopancreas sample			-	0.021
gill sample			+	0.059
gut sample			+	0.080
pleopod sample			+	0.045
Marine prawn, grow-out	03/02/03	19.1		
hepatopancreas sample			-	0.021
gill sample			+	0.064
gut sample			+	0.033
pleopod sample			+	0.050
Marine prawn, grow-out	03/02/03	16.0		
hepatopancreas sample			+	1.219
gill sample			+	0.057
gut sample			-	0.026
pleopod sample			+	0.051
Marine prawn, grow-out	03/02/03	15.6		
hepatopancreas sample			-	0.020
gill sample			+	0.062
gut sample			-	0.023
pleopod sample			+	0.047
Marine prawn, grow-out	03/02/03	13.8		
hepatopancreas sample			+	0.074
gill sample			+	0.060
gut sample			+	0.064
pleopod sample			+	0.051
Marine prawn, grow-out	03/02/03	20.5		
hepatopancreas sample			+	0.081
gill sample			+	0.071
gut sample			-	0.026
pleopod sample			+	0.052
Marine prawn, grow-out	03/02/03	19.4		
hepatopancreas sample			-	0.015
gill sample			+	0.044
gut sample			+	0.026
pleopod sample			+	0.042
Marine prawn, grow-out	03/02/03	20.4		
hepatopancreas sample			-	0.021
gill sample			+	0.040
gut sample			+	0.035
pleopod sample			+	0.040
Marine prawn, grow-out	03/02/03	17.2		
hepatopancreas sample			-	0.021
gill sample			+	0.039
gut sample			+	0.031
pleopod sample			+	0.033

Marine prawn, grow-out	03/02/03	17.5		
hepatopancreas sample			+	0.032
gill sample			+	0.040
gut sample			+	0.034
pleopod sample			+	0.038
Marine prawn, grow-out	03/02/03	18.8		
hepatopancreas sample			-	0.019
gill sample			+	0.051
gut sample			-	0.019
pleopod sample			+	0.044
Marine prawn, grow-out	03/02/03	18.8		
hepatopancreas sample			+	0.027
gill sample			+	0.046
gut sample			-	0.023
pleopod sample			+	0.046
Marine prawn, grow-out	03/02/03	17.2		
hepatopancreas sample			-	0.020
gill sample			+	0.061
gut sample			+	0.033
pleopod sample			+	0.033
Marine prawn, grow-out	03/02/03	19.2		
hepatopancreas sample			+	0.024
gill sample			+	0.043
gut sample			-	0.017
pleopod sample			+	0.035
Marine prawn, grow-out	03/02/03	17.0		
hepatopancreas sample			-	0.020
gill sample			+	0.034
gut sample			-	0.015
pleopod sample			+	0.032
Marine prawn, grow-out	03/02/03	19.2		
hepatopancreas sample			-	0.016
gill sample			+	0.028
gut sample			-	0.019
pleopod sample			+	0.035
Marine prawn, grow-out	03/02/03	24.7		
hepatopancreas sample			+	0.044
gill sample			+	0.036
gut sample			+	0.038
pleopod sample			+	0.038
Marine prawn, grow-out	03/02/03	22.2		
hepatopancreas sample			-	0.017
gill sample			+	0.031
gut sample			-	0.017
pleopod sample			+	0.036
Marine prawn, grow-out	03/02/03	23.9		
hepatopancreas sample			-	0.017
gill sample			+	0.039
gut sample			+	0.044
pleopod sample			+	0.035

Marine prawn, grow-out	03/02/03	16.5		
hepatopancreas sample			-	0.011
gill sample			+	0.013
gut sample			-	0.020
pleopod sample			+	0.043

