

White spot syndrome virus (WSSV) PCR-positive *Artemia* cysts yield PCR-negative nauplii that fail to transmit WSSV when fed to shrimp postlarvae

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ABSTRACT: Positive results were obtained with nested white spot syndrome virus (WSSV) diagnostic PCR performed on 5 commercial brands of dry-packed *Artemia* cysts using several WSSV genomic sequence-specific primers. In 2 brands, PCR and nucleotide sequence analysis found C→T and C→G point mutations in the pms 146 WSSV amplicon, but in all 5 brands, the nucleotide sequences that were successfully amplified by the *rr1*, *rr2* and *tk-tmk* gene-specific primer sets were identical to those of *Penaeus monodon* WSSV. However, despite the inarguable presence of WSSV or WSSV-like template DNA, we were unable to detect WSSV by PCR in hatched nauplii derived from PCR-positive cysts or in *P. monodon* postlarvae fed *Artemia* nauplii hatched from such cysts. Most simply, these results suggested that the cysts were externally contaminated with WSSV or WSSV-like template material that was removed during hatching and washing of the nauplii. Given the small sequence variations found, it may also have been a variety of WSSV non-infectious for *P. monodon* or *Artemia* and derived from other crustaceans or arthropods in the *Artemia* environment. However, we could not establish this conclusively and a small possibility remained that the PCR template in these tests was derived from WSSV template present internally in the cysts and derived from infected *Artemia* adults. However small, this possibility must be vigorously tested, given the impact that a positive outcome could have on the shrimp industry.

KEY WORDS: White spot syndrome virus · Brine shrimp · *Artemia* · *Penaeus monodon* · WSSV diagnostic PCR · Amplified restriction fragment length polymorphism

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INTRODUCTION

White spot syndrome virus (WSSV) is the extremely virulent causative agent of white spot syndrome, an acute, contagious disease of shrimp. It was first reported from an outbreak in cultured penaeid that occurred in NE Asia in 1993 (Nakano et al. 1994, Takahashi et al. 1994). White spot syndrome causes high mortality, and it continues to seriously affect most of the commercially cultured marine shrimp species, not

just in Taiwan and Asia but globally (Chou et al. 1995, Flegel 1997, Spann & Lester 1997).

Because WSSV has such a wide host range that extends not only to several shrimp species but also to many other crustaceans (Lo et al. 1996b), we questioned whether it would be capable of infecting or contaminating the brine shrimp *Artemia* (Crustacea, Anostraca). This crustacean is used worldwide for feeding fish and crustaceans (Bardach et al. 1972, Sorgeloos & Persoone 1975, Sorgeloos 1980, Kim et al. 1996, Naessens et al. 1997). For example, *Artemia* nauplii are routinely used in penaeid shrimp larviculture from mysis through to postlarval (PL) stages (Cook

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& Murphy 1969, Beard et al. 1977, Emmerson 1984). Thus, if infected or contaminated with WSSV, *Artemia* would constitute a serious threat to the shrimp industry. WSSV has not hitherto been positively identified either in *Artemia* or in *Artemia* cysts (sometimes mistakenly called brine shrimp 'eggs'). However, it has been speculated (Austin & Allen 1982, Overstreet et al. 1988, Mortensen et al. 1993) that *Artemia* can act as a reservoir or mechanical vector for bacterial pathogens such as *Bacillus*, *Erwinia*, *Micrococcus*, *Staphylococcus* and *Vibrio* spp. as well as the infectious pancreatic necrosis virus (Austin & Allen 1982, Mortensen et al. 1993). In addition, cytoplasmic, rod-shaped, virus-like particles were reported in the shell gland of *Artemia* ovaries as long ago as 1980 (Criel 1980) and *Artemia* has been used as a mechanical vector in an infectivity bioassay of *Baculovirus penaei* (Overstreet et al. 1988). This information suggests that *Artemia* has the potential to be a vector for pathogens including viruses.

In view of the great importance of *Artemia* in shrimp culture, we used various PCR primer pairs to screen *Artemia* cysts from several different sources for the presence of WSSV. We also used PCR tests to screen nauplii hatched from WSSV PCR-positive cysts and to examine *Penaeus monodon* experimentally fed with *Artemia* nauplii derived from WSSV PCR-positive cysts.

MATERIALS AND METHODS

Overall strategy for PCR tests. Five commercial brands of *Artemia* cysts were subjected to 2-step WSSV diagnostic PCR (Lo et al. 1996a, Lo & Kou 1998) using either the primer set pms 146F1/R1-F2/R2 (Lo et al. 1996a) or the primer set IQ2000 (Farming IntelliGene Tech, Taipei, Taiwan). Both sets target the WSSV genomic fragment pms 146 generated by pms 146F1/R1-F2/R2 but the IQ2000 set yields a smaller amplicon. With positive cyst samples, several other nested primer sets also designed from pms 146 were subsequently used to amplify fragments for sequencing. Sequences and amplified restriction fragment length polymorphism (ARFLP) results were then compared. In addition, 4 further primer sets were used to test for amplification of sequence-specific WSSV gene fragments derived from the coding regions of 3 recently identified WSSV genes. These were WSSV *rr1* and *rr2* (van Hulten et al. 2000, Tsai et al. 2000a), and the WSSV *tk-tmk* gene (Tsai et al. 2000b), which encodes a novel chimeric polypeptide of cellular-type thymidine and thymidylate kinase.

Template DNA preparation for 2-step PCR. A DNA extraction kit (Farming IntelliGene Tech) consisting of 'DTAB Solution', 'CTAB Solution' and 'Dissolving Solution' was used to prepare template DNA from

pooled 100 mg samples of *Artemia* cysts, *Artemia* nauplii, *Penaeus monodon* at the mysis stage and *P. monodon* PL₁₅ with their heads removed. The DNA extraction protocol followed the instruction manual provided with the kit, and the final DNA pellet was resuspended in 200 μ l double-distilled water. For 2-step WSSV diagnostic PCR, the DNA concentration was adjusted to 50 ng μ l⁻¹ and an aliquot (2 μ l) of DNA solution was used for each reaction. Positive control templates were prepared from the abdominal muscle (100 mg) of a seriously infected (1-step PCR positive; Lo & Kou 1998, Lo et al. 1998) cultured *P. monodon* collected from southern Taiwan in 1994 (*P. monodon* WSSV 1994 Taiwan isolate; Lo et al. 1996a).

