# Applications of shrimp immune DNA microarray in aquaculture

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## ABSTRACT

Infectious disease constitutes a major obstacle to the sustainability of shrimp aquaculture worldwide and a significant threat to natural populations of shrimp and other crustaceans. The study of the shrimp immune system, including the response to viral infection, has been hampered by a relative lack of molecular genetic information and of tools suitable for high throughput assessment of gene expression. The aim of this paper is to provide insights into the transcriptomic profile on two culture shrimps *Penaeus monodon* and *P. vannamei* responses to immune challenge. In this report, the generation of a tiger shrimp immune DNA microarray encompassing 30 immune-related genes and a cDNA microarray encompassing 6000 putative unigenes expressed in gills, circulating haemocytes, and hepatopancreas of *P. vannamei* is described. Penaeid shrimp immune microarray was applied in screening high disease-resistant broodstocks of tiger shrimp. The result indicated that higher mRNA level *hemocyanin* was found in disease-resistant shrimp broodstocks. The second application of shrimp immune DNA microarray was in nutrigenomic study. The result revealed that the expression of several immune genes (c*rustin*, *lysozyme, Mo-penaeidin*, *transglutaminase* and *Kazal-type proteinase inhibitor*) were increased significantly after injection of microbial immune elicitors (such as lipopolysaccharide, β-1-3 glucan and peptidoglycan) or other immune stimulants (such as polyI:C, Chinese medicine herbs). Shrimp immune DNA microarray analysis may provide useful clues to investigate the intracellular regulation and cross talk between many innate immune genes. Furthermore, shrimp immune DNA microarray might also provide a useful tool to identify disease resistant gene markers for marker-assisted selection program and to explore the Chinese herbs as immune stimulants for paneid shrimps.

**Key words:** cDNA microarray, innate immunity, penaeid shrimps, immunostimulants

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## INTRODUCTION

The shrimp aquaculture with its great potential to generate high export earnings has developed rapidly since 1970s as an industrial activity, but its development was regularly and seriously affected by the outbreak of viral and bacterial diseases during the last decade. From 1993 to 2000, white spot syndrome virus (WSSV), the most devastating viral pathogen of penaeid shrimp (*Penaeus* spp.), caused severe losses to the shrimp farming industry from Asia to America. Therefore, preventing and controlling the spread of disease has become a priority to the shrimp industry.

Host defense in invertebrates including shrimp is believed to rest entirely on innate, non-adaptive immunity that consists of cellular and humoral reactions. Cellular reactions involve phagocytosis, nodule formation and encapsulation, while humoral reactions involve the prophenoloxidase-activating cascade and immune-related proteins such as lysozymes, lectins, and antimicrobial peptides. Our knowledge about the innate immunity of shrimp comes mostly from the studies on crustaceans against bacteria and fungi. Two antivirus protein-pmAVP and hemocyanin from penaeid shrimp have been identified recently, however, the profile of immuno-related factors whereby shrimp defends against virus infection still remains unclear.

cDNA microarrays have emerged as a versatile technology that can be applied with relative ease to studies of differential gene expression in a wide variety of organisms. In present study we try to use of a cDNA microarray to study immunity against viral infection in host following various treatments, it can provide the information of host pathogen interactions, it also can determine which genes are expressed, and in what relative amounts during the infection process.

Nutrigenomics is a science of how the genes interact with nutrients. It is the study of how DNA and the genetic code affect the need for certain nutrients and help maintain optimal health throughout the life. Expression profiling using microarrays offers a powerful tool to gain a comprehensive view of biological systems by measuring the expression of thousands of genes simultaneously. The main advantage of such an approach is the ability to retain the power of microarray technology for monitoring a set of genes selected for their involvement in a specific pathway.

In this study, a cDNA microarray was used to identify genes upregulated in virus-resistant shrimp and to identify their expression profile. Some of these genes that might be involved in defense against virus were identified in the hemolymph of penaeid shrimp. The development of immunostimulants (as green feed additives) to reduce disease susceptibility, a traditional Chinese herb medicine complex formula was used to identify genes expression profile and up- and down-regulated immune-related genes.

