Gene Expression Profiles in Giant Freshwater Prawn *Macrobrachium rosenbergii* Infected with *Macrobrachium rosenbergii* Nodavirus (*Mr*NV) by DD-PCR Technique

Anan Chungjit[1,2], Suwit Wuthisuthimethavee[3], Supawadee Poompuang[4] and Suriyan Tunkijjanukij[4*]

ABSTRACT

Differential Display PCR technique (DD-PCR) was used for analysis of altered gene expression in *Macrobrachium rosenbergii* infected with *Macrobrachium rosenbergii* Nodavirus (*Mr*NV). Twenty-four combinations of arbitrary and oligo-dT primers were used to screen for differentially expressed genes. A total of 115 differentially expressed bands could be identified from 18 primer combinations. These included 67 bands (77%) whose expression levels increased and 48 bands (33%) which decreased in expression after *Mr*NV challenge. Fifteen differential display fragments were successfully re-amplified, cloned and sequenced with 15 clones, 5 matched with sequences in the GenBank database. There were genes with known function, including Crustacyanin-like lipocalin, Ferritin 2 and dLp/HDL-BGBP. The other two genes were Ribosomal protein S2-like isoform 3 and Ribosomal protein L8. Crustacyanin-like lipocalin and ferritin 2 were selected for further confirmation for their differentially expression patterns by Quantitative Real-time PCR. The results indicated that these genes up regulated *M. rosenbergii* upon *Mr*NV challenge. Crustacyanin-like lipocalin and Ferritin 2 were involved in shrimp defense immunity.

Keywords: Differential display PCR, Differentially expressed genes, *Macrobrachium rosenbergii*, Real-time PCR, *Mr*NV

INTRODUCTION

The giant freshwater prawn, *Macrobrachium rosenbergii* (de Man), is an economically important prawn species in Thailand. In recent years, white tail disease (WTD) or white muscle disease was the main disease afflicting them. This disease has caused high mortalities and huge economic losses (Sahul Hameed *et al*, 2004). This was first reported in the Guadeloupe Island in French West Indies in 1997 with mass mortality in hatchery post-larvae (Arcier *et al*., 1999), then later in Taiwan (Tung *et al*.,

[1]Center for Agricultural Biotechnology, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom 73140, Thailand
[2]Center of Excellence on Agricultural Biotechnology: (AG-BIO/PERDO-CHE), Bangkok 10900, Thailand
[3]The Program of Aquatic Animal Production Technology, School of Agricultural Technology, Walailak University, Tarsala, NakornSrithammarat, 80160 Thailand
[4]Department of Aquaculture, Faculty of Fisheries, Kasetsart University, Bangkok 10900, Thailand
*Corresponding author, e-mail: ffissrt@ku.ac.th

1999), China (Qian *et al*., 2003), India (Sahul Hameed *et al*., 2004), Thailand (Yoganandhan *et al*., 2006) and Australia (Owens *et al*., 2009). The causative agent of WTD has been identified as *M. rosenbergii* nodavirus (*Mr*NV) (Arcier *et al*., 1999) However, Qian *et al*. (2003) subsequently reported that the occurrence of an additional extra small virus (XSV) may be a satellite virus that depends on *Mr*NV for its replication and causes WTD.

Prawns do not have an acquired immune system, but they have developed an innate immune system to protect themselves against pathogens. For immediate immune response to pathogens, several proteins and peptides are synthesized. However, in recent years, knowledge about prawn defense at the molecular level in *M. rosenbergii* has been limited to few studies such as toll receptor gene in *M. rosenbergii* (Srisuk *et al*., 2014), Cathepsin L gene response to viral and bacterial infections (Arockiaraj *et al*., 2013), and protein interaction of caspase gene (Youngcharoen *et al*., 2015). Therefore, to better understand the innate immune system, studies on the changes in gene expression profiles may help in identifying the genes that play an important role in the prawn defense mechanisms or antiviral system. Differential display PCR (DD-PCR) is a technique to identify and characterize differentially expressed genes. DD-PCR was adopted to compare the mRNAs from hepatopancreas of virus-sensitive and virus-resistant shrimps (*Penaeus monodon*) which found the first shrimp antiviral genes, *PmAV* (Luo *et al*., 2003). Somboonwiwat *et al*. (2006) adopted DD-PCR to determine changes in gene expression patterns in hemocytes of *P. monodon* upon *Vibiro harveyi* infection and found ALF*Pm*3 involved with bacterial response in shrimp.

