



Immune and bacterial toxin genes expression in different giant tiger prawn, *penaeus monodon* post-larvae stages following AHPND-causing strain of *vibrio parahaemolyticus* challenge

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ABSTRACT

Acute Hepatopancreatic Necrosis Disease (AHPND), a disease caused by *Vibrio parahaemolyticus* (V_{PAHPND}), kills Penaeid shrimps worldwide, resulting in severe economic losses during aquaculture. To further understand how *Penaeus monodon* post-larvae (PL) respond towards infection of this pathogenic bacterium, the expression of several important immune and bacterial toxin genes in three stages of *P. monodon* PL (PL15, PL30 and PL45) upon V_{PAHPND} challenge, were determined. A 20-hrs challenge test with 2.7×10^7 cfu ml⁻¹ of V_{PAHPND} resulted 81, 65 and 1.7% mortality respectively for PL30, PL15 and PL45, indicating that PL30 was most vulnerable to V_{PAHPND}. The immune response of shrimp PL at this stage was robust, with Toll-like receptor (TLR), prophe-noloxidase (proPO), lysozyme (lyso) and penaeidin (PEN) augmented approximately 10.7, 4.7, 6.5 and 3.2-fold, respectively. The expression initiated at one hour post-infection (h.p.i), peaked at 16 h.p.i and 20 h.p.i, and decreased at 18 h.p.i, indicating the crucial involvement of these immune related genes in the defence and recovery of the first-line defence mechanisms during V_{PAHPND} infection. This work also revealed that toxR gene represents a good indicator gene for *Vibrio* detection whereas PIR A, for V_{PAHPND} pathogenicity determination of *P. monodon*. Overall, these findings provided novel insights into the immune response and V_{PAHPND} susceptibility of different *P. monodon* PL stages during infection, with outcomes potentially useful in enhancing the application of health therapy and biosecured aquaculture practices to minimize the damaging risk of AHPND towards sustainable production of *P. monodon*.

1. Introduction

Penaeus monodon, commonly referred to as the Giant tiger prawn, is a valuable crustacean species used in aquaculture. The global production of the Penaeid shrimp reached 4200 metric tonnes, valued at USD 4.8 billion in 2016 (GAA, 2017). Disease represents one of the major impediments to the development of *P. monodon* culture (FAO, 2012) and in this context, the Acute Hepatopancreatic Necrosis Disease (AHPND), also known as the Early Mortality Syndrome (EMS), is an epidemic disease to both *Penaeus vannamei* and *P. monodon* (Dabu et al., 2015; Peña et al., 2015). Outbreak of AHPND was first reported in

China in 2009, and the disease then spread to Vietnam, Thailand, Philippines, Malaysia, and Mexico (Lightner, 2012; FAO, 2013; Tran et al., 2013; Joshi et al., 2014; Nunan et al., 2014; Peña et al., 2015; Chu et al., 2016). AHPND occurs within 20–30 days after stocking in grow-out ponds and often causes total mortalities in shrimp post-larvae (De Schryver et al., 2014). The clinical signs of AHPND include the formation of atrophied pale hepatopancreas coupled with abnormal swimming behavior and retarded growth.

The real cause of AHPND remained vague but it was reported that virulent *Vibrio parahaemolyticus* strains possessing plasmids encoded binary toxin PIR ABvp are the main causative agent of V_{PAHPND} (Lai

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et al., 2015; Tran et al., 2013). Vp_{AHPND} infect shrimp by oral transmission and colonization in shrimp gut, followed by production of PIR A- and PIR B-like toxins which destroy the hepatopancreas (Lee et al., 2015; Zorriehzahra and Banaederakhshan, 2015). To date, molecular analysis (PCR) of PIR AB gene is used to assess the presence of Vp_{AHPND} in Penaeid shrimp during culture (Han et al., 2015).

