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Macrobrachium rosenbergii nodavirus disease (white tail disease) in Australia

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ABSTRACT: The index case of white tail disease (WTD) is presented in adult broodstock prawns *Macrobrachium rosenbergii* from the Flinders River in western Queensland, Australia, in mid-2007. Histological examination revealed extensive myonecrosis with massive infiltration of myonuclei and some haemocytes. Juveniles from the same broodstock but not from 3 other families displayed white muscle lesions. Low-grade chronic mortalities approaching 100% over 1 yr occurred. Reverse transcriptase polymerase chain reactions (RT-PCR) were attempted for both *M. rosenbergii* nodavirus (*Mr*NV) with 2 sets of primers and for the satellite virus, extrasmall virus (XSV). All 3 PCRs generated amplicons of the expected sizes. Basic local alignment search tool (BLAST) analyses of the 3 consensus sequences identified a 91% match with *Mr*NV viral capsid protein gene, 96% match with *Mr*NV RNA-directed RNA polymerase gene, and a 99% match with *M. rosenbergii* XSV capsid protein gene. The clinical signs, histopathological lesions and RT-PCR amplicons could be reproduced in *M. rosenbergii* inoculated with cell-free extracts fulfilling River's postulates. We conclude that this is an endemic strain of *Mr*NV as the sequences are dissimilar to strains of *Mr*NV circulating around Asia and the Americas. This case only poorly meets the Office International des Epizooties (OIE) case definition for WTD due to the age of the prawns involved and the nature of the inclusion bodies. Perhaps the OIE case definition needs broadening.

KEY WORDS: White tail disease · WTD · *Macrobrachium rosenbergii* · Broodstock · *Macrobrachium rosenbergii* nodavirus · *Mr*NV · Extrasmall virus · XSV

INTRODUCTION

The giant freshwater prawn *Macrobrachium rosenbergii*, sometimes called cherabin in Australia, or scampi internationally, is farmed throughout Asia and the Pacific mainly for domestic consumption. It has been distributed widely around the tropical regions of the globe (Qian et al. 2003, New 2005). During aquaculture of the giant freshwater prawn, diseases have arisen, including the widespread white tail disease (WTD; Arcier et al. 1999, Tung et al. 1999, Qian et al. 2003). The causative agent of WTD has been identified as *Macrobrachium rosenbergii* nodavirus (*Mr*NV), which is a small, icosahedral, non-enveloped virion, 26 to 27 nm in diameter, containing 2 segments of RNA.

In Australia where *Macrobrachium rosenbergii* is endemic west of the Great Dividing Range, a number of attempts have been made to grow this species commercially. All attempts have ultimately failed due to disease problems. The first recorded failure of a farm established in northern Queensland was due to microsporidian infections that made the product unpalatable (Bergin 1986). Later, mortalities from mid-cycle disease caused by Gram-negative bacteria, mostly *Enterobacter aerogenes* and *Vibrio alginolyticus*, affected commercial farms in Western Australia and northern Queensland, which forced their closure (Owens & Evans 1989).

Recently, the worldwide upsurge in interest in *Macrobrachium* culture has prompted research in Australia, particularly on the problematical hatchery phase. Re-

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cent analysis of 18S rRNA gene sequences suggested that *M. rosenbergii* can be divided into 'eastern' and 'western' lineages separated by the Huxley's Line biogeographic barrier (De Bruyn et al. 2004). Based on these findings, Australian *M. rosenbergii* belong to the 'eastern form' and differ from the widely cultured 'western form'. The research further found that within Australia, *M. rosenbergii* distributed throughout the northern tropical and sub-tropical regions could be further categorised into 4 genetically distinguished lineages (Lineages I to IV; De Bruyn et al. 2004).

Wild broodstock belonging to Lineage II were sourced from the Flinders River area of western Queensland and were used to establish 4 families in hatchery facilities at James Cook University. Adults of one family started to display white muscle and mortalities during the cold winter months. Incidental observations suggested that nearly all broodstock infected with WTD died. However, for early juveniles that showed minor WTD symptoms, which subsequently subsided, their continued growth was reduced substantially compared to normal juveniles. Here we describe the histopathology, experimental infections, incorporating reverse transcriptase polymerase chain reaction detection of *Mr*NV and genome sequencing.