In the present study, DD-PCR technique was used to compare the differences in gene expression patterns of *M. rosenbergii* with *Mr*NV infected. We have identified several up and down regulated genes and confirm differential expression profiling by real-time RT-PCR. These data from the study will enhance understanding in defense mechanisms against virus infection of shrimp and prawns.

MATERIALS AND METHODS

Preparation of viral inoculum

Post-larvae (PL) of *M. rosenbergii* infected with WTD were collected from hatcheries with recorded WTD outbreak, such as those located in Nakhon Prathom and Suphanburi provinces, Thailand and used as the source of viral inoculum for infectivity experiments. Frozen infected PL were thawed and homogenized in a sterile homogenizer and a 10 % (w/v) suspension was made with TN buffer (20 mMTris-HCL and 0.4 M NaCl, pH 7.4). The homogenate was centrifuged at 4,000 x g for 20 min at 4^{o}C and its supernatant was re-centrifuged at 10,000 x g for 20 min at 4^{o}C. The final supernatant was filtered through a 0.45 µm pore membrane. Then, the presence of *Mr*NV in tissue suspension was confirmed by RT-PCR (Saul Hameed *et al*., 2004). The filtrate was then stored at -80^{o}C for infectivity studies.

Collection and maintenance of experimental animals

For experimental transmission, healthy post-larvae (PL 1) of *M. rosenbergii* were collected from a hatchery in the vicinity of Ruamchok farm in Nakorn Pathom Province, Thailand with no record of WTD. They were randomly sampled and screened for WTD by RT-PCR assay. The post-larvae were maintained in 120-Lglass aquarium tank with continuous aeration at room temperature (28-32^{o}C) in freshwater. The animals were fed twice a day with commercial pellet feed (TNT#2 Aqua-progress Co., Ltd.).

Infectivity experiments

Six hundred prawns (N = 100 per tank) at age PL 12 were randomly divided and transferred into 6 glass tanks (1.2-L). Three replicate tanks were assigned as challenge group, with each tank immersed with 600 µl of the $1X10^{-3}$ dilution of the virus stock. Whereas 3 tanks of the control group tanks were immersed with 600 µl TN buffer. The samples were randomly collected from experimental animals (6 animals per time point) at 6, 12, 24, 48 and 72 hours post immersion. The prawns were tested whether the infection was successful by RT-PCR.

Total RNA preparation

Total RNA was isolated using TRIzol™ reagent (Invitrogen) according to the manufacturer's instruction. Briefly, 3 individual prawns were collected for differential display PCR experiments and pooled after isolation, while 3 individual prawns were stored at -80^{o}C for real-time PCR experiments. TRIzol reagent was homogenized with whole shrimp individually and incubated at room temperature for 5 min. Homogenates were mixed with chloroform and incubated at room temperature for 3 min. then centrifuged at 12,000 x g for 15 min at 4^{o}C. The clear upper lysates were transferred to a new tube and isopropanol was added to precipitate the RNA incubated at room temperature for 10 min. RNA pellets were collected by centrifuging at 12,000 x g for 10 min at 4^{o}C followed by washing with 75% ethanol in DEPC water, then briefly air dried for 15 min. The total RNA was dissolved in 30 µl of DEPC water.

The concentration of total RNA sample was quantified by measuring the absorbance at 260 nm (A260). The purity of total RNA was checked by measuring the ratio of optical density OD_{260} nm /OD_{280} nm. The quality of total RNA was analyzed by 1% agarose gels. Test infection was successful by RT-PCR with specific primer for *Mr*NV-F 5'GCG TTA TAG ATG GCA CAA GG 3' and *Mr*NV-R 5'AGC TGT GAA ACT TCC ACT GG 3' (Sahul Hameed *et al*., 2004). The protocol followed Sahul Hameed *et al*. (2004) with the size of DNA amplified product at 425 bp.