## MATERIALS AND METHODS

### Preparation of WSSV-resistant shrimp

Forty shrimp, *Penaeus monodon* (Crustacea, Decapoda) (about 20–25 g), were obtained from a shrimp farm in PinTon county, Taiwan, and cultured in our laboratory in 500 l tanks (at 25 °C) filled with air-pumped circulating sea water. In order to obtain virus-resistant shrimp, WSSV challenge test was performed. A WSSV inoculum was prepared with virus-infected shrimp tail muscle that tested positive by PCR. Frozen infected tissue was homogenized in 1X PBS (1:5, wt/vol) and centrifuged at 5000 rpm for 10 min. The supernatant was filtered through a 0.45-μm pore-size filter and used for injecting the animals. The surviving shrimps were injected with100 μl of WSSV inoculums between the second and third tail tarsal plates on the lateral side of the shrimp with a 1-ml sterile syringe. Shrimps were challenged with WSSV three times for the period of one month. The 2–3 survivals were collected as WSSV-resistant shrimp. The unchallenged shrimps were used as control (virus-sensitive) shrimp.

Pacific white shrimp (*P. vannamei*) weighing 15-20 g were obtained from a local shrimp farm in I-Lan county, Taiwan. The shrimps were divided into each group of 30 animals. Each was assigned randomly to the three dietary treatments. Each shrimp was housed individually in an 18 l glass tank containing 12 l water. Constant aeration was provided in all tanks during the experiment. Temperature of culture water was approximately 25° C. The shrimps were fed the test diets twice daily throughout the 3 day feeding trial. Six shrimps from each treatment were sampled at 0 h (after feeding), 12 h, 24 h and 72 h (3 days) after the feeding trial began. The moulting stage of each sampled shrimp was determined. Only those in the intermolt or early premolt stages were evaluated in the study.

### Chinese herbs compound feeds preparation

Two kinds of Chinese herbs compound (the principal constituents were: *Astragalus membranaceus* Bge*, Lonicera japonica* Thunb*, Isatis indigotica* Fort and *Glycyrrhiza glabra* L.) were added as a solution into normal feeds; for each 1 kg feed 150 ml Chinese herbs solution were added.

### Construction of shrimp immune-related DNA microarrays

A *P. monodon* shrimp cDNA microarray that contained 30 unique EST clones was constructed from from cDNA library of *P. monodon* prepared from haemocytes (Table 1). Each EST was spotted in quarters.

The *P. vannamei* oligonucleotide microarray of 5885 elements was constructed. The genes spotted onto the microarray from the haemocytes cDNA library, including the accession number of ESTs, have been submitted to the NCBI GEO. The clones of the unique genes from ESTs were selected based on bioinformatics analysis of the haemocytes ESTs. The probes were design and spotted by Agilent Technologies. Each EST and gene were spotted in duplicate.

**Source of samples for microarray hybridization**

Two groups of samples were set for hybridization, each containing experimental shrimps and control, to prepare the haemocytes for challenge, Haemolymph was collected from the shrimp by inserting a 26-gauge needle into the ventral sinus cavity and withdrawn into a syringe rinsed by precooled anticoagulant solution (0.1 M sodium citrate, 0.25 M sucrose, 0.01 M Trise HCl, pH 7.6). The diluted haemolymph was centrifuged at 3 000 rpm and 4° C for 15 min to separate the haemocytes from the plasma. The resulting haemocytes pellet was washed with the anticoagulant solution and suspended in Trizol (Invitrogen, Carlsbad, CA) for total RNA isolation.