Table 10.3: Individual results of SMV testing for sampling at facility C

Sample	Collection Date	Weight (g)	Decapod PCR	Absorbance
Marine prawn, spawner, faecal sample	21/10/03 to 24/10/03	ND	+	0.020
Marine prawn, spawner, faecal sample	21/10/03 to 24/10/03	ND	-	0.019
Marine prawn, spawner, faecal sample	21/10/03	ND	-	0.020
Marine prawn, spawner, faecal sample	21/10/03	ND	-	0.019
Marine prawn, spawner, faecal sample	21/10/03	ND	-	0.017
Marine prawn, spawner, faecal sample	21/10/03	ND	-	0.018
Marine prawn, spawner, faecal sample	21/10/03	ND	+	0.017
Marine prawn, spawner, faecal sample	21/10/03	ND	-	0.020
Marine prawn, spawner, faecal sample	21/10/03	ND	-	0.018
Marine prawn, spawner, faecal sample	21/10/03	ND	-	0.017
Marine prawn, spawner, faecal sample	21/10/03	ND	-	0.020
Marine prawn, spawner, faecal sample	22/10/03	ND	+	0.105
Marine prawn, spawner, faecal sample	22/10/03	ND	-	0.018
Marine prawn, spawner, faecal sample	22/10/03	ND	+	0.016
Marine prawn, spawner, faecal sample	22/10/03	ND	-	0.017
Marine prawn, spawner, faecal sample	22/10/03	ND	-	0.015
Marine prawn, spawner, faecal sample	22/10/03	ND	+	0.018
Marine prawn, spawner, faecal sample	22/10/03	ND	-	0.017
Marine prawn, spawner, faecal sample	22/10/03	ND	+	0.017
Marine prawn, spawner, faecal sample	22/10/03	ND	+	0.018
Marine prawn, spawner, faecal sample	22/10/03	ND	-	0.016
Marine prawn, spawner, faecal sample	22/10/03	ND	+	0.017
Marine prawn, spawner, faecal sample	22/10/03	ND	+	0.017
Marine prawn, spawner, faecal sample	23/10/03	ND	-	0.017
Marine prawn, spawner, faecal sample	23/10/03	ND	-	0.017
Marine prawn, spawner, faecal sample	23/10/03	ND	-	0.019
Marine prawn, spawner, faecal sample	23/10/03	ND	+	0.017
Marine prawn, spawner, faecal sample	23/10/03	ND	-	0.016
Marine prawn, spawner, faecal sample	23/10/03	ND	-	0.017
Marine prawn, spawner, faecal sample	23/10/03	ND	-	0.018
Marine prawn, spawner, faecal sample	24/10/03	ND	-	0.016
Marine prawn, spawner, faecal sample	24/10/03	ND	-	0.017
Marine prawn, spawner, faecal sample	24/10/03	ND	+	0.016
Marine prawn, spawner, faecal sample	24/10/03	ND	-	0.016
Marine prawn, spawner, faecal sample	24/10/03	ND	+	0.016
Marine prawn, spawner, faecal sample	24/10/03	ND	-	0.017
Marine prawn, spawner, faecal sample	24/10/03	ND	-	0.017
Marine prawn, spawner, faecal sample	27/10/03	ND	+	0.013
Marine prawn, spawner, faecal sample	27/10/03	ND	+	0.015
Marine prawn, spawner, faecal sample	27/10/03	ND	+	0.015
Marine prawn, spawner, faecal sample	27/10/03	ND	+	0.015
Marine prawn, spawner, faecal sample	27/10/03	ND	+	0.015

