Outbreaks of infectious myonecrosis virus (IMNV) in Indonesia confirmed by genome sequencing and use of an alternative RT-PCR detection method☆

Saengchan Senapin [a,b], Kornsunee Phewsaiya [a,b], Matthew Briggs [c], Timothy W. Flegel [b,*]

[a] *National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, Pathumthani, 12120, Thailand*

[b] *Center of Excellence for Shrimp Molecular Biology and Biotechnology, Mahidol University, Rama VI Rd., Bangkok, 10400, Thailand*

[c] *Epicore BioNetworks Inc., 4 Lina Lane, Eastampton, New Jersey, 08060, USA*

Abstract

Outbreaks of disease due to infectious myonecrosis virus (IMNV) were first reported from Brazil in 2004 in Pacific white shrimp *Penaeus* (*Litopenaeus*) *vannamei*, an exotic species to Brazil that was relatively recently imported for cultivation in earthen ponds. In Indonesia, this non-native species has also been imported and successfully cultivated on a large scale since 2003. Starting in early 2006, there were anecdotal reports from Indonesian shrimp farmers of high mortality in cultivated *P. vannamei* with gross signs of white muscle, similar to those reported for IMNV outbreaks in Brazil. We obtained a sample of shrimp from one of these outbreak ponds and it gave a positive result for the presence of IMNV using a standard commercial detection kit. After sequencing the PCR fragment to confirm the presence of IMNV, additional primers were designed for cloning and sequencing the full 7.5 kb IMNV genome. Subsequent analysis (GenBank accession no.EF061744) revealed that the Indonesian IMNV sample had 99.6% nucleic acid sequence identity (a total of 29 differences in 7.5 kb) to that of Brazilian IMNV reported at GenBank. It is interesting that one of these differences was a single base insertion at nucleotide 7431 leading to the creation of a delayed termination (stop) codon that led to 13 additional amino acid residues in the deduced RdRp (RNA-dependent RNA polymerase) protein product. Due to some difficulty with very weak false positive results obtained using the commercial detection kit with some samples, we designed an alternative, nested RT-PCR detection method with specific primers to target the viral RdRp region instead of the capsid gene targeted by the commercial kit. In our hands, this protocol gave more consistent results and higher sensitivity that did the kit.

Keywords: Detection; IMNV; *Penaeus vannamei*; Shrimp; Virus

1. Introduction

Although the Pacific white shrimp *Penaeus* (*Litopenaeus*) *vannamei* is a non-native (exotic) penaeid shrimp

☆ The GenBank accession number for the sequence reported in this paper is EF061744.

* Corresponding author. Tel.: +66 2 2015876; fax: +66 2 3547344.
E-mail address: sctwf@mahidol.ac.th (T.W. Flegel).

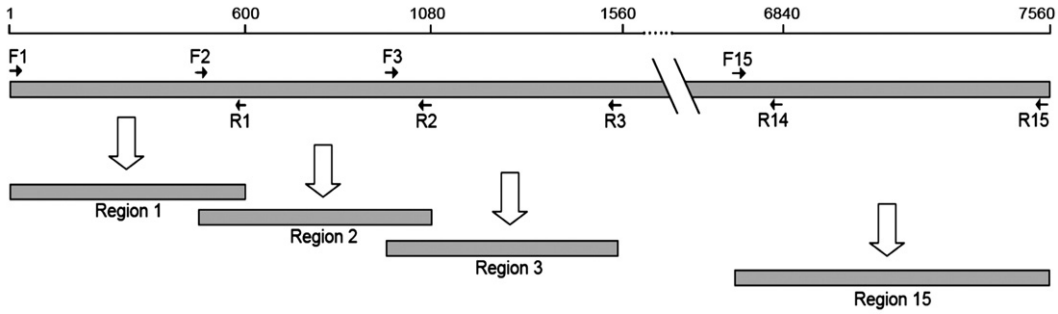

Fig. 1. Illustrated diagram for primer design to amplify 15 regions of the IMNV genome. A fragment of 600 bp was amplified from each region except for region 15 that was 840 bp. Numbers indicate nucleotide positions in the genome.

