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Short communication

# A cDNA microarray approach for analyzing transcriptional changes in *Penaeus monodon* after infection by pathogens

Siriporn Pongsomboon [a,b], Sureerat Tang [a,b], Suleeporn Boonda [a], Takashi Aoki [c], Ikuo Hirono [c], Anchalee Tassanakajon [a,*]

[a] *Center of Excellence for Molecular Biology and Genomics of Shrimp, Department of Biochemistry, Faculty of Science, Chulalongkorn University, 254 Phayathai Road, Bangkok 10330, Thailand*
[b] *National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Pathumthani 12120, Thailand*
[c] *Laboratory of Genome Science, Tokyo University of Marine Science and Technology, Tokyo, Japan*

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ABSTRACT

A cDNA microarray comprised of 9990 different ESTs obtained from the *Penaeus monodon* EST project (http://pmonodon.biotec.or.th) was employed to identify viral (white spot and yellow head viruses) and bacterial (*Vibrio harveyi*) responsive genes in the hemocytes of *P. monodon* at 6, 24 and 48 h post-injection (hpi). The number of differentially expressed genes found was highest in shrimps infected with white spot virus (1954 genes) followed by yellow head virus (1136 genes) and *V. harveyi* (420 genes). Changes in shrimp gene expression were highest at the late infection stage for both viruses, whilst that for *V. harveyi* induced gene expression was mainly found at the early infection stage, but the repression of genes was mainly found in the mid stage of infection. Shrimp genes specifically upregulated by each particular pathogen are identified and are summarized.



## 1. Introduction

Shrimp farming is currently affected by outbreaks of infectious diseases that frequently result in high mortality rates and economic losses. Viruses are the major pathogen problem, being responsible for the most devastating losses [1]. The major viruses that affect the cultivation of the black tiger shrimp, *Penaeus monodon*, in Thailand are the white spot syndrome virus (WSSV), yellow head virus (YHV) and hepatopancreatic parvovirus (HPV) [1,2]. Vibriosis is also an important disease of farmed shrimps and especially in the larval stage. Some of the *Vibrio* species, such as *Vibrio harveyi* and *Vibrio parahaemolyticus*, are opportunistic pathogens that cause symptomatic infections when the shrimps are stressed [3,4].

Due to the serious impact of infectious diseases on shrimp cultivation, an understanding of the shrimp's immune system responses to pathogen infection would potentially allow for the development of management strategies to control virulent or problematic pathogens encountered on shrimp farms. Analysis of the differential gene expression in pathogen-infected shrimps by microarray technology can reveal host immune mechanisms that are essential for their defense against pathogens. However, to date the simultaneous analysis of expression of a large number of genes, that is to look at coordinate expression of genes by using the microarray technique, has been limited to the screening of a small number of shrimp genes [5–9].

In this study, a shrimp cDNA microarray chip comprising unique contigs of 9990 cDNAs from an EST collection of the *P. monodon* EST database (http://pmonodon.biotec.or.th) [10] was used to study the expression profile of *P. monodon* genes that are differentially expressed in response to viral (WSSV and YHV) or bacterial (*V. harveyi*) infections. This global analysis of gene expression provides useful information on shrimp immune responses to viral and bacterial infections.

## 2. Materials and methods

### 2.1. Animals and experimental infection

Juvenile *P. monodon* shrimps (approximately 20 g each) were obtained from the shrimp Genetic Improvement Center, BIOTEC, Thailand. For viral infection, shrimps in the experimental group

* Corresponding author. Tel.: +66 2 2185439; fax: +66 2 2185418.
*E-mail address:* anchalee.k@chula.ac.th (A. Tassanakajon).

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were injected with 100 μl of a 1500- and 2000-fold diluted hemolymph sample, previously isolated from a moribund YHV- or WSSV-infected *P. monodon* shrimp, respectively. The control group received 100 μl of lobster hemolymph medium, which was the media used as a diluent for these viruses. At these viral infection doses, shrimps were moribund at 48 h post-injection (hpi). For *V. harveyi* infection, the experimental shrimps were injected with 100 μl of *V. harveyi* 639 ($10^5$ CFU) in 0.85% (w/v) NaCl solution, whilst the control group received 100 μl of just the 0.85% (w/v) NaCl solution. Hemolymph was collected at 6, 24 and 48 h post-injection, representing the early-, mid- and late-stages of infection, respectively. Total RNA was isolated from the hemocytes using TRI REAGENTs (Molecular Research Center, USA) and then treated with DNase I (Promega, USA) following the manufacturer's protocol. WSSV and YHV infections were detected by PCR or RT-PCR, according to Kiatpathomchai et al. [11] and Wongteerasupaya et al. [12], respectively. For *V. harveyi* infection, shrimps were tested whether the infection was successful by culturing the suspensions of hepatopancreas on TSA plates supplemented with 2% NaCl, and examining the strong luminescence from the colonies.