Two-step WSSV diagnostic PCR. The IQ2000 primer set and all the pms 146 primer sets used here were derived from the nucleotide sequence of a cloned WSSV *SalI* 1461 bp (pms 146) fragment (Lo et al. 1996a). The nucleotide sequence of the pms 146 fragment has been deposited in GenBank (accession number U50923). The pms 146F1/R1-F2/R2 nested primer set (i.e. the outer primer pair pms 146F1/R1 and the inner primer pair pms 146F2/R2) has already been described (Lo et al. 1996a). The other primer set (IQ2000) was provided as part of a commercially available WSSV detection kit (Farming IntelliGene Tech; Lo et al. 1998). It consists of an outer primer pair, 146Fup/WNFdown, and an inner primer pair, Nestup/Nestdown2 (Farming IntelliGene Tech). The first and second steps of amplification with the IQ2000 primer set were expected to amplify DNA fragments of 910 bp and 296 bp, respectively.

The quality of the DNA extracted from the tested *Artemia* and *Penaeus monodon* specimens was checked with a primer pair (143F/145R) designed by Lo et al. (1996a) and based on a highly conserved region of the 18S rRNA gene sequence of decapods (Kim & Abele 1990). The thermal cycling programs and reaction conditions for 2-step WSSV diagnostic PCR and DNA quality testing PCR were as described in Lo et al. (1996a).

WSSV screening of *Artemia*. Primer sets pms 146F1/R1-F2/R2 and IQ2000 were used for 2-step WSSV diagnostic PCR screening of samples from 3 commercial brands of dry-packed *Artemia* cysts. These packets of cysts were obtained from the Taiwan Fisheries Research Institute Tung Kang Marine Laboratory, and they were all originally intended for general aquacultural use. The labeling information on Brand A was no longer legible, but its production date was deduced to be no later than 1987 based on other extrinsic information. Brand B was packed in 1997 and Brand C was packed in 1998. Nauplii hatched from Brands B and C *Artemia* cysts were also subjected to 2-step WSSV PCR screening. The Brand A cysts did not hatch and so no Brand A nauplii could be tested.

Cysts A, B and C together with cysts from 2 additional samples (D and E, packed in 1999 and obtained from a local commercial source) were subjected to further PCR tests using 3 nested primer sets based on the pms 146 fragment. These were pms 146F1/R4-F3/R5, pms 146F2/R2-F5/R4 and pms 146F4/R2-F5/R4 (Lo et al. 1996a). In addition, 4 other nested PCR primer sets were used. These targeted 2 sequences within the WSSV-*rr1* coding region (1 by primer set rr1F809/rr1R1426–rr1F863/rr1R1227 and 1 by primer set rr1F1551/rr1R1980–rr1F1726/rr1R1945), 1 sequence in the WSSV-*rr2* coding region (by primer set rr2F704/rr2R1423–rr2F806/rr2R1008) and 1 sequence in the WSSV-*tk-tmk* coding region (by primer set tkF310/tkR779–tkF351/tkR707). The latter primer set yielded an amplicon that included the *tk-tmk* junction region, which is a specific WSSV identification marker (Tsai et al. 2000b). The PCR conditions were the same as described in Lo et al. (1996a) except that the annealing temperature for primer set rr1F809/rr1R1426–rr1F863/rr1R1227 was 58°C, and the one for primer set tkF310/tkR779–tkF351/tkR707 was 52°C.

The 2-step PCR amplification products yielded by each of the above primer sets were subjected to direct nucleotide sequencing using the respective internal primer pairs. Sequence analysis was performed on an automated ABI Model-377 sequencing apparatus (Applied Biosystems) and carried out by Mission Biotech. DNA samples extracted from nauplii hatched from Brand D and Brand E *Artemia* cysts were also tested by 2-step WSSV PCR with primer set pms 146F4/R2-F5/R4.

Nucleotide sequence comparison and ARFLP analysis. The nucleotide sequences of the 2-step PCR products amplified from *Artemia* cysts using WSSV-specific primers were compared with the sequence of *Penaeus monodon* WSSV 1994 Taiwan isolate (Lo et al. 1996a). Multiple nucleotide sequences were aligned using the programs CLUSTAL_X (Thompson et al. 1997) and GeneDoc (Nicholas et al. 1997). Restriction enzyme digestion sites were analysed with the computer software GeneWorks version 2.5.1 for Macintosh (IntelliGenetics).

On the basis of the sequence analysis data, an ARFLP analysis using either *MnII* or *BsrI* restriction enzyme was developed to distinguish samples from different sources. Two-step PCR products from all brands of *Artemia* cysts and positive controls were selected for *MnII* or *BsrI* ARFLP analysis. Aliquots of 10 to 15 µl of PCR products were digested in a 20 µl reaction volume containing the restriction enzyme and an appropriate buffer supplied by the enzyme manufacturer (New England Biolabs). The mixtures were incubated at 37°C (*MnII*) or 65°C (*BsrI*) for 1 h, and

10 µl aliquots were loaded for electrophoresis. Digested DNA fragments were separated on 5% Tris-borate-EDTA agarose (NuSieve 3:1 agarose, FMC BioProducts), stained with ethidium bromide and examined on a UV transilluminator.

Feeding of nauplii hatched from WSSV-positive *Artemia* cysts to *Penaeus monodon*. Uninfected *Penaeus monodon* larvae from a single WSSV-free (2-step PCR-negative) brooder were used for this challenge test. Special attention was paid to diet. In particular, these larvae were not fed *Skeletonema costatum* because naturally harvested *S. costatum* is often contaminated with copepods, and Lo et al. (1996b) have shown that copepods are potential reservoir hosts for WSSV. Before the experimental challenge, the larvae were therefore fed exclusively monocultures of the microalgae *Isochrysis galbana* and *Chaetoceros gracilis* and a commercial dry diet. As a further precaution before the infectivity trials began, pooled samples from the shrimp larval population were tested for the presence of WSSV, and their 2-step PCR negative status was reconfirmed with both the pms 146F1/R1-F2/R2 and IQ2000 primer sets.