Like other invertebrates, shrimp depends on innate immunity, the first-line defence mechanism to battle against pathogenic infection. This defence system eliminates invading microorganisms through the humoral and cellular innate immune responses (Söderhäll and Cerenius, 1998). The defence against infectious pathogens begins with humoral responses which relies on a repertoire of germ line encoded receptors, known as Pattern Recognition Receptors (PRRs) to identify generic Pathogen Associated Molecular Patterns (PAMPs) present in the pathogens (Rowley and Powell, 2007). This microbial recognition step triggers signal transduction of both Toll-like receptors (TLRs) and Immune Deficiency (IMD) pathways to induce the synthesis of immune effectors such as potent Antimicrobial Peptides (AMPs) in the haemolymph (Sritunyalucksana and Söderhäll, 2000; Hultmark, 2003). TLRs are transmembrane glycoproteins and a type of PRR that detect microbial pathogens and binds to the microbial molecule to mediate a series of immune responses from extracellular to intracellular regions in shrimp (Mekata et al., 2008; Cerenius et al., 2008). Lysozyme (lyso) and Penaeidin (PEN) are common AMPs which destroy microbial cell wall via hydrolysis (Destoumieux et al., 2000; Tassanakajon et al., 2013). The cellular immune response, on the other hand, is initiated by a cascade of prophenoloxidase (proPO) activating systems leading to phagocytosis, encapsulation, coagulation, and melanization of intruding pathogens (Cerenius et al., 2008; Sritunyalucksana and Söderhäll, 2000).

Notably, the enzymes such as lyso in Penaeid shrimps have antimicrobial activities and prominent resistance against important bacterial pathogens for the Penaeid shrimp such as *Vibrio alginolyticus*, *V. cholerae* and *V. parahaemolyticus* (de La Vega et al., 2007). Therefore, the gene expression levels of these enzymes could best reflect the immune response of shrimp towards specific microbial pathogenicity, providing vital knowledge of innate immunity at molecular basis. The massive loss in total shrimp production caused by AHPND has prompted continuous global investigation of this disease. Our approach is to explore the immune response of *P. monodon* towards Vp_{AHPND} via innate immune molecular response. To our knowledge, information about the activation and modulation of innate immune response in post-larvae of different stages following Vp_{AHPND} infection is still lacking, though it has already been reported that, AHPND generally affects PL stages of between 20–30 days. In this study, we conducted Vp_{AHPND} challenge tests on *P. monodon* post-larvae (PL) 15, PL30 and PL45 using immersion method. Subsequently, we estimated the survival and immune genes expression level to determine which PL stages are most susceptible toward Vp_{AHPND} as well as identification on the status of bacterial genes expression during pathogen exposure.

2. Material and Methodology

2.1. Experimental and rearing condition of *Penaeus monodon* PL

Penaeus monodon PL stage 10 (PL10) purchased from local hatcheries were transferred to the hatchery of the Institute of Tropical Aquaculture and Fisheries Research (AKUATROP), Universiti Malaysia Terengganu, and grown in aerated 1.2 ton fibre tanks up to PL15, PL30 and PL45 prior to Vp_{AHPND} challenge. The shrimp post-larvae stage of PL10 to PL15 were provided with a combination diet of live feed (*Artemia* spp.) and artificial diet. Upon reaching PL16, they were fed with 100% of artificial diet till they reached PL45. This feeding regime was applied to mimic the real aquaculture setting. Excessive food and faeces were removed daily and water were changed once every three days to maintain optimal water quality. Water temperature was

maintained at 28 °C, salinity at 28 g/L, dissolved oxygen above 5 ppm, nitrite concentration below 0.05 ppm and total ammonia concentration below 0.1 ppm.

2.2. Bacteria preparation and culture

Vp_{AHPND} cells were inoculated on tryptic soy agar (TSA; Miller) with 1.5% sodium chloride (NaCl) and grown for 24 h at 35 °C. Bacterial colonies were transferred to tryptic soy broth (TSB) with 1.5% NaCl and subsequently grown for 24 h to stationary phase at 35 °C. Cells were harvested by centrifugation at 10,000 × g for 15 min, rinsed with phosphate buffer saline (PBS), and re-suspended into the rearing water of *P. monodon*. The density of bacteria has been determined following León et al (2016) using spectrophotometer (Shimadzu, Japan).