species to Brazil, it was imported for commercial shrimp culture beginning in 1983 (Briggs et al., 2004). Disease outbreaks due to infectious myonecrosis virus (IMNV) were first reported in farmed Pacific white shrimp from Brazil in 2004 and characterization of the viral particles was subsequently described in 2006 (Poulos et al., 2006). Gross signs of IMNV infected shrimp include necrosis of striated muscles, primarily in the distal abdominal segments and the tail fan, and the appearance of white discoloration of the affected muscle similar to the color of cooked shrimp. IMNV has non-enveloped, icosahedral virions 40 nm in diameter and a genome of double-stranded RNA comprised of 7560 nucleotides containing two open reading frames, designated ORF1 and ORF2. ORF1 encodes a putative RNA-binding protein and a capsid protein and ORF2 codes for a putative RNA-dependent RNA polymerase (RdRp). This virus has been classified in the family *Totiviridae*. Apart from naturally infected species *P. vannamei*, experimentally susceptible shrimp species to IMNV are *Penaeus monodon* and *Penaeus* (*Litopenaeus*) *stylirostris* (Tang et al., 2005). However, no mortalities were observed in these two species. So far, IMNV detection systems using RT-PCR (Farming IntelliGene Tech. Corp., Taiwan), *in situ* hybridization (Tang et al., 2005) and real-time RT-PCR (Andrade et al., in press) have been established.

Many viral pathogens are restricted in their geographical distribution and their spread to new areas can have disastrous consequences. It is believed that unregulated trans-boundary movement of broodstock and postlarvae for aquaculture is the major cause of pathogen spread between regions (Flegel, 2006). Here we report the occurrence of IMNV infected shrimp from a farm in Indonesia and an alternative RT-PCR method for its detection. Genome sequence analysis indicated very little sequence difference between the Indonesian and Brazilian IMNV isolates.

2. Materials and methods

2.1. Shrimp samples

Pacific white shrimp *Penaeus vannamei* with opaque, whitish abdomens were obtained from a shrimp farm in the Situbondo District of East Java Province in Indonesia mid June 2006. They were preserved in 95% ethanol at the farm and transported to Bangkok, Thailand for analysis within 1 week of collection.

2.2. RNA extraction

Shrimp muscle tissue from the 6th abdominal segment was homogenized in Trizol reagent (Invitrogen) and RNA was extracted following the manufacturer's instructions. RNA concentration and quality were measured by spectrophotometric analysis at 260 and 280 nm.

2.3. Primer design and single-step RT-PCR

Primers were designed based on an IMNV genome sequence (GenBank accession no.AY570982). Altogether 15 regions of the genome were amplified by RT-PCR to produce 14 fragments of 600 bp each and 1 fragment of 840 bp (*i.e.*, to cover the entire viral genome). Except for region 15 (840 bp), each amplified fragment contained 120 bp at the 3′-terminus in order to overlap with the 5′-terminus of the adjacent region. The strategy in primer design is depicted in Fig. 1 and primer sequences are summarized in Table 1. RT-PCR was performed using a SuperScript One-Step RT-PCR kit with Platinum® Taq (Invitrogen). The protocol comprised one initial step at 50 °C for 30 min and 94 °C for 2 min followed by 30 cycles of 94 °C for 30 s, 41 °C for 45 s, 72 °C for 45 s and final extension at 72 °C for 5 min. PCR products were analyzed using 1.2% agarose gels.