At stage 2 of mysis, 3000 of these *Penaeus monodon* specimens were divided evenly into three 150 l tanks containing chlorinated, aerated 30 ppt seawater. *P. monodon* in 2 tanks were then fed *Artemia* nauplii hatched from WSSV PCR-positive cysts Brand B and Brand C while the third tank served as the control. The *Artemia* cysts were hatched in sterilized 30 ppt seawater at room temperature (25 to 29°C) with full aeration for 24 h. After hatching, the nauplii were separated from the empty shells and washed thoroughly with sterilized seawater before being used to feed the *P. monodon* larvae. Twice daily the *P. monodon* larvae were fed 1 meal each of Brand B *Artemia* nauplii plus commercial dry diet and Brand C *Artemia* nauplii plus commercial dry diet or 2 meals of dry diet only (control). At PL₁₅, randomly selected specimens were collected and had their heads removed before being placed in pooled samples (10 samples of 100 mg each), which were then independently subjected to both of the 2-step WSSV diagnostic PCR protocols described above (2 µl DNA template per reaction). A positive control DNA template was prepared from the abdominal muscle tissue (100 mg) of a 1-step PCR-positive adult *P. monodon*.

RESULTS

Two-step WSSV PCR tests of *Artemia* cysts and nauplii

Examples of the PCR detection results with the IQ2000 primer set in the first 3 tested brands of

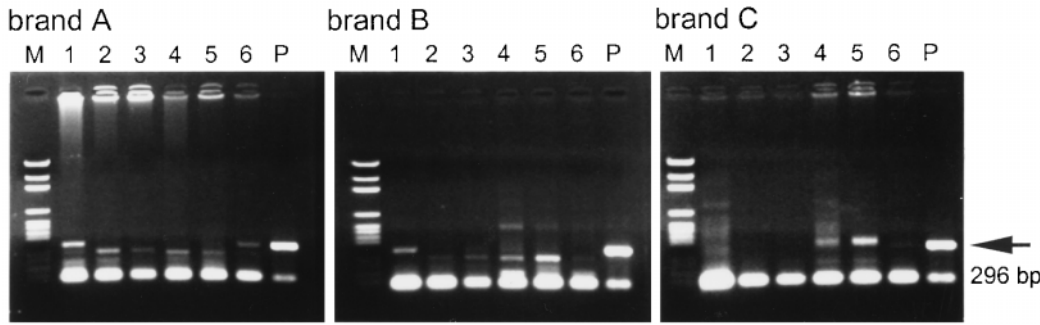


Fig. 1. Typical results of 2-step PCR screening of *Artemia* cysts using the IQ2000 primer set. PCR amplicons of the expected molecular weight (296 bp; arrow) can be seen in Lanes A1, A6, B1 and C6. M: pGEM DNA markers (Promega); P: PCR-positive control

Artemia cysts are shown in Fig. 1. In total, 4 out of 21 tested samples of Brand A gave WSSV positive reactions in the second step of the 2-step PCR while Brands B and C each gave 6 positive in 21. However, the primer set pms 146F1/R1-F2/R2 gave negative results for every tested sample of these 3 brands. WSSV negative results were also obtained with all of the hatched *Artemia* nauplii tested (Brands B and C with the IQ2000 primer set and Brands D and E with the pms 146F4/R2-F5/R4 primer set). All of the above results and results for the other 3 pms 146 primer sets (i.e. pms 146F1/R4-F3/R5, pms 146F2/R2-F5/R4 and pms 146F4/R2-F5/R4) for all of the 5 brands of *Artemia* cysts are summarized in Table 1.

Nucleotide sequence comparison and ARFLP analysis of the pms 146 fragments

Sequences and alignment (with *Penaeus monodon* WSSV 1994 Taiwan isolate; accession number U50923) of the amplified pms 146F3/R5 and pms 146F5/R4 fragments from each brand of *Artemia* cyst WSSV are shown in Fig. 2. No sequence differences were found in pms 146F5/R4 amplicons from any brand of *Artemia* cyst. Single nucleotide variations were found in the pms 146F3/R5 amplicons from

Brand B and Brand E (Fig. 2B). A point difference ($C^{296} \rightarrow T$) in a fragment from Brand B *Artemia* cysts blocked an *MnlI* recognition site. In consequence, the *MnlI* digestion profile of Brand B pms 146F3/R5 amplicons consisted of 3 bands (136, 116 and 18 bp) rather than the normal 4 (136, 92, 24 and 18 bp; Fig. 3A). In Brand E a $C^{267} \rightarrow G$ difference generated a new recognition site for *BsrI*, *Sau96I* and *HaeIII*. In this study *BsrI* was selected for ARFLP analysis. The Brand E *BsrI* digestion profile showed a 2 band pattern (216 and 54 bp) compared with the normal WSSV 270 bp single band pattern (Fig. 3B).

Other WSSV gene fragments amplified from *Artemia* cysts

As shown in Table 2, the other 4 WSSV gene-specific primer sets all produced positive results with Brand A *Artemia* cysts. One of the *rr1* primer sets (*rr1F809/rr1R1426-rr1F863/rr1R1227*) gave no amplicons with cyst Brands C, D and E, and the *rr2* primer set gave no amplicons with Brands B and D. The other *rr1* primer set (*rr1F1551/rr1R1980-rr1F1726/rr1R1945*) and the *tk-tmk* primer set both yielded amplicons with all 5 brands of *Artemia* cysts tested (Table 2).