Differential display PCR

The first-stranded cDNAs were generated from 1 µg of total RNA and 1 µl of 20 µM oligo(dT) (T) primer (Table 1) according to RevertAid™ First-stranded cDNA synthesis kit (Fermentas) protocol, and added DEPC-treated water to a final volume of 12.5 µl. The reaction was incubated at 65^{o}C for 5 min and immediately placed on ice for 2 min. After that, 4µl of 5x reaction

Table 1. List of primers Oligo (dT) and Arbitrary used for DD-PCR

Primer		Primer sequence (5'-3')
Oligo (dT)1	T1	5'-CATTATGCTGAGTGATATCTTTTTTTTTAC-3'
Oligo (dT)2	T2	5'-CATTATGCTGAGTGATATCTTTTTTTTTAG-3'
Oligo (dT)3	T3	5'-CATTATGCTGAGTGATATCTTTTTTTTTCA-3'
Oligo (dT)4	T4	5'-CATTATGCTGAGTGATATCTTTTTTTTTAA-3'
Arbitrary1	P1	5'-ATTAACCCTCACTAAATCGGTCATAG-3'
Arbitrary2	P2	5'-ATTAACCCTCACTAAATGGAGCTGG-3'
Arbitrary3	P3	5'-ATTAACCCTCACTAAATGTGGCAGG-3'
Arbitrary4	P4	5'-ATTAACCCTCACTAAAGCACCGTCC-3'
Arbitrary5	P5	5'-ATTAACCCTCACTAAATGCTGGGGA-3'
Arbitrary6	P6	5'-ATTAACCCTCACTAAATGCTGGTGG-3'

buffer, 0.5 µl of RiboLock^{TM}RNase Inhibitor (40 units/µl), 2 µl of dNTP mix (10 mM each), and 1 µl of M-MuLV Reverse Transcriptase (200 units/µl) were added and gently mixed. The reaction was incubated at 42°C for 60 min. Then, the reaction was incubated at 70°C for 10 min to terminate reverse transcriptase activity. The cDNA was then diluted with sterile water to 1:1 dilution and used as template for differential display PCR (DD-PCR) reaction. Twenty-four combinations of oligo(dT) (T) primer and arbitrary (P) primer (Somboonwiwat *et al.*, 2006) were used for DD-PCR reactions (Table 1).

For each differential display experiment, 1 µl of each diluted cDNA sample, 1 µl of 10 µM P primer and 1 µl of 10 µM T primer were mixed with 17 µl of master mix containing 2 µl of 10x Dream Taq PCR reaction buffer, 13.4 µl of sterile H_2O, 0.2 µl of dNTP mix (5 mM each; final concentration of 50 µM), 1.2 µl of $MgCl_2$, and 0.2 µl Dream Taq Polymerase (Fermantas). The cycling parameters were one cycle at 94°C for 5 min, 10 cycles at 94°C for 30 sec, 35°C for 30 sec, 72°C for 1.30 min, 40 cycles at 94°C for 30 sec, 58°C for 30 sec, 72°C for 1.30 min and a final extension at 72°C for 7 min. The PCR products and the labeled molecular weight marker were electrophoresed through a 6 % denaturing polyacrylamide gel. DD-PCR patterns with visually different signal intensities between normal and infected shrimps were compared for each primer combination.

To isolate differential expressed cDNA fragments, regions of dried gels corresponding to the cDNAs were excised. Gel slices were rehydrated in 50 µl T.01 E (10 mM Tris-Cl pH 8, 0.1 mM EDTA pH 8) and incubated at 100°C for 10 min. The eluted cDNAs were re-amplified with the same pairs of primer and conditions used in DD-PCR reaction. Amplified cDNAs were purified using QIAGEN gel extraction kit (QIAGEN) and directly cloned into the pGEM-T Easy vector (Promega). Recombinant clones were screened by colony PCR using T7 primer and 3 clones for each cDNA were randomly selected for further nucleotide sequencing.

DNA sequencing and data analysis

DNA sequences were edited to remove the vector and ambiguous sequences. The sequences were compared with sequences in GenBank (National Center of Biotechnology and Information, NCBI) using BLASTN and BLASTX (Anderson and Brass, 1998; Altschul *et al*., 1997). Sequences were considered to be significantly matched when the possibility (E) value is $< 10^{-4}$ with a match of more than 100 nucleotides for BLASTN and a match of more than 10 amino acid residues for BLASTX.