### 2.2. Microarray analysis

The *P. monodon* cDNA microarray chip, containing 9990 non-redundant cDNA sequences, was constructed from unique contigs of the EST collection of the *P. monodon* EST database (http://pmonodon.biotec.or.th). Each EST clone was spotted in duplicate. Microarray hybridization and analysis were performed as described by Pongsomboon et al. [8]. Briefly, 10 μg of total RNA from 10 individual shrimps was reverse-transcribed and the synthesized cDNA obtained was labeled with Cy3 or Cy5 using a Labelstar Array kit (QIAGEN, Germany). Each microarray analysis was carried out with duplicate experimental samples. The obtained microarray data analysis was performed using MIDAS (http://www.tigr.org/software). A flip dye consistency check was performed. A gene was considered differentially expressed by infection if its expression ratio increased or decreased by two-fold or more compared to the control samples.

### 2.3. SYBR Green I real-time RT-PCR

SYBR Green I real-time RT-PCR was employed to confirm the microarray results. The hemocyte total RNA from each duplicated sample was pooled with equal amounts from 10 individual shrimps. One μg of total RNA was reverse transcribed into cDNA using the ImPromp II Reverse Transcription System (Promega, USA) according to the manufacturer's instructions. The real-time PCR was performed by an iCycler iQTM Real-Time Detection system using iQ SYBR Green Supermix (Bio-Rad, USA) as described by Pongsomboon et al. [8]. The primers and annealing temperatures of the six genes selected to check the microarray expression results are shown in the Table 1. Each PCR reaction was done in triplicate. The relative quantification values for each gene were calculated by the $2^{-\Delta\Delta CT}$ method using β-actin as an internal reference gene.

**Table 1**
Primers used for real-time PCR.

| Putative gene[a] | GenBank accession | Primer sequence 5′–3′ | Annealing (°C) |
|---|---|---|---|
| AGAP000432-PA | DW042937 | F CGTCCTTCAGT GCGCTTCCATA<br>R ACAGCGACTCC AAGGTCTACGA | 60 |
| Mitochondrial 2-oxoglutarate/malate carrier protein | GW996584 | F GGCAGTCTTA GGCATGACAG<br>R CTTGTAGTTG CGCCTCTCAG | 50 |
| Cathepsin L | ES609112 | F ATCAGGGCTA CGTTAAGATGG<br>R CAAGGTCTAG TGCTCAGAAT | 65 |
| Unknown | HO004745 | F CACTCTGAT CGCCAGTGA<br>R CATACAGC CTTCGTTGTC | 50 |
| Penaeidin 5 | GH717907 | F AGTGGCTGG ACGACTGATT<br>R GGCGGTTAC ACAGGTTCAT | 55 |
| Hemocyte kazal-type proteinase inhibitor (Pm2) | BI018075 | F ATGCAACCAC GTCTGTACTG<br>R CTGCAAGG TTCCACATCT | 55 |
| β–actin | HO113015 | F GAACCTCTCGT TGCCGATGGTG<br>R GAAGCTGTGC TACGTGGCTCTG | 60 |

[a] is based on expected values of BlastX and BlastN matches less than $E^{-4}$.

## 3. Results and discussion

### 3.1. Clinical signs of pathogen-infected P. monodon shrimps

After injection with the different pathogens, the infected shrimps showed obvious signs of infections. At 24 h post-injection (hpi) of the virus, the shrimps displayed very little feeding and a faded or reddish body color was observed in the YHV or WSSV injected shrimps, respectively. By 48 hpi, some of the infected shrimps were dead whereas the remaining ones appeared weak to moribund. In addition, the shrimps clearly showed the typical gross signs of yellow head or white spot diseases [12,13]. Additionally, PCR or RT-PCR screens were positive for the presence of WSSV or YHV, respectively, in the injected shrimps as early as 6 hpi. For shrimps systemically infected (injected) with *V. harveyi*, some of the shrimps were found dead after 6–24 hpi, with a mortality rate of about 50%. However, no shrimp mortality was observed before 6 or after 24 hpi. Several of the shrimps that had died between 6 and 24 hpi had a clear luminescence appearance, as is typical of vibriosis.

### 3.2. Microarray analysis of differentially expressed genes in pathogen challenged shrimps

The numbers of differential expressed genes, that is those whose expression level changed more than two-fold in at least one of the three assayed time points after systemic infection (injection) with the virus (WSSV or YHV) or bacteria (*V. harveyi*), were 1954, 1136 and 420 genes, respectively. Thus, WSSV and YHV viral infections affect the expression of a 4.6- and 2.7-fold, respectively, higher number of shrimp genes than that induced by *V. harveyi* bacterial infection. This finding is in accordance with the study by Wang et al. [9] who reported that the number of WSSV responsive genes in the Chinese shrimp, *Fenneropenaeus chinensis*, was 1.7-fold higher than that found in *Vibrio anguilarum* infected shrimps. Comparison of the number of responsive genes between WSSV and YHV infected shrimps revealed a 1.7-fold higher number for WSSV infection. Interestingly, this correlates to the higher virulence of WSSV, which causes the highest mass mortality in shrimp aquaculture worldwide [1,3,14].

The numbers of genes that showed an altered expression in viral infections were highest at the late infection stages (48 hpi), whilst that for the *Vibrio* infection was highest at the early infection stage (6 hpi) for induced genes and at the mid infection stage (24 hpi) for repressed genes. The majority of the upregulated genes at the early stage of *Vibrio* infection might be important in the immune