Table 1. White spot syndrome virus (WSSV) PCR screening results for *Artemia* cysts and nauplii using 5 primer sets derived from the WSSV pms 146 genomic fragment. Data are the number of 2-step positive results out of the number of samples tested. -: not tested

Cyst data WSSV pms 146 primer sets	Amplicon size (bp) 1st-step / 2nd-step	Brand and year of packaging				
		A before 1987	B 1997	C 1998	D 1999	E 1999
F1/R1-F2/R2	1447 / 941	0/21	0/21	0/21	-	-
IQ2000	910 / 296	4/21	6/21	6/21	-	-
F1/R4-F3/R5	839 / 269	1/15	4/15	3/15	1/15	1/15
F2/R2-F5/R4	941 / 237	2/15	3/15	1/15	1/15	4/15
F4/R2-F5/R4	736 / 237	5/15	5/15	6/15	4/15	1/15
Hatched nauplii data						
IQ2000		-	0/21	0/21	-	-
F4/R2-F5/R4		-	-	-	0/5	0/5

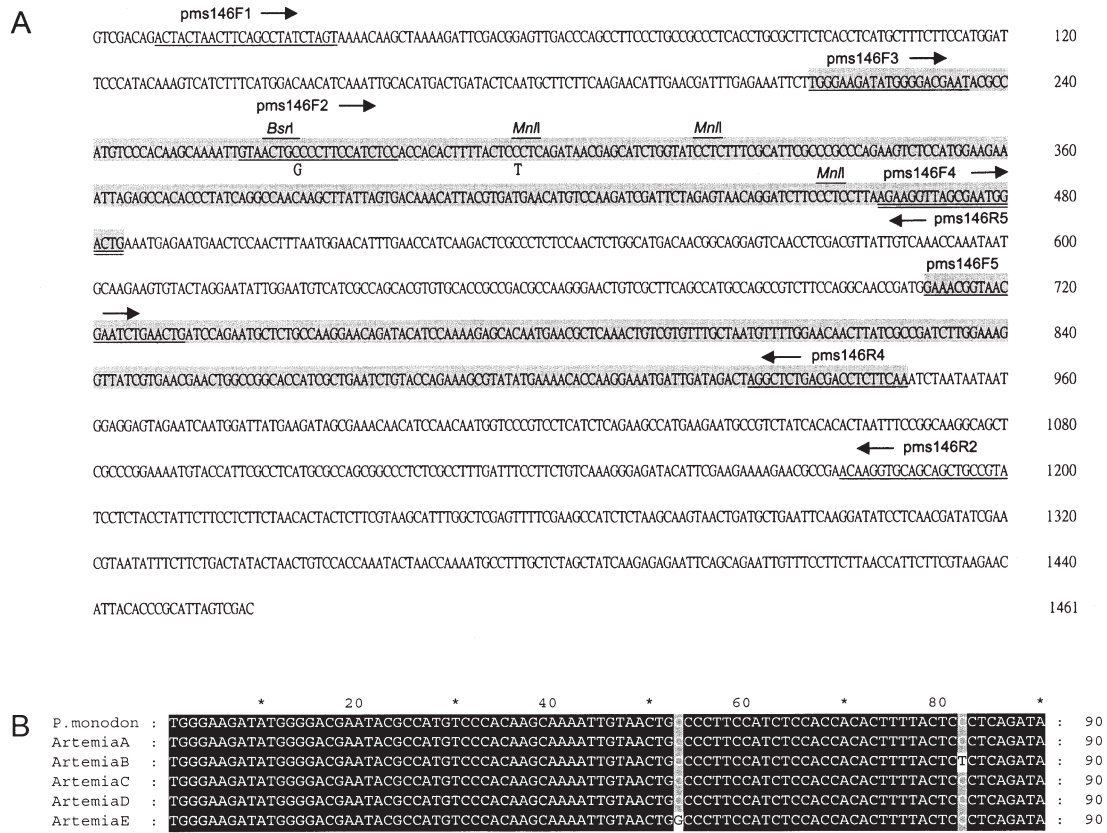


Fig. 2. (A) Full nucleotide sequence of the *Penaeus monodon* WSSV pms 146 fragment (accession number U50923) showing the PCR primer positions, *Bsr*I and *Mnl*I restriction enzyme recognition sites and the regions in *Artemia* cyst WSSV that were sequenced (shaded). (B) Nucleotide sequence alignment of pms 146F3/R5 amplicons of *Artemia* cyst WSSV with *P. monodon* WSSV 1994 Taiwan isolate. Nucleotide variations were observed in Brand B at location C²⁹⁶→T and in Brand E at location C²⁶⁷→G

Nucleotide sequence analysis showed that all of the successfully amplified WSSV gene fragments (Table 2) exactly matched the corresponding sequences in the coding regions of the *Penaeus monodon* WSSV 1994 Taiwan isolate. For reference, full nucleotide and de-

duced amino acid sequences of the *P. monodon* WSSV *rr1*, *rr2* and *tk-tmk* coding regions are shown in Fig. 4A, B and C, respectively. Also indicated in Fig. 4 are the PCR primer sets used and the location of the sequenced regions.

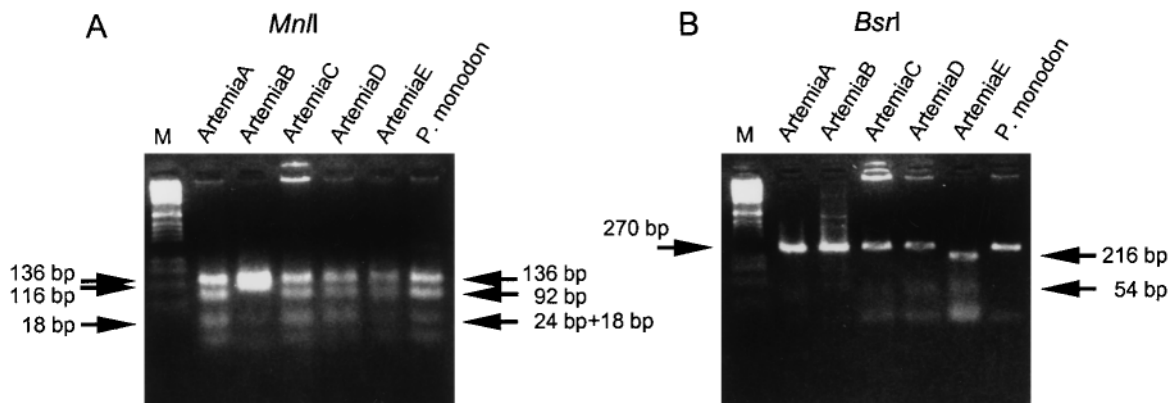


Fig. 3. Restriction profiles of the 270 bp WSSV fragment amplified by pms 146F3/R5. (A) *Mnl*I-digested DNA fragments; (B) *Bsr*I-digested DNA fragments. The *Penaeus monodon*-positive control DNA fragments were derived from *P. monodon* WSSV 1994 Taiwan isolate. M: pGEM DNA markers (Promega)