For *P. monodon* experiment groups to be injected with lipopolysaccharide, peptidoglycan and β-1,3-glucan, each shrimp was injected with 1μg immunostimulant per 1 g og shrimp weight. Haemolymph was collected after injection 48 h for microarray analysis. For *P. vannamei* experimental group to be with injected Poly I:C, each shrimp was injected with 1μg immunostimulant per 1 g of shrimp weight. Haemolymph was collected after injection 0 h, 12 h, 24 h, 48 h and 72 h for semiquantitative RT-PCR. The feeding experiments have two groups for Chinese herbs compound. Each 1 kg feed was added with 150 ml Chinese herbs solution. The shrimp were fed the test diets twice daily throughout the 3 day feeding trials. Uneaten food and excreta were removed each morning before feeding, and the haemolymph was collected after feeding 0 h, 12 h, 24 h, 48 h and 72 h for microarray and semiquantitative RT-PCR.

**Microarray analysis**

The cDNA transcribed from 20 μg of total RNA from 10 individual *P. monodon* was labeled with Cy3 or Cy5 using a SuperScript™ Plus Indirect cDNA Labeling System (Invitrogen). The arrays were hybridized with the labeled cDNA sample pairs (one with Cy3 and the other with Cy5) overnight at 42° C. After hybridization, the arrays were washed at 30° C in 5X SSC/0.1% (w/v) SDS for 10 min, then twice in 0.5X SSC for 2 min each, and finally briefly in 0.5X SSC/0.01% (v/v) Tween 20. The arrays were scanned using a GenePix 4000B, and the raw images were analyzed with GeneSpring Analysis Platform. After normalization, the values of spot replicates were averaged. A gene was considered differentially expressed by each experiment if its expression ratio increased or decreased by 2-fold or more compared to the control samples.

**Semi-quantitative RT-PCR**

Total RNA was extracted from a pool of hemocyte pellets using TRI reagent (Invitrogen). The first-strand cDNA was prepared from total RNA with primer. Briefly, the total RNA (5 μg) was mixed with 0.5 μg of oligo dT. RNase-free water was used to make a final volume of 10 μL. The mixture was heated at 65° C for 2 min and then cooled immediately on ice. Thereafter, the volume was adjusted to 20 μL using 10 reaction buffer containing 3 mM $MgCl_2$, 0.5 mM dNTPs, 3 U MMLV reverse transcriptase (EPICENTRE Biotechnologies, USA) and RNase-free water. The first-strand cDNA was then used as the template for PCR amplification with the appropriate primers designed by the Primer Premier 5 of immune-related genes. Semi-quantitative RT-PCR analysis was performed on total RNA isolated

from control and infected hemocyte at various time-points. RT-PCR of elongation factor-1 was performed and used for normalization of total RNA variation. RNA expression levels were quantitatively analyzed using Kodak Electrophoreisis Documentation and Analysis System 290 (Kodak EDAS 290).

**Data analysis**

A multiple comparison (Tukey) test was conducted to compare the significant difference for each gene expression among different time courses using a SAS computer software (SAS Institute Inc., Cary, NC, USA). A significant level of $p = 0.05$ was chosen.

## RESULTS

In order to find the immune-relevant factors responsible for the virus resistance in the WSSV-resistant shrimp, a cDNA microarray method was employed to identify differentially expressed genes and their expression profile in the hemolymph of the virus-resistant penaeid shrimp. Twenty five genes were identified from more than 8000 clones, of which are related to innate immunity in penaeid shrimp.

**Application of penaeid shrimp immune cDNA microarray in screening high disease-resistant broodstocks of tiger shrimp**

Hemocyanin is the most abundant gene in our cDNA microarray analysis except penaeidin, crustin, lectin, ferritin, oxygenase and chitinase of the virus-resistant black tiger shrimp; all showed up-regulation in expression compared with those of normal. The result indicated that higher mRNA level *hemocyanin* and *5HT1-receptor* were found in disease-resistant shrimp broodstocks (Fig.1).