Table 1
List of primers used in this study

Region	Expected product size (bp)	Forward primer name/sequence	Reverse primer name/sequence
1	600	F1/GGCAATTTCAACCTAATTCTAAAAC	R1/TGAAAAATAAGCTGTGCCCCATGTT
2	600	F2/AATACTACATCATCCCCGGGTAGAC	R2/GACTTTCTTCCCAAGATGGAGTCTC
3	600	F3/GAAGTTAAAGATGTAACACTTGCCT	R3/ATACTCCTTCTCCAAAGGGTGTACG
4	600	F4/GATCCAGTTCTAACTAGAGAAGATA	R4/TCCAGATACAATTACCATGCTGGTT
5	600	F5/GCAGCTTGGCTAAACAACAGACCAT	R5/ATTCAATCCACGAATTTGTCTTGGT
6	600	F6/CTTCGTGATAATGACTCTATTAGGG	R6/CTGTGGAACAGATTGTAAAGTAAGA
7	600	F7/ATAAATAATGGTGTTAATATATTTG	R7/ACTAATTGGCAGTGTTGTTTTCATT
8	600	F8/GTTGGTGTGGCCCTGCCAACTGTAA	R8/ACTACCTTGCATTGAACTCCACGAA
9	600	F9/GTTTGGTATTCAACAAGACGTATTT	R9/AACATTAATACAACTCTCATCATGA
10	600	F10/GAGACAGGCAATGTATTCAGACCAT	R10/CTCTTGCTGACTCGGCTGTTCGATC
11	600	F11/TCGGGTTTTATGAATGCCCGTTCCA	R11/TTGATAACTGTTTTGCAATTTCAAT
12	600	F12/TTGTACAAAACATTTGTATCTATAT	R12/CTTCGATGTTAGATGCCACAGCAAG
13	600	F13/TTTATACACCGCAAGAATTGGCCAA	R13/AGATTTGGGAGATTGGGTCGTATCC
14	600	F14/GAGTACCATCAGGAGTGAGAATAAC	R14/GATGTATGTCCTCTACGTTAACCAA
15	840	F15/AATATCTAGAATTGCCAAAACGACT	R15/CATGGCTGGCCACAAAACCCAACTG
Nested	282	F13N/TGTTTATGCTTGGGATGGAA	R13N/TCGAAAGTTGTTGGCTGATG

2.4. DNA cloning and sequence analysis

Amplified cDNA was purified using a QIAGEN QIAquick PCR Purification Kit and cloned into pDrive cloning vector (QIAGEN). Recombinant plasmids were sequenced by Macrogen Co. Ltd. (South Korea). DNA and protein analysis were carried out using the EXPASY web server (http://au.expasy.org/). To identify related sequences, a protein–protein basic local alignment search tool (blastp) search was carried out using the NCBI protein database (http://www.ncbi.nlm.nih.gov/BLAST/). Alignments of protein sequences were performed by ClustalW (http://www.ebi.ac.uk/clustalw/). PCR primer were designed using Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

2.5. Diagnostic tests

Diagnostic tests using a commercially available IQ2000, IMNV Detection and Prevention System kit (Farming IntelliGene Tech. Corp., Taiwan) were carried out according to the supplier's protocol. An additional nested RT-PCR method was developed and carried out as follows. In the first reaction, designed primers F13 and R13 (Table 1) were used (0.25 μM each) in 20 μl reaction solution containing 0.2-2 μl of RNA template, 0.4 μl of SuperScript One-Step RT/Platinum Taq mix, and 1X reaction buffer. The RT reaction was carried out at 50 °C for 30 min. The mixture was then denatured at 94 °C for 2 min, and amplification was carried out using 30 cycles of 94 °C for 40 s, 45 °C for 40 s, and 72 °C for 40 s. The expected amplicon was 600 bp. A second, nested amplification was carried out in 20 μl reaction solution containing 10 μl from the first RT-PCR reaction vial, 0.25 μM of each primer F13N and R13N (Table 1), 1 unit of Taq polymerase (Invitrogen), 0.2 mM dNTPs, and 1X reaction buffer. The reaction protocol comprised denaturation at 94 °C for 5 min followed by 25 cycles of denaturation at 94 °C for 45 s, annealing at 50 °C for 45 s, and extension at 72 °C for 30 s. The expected amplicon was 282 bp. The PCR product of region 13 cloned into pDrive plasmid was used as a positive control and a reaction without DNA was used as a negative control. PCR products were analyzed using 1.2% agarose gels.