A	ATGGGTCTTAACACGCAACATCACTCTCAAAGGGAATGGCACTAAGCAAGAAATAGTCTTGAAAAGATAATCAAGAGGATTGAAAATGCCTGTCTCCAGTCAACAGGATGTG	120
	M G S N Q Q Q S F I S K R N G T K Q E I S L E K I I K R I E N A C L P V N Q Y V	
	CCCAAGCTTGACAAGAACGCAATTAACCCCTCAAGAAGCTGCATCTCACATCATGGACCGCTCTCCCGCTACCATCTCCTTCCAAGAAATGGAGGATTTCTGGCCGATTATGCAAGACA	240
	P K L D K N A I N P Q E L A S H I M D R L P A T I S F Q E M D D F L A D Y A K T	
	AAAATGTTGACCACCTGATTTTGGAAAATGGCAGGAAGATTCATCTGTGGAACATCCAAAAACACCAAGAGTGGAAATAGTTTATGTGCAACACTCAGAAAATGGAGCAGCA	360
	K I V D H P D F G K L A G R F I C S N I H K N T K E W N S F S A T T Q K L R H A	
	ATCCACCCCTGGAACCTGGTAAACACGACATCAGTAGTAAATGATACCTACTATGAAAATGTTATGGCCAATGCTGAAATTCOGATGCTGTTATCGATTACAAAATGGATTATCTCTCAC	480
	I H P G T G K P A S V V N D T Y Y E N V M A N A E I L D A V I D Y K M D Y L F T	
	TGTTTCGAGCTGAGGACCTAGAACTACTCTTATTTGATCAAGATGTTGCCCACTGATAGAAAAGAGAAATCTGGTTGAGCGCCCTCAGGACATGATTACGCTGTGGCTGTCCGGC	600
	C F G L R T L E Y S Y L I K I G S P T D R K K R I L V E R P Q D M I M R V A V G	
	ATTCCAGGATCAGACATCAAATCTGTCAATGAAACATATGATCTCATGTGGAGGCACTATTTACCCATGCTTCCCCACACTGTTTAAATTTGGAAACAGTCAACCCCACTTCTCTCG	720
	I H G S D I K S V I E T Y D L M S R H Y F T H A S P T L F N C G T V T P Q L S S	
	TGCTTCCTCTGGCCCTCAAGATGATAGCATTGAGGGTATTTATGATCTCTTAAGGAGCGGCAATCATCTCCAAGACTGCTGGAGGACTCGGCATCCACTTTCATGATTGAGGGCA	840
	C F L L G L Q D D S I E G I Y D T L K E A A I I S K T A G G L G I H F H D L R A	
	AAAGGAAGCCCAATTCGTCATGGAGTGGTACCCACCTGGTCTCATGGGGTTCCTCCAAATCTTAAAGCTCTCTGTGAAAAGGTTAGCCAGGGAGGAGACAAGAGGAGGAGGCTGCA	960
	K G S P I S S W S G T H P G L M A F L Q I F N V S V K K V S Q G G D K R R G A A	
	GCACATATATTTCTGATTGGCATCGGACGTGAAGACTTTATGGACTGCAGAAAGAATGGCGTAATGAAGATTGAGGAGGAGGATCTTTCCACGATATCTGGGTATCTGATCTC	1080
	A I Y I S D W H L D V K D F I D C R K N A G N E D L R T R D L F P A I W V S D L	
	TTACGGAGAGAGTGAAGGCTGGGAAGAATTTGCTCCTGATGTGCCCCACGAGTGCCTGGCCCTTCCGACGTCATGGGAGAGTTTAAAGGCTGTGACGAGAAATATGAGGCTGAA	1200
	F M E R V K A G K N W S L M C P H E C P G L S D V H G E E F K A L Y E K Y E A E	
GCACAAAGCTAAAGAGCTGGTGAAGGCACTGTCATTATCGACCAAAATTAATCTGCACGATCGAACTGGAACCTTATGTGTCTTAAAGGATACCATCAATAGAAAATCTAACCA	1320	
G K G K E V V K A R A L F D Q I N S A R I E T G T P Y V C F K D T I N R K S N Q		
GAAAATGTCGGCATCATCAAGTCTTCAAATTTGTCACCTGAAATGTCAGTACAGTATCGGAGGAACTGCAAGTGTGCAATTTGGCTTATCGCAGTCAACAAGTTTGTGAAGTAT	1440	
E N V G I I K S S N L C T E I V Q Y S D S E E T A V C N L A S I A V N K F V K Y		
TCTCCCATCCCTCCCTAAGCCCTATGTTGATTACCCGGAGATGAAGGGTGTGAAAAATCATGACCAGAAATCTCGACAAGGTGATTGATGTCAATTTCTATCGAGTTGACAAGACC	1560	
S P I P S L R P Y V D Y R E M K R V V K I M T R N L D K V I D V N F Y A V D K T		
CGCATTTCCAAATGAAAATAGGCCAATGGGATGGGTGTCAGGACTAGCAGATTGTTCTTCAAATCAGAAATCCCTTCGAATCTGAAGAAGGGCACTAATTAACAAGAGGATT	1680	
R I S N M K T R P M G L G V Q G L A D L F F K L R I P P E S E E A A L I N K R I		
TTTGAAATATATACTATGTCCTGGAAAGCTTCAATGAAAATTTCCAAAGAAAAGGAGAAACATATGAGCTGTTTGAAGGTAGTCTTTGAGCAAGGGGATTTTCCAATTTGACATG	1800	
F E T I Y Y G A L E A S C E I A K E K G E T Y E L F E G S P L S K G I F Q F D M		
GGGAAGAAAATATTAAGAATAGGACATATATTTCAACTCTTTGCCAATTCAGATTGGAGCAATGGAAGGACATTATGAAGTATGGTGTTCACAATCAATGTTTGTGCTCCC	1920	
G K E N I K N R D I Y F N S L P I H D W E Q L R R D I M K Y G V H N S M F V A P		
ATGCTACTGCATCCACTGACAGATCCTGGCAACTCTGAATCCTTTGAGCCTTAAAGTCCCAACATGATAATCGTAATGTACTTTCAGGATCATTCGAAGTGGTGAAGCAATATGTG	2040	
M P T A S T A Q I L G N S E S F E P L T S N M Y N R N V L S G S F Q V V N E Y V		
ATTAGAGAGCTCATAAACTGGGAGAAATGGAATTCAGTAACTAAACAGAGGATTATGGCAAGTGGTGGATCTATTTCAGACGCTTCTAATAATCCCTAAATGACCAAGGAACTATTCAA	2160	
I R E L I K L G E W N S V T K Q R I M A S G G S I Q T L P N I P K S T K E L F K		
ACTGATGGGAAATTAATCCTGCTACTACTTTGGACATGGCTATTCAGAGAGGTATGTTGTTGACCAAGCTCAATCCCTCAACTGTTTGTGGAGAAACCCGAACTCAGCAAGGTGGG	2280	
T V W E I N P R T T L D M A I Q R G M F V D Q A Q S L N L F V E E P E L S K V R		
TCAATGACTATGATGCATGGGAGAGGGGATCAAGACTCTCTATTATCTACGCACAAGGGCGAGCTAGAGCTGTCCAGTTCACAGTGCACAAGAATGTACTCCAAGAAGTCAAGAAG	2400	
S M T M Y A W E K G I K T L Y L R T K G A A R A V Q F T V D K N V L Q E V K K		
GAAGCTCCCTCTCTGTGACGCTTTTCTGCTCCTGTCGAGAAGAAGAAGGAGAAGAAGTCTCTATTGTTGTTCCAGATCCTGCTGCTCTTTTATGTTCTATCAATAACCCCT	2520	
E A P S P V A A F S A P V R E E E E K K S S I V V P D P A A A L L C S I N N P		
GGTCTTGTGAAATGTTCTTCTCTAG	2547	
G A C E M C S S *		