Shrimps followed by feeding of WSSV-containing tissue at 10 % biomass daily for 3 consecutive days or injected intramuscularly with 100 µl WSSV inoculums ($10^{-6}$ dilution) resulted in a cumulative >80% death of the tiger shrimps at 4 days post infection (Fig. 2a, 2b). Control shrimp injected with dsRNA (shrimp hemocyanin siRNA) showed higher mortality from the control at 2 days following both viral infection methods. On the other hand, WSSV-resistant shrimp fed with 3% complex herbs formula showed increase in survival rate from the control start from 4 days post infection. No shrimp death was observed through the whole experimental period, indicating that shrimp hemocyanin siRNA and complex Chinese herbs formula did not give any cytotoxicity.

**Application of shrimp immune cDNA microarray in nutrigenomic study**

After the treatment with Chinese herbs complex formula, the hemolymph were collected to isolate total RNA and analyzed using the immune-related cDNA microarray which contained approximately 6 000 shrimp genes. Chinese herbs changed the expression of the genes related to immune response, cell growth and cell proliferation, signal transduction in *P. vannamei*. In this study, up-regulation of innate immunity genes was the most remarkable and it was suggested the mediation of *crustin*, *lysozyme*, *Mo-penaeidin*, *transglutaminase*

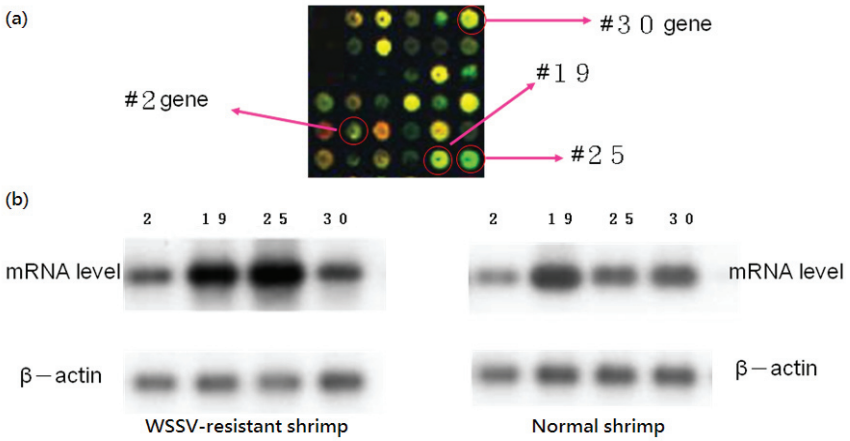


**Figure 1.** Selection of higher expression of endogenous innate immune genes as marker genes. (a) Pseudocolour microarray images (Cy3 and Cy5 are coloured green and red, respectively) of one of the on-array duplicate sets of probes. An equal mix of red and green results in a yellow pseudocolour; other ratios result in intermediate colours. Probes for WSSV- resistant shrimp and normal shrimp genes, as well as background and luciferase controls, are distributed randomly over the array. For probes representing shrimp genes (examples are boxed) that are red at #2 (*BGBP*), #19 (*5HT-1 receptor*), #25 ( *haemocyanin)*, #30 (*Transglutaminase*) and gene in WSSV-resistant shrimp. White probes (saturation) indicate a very high Cy3/Cy5 signal. (b) Expression profile of four genes in haemocyanin was analyzed by RT-PCR; β-actin cDNA fragment amplified from the same RNA samples was used as an internal loading control.

**Table 1**. Innate-immune related EST tags of tiger shrimp

| Gene Name | Gene Name |
|---|---|
| 1: Anti-lipopolysaccharide factor (ALF) | 16: Intracellular fatty acid binding protein |
| 2: Beta-1,3-glucan binding protein (BGBP) | 17: Kazal-type proteinase inhibitor |
| 3: 11.5 kDa antibacterial peptide | 18: Glutathione peroxidase |
| 4: Prophenoloxidase | 19: 5-HT1 receptor |
| 5: Prophenoloxidase activating factor | 20: Cytochrome b |
| 6: Peroxinectin | 21: Innexin 1 |
| 7: Serine proteinase | 22: α- tubulin |
| 8: α-2-macroglobulin | 23: Ribosomal protein L27a |
| 9: Cytosolic manganese superoxide dismutase | 24: RNA virus putative RNA-dependent RNA polymerase |
| 10: Lysozyme | 25: Hemocyanin |
| 11: Chelonianin | 26: Syntenin |
| 12: PMAV (virus resistance) | 27: Mo-penaeidin |
| 13: Astakine | 28: Clottable protein |
| 14: Heat shock protein 90 | 29: Heat shock protein 70 |
| 15: Heat shock protein 10 | 30: Transglutaminase |