Marine prawn, spawner, faecal sample	27/10/03	ND	+	0.001
Marine prawn, spawner, faecal sample	27/10/03	ND	+	0.017
Marine prawn, spawner, faecal sample	27/10/03	ND	-	0.015
Marine prawn, spawner, faecal sample	27/10/03	ND	+	0.016
Marine prawn, spawner, faecal sample	27/10/03	ND	+	0.017
Marine prawn, spawner, faecal sample	28/10/03	ND	-	0.014
Marine prawn, spawner, faecal sample	28/10/03	ND	+	0.015
Marine prawn, spawner, faecal sample	28/10/03	ND	-	0.016
Marine prawn, spawner, faecal sample	28/10/03	ND	-	0.014
Marine prawn, spawner, faecal sample	28/10/03	ND	+	0.017
Marine prawn, spawner, faecal sample	28/10/03	ND	+	0.014
Marine prawn, spawner, faecal sample	28/10/03	ND	+	0.014
Marine prawn, spawner, faecal sample	28/10/03	ND	+	0.014
Marine prawn, spawner, faecal sample	28/10/03	ND	+	0.016
Marine prawn, spawner, faecal sample	29/10/03	ND	+	0.015
Marine prawn, spawner, faecal sample	29/10/03	ND	+	0.014
Marine prawn, spawner, faecal sample	29/10/03	ND	-	0.014
Marine prawn, spawner, faecal sample	29/10/03	ND	+	0.014
Marine prawn, spawner, faecal sample	29/10/03	ND	+	0.015
Marine prawn, spawner, faecal sample	29/10/03	ND	-	0.015
Marine prawn, spawner, faecal sample	29/10/03	ND	-	0.016
Marine prawn, spawner, faecal sample	30/10/03	ND	+	0.015
Marine prawn, spawner, faecal sample	30/10/03	ND	-	0.017
Marine prawn, spawner, faecal sample	30/10/03	ND	-	0.016
Marine prawn, spawner, faecal sample	30/10/03	ND	-	0.015
Marine prawn, spawner, faecal sample	31/10/03	ND	+	0.015
Marine prawn, spawner, faecal sample	31/10/03	ND	-	0.015
Marine prawn, spawner, faecal sample	31/10/03	ND	+	0.017

ND=Not done

Table 10.4: Individual results of SMV testing for sampling at facility D

Sample	Collection Date	Weight (g)	Decapod PCR	Absorbance
Marine prawn, spawner	04/09/02	44.8		
hepatopancreas sample			+	1.883
gill sample			+	0.080
gut sample			+	2.318
pleopod sample			+	0.181
Marine prawn, spawner	04/09/02	45.4		
hepatopancreas sample			+	0.258
gill sample			+	2.240
gut sample			+	0.791
pleopod sample			+	0.109
Marine prawn, spawner	04/09/02	57.0		
hepatopancreas sample			+	0.188
gill sample			+	0.078
gut sample			+	0.075
pleopod sample			+	1.664
Marine prawn, spawner	04/09/02	48.6		
hepatopancreas sample			+	1.822
gill sample			+	0.152
gut sample			+	0.068
pleopod sample			+	0.065
Marine prawn, spawner	04/09/02	51.0		
hepatopancreas sample			+	2.004
gill sample			+	0.155
gut sample			+	0.633
pleopod sample			+	0.133
Marine prawn, spawner	04/09/02	39.8		
hepatopancreas sample			+	1.295
gill sample			+	0.171
gut sample			+	0.060
pleopod sample			+	0.079
Marine prawn, spawner	04/09/02	48.9		
hepatopancreas sample			+	0.027
gill sample			+	1.048
gut sample			+	0.024
pleopod sample			+	0.095
Marine prawn, spawner	04/09/02	46.9		
hepatopancreas sample			+	0.147
gill sample			+	0.087
gut sample			+	0.064
pleopod sample			+	0.066