Fig. 4. (Above and facing page). Nucleotide and deduced amino acid sequences of the coding regions of 3 genes of *Penaeus monodon* WSSV 1994 Taiwan isolate. (A) *rr1*; (B) *rr2*; (C) *tk-tmk*. The positions of the PCR primer sets are shown. Shading is used to indicate the sequenced regions. The boxed sequence in (C) indicates the *tk-tmk* junction region

B

ATGGAGTCAATCAAAGTGTTCACCGTTGCTGGTCTGAATATGGAGCAAGCCAAACAGTGGCTGAAGAAATCAAGTCAGAATATAAACCGAGGAGAAAAGGATTGCCAGGAAATG 120
M E S I K L F T V A G L N M E Q A N Q V A E E I K S E Y K T E E E K R I A Q E V

TTTGACAAATTCACCAAAAACTCATTATGCAAGTAGATACGTCTAAACACTTACTTACAAGAGAAAACCCCAACCGTTTGTATCCCGCCCATTTGCCATGAAGATCTCTGGGAAATG 240
F D K F T K K L I M Q V D T S K H L L T R E N P N R F V S R P I V H E D L W E M

TACAAAAAAGAGGTTCGCTGTTTGGACATGGAAAGAGATGATTCGAAAGGGATCTTAAGATTTGGGAGAACTCACTCAAGATGAGAAGGATTCATCTCCAGATCTCGCGTTC 360
Y K K E V A C F W T L E E I D F E R D P K D W E K L T Q D E K D F I L Q I L A F

TTTGCATCCTCTGACCGAATTTGAATGAAAACTTACAACACCTCTTCGTCAAGTGGCGCAGATCCAGAAAGGAGGATTTCTTTGACTTCCAAGTTGGAAATGGAGATTCATGGC 480
F A S S D G I V I E N L T T R L R Q V A Q I P E A R S F F D F Q V G M E S I H G

AAAGTCTACGGAGAACTGATTGATAGACTGGTCCCGACGAAAAAGCAAGGCTATCTTGTGTTAACCGTGCACAACACTTCCCCGCCATCAAGAAGAGGAGCAGTGGGTATTAATTTG 600
N V Y G E L I D R L V P D E K D K A I L F N A A Q H F P A I K K K E Q W A I N W
WSSVrr2F704 →

ATGCAAGCAATAACGATTTGGCGGAATAATTGTGCTGTCAGTTGAAGGAATCTTCTTGTAGTGGTGCATTGCAATCCATTTCTGGATCAAGAACAGGGTATTTGGCTGGT 720
M Q S N N D L A E L I V A F A A V E G I F F S G A F A S I F W I K N R G I L P G
WSSVrr2F806 →

CTCACCTCCTCAATGAGTTCAATTTCTAGGGACGAAGGTCTTCAATCCGACTTTGCATGCATGCTGTGAAAAAGGTTTGTGTATACCCCATCAAGAGAAAGGATTCCTGAAATTTGC 840
L T S S N E F I S R D E G L H R D F A C M L L K K G F V D T P S R E R I L E I V

ACTGAAGCGTCCGAAATGAAACAAGAATTTCTCACAGTTCCCTGGCTGTTAAATAGTGGGAATGAACTGCAAGTTGATGAGCCAGTACATTTGAATTTGTGGCAGATAAATATTGGTT 960
T E A V R I E Q E F L T V S L P V K L V G M N C K L M S Q Y I E F V A D K L L V
← WSSVrr2R1008