**Table 2**. 2x expression up and down from β-glucan in *Penaeus monodon*

| Gene Name | Normalized Data | Gene Name | Normalized Data |
|---|---|---|---|
| 11.5 kDa antibacterial peptide (Crustin) | 37.61 | Cytosolic manganese superoxide dismutase | 0.399 |
| Kazal-type proteinase inhibitor | 10.27 | Astakine | 0.0594 |
| Prophenoloxidase activating factor | 9.111 | RNA virus putative RNA-dependent RNA polymerase | 0.0399 |
| ribosomal protein L27a | 7.016 | Serine proteinase | 0.0355 |
| Mo-penaeidin | 6.851 | Heat shock protein 10 | 0.0304 |
| beta-actin | 5.714 | syntenin | 0.0278 |
| Lysozyme | 5.163 | Peroxinectin | 0.0277 |
| alpha-2-macroglobulin | 4.982 | Beta-1,3-glucan binding protein (BGBP) | 0.0276 |
| cytochrome b | 3.9 | Intracellular fatty acid binding protein | 0.01 |
| Anti-lipopolysaccharide factor (ALF) | 3.735 | Clottable protein | 0.01 |
| Transglutaminase | 3.663 | PMAV (virus resistance) | 0.01 |
| beta-actin | 3.237 | hemocyanin | 0.01 |
| Chelonianin | 2.629 | | |
| 5-HT1 receptor | 2.034 | | |

**Table 3.** 2x expression up and down from peptidoglycan in *P. monodon*

| Gene Name | Normalized Data | Gene Name | Normalized Data |
|---|---|---|---|
| 11.5 kDa antibacterial peptide (Crustin) | 2.733 | 5-HT1 receptor | 0.151 |
| Heat shock protein 90 | 0.363 | Peroxinectin | 0.148 |
| alpha-tubulin | 0.36 | Astakine | 0.139 |
| Prophenoloxidase activating factor | 0.356 | syntenin | 0.125 |
| Glutathione peroxidase | 0.303 | Clottable protein | 0.105 |
| Prophenoloxidase | 0.268 | hemocyanin | 0.102 |
| Heat shock protein 10 | 0.229 | RNA virus putative RNA-dependent RNA polymerase | 0.0982 |
| Serine proteinase | 0.201 | Beta-1,3-glucan binding protein (BGBP) | 0.0685 |
| PMAV (virus resistance) | 0.178 | 5-HT1 receptor | 0.151 |
| innexin 1 | 0.175 | Peroxinectin | 0.148 |
| Intracellular fatty acid binding protein | 0.167 | | |

**Table 4**. 2x expression up and down from lipopolysaccharide in *P. monodon*

| Gene Name | Normalized Data | Gene Name | Normalized Data |
|---|---|---|---|
| 11.5 kDa antibacterial peptide (Crustin) | 5.314 | Cytosolic manganese superoxide dismutase | 0.255 |
| Lysozyme | 4.326 | Astakine | 0.219 |
| cytochrome b | 3.55 | Intracellular fatty acid binding protein | 0.189 |
| Peroxinectin | 0.406 | Heat shock protein 10 | 0.147 |
| innexin 1 | 0.357 | Beta-1,3-glucan binding protein (BGBP) | 0.145 |
| Glutathione peroxidase | 0.347 | syntenin | 0.108 |
| Prophenoloxidase | 0.326 | 5-HT1 receptor | 0.0995 |
| alpha-tubulin | 0.296 | PMAV (virus resistance) | 0.0772 |
| Serine proteinase | 0.293 | RNA virus putative RNA-dependent RNA polymerase | 0.0757 |
| Heat shock protein 90 | 0.268 | Clottable protein | 0.0692 |
| hemocyanin | 0.0427 | | |