Quantitative real-time RT-PCR

The first-strand cDNAs were generated from 1µg of total RNA sample and 1 µl of oligo$(dT)_{18}$ primers and RevertAid™ M-MuLVReverase Transcriptase Kit (Fermentas, USA) according to the manufacturer's protocol. The SYBR Green I real-time RT-PCR assay was carried out using the 7300 Real-Time PCR System (Applied Biosystems, Singapore). The amplifications were performed in a 96-well plate using a 20 µl reaction volume containing 2µl of cDNA from each reverse transcription reaction as template, 1 µl each of 10 µM gene-specific forward and reverse primers (Table 2), 10 µl of 2x SYBR Green supermix (Bio-Rad), and Nuclease-free water to adjust the reaction volume. The real-time PCR program was 95°C for 2 min followed by 40 cycles of 95°C for 30s, 55°C for 30s and 72°C for 30s. Melting curve analysis of amplification products was performed at the end of each PCR reaction. The specificity of PCR amplification of each primer pair was determined by constructing a melting curve immediately after the PCR amplification that only one of PCR product.

cDNA of each sample from the experimental prawns per time point was used for analysis. Each sample was run in two replicates and the amplification of elongation factor 1-alpha gene (EF-1α) was used as the internal control. For each *Mr*NV-infected sample, the threshold cycle or Ct value was normalized with the TN buffer control. The real-time standard curve of each gene was prepared using the pooled cDNA of normal animal as a template. Serial 10-fold dilutions of known qualities, ranging from 10^{0}-10^{-3} copies were made. Fluorescence signal was analyzed by the 7300 System SDS Software (Applied Biosystems, Singapore) using PCR base line subtracted curve fit method. The comparative Ct method was used to analyze the expression level of different genes in *Mr*NV infected and control of samples was calculated according to the $2^{-\Delta\Delta CT}$ method (Pfaffl, 2001).

Table 2. Specific primers use for real-time PCR

Gene name	Primer sequence (5'-3')	Product (bp)	Tm (°C)
Crustacyanin-like lipocalin	CATGCAGGCGTAGTTGTTGT	335	55
	TCATCAAAGGCAAATGTCCA		
Ferritin 2	ATGCTGGGGACGACTTACAG	461	55
	GCAGCGTTGAGCTTCATAAC		
EF-1α	GGTGCTGGACAAGCTGAAGGC	148	55
	CGTTCCGGTGATCATGTTCTTGAT		

Statistical analysis

The data are presented as means ± standard error (S.E.). Statistical significance was determined by one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests, the significance level was set at $P< 0.05$.

RESULTS

The differential display PCR technique was carried out using 24 combinations of primers. The DD-PCR patterns of normal and *Mr*NV infected *M. rosenbergii* were compared for each primer combination. A total of 15 differential expressed bands from 18 combinations of primers were identified. The differential expression bands could be divided into two groups, 67 bands showing increased expression and 48 bands showing reduced expression. The approximate sizes of differentially expressed cDNA bands ranged from 200 to 1,300 bp.

Twenty-five of these 115 differential expressed bands were chosen based on the level of differential expression and/or mostly of time point was expression when compared with two group experiments, fifteen out of 25 differential expressed bands were successfully extracted from the acrylamide gels, re-amplified and cloned. Among these, 10 bands were up regulated, while 5 bands were down regulated. These 15 differentially expressed cDNA fragments were subjected to BLASTN and BLASTX searches of the GenBank database. The result showed 5 bands were similar with Crustacyanin-like lipocalin, Ferritin 2 and dLp/HDL-BGBP, Ribosomal protein S2-like isoform 3 and Ribosomal protein L8 (Table 3).

Analysis of gene expression by real-time RT-PCR

To confirm the differential expression of genes identified from DD-PCR, Crustacyanin-like lipocalin and Ferritin 2 were further analyzed by real-time PCR.

Table 3. Expression patterns of cDNA fragments in response to *Mr*NV infected of *M. rosenbergii* by DD-PCR

DNA fragment	Gene name	Scientific name	Accession No.	E-value	% Identity	Up/ down*
DD1	Ribosomal protein S2-like isoform 3	*Callorhinchus milii*	AFM86774.1	3e-128	193/218 (89)	+
DD5	Crustacyanin-like lipocalin	*Macrobrachium rosenbergii*	ABC88388.1	2e-79	113/118 (96)	+
DD6	Ribosomal protein L8	*Litopenaeus vannamei*	ABC48600.1	2e-49	83/89 (93)	-
DD10	Ferritin 2	*Eriocheir sinensis*	ADF87491.1	6e-28	54/84 (64)	+
DD12	dLp/HDL-BGBP	*Pontastacus leptodactylus*	AHJ78589.1	8e-54	89/153 (58)	+