GAAATGGGACTAGAAAAGCATAAATGTTACCAACCCCTTCCCATTCATGGACAATATTTCCCTCGAATAAGACCACTTTTTTTGAAAAGAGAGTCGCCGATTAACAGTCCCGCAG 1080
E M G L E K H Y N V T N P F P F M D N I S L E N K T N F F E K R V A E Y Q R A Q

GTATGGCTCTATCAATAAGATCAAGAAGCAACAAACCAAGAACTGGTTCCTCTCCCAATTTGACTGCACCTCTCCAGTCTCTCCCTCATCAACGAAAGAGATGTT 1200
V M A S I N K I K K D Q Q T Q E T G S P L P I L T A P P P V S S S S S E Q E D V

GAAGACGGCTCGGGACTACATCAGTTATGACGATTTTGTAGTCCACTATTGTGCAATAGTGTGTATTGTATTATTGTTATAATTTTTAAAAATAAATGTTCTATAAGAC 1320
E D G V G D Y I S Y D D F *
← WSSVrr2R1423

TAAAAAATGAATTACTTCCAATATCTCTGACAACTTTTTTGTGGGTAGATGCATGCTCTGCTCACTGCCTTTTACCTGATGGGAGAAACAACCTTGGTTTTGATTC 1440

C

ATGGCTGGTGTAGAGCTGTCACCTGGACCCATGTTTGGCGCAAGTCTACCTACCTGAAAAACATATACCAACAAGAAAATGGAGGCAATAAACATTGCCGTTTGTCAAACTCC 120
M A G R V E L V T G P M F A G K S T Y L K N I Y Q Q E N G G N K H C L F V K H S

CTAGAACTAGTACGGTGTGGAAGCTGGAACAATAGTCACCTATGCCGAGAAAGTGAAGGTTGTACTACAGTTCTTCTATCAAGGAATAATCAGTGTGTACCAGAAATTTGTG 240
L E T R Y G C G T G T I V T H A G E V I E G C T T V S S I K E L I S V L P E V V
WSSVtkf310 → WSSVtkf351

GATGTGATTTCTATTGACGAGGGCAATTTCTTCAAGGATTTGGTCTAGTCAATAGACTGGCTGACAAGGGGAAAAGGATTTGATTTGCAGCACTTGATGGAATCTCTGACCAAAATG 360
D V I L I D E G Q F F T D L V L V N R L A D K G K R I V I A A L D G T S D Q Q M
→

TTCACTCCTAATTCATAAGCTATTGCCCTTATCAAAATCCATTTGTAAGCTAGCATTAATGTATGATTTGTAATAATGATACCAAGAAGCTCCTTTTACTGTAAGTTTGGTAATGAC 480
F S P I H K L L P Y T N S I V K L A S K C M I C K I D T K E A P F T V R F G N D

AATGATAAATAATGTTATGTGTAGGAGGAGCTGAAATGTACGCTGCCTGCCGAGCTGTTACAAAAAATAACAAGAAAAGAAAGGGGAACTTGTGTACTTGAAGGAGGT 600
N D N N V I C V G G A E M Y A A A C R D C Y K K I N K K K N K G K L V V L E G G
← WSSVtkr707

GACAGTGGGTAAAGATGACCAAGCAAACTCTTGTGACCAATAAAAACTCGCTCTTTATGGAGGAGAATACATGTGCTTTCCCGACAGGAGCAGCCATACGGGTAACACTCAAT 720
D R C G K S T Q A K L L L T N K N S P L Y G G E Y M C F P D R S S H T G K L I N
← WSSVtkr779

GATTATTAACATAAGAAATGAACTAGATGATCATGACGCTCACTTGTATTTCTGCAAAATAGATGGGAAAGTTGTAGTAAAATTAAGCAGTTGTAGACGATGGAATCCATGTTGTG 840
D Y L T K K I E L D D H A A H L L F S A N R W E V C S K I K Q L L D D G I H V V

ATGGATAGATTAATCTACTCGGGATGTTTCTTTAGCTAGAGGAGTGGATACCGTTGAGTGGTGTCTGCTGACGATGAGGAGCTTCCTCAGCCGATCTGTATTGTGATGCTTT 960
M D R Y Y Y S G I V F S L A R G V D T V E W C S A S D E G L P Q P D L V L L M L

TTAGATGTTGAAAAGTGTCAAAATAGGATACTTTTGGTGTGAAAGATTTGAGACAAATCCATTCAGAAACCTGTAGAGCCCTATTCTAGACCTCGCAAAATAGGACGAAAAGAA 1080
L D V E K C S N R D T F G V E R F E T N S I Q E R A R A L F L D L A N K D E K N

GTATGGATTAAGGTAGAGCTCGCGCACCAATGAGGAGTGCAAACTAAAAATATAAATATTGTATATAATATTGTTGAAGAATAA 1167
V W I K V D A R G T I E E V Q T K I I N I V Y N I V E E *

Table 2. Recognition of specific WSSV *rr1*, *rr2* and *tk-tmk* gene fragments in *Artemia* cyst WSSV. Two-step PCR was performed on DNA samples from WSSV-positive *Artemia* cysts using primer sets for 4r WSSV gene fragments. Pooled samples from each brand were tested up to 5 times with each primer set. Data show the number of times a sample was tested (n) before a PCR-positive signal was seen (1/n) or indicate that a sample was tested 5 times without a positive reaction (0/5)

WSSV primer sets	Brand and year of packaging				
	A before 1987	B 1997	C 1998	D 1999	E 1999
rr1F809/rr1R1426– rr1F863/rr1R1227	1/5	1/3	0/5	0/5	0/5
rr1F1551/rr1R1980– rr1F1726/rr1R1945	1/4	1/1	1/4	1/3	1/1
rr2F704/rr2R1423– rr2F806/rr2R1008	1/5	0/5	1/5	0/5	1/5
tkF310/tkR779– tkF351/tkR707	1/5	1/4	1/5	1/4	1/5

Results from feeding *Penaeus monodon* with nauplii hatched from WSSV-positive cysts

All tested *Penaeus monodon* PL from the 2 test groups and the control group were 2-step WSSV PCR-negative with the pms 146F1/R1-F2/R2 and the IQ2000 primer sets (data not shown). The survival rates from stage 2 mysis to PL₁₅ were about 50 % in both of the groups fed *Artemia* while that in the control group (commercial diet only) was approximately 20 %. Growth rates were also very similar in the 2 *Artemia*-fed groups and slightly lower in the group fed commercial diet only.