MATERIALS AND METHODS

Source of *Macrobrachium rosenbergii* broodstock. Wild broodstock were collected from the Flinders River system and its tributaries, near the Gulf of Carpentaria region (17° 52.522' S; 140° 46.837' E), Queensland, Australia. Samples of the prawns were sent to Queensland University of Technology, Brisbane, Australia, for lineage identification and were confirmed to belong to Lineage II from the rivers flowing into the Gulf of Carpentaria (De Bruyn et al. 2004). Broodstock were held in several 2500 l tanks subject to ambient changes in temperature.

Histology. The cephalothoraxes of all prawns were prepared for histology by splitting laterally. The cephalothoraxes were placed in Davidson's fixative for 48 h, and the tail of each prawn was stored in 95% ethanol for RT-PCR. After 48 h, fixed tissues were transferred to 70% ethanol and processed for histology using standard methods before being embedded in paraffin wax. Sections were cut at 5 µm and stained with Mayer's haematoxylin and eosin. Special stains including methyl-green pyronin, Feulgen's and phloxine tartrazine were used to check for inclusion bodies. Light microscopy (Olympus E C microscope) was used to view the sections. Photographs were taken using an Olympus Camedia 5.0 Megapixel Digital Camera with a C-5050 zoom.

Preparation of inoculum. Tail muscle tissues from the index case of *Macrobrachium rosenbergii* WTD were homogenised in phosphate-buffered saline (PBS) in a stomacher bag with a hammer. The homogenate was partial clarified by centrifugation at 7000 × *g* in an Eppendorf 5424 centrifuge. A 0.45 µm syringe filter was then used to make a cell-free extract.

RNA extraction. Total RNA was extracted from tail muscle of 10 prawns from the diseased family and from experimental prawns using the SV Total RNA Isolation System (Promega) according to the manufacturer's instructions, and RNA was used immediately for reverse transcription-polymerase chain reaction (RT-PCR).

RT-PCR. As the histopathology was consistent with WTD (see 'Results' below) and not consistent with other viruses found naturally or experimentally in *Macrobrachium* spp., PCRs targeted only *Mr*NV and extra small virus (XSV). cDNA was synthesised using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). cDNA was amplified using *Mr*NV primers described by Sri Widada et al. (2003) and Yoganandhan et al. (2006) and for XSV by Sri Widada et al. (2004). The sequence of Sri Widada et al.'s (2003) oligonucleotide primers were forward (1A775) 5'-CCA CGT TCT TAG TGG ATC CT-3' and reverse (1B690) 5'-CGT CCG CCT GGT AGT TCC-3'. The oligonucleotide primers from Yoganandhan et al. (2006) were *Mr*NV (no name) forward 5'-GAT ACA GAT CCA CTA GAT GAC C-3' and *Mr*NV (no name) reverse 5'-GAC GAT AGC TCT GAT AAT CC-3', whilst the XSV primers from Sri Widada et al. (2004) were (XS-1) forward 5'-GGA GAA CCA TGA GAT CAC G-3' and (XS-5) reverse 5'-CTG CTC ATT ACT GTT CGG AGT C-3'.

Each PCR mixture contained 1× *Taq* buffer (750 mM Tris-HCl pH 8.8, 200 mM $[NH_4]_2SO_4$, 0.1% Tween 20), 2.5 mM $MgCl_2$, 0.75 U *Taq* polymerase (MBI Fermentas), 200 µM each dNTP, 50 pmol of each primer and 20 to 50 ng of DNA template. The PCR volume was adjusted with sterile distilled water to a final volume of 25 µl. Amplification was performed in an Eppendorf Mastercycler Gradient Thermocycler with a PCR profile consisting of an initial 94°C for 7 min, 35 cycles at 94°C for 45 s, 55°C for 45 s and 72°C for 1 min, and 5 min at 72°C on the last cycle. The PCR products were analysed by electrophoresis in 1.0% agarose gels containing 0.5 µg ml^{-1} ethidium bromide.

Cloning and sequencing. RT-PCR amplicons were purified from agarose gels using the Wizard® SV Gel and PCR Clean-Up System (Promega), ligated directly into the pGEM-T® Easy Vector (Promega) and transfected into *Escherichia coli* JM 109 cells. Blue/white screening was used to identify *E. coli* cells containing recombinant plasmids that were purified from 4 white

colonies using the Wizard® Plus SV Minipreps DNA Purification System (Promega), according to the manufacturer's instructions. Plasmid DNA was digested with *Spe*I, followed by electrophoresis in 1.0% agarose gels to screen for DNA inserts. Plasmids containing DNA inserts were sent to Macrogen Inc. for sequencing using M13 universal primers. Three forward and 3 reverse sequencing reactions were performed on each clone. Sequencher™ software (Gene Codes Corporation) was used to analyse and align overlapping sequences for each clone. Sequence results were compared to the GenBank database using the basic local alignment search tool (BLAST) at the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov/).

