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Polymerase chain reaction detection of Taura Syndrome Virus and infectious hypodermal and haematopoietic necrosis virus in frozen commodity tails of *Penaeus vannamei* Boone

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Abstract

The prawn viruses White Spot Syndrome Virus and yellow head virus have been detected in frozen commodity prawns. However, no studies on the occurrence of two other Office International des Epizooties-listed viruses, Taura Syndrome Virus (TSV) or infectious hypodermal and haematopoietic necrosis virus (IHHNV), have been published. Therefore, a polymerase chain reaction (PCR) study on whether these two viruses could be found in frozen commodity prawns imported into Australia was undertaken. Amplicons indicative of TSV were found in 6/10 prawns collected at a retail outlet in Townsville and 10/10 prawns from Coff's Harbour using two different reverse transcriptase PCR protocols. Nucleotide sequences from the amplicons were between 97% and 99% similar to various TSV isolates from Asia. Amplicons indicative of the Philippine/American lineage of IHHNV were found in 5/5 and 5/6 prawns collected from Townsville and Coff's Harbour respectively. Clearly, frozen commodity *Penaeus vannamei* Boone from China can carry TSV and IHHNV with nucleic acid intact enough to be detected using PCR.

Keywords: TSV, IHHNV, commodity prawns, shrimp, vannamei

Introduction

Frozen commodity prawns are known carriers of viable prawn viruses (Nunan, Poulos & Lightner 1998; Durand, Tang & Lightner 2000; Reville, Al-Beik, Meehan-Meola, Xu, Goldsmith & Alcivar-Warren, 2005). They have been shown to carry both White Spot Syndrome Virus (WSSV) and yellow head virus (YHV) (Nunan *et al.* 1998; McColl, Slater, Jeyasekaran, Hyatt & Crane 2004). Furthermore, wastes from processing commodity shrimp have been implicated in the spread of viruses from rubbish dumps to prawn farms by birds. This theory has received support from studies that show that viable, non-enveloped viruses can pass through the gut of seagulls (Garza, Hasson, Poulos, Redman, White & Lightner 1997; Vanpatten, Nunan & Lightner 2004) and chickens (Vanpatten *et al.* 2004).

In Australia, there have been at least three major incidents involving commodity prawns infected with WSSV as well as some minor events involving false-positive diagnoses. The first incident involved prawns from Asia being sold for bait that were confirmed to be WSSV positive by this laboratory and CSIRO Long Pocket Laboratory. This incident ultimately led to a ban on using imported prawns for bait. The second incident was initiated by a concerned member of the public who purchased prawns from a retail noodle outlet and submitted them to this laboratory, where they were subsequently found to be WSSV positive by the diagnostic Office International des Epizooties (OIE) polymerase chain reaction (PCR) and by sequencing. These results were confirmed by CSIRO Long Pocket Laboratories and ultimately Australian Animal Health Laboratory, which undertook infection trials demonstrating infectivity

and published the results (McColl *et al.* 2004). That paper also reported the PCR presence of YHV in those same prawns. The third incident also involved bait prawns being fed to prawns as maturation feed. The subsequent action led to quarantining, destruction of animals and discovery of the WSSV PCR signal in wild crabs near the facility. In time, an Australia wide survey was undertaken, which maintained Australia's WSSV-free status (East, Black, Findlay & Bernoth 2005). Several false-positive incidents were due to white spot lesions occurring in prawn farms that were shown to be not viral in aetiology but possibly bacterial. Australia has had a history of rapid, strong legislative and expensive responses to incursions of aquatic viruses and is currently declared free of both WSSV and Asian strain YHV.

Despite commodity prawns being shown to carry WSSV and YHV Asian strain both overseas and in Australia, no study has been published on whether commodity prawns can carry Taura Syndrome Virus (TSV) or infectious hypodermal and haematopoietic necrosis virus (IHHNV). They are both non-enveloped and therefore are likely to be more resistant to degradation than either WSSV or YHV. Therefore, there is an expectation that they would be found in commodity prawns. Both these viruses are notifiable under the auspices of the OIE but no published data exist on their presence in commodity prawns. This study was initiated to see whether TSV in particular or IHHNV adventitiously could be detected in imported tail meat from Asia arriving at commercial retail outlets in Australia.

Materials and methods

Prawns

Peeled tail meat of *Penaeus vannamei* Boone was used in this study. They were labelled on the retail packs as imported from China and purchased from commercial retail outlets (fish shops) in Australia at Coff's Harbour (New South Wales) and Townsville (Queensland). Ten specimens from each geographical sample were processed and examined. The tissue in the uropods of each specimen from Townsville and muscle from the sixth abdominal segment from Coff's Harbour prawns was used for DNA and RNA extraction. Total RNA was extracted using TRIzol reagent (Invitrogen, Melbourne, Australia).