3. Results and discussion

3.1. Shrimp from a farm in Indonesia were positively IMNV infected

In June 2006, a disease outbreak with high mortality occurred on a Pacific white shrimp *P. vannamei* farm in the Situbondo District of East Java Province in

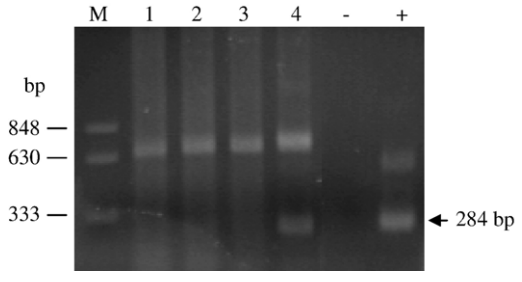

Fig. 2. Agarose gel of amplicons obtained using the IQ2000 kit with 4 Indonesian shrimp samples showing gross signs of white muscle tissue. M, DNA marker; −, negative control; +, positive control. The band at 284 bp for sample 4 (arrow) indicates a light IMNV infection according to the kit instructions.

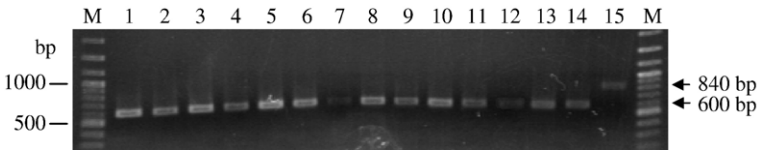

Fig. 3. Agarose gel of RT-PCR amplicons obtained using primers that target 15 regions of the IMNV genome. M, DNA marker; numbers indicate regions in the genome described in Fig. 1. Expected product sizes of 600 and 840 bp are indicated by arrows.

Indonesia. Moribund shrimp exhibited opaque, whitish abdominal muscles similar to those of white shrimp infected with infectious myonecrosis virus (IMNV) previously reported from Brazil (Poulos et al., 2006). Four shrimp were collected, preserved in 95% alcohol and transported to Bangkok where they were tested for the presence of IMNV using a commercial RT-PCR assay kit. The template used was RNA extracted from muscle tissue of the 6th abdominal segment. Shrimp sample 4 gave a single band (284 bp) indicating a light IMNV infection according to the kit instructions (Fig. 2) while the other 3 samples gave negative test results. Since the negative samples showed the internal kit control band at 680 bp, the integrity of the RNA extract was confirmed. Sequencing of the 284 bp amplicon from the positive test sample confirmed that it originated from the IMNV capsid protein gene. Since we could find no other reports of IMNV from Indonesia or Asia, it suggested that IMNV may have been introduced to Indonesia from Brazil. We reported the case to the Network of Aquaculture Centres in Asia-Pacific (NACA) and the information has been available at the NACA's website (http://www.enaca.org/) since August 2006.

3.2. Amplification of 15 regions of IMNV genome

To better determine the relationship between IMNV from Indonesia and Brazil, we employed a one-step RT-PCR and DNA cloning strategy to obtain the complete nucleotide sequence of the Indonesian IMNV isolate. Using the RNA extracted from IMNV-positive shrimp (sample 4) and 15 primer pairs designed to cover the entire viral genome, the 15 expected amplicons were obtained, although band intensities differed (Fig. 3). However, the fact that these products were obtained by non-nested RT-PCR for 30 cycles suggested that the primer targets (*i.e.*, IMNV genome) were present at relatively high copy number. After purification, the PCR products were ligated to pDrive cloning vector (QIAGEN). Recombinant plasmids were verified by colony PCR (data not shown) prior to DNA sequencing by Macrogen (South Korea). All fragments were fully sequenced from both strands of the DNA.