The 861 nucleotides (nt) consensus sequence for *Mr*NV using Sri Widada et al.'s (2003) primers was generated from 12 overlapping reads. With Yoganandhan et al.'s (2006) primers, the 682 nt consensus sequence was generated from 5 reads. The 508 nt consensus sequence for XSV using Sri Widada et al.'s (2004) primers was from 8 reads. *Mr*NV sequences from GenBank were trimmed to be the same number of nt as the Australian isolate, and ClustalW2 with a neighbour-joining algorithm was then used to produce the phylograms.

Experimental infections. Juvenile *Macrobrachium rosenbergii* approximately 3 to 4 cm in length and 2 g in weight were obtained from one uninfected family at the Marine & Aquaculture Research Facilities Unit (MARFU), James Cook University. Prawns were divided at random into 3 treatment groups: (1) control, (2) per os exposure, and (3) inoculated, each with 3 replicates of 10 prawns. Water exchanges were undertaken daily to maintain water quality.

Control prawns were fed only commercial pelleted food. After being starved for 24 h, prawns in the feed exposure were fed muscle tissue of an individual displaying WTD clinical signs at 5% of body weight on Day 0 and fed thereafter on pelleted food. For the inoculated treatment, the cell-free extract was diluted with PBS, and 25 µl were inoculated intramuscularly into the first abdominal segment. The experiment was terminated on Day 30 post injection.

Statistical analysis was undertaken using the Statistical Package for the Social Sciences version 14 with a 1-way analysis of variance (ANOVA) conducted after testing for normality. The significance level was set at $p < 0.05$.

RESULTS

Inoculated *Macrobrachium rosenbergii* and, to a lesser extent, fed prawns developed the same gross clinical signs consistent with WTD (Fig. 1). Prawns from the uninfected family possessed normal muscle tissues (Fig. 2). The index case adults had extensive myonecrosis with massive infiltration of myonuclei and some haemocytes (myositis; Figs. 3 to 5). Similarly, prawns fed infected tissue and prawns injected with muscle extract showed the same myonecrosis of the tail and myositis. Approximately half (10/25 fed and 15/23 inoculated) of the exposed prawns showed mostly limited

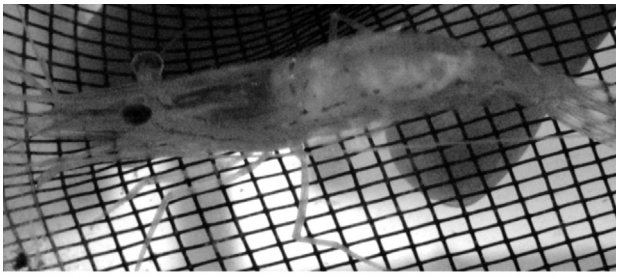

Fig. 1. *Macrobrachium rosenbergii.* Gross signs of white tail disease in a juvenile on Day 25 after injection with a cell-free extract

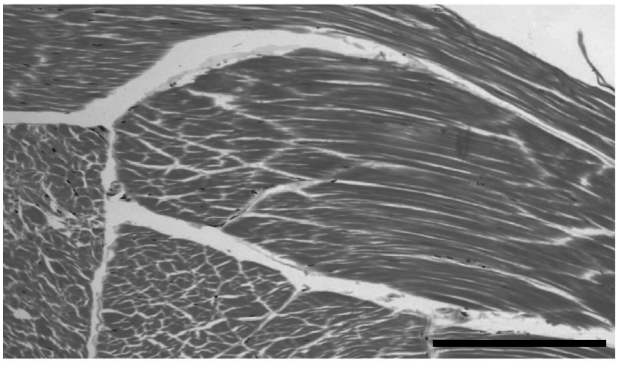

Fig. 2. *Macrobrachium rosenbergii.* Normal abdominal muscle of a control (scale bar = 125 µm)