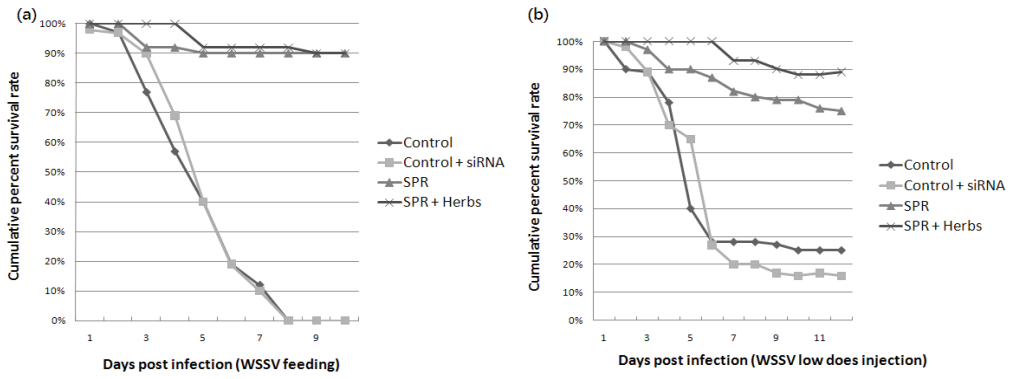


**Figure 2.** Viral challenge test of various treatments in SPR and control shrimp (*L. vannamei*). (a) Shrimp (5 to 10 g) were injected intramuscularly with either saline (control), 12µg of dsDNA for the shrimp haemocyanin gene, SPR shrimp and SPR shrimp with 3 % herbs added. A collective challenge system was used (n=30 to 35). (b) Control, SPR and SPR shrimp with 3% herbs added was as described above for (a). One microgram of the haemocyanin siRNA was injected per shrimp, followed by feeding of WSSV-containing tissue at 10% biomass daily for 3 consecutive days. Herbs addition = 3% combined Chinese herbs complex formula ( containing *Astragalus membranaceus Bge, Lonicera japonica Thunb, Isatis indigotica Fort* and *Glycyrrhiza glabra L.* )

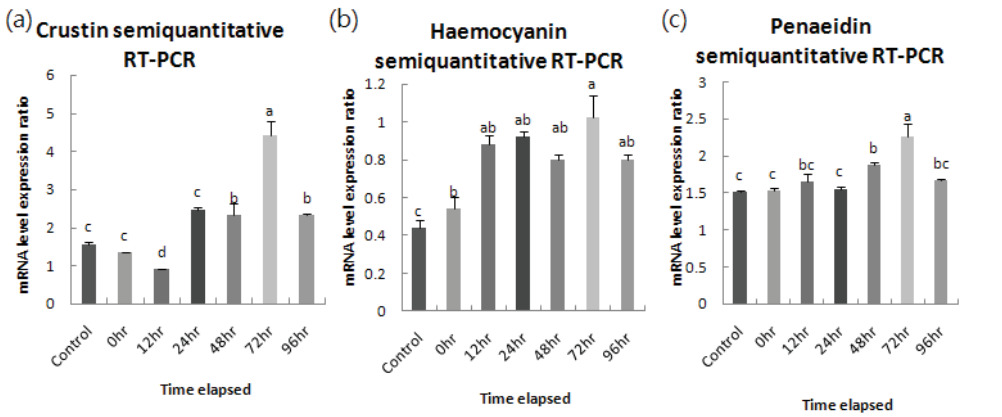


**Figure 3.** Semi-quantitative RT-PCR analysis of (a) *crustin*, (b) *haemocyanin* and (c) *penaeidin* mRNA gene expression in *L. vannamei*, β-actin served as a reference gene for the poly-I:C injection in different time course, 0hr, 12hr, 24hr, 48hr, 72hr, 96hr and normal haemocytes (control) as a positive control. Error bars represent standard error of mean (n=6, $p$=0.05).