3.3. IMNV sequence comparisons

Since each amplified fragment was designed to contain ends that overlapped with adjacent fragments, only internal fragment sequences internal to the primers were used to produce the concatenated genome sequence for Indonesian IMNV, except for the first and the last 25 nucleotides where overlaps were not possible. The consensus genome sequence of the Indonesian IMNV isolate has been submitted into the GenBank under accession number EF061744. The sequence shares 99.6% sequence identity with that of the Brazilian IMNV isolate sequence deposited at GenBank (accession no. AY570982). There were totally 29 base differences throughout the genome (Table 2). Excluding a frame shift difference, 28 of the changes resulted in only 11 changes in amino acids. However, a single base (frame shift) insertion at position 7431 resulted in a change of 19 amino acids including an addition of 13 amino acid residues to the deduced viral RdRp protein product of ORF2 (Fig. 4). The frame shift was confirmed by sequencing 10 different clones of the PCR product from this region (region 15). Since the frame shift mutation occurred at amino acid position 731 and since three other mutations in ORF2 did not alter amino acids (see GenBank submission), the eight RdRp motif sequences located at residues 46–513 (Poulos et al., 2006) were not affected. The RdRp function of the Indonesian IMNV was therefore presumed to be intact. This was in accordance with relative high mortality rate

Table 2
Sequence comparison of two IMNV isolates

Region in the genome	Nucleotide position		Analysis compared to the Brazilian isolate	
	In the Brazilian isolate	In the Indonesian isolate	Nucleotide changes	Amino acid changes
5′ UTR	1–135	1–135	2	NA
ORF1	136–4953	136–4953	22	11
Interspace	4954–5240	4954–5240	1	NA
ORF2	5241–7451	5241–7490	4	19
3′ UTR	7453–7560	7491–7561	0	NA

Fig. 4. Sequence analysis of the RdRp region of the Indonesian IMNV isolate. (a and b) Chromatograms in both sequence orientations showing positions of a base insertion marked by arrows. Black letters above indicate deduced amino acid residues. (c) Sequence comparison at the C-terminal ends of the deduced RdRp sequences of IMNV from Indonesia and Brazil showing 19 amino acid changes including 13 additional residues in the Indonesian isolate.

of collected shrimp, suggesting similar virulence to the original IMNV isolates.

3.4. Preliminary comparison of diagnostic systems

RdRp sequences of RNA viruses show a reasonably high level of conservation due to their crucial replication function. Sequences covering the conserved motifs characteristic of RdRp have been used for virus classification and phylogenetic taxonomy (Koonin and Dolja, 1993; Culley et al., 2003). Therefore, we chose to design primers corresponding to the RdRp region for IMNV detection. Nested primers, F13N and R13N, were designed to amplify an internal fragment of 282 bp from the 600 bp amplicon obtained using primers F13 and R13 that target the IMNV RdRp. When this method was compared to that of the IQ2000 commercial kit using equal amounts of RNA template and a plasmid containing the 600 bp amplicon as a positive control (Fig. 5), it was found that our newly designed method gave positive results for IMNV (*i.e.*, a 282 bp amplicon) with all 4 of the Indonesian shrimp samples while the IQ2000 kit gave positive results for shrimp sample 4 only. Sample 4 appeared to be more heavily infected than the other 3 since the first PCR product band of 600 bp could also be observed (Fig. 5). The 282 bp amplicons from samples 3 and 4 were cloned, sequenced and confirmed to originate from the IMNV RdRp gene (100% identity). Although our newly developed RT-PCR protocol gave higher sensitivity

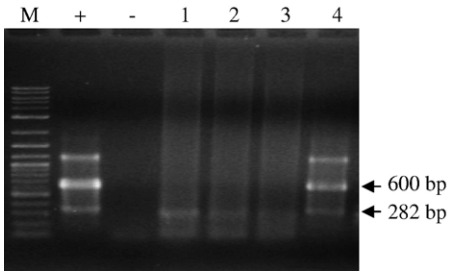

Fig. 5. Agarose gel of IMNV detection using our newly developed RT-PCR protocol with RNA template from the 4 shrimp collected from a farm in Indonesia. All 4 give positive results for the 282 bp amplicon. M, DNA marker; +, positive control; −, negative control. Arrows mark bands of 600 bp and 282 bp representing amplicons from the first and nested PCR, respectively.