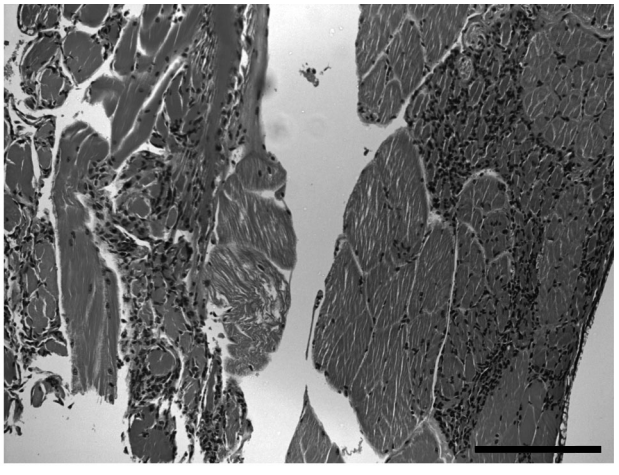

Fig. 3. *Macrobrachium rosenbergii.* Cellulitis in abdominal muscles of index case broodstock (scale bar = 250 µm)

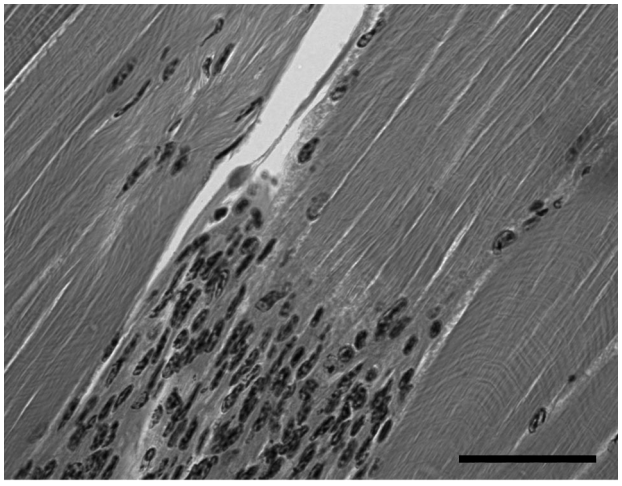

Fig. 4. *Macrobrachium rosenbergii.* Myonecrosis and myolitis of abdominal muscles of index case broodstock (scale bar = 50 µm)

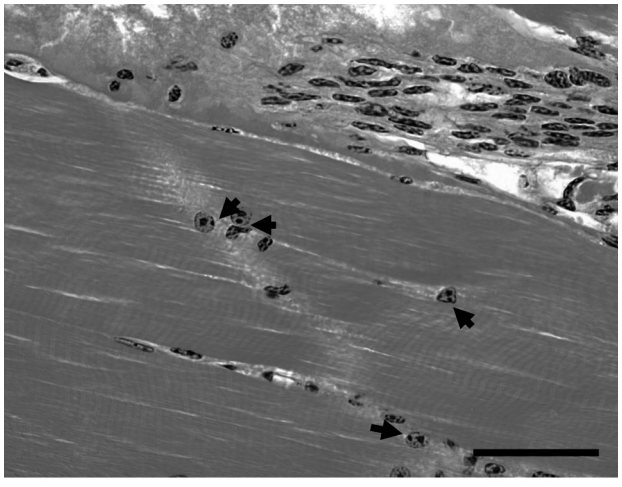

Fig. 5. *Macrobrachium rosenbergii.* Myonecrosis and myolitis of abdominal muscles of the index case broodstock (scale bar = 50 µm). Inclusion bodies of unknown significance were present in many of the haemocytes (arrows)

muscle necrosis and myositis but some displayed extensive areas of lesions. With methyl green pyronin, nuclear debris stained green whilst some cytoplasmic inclusion bodies stained pink (pyroninophilic; data not shown).

When the challenge experiment was finalised at 30 d, the control prawns had the highest survival (90%, 27/30), while the per os exposed prawns had slightly lower survival at (83%, 25/30) and the injected prawns had the lowest survival (77%, 23/30). However, these differences between exposure treatments were not significant ($p > 0.05$). As prawns that died were cannibalised with only the exoskeleton being found the next morning, histology and RT-PCR were not possible on these remains. Of interest, approximately 400 juvenile *Macrobrachium rosenbergii* displaying WTD in a holding tank slowly perished over 12 mo.

All RT-PCRs produced amplicons of approximately the size expected (see below). Seven of 10 progeny were positive with the Sri Widada et al. (2003) primers for *Mr*NV; 6/10 of the same prawns were positive with the Yoganandhan et al. (2006) primers and 5/10 of the same prawns were positive for the XSV (Fig. 6).