RT-PCR for TSV detection

Two different PCR methods were applied to each sample to detect TSV. They were the one-step RT-PCR method promulgated by OIE (2003) and the semi-nested RT-PCR method established by Chang, Peng, Yu, Liu, Wang, Lo and Kou (2004). With the OIE (2003) method, the GeneAmp, EZrTth RNA PCR kit (Applied Bioscience, Mumbai, India) was used for all amplification reactions. Primers 9195 (5′-TCA ATG AGA GCT TGG TCC-3′) and 9992 (5′-AAG TAG ACA GCC GCG CTT-3′) yielding a 231 bp product were used. The reaction mixture contained 300 μM of each dNTP, 0.5 μM of each primer, 2.5 U of rTth DNA polymerase, 2.5 mM manganese acetate, in 5 × EZ buffer (25 mM bicine, 57.5 mM potassium acetate, 40% [w/v] glycerol, pH 8.2), 1 μL extracted sample template and sterile-distilled water to 50 μL total volume. The RNA template and all the reagents were combined and reverse transcription was allowed to proceed at 60 °C for 30 min, followed by 94 °C for 2 min. At the completion of reverse transcription, the samples were amplified for 35 cycles under the following conditions: denaturation at 94 °C for 45 s, annealing/extension at 60 °C for 45 s and a final extension at 60 °C for 7 min. All PCR-amplified products were visualized using electrophoresis with 2% agarose gels containing ethidium bromide at a concentration of 0.5 μg mL^{-1}.

The procedure of a nested RT-PCR for TSV detection from Chang *et al.* (2004) was also used to maximize the chances of detecting TSV. First-strand cDNAs of extracted RNAs were synthesized using an oligo-dT primer (Roche Diagnostics, Castle Hill, Australia) and Superscript III reverse transcriptase (Invitrogen) using the manufacturer's protocol (65 °C for 5 min, 37 °C for 1 h, followed by 70 °C for 15 min). A TSV-specific semi-nested primer set, TSVF2, TSVF3 and TSVR2, was used (Chang *et al.* 2004) for amplification. The primers TSVF2 (5′-ACC CCA GAA ATG TGA ATA ACC-3′) and TSVR2 (5′-GGA AAA GCA ATG TCA ATA CCC-3′) served as the outer primer pair for the first PCR step and the primer TSVF3 (5′-ATA CTT AGC ACA GCG ACC ATA-3′) combined with TSVR2 served as the inner primers for the semi-nested amplification. Amplicons resulting from TSVF2/TSVR2 and TSVF3/TSVR2 were 910 and 360 bp in length.

In the first step of amplification, the reaction mixture contained 200 μM each dNTP, 100 pM of each primer (TSVF2 and TSVR2), 1.5 mM $MgCl_2$, PCR buffer (100 mM Tris–HCl, 200 mM $(NH4)_2SO_4$, 0.1%

Tween 20, pH 8.8), 2 U *Taq* polymerase (MBI Fermentas; Progen, Brisbane, Australia), 10 μL extracted sample template and sterile-distilled water to a 100 μL total volume. Amplification consisted of an initial denaturation at 94 °C for 4 min, followed by 40 cycles of denaturation at 94 °C for 1 min, primer annealing at 55 °C for 1 min and extension at 72 °C for 2 min and a final extension at 72 °C for 5 min.

After completion of the first step, 10 μL of the reaction mixture was added to 90 μL of the second reaction mixture containing 200 μM each dNTP, 100 pM each primer (TSVF3 and TSVR2), 1.5 mM $MgCl_2$, PCR buffer (100 mM Tris–HCl, 200 mM $(NH4)_2SO_4$, 0.1% Tween 20, pH 8.8), 2 U *Taq* polymerase (MBI Fermentas; Progen). Cycling conditions were as in the outer nested step.

Cloning of the PCR products

The procedure of cloning PCR products involved three main steps: cleaning up and extraction of nucleic acid from PCR products, ligation of extracted nucleic acid into cloning vector and transformation of clones containing insert in *Escherichia coli* cells (Mezei & Storts 1994). Cleaning up and extraction of nucleic acid from PCR products was performed using the HiYield Gel/PCR Extraction Kit (Real Biotech Corporation, ChungHo, Taiwan). The extracted nucleic acids were ligated using the T&A Cloning Vector Kit (Real Biotech Corporation). At the end of the cloning stage, the ligated products were transformed into *E. coli* HIT Competent Cells™ (Real Biotech Corporation) using the manufacturer's protocol. Three clones were selected based on blue/white colony screening on the LB/amplicillin/IPTG/X-Gal agar. Then the clones were grown over-night at 37 °C in 10 mL of LB media containing 100 μg amplicillin. Plasmids were harvested after growing HIT Competent Cells™ overnight based on the protocol of the Hi Yield Plasmid Mini Kit (Real Biotech Corporation) and sequenced.