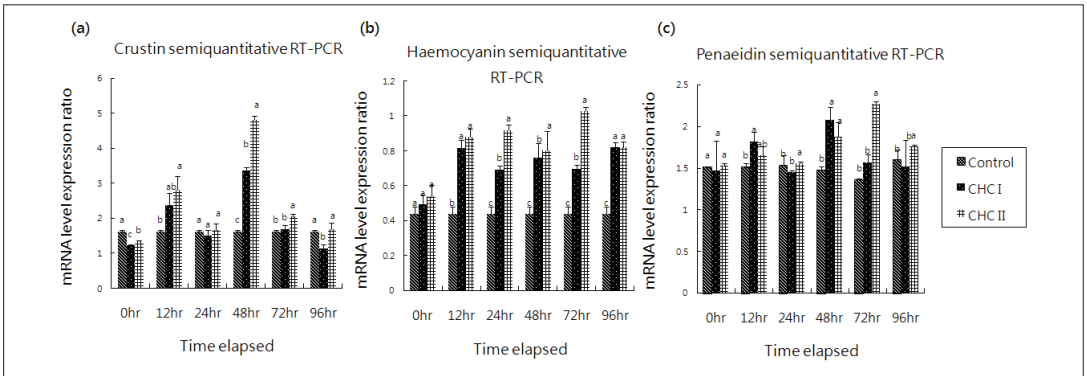


**Figure 4.** Expression profiles of immune marker genes following two kinds Chinese herbs compound (CHC) treatment in *L. vannamei.* Semi-quantitative RT-PCR analysis of (a) *crustin*, (b) *haemocyanin* and (c) *penaeidin*, β-actin served as a reference gene for feeding CHC I and CHC II after the different time course, 0hr, 12hr, 24hr, 48hr, 72hr, 96hr and normal haemocytes (control) as a positive control. Error bars represent standard error of mean (n=6, $p$<0.05)

and *Kazal-type proteinase inhibitor* responded significantly by microbial immune elicitors (such as lipopolysaccharide, β-1-3 glucan and peptidoglycan) (Tables 2, 3, 4) and other immune challenges (such as polyI:C , Chinese medicine herbs) treatment in black tiger shrimp and Pacific white shrimp (Fig. 3 and Fig. 4). On the other hands, Chinese herbs also up- and down-regulated the mRNA expression of some immune-related genes (Fig. 5 ). The data also gave new information about the regulation by Chinese herbs in penaeid shrimp.