The 861 nt viral capsid protein gene (RNA-2) consensus sequence obtained from the PCR amplicons produced with the Sri Widada et al. (2003) primers (expected size 859 nt) had 91.0% identity to *Mr*NV from the French West Indies and 90.9% identity to *Mr*NV from China (the highest 2 matches in BLAST searches; Table 1). This sequence for the Australian isolate of *Mr*NV has been logged in GenBank (accession FJ379531). The 682 nt RNA polymerase gene (RNA-1) consensus sequence (GenBank FJ379530) obtained from the PCR amplicons using Yoganandhan et al.'s (2006) primers (expected size 681 nt) for a different part of the genome had 95.9% identity to *Mr*NV from India and 95.6% identity to *Mr*NV from the French West Indies. The 508 nt consensus sequence (GenBank FJ379532) obtained from the PCR amplicons for XSV with Sri Widada et al.'s (2004) primers (expected size 507 nt) had 99.4% identity to XSV from 2 separate XSV sequences from Thailand.

The phylograms gave similar results in that the Australian isolate of *Mr*NV is the most distant from all other isolates with both the sequences of the RNA-dependent RNA polymerase gene (Fig. 7a) and the capsid gene (Fig. 7b). Due to how concurrent nt changes are weighted in ClustalW, the phylogenetically closest isolate to the Australian isolate was from China (AY231436, AY231437).

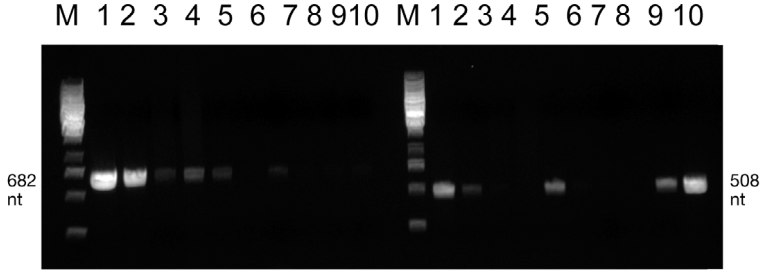

Fig. 6. *Macrobrachium rosenbergii.* RT-PCR amplicons of 10 randomly selected juvenile progeny from infected broodstock for *M. rosenbergii* nodavirus (*Mr*NV) using the primers of Yoganandhan et al. (2006). Lanes labelled M contain the 250 nucleotide (nt) ladder markers (Fermentas). Sizes of the amplicons are marked. Left half of the gel depicts amplicons using primers for *Mr*NV; right half of the gel depicts amplicons using primers for extra small virus produced by the primers of Sri Widada et al. (2004)

Table 1. Top 2 alignments giving the highest scores for sequence identity between Australian *Macrobrachium rosenbergii* nodavirus (*Mr*NV) and other geographical isolates

Primer set	Location	Accession no.	Description	Identity (%)
Sri Widada et al. (2003)	French West Indies	AY222840.1	*Macrobrachium rosenbergii* nodavirus segment RNA-2, complete sequence	91.04
	China	AY231437.2	*Macrobrachium rosenbergii* nodavirus capsid protein gene, complete coding sequence	90.89
Yoganandhan et al. (2006)	India	DQ146969.1	*Macrobrachium rosenbergii* nodavirus RNA-directed RNA polymerase gene, partial coding sequence	95.92
	French West Indies	AY222839.1	*Macrobrachium rosenbergii* nodavirus segment RNA-1, complete sequence	95.58
Sri Widada et al. (2004)	Thailand	EU150133.1	*Macrobrachium rosenbergii* XSV isolate M23 capsid protein gene, complete coding sequence	99.38
	Thailand	EU150132.1	*Macrobrachium rosenbergii* XSV isolate M308 capsid protein gene, complete coding sequence	99.38