Sequencing PCR

The Amersham Pharmacia Biotech, Sydney, Australia, protocol from the DYEnamic ET dye terminator cycle sequencing kit for MegaBACE DNA Analysis Systems was used for the sequencing PCR. Three clones were sequenced using pUC/M13 universal primers (M13F-17; 5′-GTTTTCCCAGTCACGAC-3′ and M13R-17; 5′-CAGGAAACAGCTATGAC-3′) (Promega, Sydney, Australia). Although both three forward and three reverse reactions were performed for most of the clones, two or only one forward and reverse reactions were performed on some samples (mentioned in the results of each sample).

Sequencing PCR, which included 25 cycles of 95 °C for 20 s, 50 °C for 15 s and a final 60 °C for 1 min, was conducted. In order to remove unnecessary materials from the sequencing PCR products, the post-reaction clean-up procedure using the D50 Dye Terminator Removal Kit (Real Biotech Corporation) protocol was performed. Sequencing products were then analysed either at the Advanced Analytical Center, James Cook University, Townsville, Australia, or at Macrogen, Seoul, South Korea.

Infectious hypodermal and haematopoietic necrosis virus

Nucleic acid extraction

Total DNA was extracted from approximately 50 mg of muscle tissue using the High Pure PCR Template Preparation Kit (Roche Diagnostics) according to the manufacturer's protocol. The quality of the extracted DNA was assessed using PCR amplification based on the 18SrRNA sequence of decapods as a multiplex PCR (Krabsetsve unpublished) with the IHHNV392 primers. Primers 143F (5′-TGC CTT ATC AGC TNT CGA TTG TAG-3′) and 145R (5′-TTC AGN TTT GCA ACC ATA CTT CCC-3′) were used, yielding an 848 bp product (Lo, Leu, Ho, Chen, Peng, Chen, Chou, Yeh, Huang, Chou, Wang & Kou 1996).

The method for detecting IHHNV followed exactly that of Krabsetsve, Cullen and Owens (2004). Briefly, primers IHHNV392F and IHHNV392R, which yield a 392 bp amplicon (5′-GGGCGAACCAGAATCACTTA 3′; 5′-ATCCGGAGGAATCTGATGTG-3′, Tang, Durand, White, Redman, Pantoja & Lightner 2000), and IHHNV primers 389F and 389R (5′-CGGAACA CAACCCGACTTTA-3′, 5′-GGCCAAGACCAAAATAC GAA-3′, OIE 2003), which yield a 389 amplicon, were used. The reaction mixture contained 200 μM each dNTP, 0.3 mM each primer, 2 mM $MgCl_2$, PCR buffer (100 mM Tris–HCl, 200 mM $(NH_4)SO_4$, 0.1% Tween 20, pH 8.8), 1 U *Taq* polymerase (MBI Fermentas; Progen), 1 μL extracted sample template and sterile-distilled water to 50 μL total volume. Amplification consisted of an initial denaturation at 94 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 55 °C for 30 s and extension at 72 °C for 1 min and a final extension at 72 °C for 7 min.

Results

Taura Syndrome Virus

All the samples bought in Coff's Harbour (10 of 10 specimens) were detected as being TSV positive in one-step RT-PCR (Fig. 1) using the OIE (2003) method (Table 1). Only example gels are depicted; the OIE method on the Coff's Harbour samples (Fig. 1) and the Chang *et al.* (2004) nested method for the Townsville samples (Fig. 2). In the case of semi-nested RT-PCR (Chang *et al.* 2004), although only one (lane 3) of 10 samples was positive in outer PCR, all the samples were TSV positive in inner semi-nested PCR. The sample that showed a positive result in outer PCR showed a strong positive result in inner PCR.

Six of ten samples bought in Townsville were detected as being TSV positive in one-step RT-PCR (OIE 2003) although some samples showed a very weak reaction. In case of semi-nested RT-PCR (Chang *et al.* 2004), although only one (lane 3) of 10 samples was positive in outer PCR (Fig. 2), six of ten samples were TSV positive in inner nested PCR. The sample that showed a positive result in outer PCR showed an extremely strong positive reaction in inner PCR.