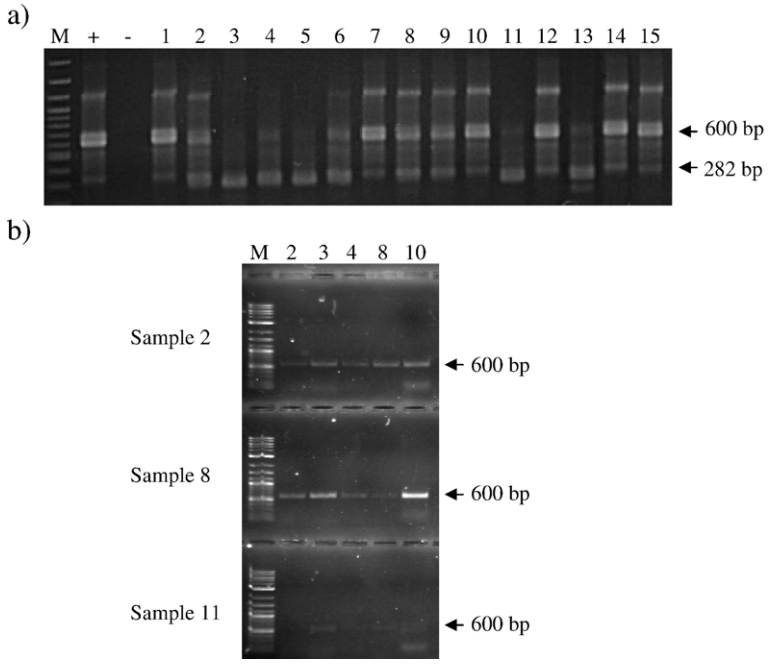

Fig. 6. Agarose gel of amplicons from IMNV detection tests with 15 additional shrimp samples obtained from Indonesia in October 2006. (a) Results using our newly developed RT-PCR protocol. (b) Results using primer pairs for regions 2, 3, 4, 8, and 10 of the IMNV genome with RNA template from samples 2, 8, and 11 from (a). M, DNA marker; +, positive control; −, negative control.

when compared to the IQ2000 IMNV detection kit, it was a less convenient 2-tube protocol. It is possible that improving convenience and serviceability by adopting a single-tube, non-interrupted, nested RT-PCR protocol and adding an internal control would reduce sensitivity and remove this apparent advantage.

Later in October, 2006, 15 *P. vannamei* exhibiting white muscle tissue were collected from the same area in Indonesia and assays were conducted using our newly developed detection method. All samples gave positive results for IMNV infection (Fig. 6a). Using the presence of the 600 bp amplicon as an indicator of heavy infections and absence as an indicator of light, representative heavy infection samples 2 and 8, and light infection sample 11 were randomly chosen for further confirmation of IMNV infections by one-step RT-PCR amplification using 5 primer pairs for other regions (2, 3, 4, 8, and 10) of the IMNV genome. The expected amplicons were obtained for all of the samples (Fig. 6b) and the light intensity of bands for sample 11 supported the contention that it was a lightly infected sample.

In conclusion, this study provides evidence that disease outbreaks caused by IMNV infection have occurred in farmed shrimp in Indonesia. We have been informed by a contact in Indonesia who understandably wishes to remain anonymous that *P. vannamei* broodstock from Brazil have been smuggled onto Java island for use in a commercial hatchery. If this is true, it may explain the high identity of the genome sequences of IMNV from Indonesia and Brazil and may constitute another example of the unfortunate transfer of shrimp pathogens over large geographical distances by careless movement of contaminated stocks for aquaculture (Flegel, 2006). It is hoped that awareness of this newly emerging pathogen in Asia will motivate the relevant governmental authorities to heighten measures against its further dispersal in the region. Our new, more sensitive detection method may be helpful in implementing surveillance and control measures.

Acknowledgements

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