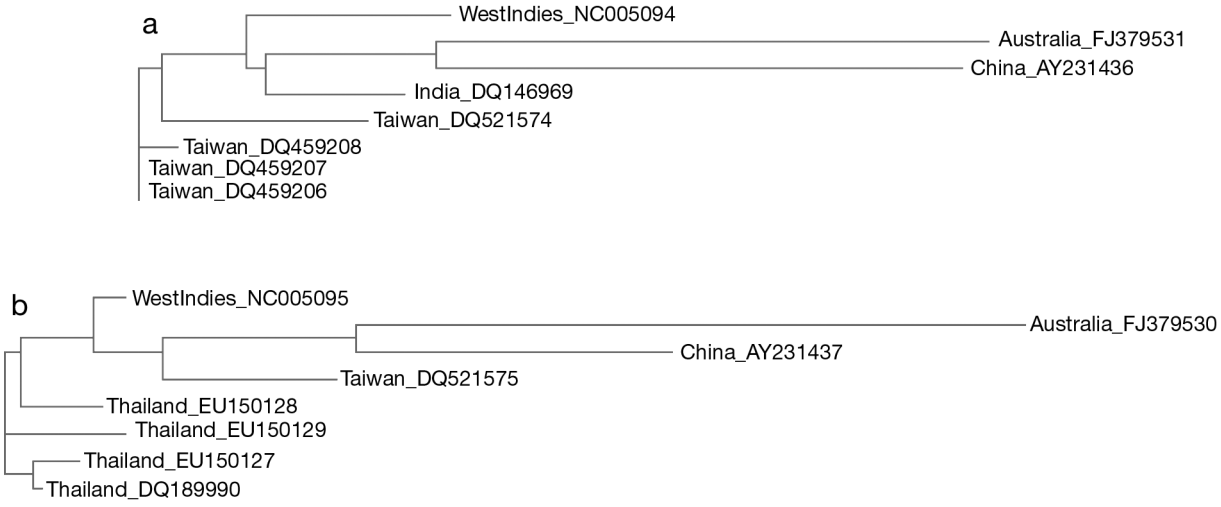

Fig. 7. Phylograms of the sequence the Australian isolate of *Macrobrachium rosenbergii* nodavirus (*Mr*NV) compared to other isolates of *Mr*NV using both sequences from RNA-1 (a) 861 nt and RNA-2 (b) 692 nt. Phylograms were produced by ClustalW2

The RT-PCR results for prawns from the experimental infections were poor, and only 2 inoculated and 1 fed prawn of the 48 exposed prawns that remained generated PCR amplicons using the primers from Yoganandhan et al. (2006). All of the 27 control prawns remaining alive were negative by RT-PCR and histology for evidence of *Mr*NV.

DISCUSSION

Due to the small size of the juvenile prawns, the inoculum was both diluted and reduced in volume so that the mortality due to the injection procedure would be minimised. This may have reduced the mortality rate and the number of positive PCRs from the experimental groups. Other explanations for the limited effect of the inoculum are possible, such as the life stage of the prawns, virulence of the virus and the sensitivity of the PCR. Further research should disclose the probable causes.

The data reported here are consistent with WTD in Australian *Macrobrachium rosenbergii* caused by *Mr*NV. The gross clinical signs, the histopathology, both of which are not pathognomonic for WTD, the genomic RNA sequence relationships and the ability to fulfil River's postulates support the conclusion that *Mr*NV is present in Australia, which was previously declared free of this disease (Ernst 2007). WTD is a notifiable disease to the Office International des Epizooties (OIE) and as such, this case was reported to government authorities and confirmed by analyses undertaken at the Australian Animal Health Laboratories. OIE was notified formally in February 2008.

This case does not quite meet the criteria defined currently in the OIE WTD disease card, mainly due to

the age of the prawns involved. Most previous reports have involved postlarvae or very young juveniles, whereas this case involved adults and older juveniles. The gross signs, the histopathological lesions in muscle and the confirmatory genome sequence are all consistent with the OIE definitions. The mortality in experimental prawns was chronic and limited over the time frame of the study, and inclusion bodies were not seen in connective tissue but rather in the muscle, staining more like RNA (pyroninophilic). Based on the findings in this case, perhaps the OIE definition needs to be broadened somewhat to include adult life stages, and the staining characteristics should be reevaluated.

WTD has been observed in many countries including Taiwan (Tung et al. 1999), French West Indies (Arcier et al. 1999), China (Qian et al. 2003), India (Sahul Hameed et al. 2004) and Thailand (Yoganandhan et al. 2006). This study adds Australia to the list of countries that have reported WTD.

The preliminary data herein suggest that the Australian isolate of *Mr*NV is most closely related to the Chinese isolate. At this time, this suggests that the Australian isolate was introduced at some time by human activities rather than being a reflection of the ancient zoogeographical spread of the virus with its natural host, *Macrobrachium rosenbergii*.

The level of nucleotide sequence identity (91 to 96%) between the genome sequences of the Australian *Mr*NV strain compared to strains from other geographic localities suggests that *Mr*NV has been in Australia for some time and that it is therefore now endemic rather than very recently introduced. However, only sequencing of the full genome of Australian *Mr*NV will determine the complete similarity. This will allow a full analysis of the amino acid changes and a better understanding of the true phylogenetic relationships.

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