Sequencing

Two samples of 231 bp amplicons produced using one-step RT-PCR (OIE 2003) and three samples of 360 bp amplicons produced using semi-nested RT-PCR (Chang *et al.* 2004) were sequenced. Three clones were sequenced in each sample. The number of forward and reverse reactions was different for each sample.

Three clones (TSV1a, TSV1b and TSV1c) of the 231 bp amplicons from the Coff's Harbour sample, (OIE 2003 protocol) produced using the one-step

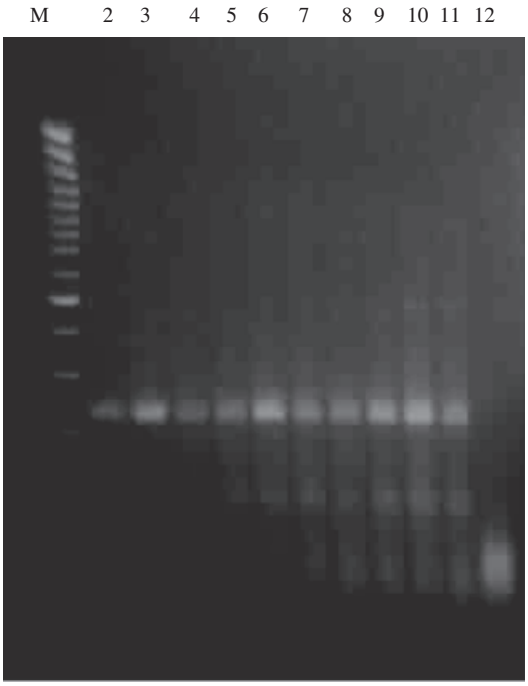

Figure 1 Polymerase chain reaction amplicons using primers 9195 and 9992 from OIE (2003) (231 bp expected product). Lane M: molecular weight marker (100 bp); lane 2–11: *Penaeus vannamei* uropod samples, Coff's Harbour; lane 12: no template (negative control).

Table 1 The results of the two polymerase chain reactions for Taura Syndrome Virus in the *Penaeus vannamei* imported into Australia

	Source of primers	**OIE (2003)**	**Chang *et al.* (2004)**		
Source of Chinese *P. vannamei*	**Size of amplicon**	**(231 bp)**	**Outer nest (910 bp)**	**Inner nest (360 bp)**	
Coff's Harbour ($n = 10$)	Number positive	10	1	10	
	Accession nos.				Accession nos.
	DQ104696 China	97%		99%, 99%	AY997025 Thailand
	AY694136 Vietnam	97%		99%, 99%	AY355309 Taiwan
Townsville ($n = 10$)	Number positive	6	1	6	
	Accession nos.				Accession nos.
	DQ104696 China	98%		99%	AY997025 Thailand
	AY694136 Vietnam	98%		99%	AY355309 Taiwan

Included are the percentage similarities of the sequenced amplicons to Taura Syndrome Virus entries into NCBI GenBank with their accession numbers.

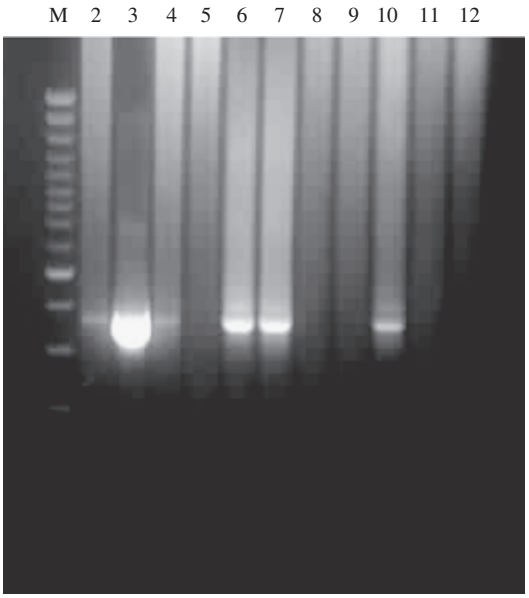

Figure 2 Results of inner semi-nested polymerase chain reaction using primers TSVF3 and TSVR2 of Chang *et al.* (2004) (360 bp expected product). Lane M: molecular weight marker (100 bp); lanes 2–11: *Penaeus vannamei* uropod samples, Townsville; lane 12: no template (negative control).

RT-PCR were sequenced with two forward and two reverse reactions on each clone. Only one of three clones (TSV1a) had a clear sequence. Basic local alignment search tool (BLAST) analyses of this sequence in the GenBank NCBI database produced alignments with previously reported TSV sequences in China (complete genome of TSV isolate ZHZC3TSV, accession no. DQ104696) with a nucleotide similarity of 97% (225/231) and it also had the same similarity to a Vietnamese isolate (AY694136).