Marine prawn, spawner	04/09/02	48.3		
hepatopancreas sample			+	0.167
gill sample			+	0.092
gut sample			+	0.777
pleopod sample			+	0.092
Marine prawn, spawner	04/09/02	45.2		
hepatopancreas sample			+	0.085
gill sample			+	0.061
gut sample			+	0.124
pleopod sample			+	0.103
Marine prawn, spawner	04/09/02	43.3		
hepatopancreas sample			+	0.195
gill sample			+	0.138
gut sample			+	0.074
pleopod sample			+	0.154
Marine prawn, spawner	04/09/02	46.8		
hepatopancreas sample			+	0.031
gill sample			+	0.160
gut sample			+	0.057
pleopod sample			+	0.106
Marine prawn, spawner	04/09/02	53.2		
hepatopancreas sample			+	0.043
gill sample			+	0.211
gut sample			+	0.072
pleopod sample			+	0.126
Marine prawn, spawner	04/09/02	42.3		
hepatopancreas sample			+	0.031
gill sample			+	0.128
gut sample			+	0.072
pleopod sample			+	0.097
Marine prawn, spawner	04/09/02	45.0		
hepatopancreas sample			+	0.127
gill sample			+	0.103
gut sample			+	1.644
pleopod sample			+	0.043
Marine prawn, spawner	04/09/02	53.2		
hepatopancreas sample			+	0.126
gill sample			+	0.150
gut sample			+	0.078
pleopod sample			-	0.070

Marine prawn, spawner	04/09/02	41.9		
hepatopancreas sample			+	0.040
gill sample			+	0.107
gut sample			+	0.123
pleopod sample			+	0.080
Marine prawn, spawner	04/09/02	46.8		
hepatopancreas sample			+	0.158
gill sample			+	0.153
gut sample			+	0.050
pleopod sample			-	0.078
Marine prawn, grow-out	19/05/03	12.3		
hepatopancreas sample			+	0.036
gut sample			+	0.026
Marine prawn, grow-out	19/05/03	17.7		
hepatopancreas sample			+	0.026
gut sample			+	0.027
Marine prawn, grow-out	19/05/03	19.8		
hepatopancreas sample			+	0.027
gut sample			-	0.019
Marine prawn, grow-out	19/05/03	11.1		
hepatopancreas sample			+	0.015
gut sample			+	0.034
Marine prawn, grow-out	19/05/03	17.7		
hepatopancreas sample			-	0.014
gut sample			+	0.019
Marine prawn, grow-out	19/05/03	15.9		
hepatopancreas sample			+	0.025
gut sample			-	0.018
Marine prawn, grow-out	19/05/03	24.3		
hepatopancreas sample			+	0.018
gut sample			-	0.017
Marine prawn, grow-out	19/05/03	16.9		
hepatopancreas sample			-	0.014
gut sample			+	0.021
Marine prawn, grow-out	19/05/03	13.3		
hepatopancreas sample			+	0.017
gut sample			-	0.016
Marine prawn, grow-out	19/05/03	18.4		
hepatopancreas sample			+	0.014
gut sample			+	0.028
Marine prawn, grow-out	19/05/03	17.7		
hepatopancreas sample			+	0.029
gut sample			+	0.029

Marine prawn, grow-out	19/05/03	17.0		
hepatopancreas sample			+	0.026
gut sample			+	0.023
Marine prawn, grow-out	19/05/03	14.2		
hepatopancreas sample			+	0.030
gut sample			+	0.025
Marine prawn, grow-out	19/05/03	13.0		
hepatopancreas sample			+	0.029
gut sample			+	0.024
Marine prawn, grow-out	19/05/03	17.7		
hepatopancreas sample			+	0.051
gut sample			+	0.024
Marine prawn, grow-out	19/05/03	15.8		
hepatopancreas sample			+	0.030
gut sample			+	0.032
Marine prawn, grow-out	19/05/03	17.0		
hepatopancreas sample			+	0.034
gut sample			+	0.022
Marine prawn, grow-out	19/05/03	15.3		
hepatopancreas sample			+	0.028
gut sample			+	0.030
Marine prawn, grow-out	19/05/03	18.6		
hepatopancreas sample			+	0.025
gut sample			+	0.027
Marine prawn, grow-out	19/05/03	15.6		
hepatopancreas sample			+	0.034
gut sample			+	0.028
Marine prawn, grow-out, gut sample	19/05/03	16.6	+	0.038
Marine prawn, grow-out, gut sample	19/05/03	16.0	+	0.030
Marine prawn, grow-out, gut sample	19/05/03	13.6	+	0.034
Marine prawn, grow-out, gut sample	19/05/03	18.3	+	0.030
Marine prawn, grow-out, gut sample	19/05/03	16.4	+	0.037