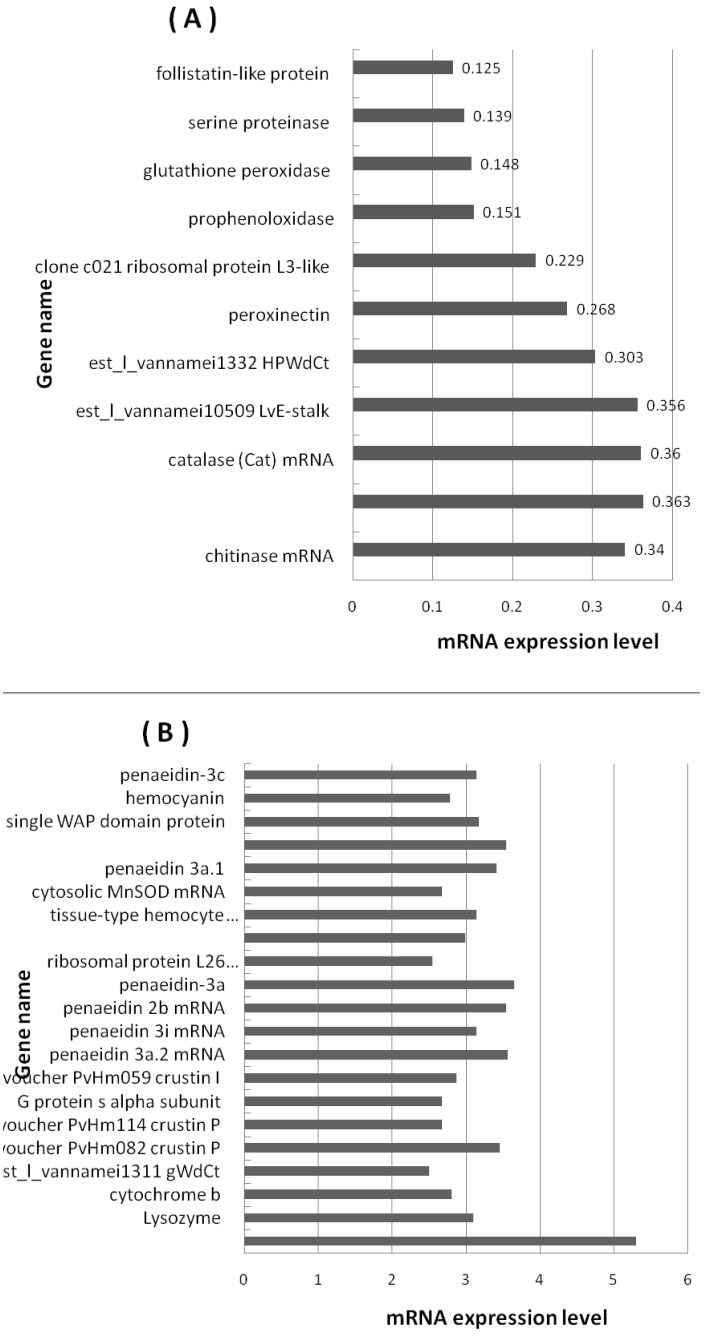


**Figure 5**. DNA microarray analysis after fed with Chinese herbs in *L. vannamei.* (A) profile for 2x down expresion after feeds with Chinese herbs; (B) profile for 2x down expresion after feeds with Chinese herbs

## DISCUSSIONS

DNA microarray analysis could provide useful clues to investigate the intracellular regulation and cross talk between many innate immune genes and regulators, and also to explore the novel function of Chinese herbs.

Control shrimp injected with dsRNA (shrimp hemocyanin siRNA) showed higher mortality from the control at 2 days following both viral infection methods demonstrating that the hemocyanin might serve a role in protection against WSSV (Fig. 2a). The WSSV-resistant shrimp fed with 3% complex herbs formula showed increase in survival rate from the control start from 4 days post infection demonstrating that WSSV-resistant shrimp and herb addition gave a better protection against WSSV infection (Fig. 2b).

The results of present study indicated that the hemolymph is a crucial system in the immune system of penaeid shrimp and cDNA microarray is an effective approach for discovering immune relevant genes. Moreover, several immune related genes (such as c*rustin*, *lysozyme, Mo-penaeidin*, *transglutaminase* and *Kazal-type proteinase inhibitor* ) showed significant up-regulation in the virus-resistant shrimp suggesting that these genes may play an important role in the virus defense response of penaeid shrimp. Transcription-based data mining of genes in QTL-limited intervals followed by efficient quantitative qRT-PCR or semi-quantitative RT-PCR methods is an effective strategy for identifying genes that may contribute to complex shrimp defense processes. After viral infection, a number of genes whose expression is altered, and thus are candidates for disease-resistant QTL and/or pathways associated with shrimp defense system. The results also confirmed that the data from cDNA microarray analysis could be correlated with the *in vivo* effect of the immune-enhancing compound (Rattanachai *et al.,* 2005; Wang, 2008).

In the present study, we have demonstrated that *hemocyanin, crustin*, *lysozyme* and *Mo-penaeidin* can serve as marker genes for screening disease-resistant shrimp broodstocks and to exploit novel immunostimulants as aquafeed additives. Here, we describe how nutrigenomics could provide new insights into animal nutrition research and innovative developments through neutraceutical products in penaeid shrimps.

## ACKNOWLEDGEMENTS

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