Three clones (TSV2a, TSV2b and TSV2c) of the 231 bp amplicons from the Townsville sample (OIE 2003 protocol) produced using the one-step RT-PCR were sequenced with three forward and three reverse reactions on each clone. Two of three clones (TSV2a and TSV2b) had the same sequence at high fidelity. Basic local alignment search tool analyses of this sequence at the GenBank NCBI database produced alignments with previously reported TSV sequences from China (complete genome of TSV isolate ZHZC3TSV, accession no. DQ104696) with a nucleotide similarity of 98% (227/230) and similarly to a Vietnamese isolate (AY694136, 98%).

Three clones (TSV12a, TSV12b and TSV12c) of the 360 bp amplicons from Coff's Harbour sample (Chang *et al.* 2004 protocol) produced using semi-nested RT-PCR were sequenced with one forward and one reverse reaction on each clone (at Macrogen, South Korea). All three clones showed an identical sequence. The BLAST analyses of this sequence at the GenBank NCBI database produced alignments with multiple previously reported TSV sequences, with the highest similarity being from Thailand (AY997025) and Taiwan (AY355309) with a nucleotide similarity of 99% (363/366). A further three clones (TSV15a, TSV15b and TSV15c) from Coff's Harbour samples were treated identically and produced the same result, but were in the reversed and complemented version of the cDNA in the clone.

Three clones (TSV22a, TSV22b and TSV22c) of the 360 bp amplicons from the Townsville sample (Chang *et al.* 2004 protocol), produced using semi-nested RT-PCR, were sequenced with one forward and one reverse reaction on each clone (at Macrogen, South Korea). All three clones showed identical sequence results, except that they were in the reversed and complemented version of the cDNA in the clone. Obviously, their BLAST analyses were identical to Coff's Harbour similarities (363/366).

Infectious hypodermal and haematopoietic necrosis virus

The opportunity to test a subset of these commodity prawn samples for IHHNV was utilized. Five *P. vannamei* from Townsville and six from Coff's Harbour were tested for DNA integrity using the 18SrRNA decapod primer set (Krabsetsve unpublished) at the same time that the samples were tested for IHHNV with the 392 primer set of Tang *et al.* (2000), i.e. a multiplex PCR. All Townsville samples produced amplicons for the decapod sequence but only four of the six Coff's Harbour samples showed DNA with an integrity high enough to produce amplicons. Nevertheless, all Townsville and five out of the six Coff's Harbour samples were positive for the IHHNV392 amplicon. A subset of three Townsville samples and one of two Coff's Harbour samples were positive for the IHHNV389 amplicon, showing that the strain of IHHNV was the Philippine/American lineage, not the endemic Australian lineage (Krabsetsve *et al.* 2004).

Discussion

Frozen commodity prawns have been shown to carry viable WSSV and YHV (Nunan *et al.* 1998; Durand *et al.* 2000; Reville *et al.* 2005). Therefore, the expectation

at the outset of this study was that commodity prawns would be carrying the more resistant, small unenveloped viruses, TSV and IHHNV. It is clear that commodity *P. vannamei* imported into Australia from mainland China and sold at retail shops have an RNA sequence consistent with TSV. Furthermore, they are also co-infected with the Philippine/American lineage of IHHNV. This is not to say that the viruses are viable and only experimental infections with susceptible hosts can determine whether the virus is infectious. However, the high prevalence of amplicons from the samples of prawns and the high fidelity of the sequences, in particular in the Townsville sample, would suggest that the likelihood of viable virus is high. If this is so, we should expect these two infectious viruses emerge in naïve crustacean populations, over time, particularly in countries where large quantities of commodity prawns are imported. If there is little emergence of clinical disease, it implies that the native animals are not highly susceptible or that the chain of infection is easily broken.

Of interest is that when *P. vannamei* was first introduced to Asia, it was largely as a specific pathogen-free (SPF) stock from Hawaii. However, the term SPF is used with considerable frequency in many publications and in advertising but is often misunderstood by farmers (Flegel & Alday-Sanz 1998). It is clear that the prawns tested in this current experiment were not SPF for TSV or IHHNV and probably represent an imported lineage that circumvented the SPF protocol as highlighted by the viral sequence similarities to viruses from other countries. Greater care and policing of the usage of the term SPF needs to occur or the advantage that real SPF animals provide will be eroded by misuse of the term. This may have trade implications on live and frozen animals as nations (e.g. Brazil) and jurisdictions (as proposed for Hawaii) start imposing strict quarantine based on OIE-notifiable diseases.

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