Changes in expression level of the selected genes upon *Mr*NV infection were observed by mRNA differential display technique and to determine their expression level at various time post-immersion. No significant differences were found in the expression of Crustacyanin-like lipocalin. However, the expression was up-regulated to those of control group from after 6 h up to 12 h post-immersion, and then the expression levels were increased to 2.30 and 3.86-fold at 24 and 72 h, respectively, compared to the control group (Fig. 1).

No significant differences were found in the expression of Ferritin 2. However, the level of expression was up-regulated from 12 h up to 72 h post-immersion by about 1.14-2.32-fold more than the control at each time point (Fig. 2).

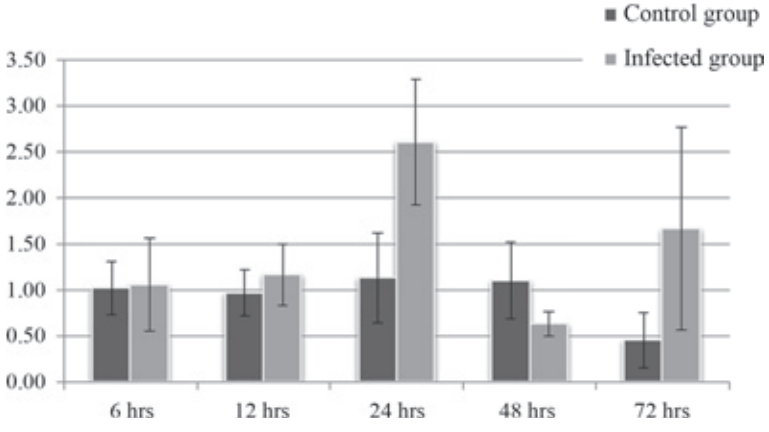

Figure 1. The relative expression ratio of Crustacyanin-like lipocalin at 6, 12, 24, 48 and 72 hours after *Mr*NV immersion determined by using real-time PCR.

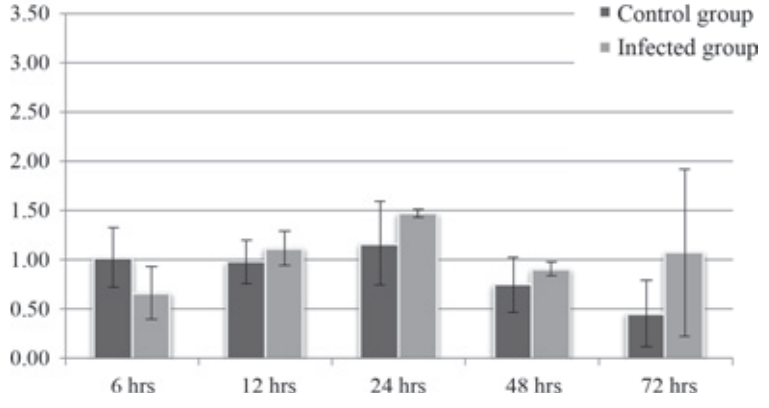

Figure 2. The relative expression ratio of Ferritin 2 at 6, 12, 24, 48 and 72 hours after *Mr*NV immersion determined by using real-time PCR.

DISCUSSION

The present study aimed to compare the patterns of expressed mRNAs present in normal and *Mr*NV-infected *M. rosenbergii* using differential display technique. The DD-PCR approaches reported several involved genes in the virus and bacteria-infected shrimp (Astrofsky *et al*., 2002, Luo *et al*., 2003, Somboonwiwat *et al*., 2006). The DD-PCR was carried out using 18 combinations of primers, with 15 clones of differentially expressed fragments identified and 5 genes significantly matched using the BLAST search program. Real-time PCR technique was used to confirm and study of relative expression of Crustacyanin-like lipocalin and Ferritin 2.