DISCUSSION

The well-known importance of *Artemia* in the larval shrimp diet was illustrated again in the present study where *Penaeus monodon* PL fed a dry commercial diet alone had a lower growth rate and survival than the 2 groups fed nauplii. We attribute this result both to the inadequacies of the artificial diet used and to the cannibalistic behavior of the shrimp larvae in the test.

Although WSSV-specific PCR tests gave positive results for all 5 of the tested brands of *Artemia* cysts, all of the tested nauplii derived from those cysts and all of the tested shrimp fed those nauplii gave negative PCR tests for WSSV. There are several possible explanations for these results. The simplest is that the cysts were externally contaminated with WSSV particles, WSSV DNA or WSSV DNA fragments of unknown derivation and that this was removed or diluted to undetectable levels when the hatched nauplii were washed with clean, sterile sea water before being fed

to the shrimp. In support of this contention was that the template DNA was present in only very low quantities as evidenced by the facts that nested PCR was required for detection and that only 58 (17%) of 351 samples tested gave WSSV PCR-positive results.

On the other hand, it was also possible that the WSSV template DNA was present within the cysts and that *Artemia* might be susceptible to WSSV infection. To accept this very unwelcome prospect it would be necessary to explain the negative WSSV PCR results with the hatched nauplii and the shrimp larvae that ate them. For the hatched nauplii, this is difficult, since any internal viral DNA should have survived washing, however vigorous, and at least some of the samples tested should have given positive results. On the other hand, any virus within the cyst membrane but outside the naupliar membrane might have been washed away. For subsequent disease transfer from the nauplii to the shrimp, it is likely that nauplii actually infected with viable WSSV would have been required at the threshold levels needed to cause shrimp infections. As already mentioned, the cysts contained only very low levels of target template and it is possible that even viable, internal WSSV at such low concentrations would not have been able to infect the shrimp larvae. Thus, despite negative WSSV PCR results for the nauplii and the larvae to which they were fed, we cannot dismiss the possibility that low-level undetectable infections might have occurred in our tests. Specific *Artemia* infection trials would be necessary, accompanied by histological examination and *in situ* DNA hybridization tests in addition to PCR tests. Finally, small detected sequence variations from the originally described WSSV of shrimp (see discussion below) open the possibility for the presence of a new variety of WSSV that may not infect shrimp.

Since the IQ2000 primer set targets a smaller amplicon, the different results from the pms 146 primer pair (F1/R1-F2/R2) with cyst Brands A, B and C in Table 1 and Fig. 1 may simply be a consequence of IQ2000's greater sensitivity. This is because primers for smaller amplicons are generally more sensitive (Lo et al. 1996b). However, since both primer sets were designed based on the sequence of the *Penaeus monodon* WSSV 1994 Taiwan isolate pms 146 fragment, it is also possible that the differences arose from sequence differences in the target DNA. Indeed, 2 point mutations were found in this region (Fig. 2, Brands B and E). More significantly, there were also possible sequence differences in the coding regions of the *rr1* and *rr2* genes since the *rr2* primer set and 1 of the *rr1* primer sets failed to detect their respective target fragments in some of the cyst brands tested (Table 2). However, other explanations of the results shown in Table 2 are also possible. For example, the quantity of target DNA

in some of the samples might simply have been below the detection threshold of the PCR assay, although the *tk-tmk* primer set did produce a positive response in all 5 cyst brands. On the other hand, the potential genetic variation found also suggests that the positive PCR results and negative bioassay results might be explained by the presence of a type or variety of WSSV DNA that is non-infectious for *Artemia* or shrimp and derived from other arthropods or crustaceans in the *Artemia* environment.

WSSV is the only organism known to have its *tk* and *tmk* genes fused together to form a chimeric *tk-tmk* gene. This gene is therefore likely to be a good marker for identifying WSSV-related viruses (Tsai et al. 2000b) and here the *tk-tmk* primer set that targeted the *tk-tmk* junction region gave positive results for all 5 brands of cysts tested (Table 2). Furthermore, sequence comparisons between the corresponding coding regions of the successfully amplified *rr1*, *rr2* and *tk-tmk* gene fragments (Table 2) showed complete identity with the *Penaeus monodon* WSSV 1994 Taiwan isolate (for sequences, see the shaded regions in Fig. 4).

We conclude that WSSV PCR-positive results can sometimes be obtained using several WSSV-specific primer pairs with batches of *Artemia* cysts and that the PCR amplicons obtained have very high sequence homology to those of WSSV from shrimp. Because of the nature of PCR testing, we cannot say whether such tests indicate the presence of a viable virus, inactivated virus or simply complete or fragmented viral DNA. Indeed, it is very unlikely that the cyst sample dating from 1987 (13 yr old) contained viable WSSV. Nor can we conclude whether the PCR target template was originally present externally or within the cysts. Fortunately, the PCR tests on hatched *Artemia* nauplii were negative for WSSV, as were the tests on PL that were fed those nauplii. We have also been informed by D. V. Lightner (pers. comm.) that because of import restrictions, *Artemia* cysts and adult *Artemia* biomass have been undergoing testing for more than a year for WSSV in his laboratory and those of several others in the USA and Mexico using various primer sets, and that no WSSV-like DNA has been detected. Again, the fact that the adult *Artemia* were negative in all those tests and in ours supports the argument that our positive PCR tests resulted from contamination of the cysts by something in the *Artemia* environment. This possibility for external contamination highlights the importance of the usual hatchery practice of washing *Artemia* cysts with chlorinated water before hatching. On the other hand, the results still leave open a small possibility that *Artemia* can be infected with WSSV, and this possibility must be vigorously tested, given the enormous impact that a positive outcome would have on the shrimp aquaculture industry.

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