Table 10.5: Individual results of SMV testing on *P. stylirostris*

Sample	Collection Date	Weight (g)	Decapod PCR	Absorbance
P. stylirostris, combined gut, gill and pleopod sample	08/10/02	≈4-5	+	PCR -ve
P. stylirostris, combined gut, gill and pleopod sample	08/10/02	≈4-5	+	PCR –ve
P. stylirostris, combined gut, gill and pleopod sample	08/10/02	≈4-5	-	PCR –ve
P. stylirostris, combined gut, gill and pleopod sample	08/10/02	≈4-5	+	PCR –ve
P. stylirostris, combined gut, gill and pleopod sample	08/10/02	≈4-5	+	PCR –ve
P. stylirostris, combined gut, gill and pleopod sample	08/10/02	≈4-5	+	PCR –ve
P. stylirostris, combined gut, gill and pleopod sample	08/10/02	≈4-5	-	PCR –ve
P. stylirostris, combined gut, gill and pleopod sample	08/10/02	≈4-5	+	PCR –ve
P. stylirostris, combined gut, gill and pleopod sample	08/10/02	≈4-5	+	PCR –ve
P. stylirostris, combined gut, gill and pleopod sample	08/10/02	≈4-5	+	PCR –ve
P. stylirostris, combined gut, gill and pleopod sample	08/10/02	≈4-5	+	PCR –ve
P. stylirostris, combined gut, gill and pleopod sample	08/10/02	≈4-5	-	PCR –ve
P. stylirostris, combined gut, gill and pleopod sample	08/10/02	≈4-5	+	PCR –ve
P. stylirostris, combined gut, gill and pleopod sample	08/10/02	≈4-5	+	PCR –ve
P. stylirostris, combined gut, gill and pleopod sample	08/10/02	≈4-5	+	PCR –ve
P. stylirostris, combined gut, gill and pleopod sample	08/10/02	≈4-5	-	PCR –ve
P. stylirostris, combined gut, gill and pleopod sample	08/10/02	≈4-5	+	PCR –ve
P. stylirostris, combined gut, gill and pleopod sample	08/10/02	≈4-5	+	PCR –ve
P. stylirostris, combined gut, gill and pleopod sample	08/10/02	≈4-5	+	PCR –ve
P. stylirostris, combined gut, gill and pleopod sample	08/10/02	≈4-5	+	PCR –ve
P. stylirostris, combined gut, gill and pleopod sample	08/10/02	≈4-5	+	PCR –ve

P. stylirostris, combined gut, gill and pleopod sample	08/10/02	≈4-5	+	PCR –ve
P. stylirostris, combined gut, gill and pleopod sample	08/10/02	≈4-5	+	PCR –ve
P. stylirostris, combined gut, gill and pleopod sample	08/10/02	≈4-5	+	PCR –ve
P. stylirostris, pleopod sample		ND	+	0.042
P. stylirostris, pleopod sample		ND	+	0.037
P. stylirostris, pleopod sample		ND	+	0.034
P. stylirostris, pleopod sample		ND	+	0.042
P. stylirostris, pleopod sample		ND	+	0.048
P. stylirostris, pleopod sample		ND	+	0.045
P. stylirostris, pleopod sample		ND	+	0.040
P. stylirostris, pleopod sample		ND	+	0.048
P. stylirostris, pleopod sample		ND	+	0.046
P. stylirostris, pleopod sample		ND	+	0.047
P. stylirostris, pleopod sample		ND	+	0.035
P. stylirostris, pleopod sample		ND	+	0.046
P. stylirostris, pleopod sample		ND	+	0.040
P. stylirostris, pleopod sample		ND	+	0.039
P. stylirostris, pleopod sample		ND	+	0.041

ND=Not done