Crustacyanin-like lipocalin

Crustacyanin-like lipocalin was identified as abundance expression transcript at 24 and 72 h after *Mr*NV immersion and showed high identity (96%) with the prawn lipocalin in subepidermal adipose tissue of *M. rosenbergii*, that it has shown identity (56%) with crustacynin C1 of European lobster (Wang *et al.*, 2007). In crustaceans, two members of lipocalin family proteins have been identified, and one member of retinol/retinoic acid binding protein in *Metapenaeus ensis* (Gu *et al*., 2002) and crustacyanin in European lobster (*Homarus gammarus*) (Cianci *et al*., 2001). Lipocalin is involved in regulation of maturation, molting and coloration processes (Wang *et al*., 2007). For maturation process, Xie *et al.*, (2010) found crustacyanin-like lipocalin expression in ovary development of adult triploid shrimp (*Fenneropenaeus chinensis*). The expression of lipocalin was detected during the molting cycle in adult prawn (*Macrobrachium rosenbergii*), especially the lipocalin signals were strongly expressed and highest at early postmolt stage. However, no signal of lipocalin mRNA expression was detected during normal larval stages (Wang *et al*., 2007). But in adult *P. monodon* the crustacyanin had very little change in expression across the moult cycle (Wade *et al*., 2012). In European lobster, crustacyanin has been identified in the carapace and shown to bind astaxanthin to form the blue color of the outlier. Crustacyanin dominates by binding astaxanthin (carotenoids) to alter the color from red to blue color in European Lobster (Cianci *et al*., 2001). In this study, there were no significant differences in crustacyanin-like lipocalin expression after prawn was infected with *Mr*NV. In fact, it had no relationship with *Mr*NV virus infected shrimp in this study.

Ferritin

Ferritin is an iron storage protein found in almost all living organisms. It is necessary for maintenance of iron homeostasis. It converts ferrous (Fe^{2+}) to ferric (Fe^{3+}) through its ferroxidase activity and subsequently stores ferric as a mineral. Ferritin is a multimeric protein consisting of 24 subunit proteins which from a hollow shell and can bind about 4500 iron atoms within the central cavity (Theil, 1987, Harrison and Arosio, 1996). In higher vertebrates, ferritin is formed by a complex of 12 heavy and 12 light polypeptide chains encoded by separate genes (Orino *et al*., 1997). While in prokaryotes and plants it is composed of 24 equal subunits (Harrison and Arosio,

1996). In vertebrate organisms, ferritin pays important roles in detoxification and resistance against oxidative stress due to its ferroxidase activity and ferric binding features (Orino *et al*., 2001). Ferritin also functions in a variety of biological processes, including inflammation (Roeser *et al*., 1980), heart protection (Chevion *et al*., 2008), neuronal differentiation (Van Landingham and Levenson, 2003), and development regulation (Levenson and Fitch, 2000). In invertebrates, ferritin was found to be up-regulated after pathogens challenge in shrimps. For example, Zhang *et al*. (2006) employed an RT-PCR which showed that the expression of *FcFer* mRNA was up-regulated after shrimp (*Fenneropenaeus chinensis*) were challenged with either WSSV or heavy metal ions (Zn^{2+} and Cu^{2+}). Pan *et al*. (2005) found that WSSV-resistant shrimp (*P. japonicus*) showed up-regulated expression of ferritin when compared with normal shrimp. Feng *et al*. (2014) found that ferritin transcripts were quickly up-regulated in WSSV-challenged *Marsupenaeus japonicus*. Ferritin was still abundant in expression in postlarvae of *P. monodon* after challenged with *Vibrio harveyi* (Nayak *et al*., 2010). A recent study by Ye *et al*. (2015) showed results of ferritin which were significantly expressed in gills of WSSV challenged shrimp (*L. vannamei*). Other investigations have demonstrated that ferritin expression increases under pH, heavy metal and antibiotics challenge in shrimp (Zhang *et al*., 2006; Zhou *et al*., 2008; Fagutao *et al*., 2009).

However, no significant differences of ferritin expression after *Mr*NV infection were found and it was not clear how to describe the function of ferritin gene relative with the *Mr*NV infected shrimp.

CONCLUSION

In the present study, DD-PCR technique provided information on *M. rosenbergii* responsive genes after infecting wtih *Mr*NV. We could identify 15 cDNA fragments from this technique to sequence analysis and BLAST program, then the result showed 5 known genes and followed with real-time PCR for confirm relative expression of 2 genes. Ferritin 2 and Crustacyanin-like lipocalin were up-regulated after being challenged but no significant differences of the expression were found.

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