2.3. Immersion challenge test of Vp_{AHPND} towards different stages of *Penaeus monodon* PL

The PLs were divided into two groups; one group was incubated with 2.7×10^7 cfu ml⁻¹ Vp_{AHPND} and another unexposed to the bacteria (control), each treatment performed with triplicates. Six hundred shrimp PL15 and PL30, as well as 200 PL45 were separately counted into the tank. The challenge tests was performed according to Soto-Rodriguez et al., (2015) using immersion method to mimic the natural bacterial infection in *P. monodon*. Shrimp individuals of the infected groups were incubated in 10 L seawater (28 g/L) containing 2.7×10^7 cfu ml⁻¹ Vp_{AHPND} for 1.5 h. Following immersion, the shrimp individuals were transferred back to experimental tanks. The survival of *P. monodon* PLs were determined. For gene expression analysis, triplicates with a pool of five individuals in each replicate were collected from tanks containing PL15 and PL30, while triplicates with a pool of three individuals in each replicate were collected from tanks containing PL45 at each of the following time points: 0, 1, 2, 4, 6, 8, 12, 16, 18, 20 h post-infection (h.p.i). All experimental procedures of shrimp were approved by the Biosecurity and Ethics Committee of Universiti Malaysia Terengganu.

2.4. Analysis of immunity genes [Toll-like receptor (TLR), prophenoloxidase (proPO), lysozyme (lyso) and penaeidin (PEN)] expression by quantitative PCR (qPCR)

Total RNA were isolated from the whole body of *P. monodon* PL using TRIzol reagent (Life Technologies, USA) according to the manufacturer's protocol. The concentration and purity of the total RNA were quantified using A_{260/280} nm ratio using Biodrop (BioDrop, UK). Approximately 100 ng of total RNA were reverse-transcribed into cDNA using iScript cDNA Synthesis Kit (Bio-Rad, USA). Full-length sequence of the immunity genes were generated using SMARTer RACE 5'/3' Kit (Clontech Laboratories, USA) following manufacturer's protocol prior to purification using Wizard Plus SV Minipreps DNA Purification System (Promega, USA) and sequencing. The full-length sequences with GenBank accession numbers listed in Table 1 were used to design species-specific qPCR primers using Primer 3 (Untergasser et al., 2012). qPCR was performed on a Mx3005 P QPCR System (Agilent Technologies, USA). The reaction mixture consisted a total volume of 20 µl containing 2x of SensiFAST SYBR Lo-ROX mix (Bioline, USA), 400 nM forward and reverse primers (Table 1), 2 µl of cDNA template, and 6.4 µl of sterile ultrapure DNase/RNase-free water (Life Technologies, USA). qPCR was performed at 95 °C for 2 min, 40 cycles of 95 °C for 5 s, 60 °C for 11 s and 72 °C for 19 s, followed by continuous heating at 55 °C to 95 °C for melting curve analysis to verify the specific amplification of a single PCR amplicon in the qPCR reactions. To test the efficiency of the primers, a standard curve was generated using a 10-fold serial dilutions of cDNA for all targeted and housekeeping genes.

Table 1
: Summary details of primers used for qPCR analysis of giant tiger prawn, *Penaeus monodon* post-larvae.

Gene	Primer Sequence (5'-3')	Annealing temperature (°C)	Primer concentration (nM)	Reference
Toll Like Receptor (TLR)	F: CTTAGCCTTGGAGACAAC R: GATGCTTAACAGCTCCTC	53	400	In this study (MK356270)
Prophenoloxidase (proPO)	F: CTCCTAGTCTTCAAGGT R: CATTCTCGGAGATACC	54	280	In this study (MK356271)
Lysozyme (lyso)	F: TGGTGTGGCAGGATTATG R: GATCGAGGTCGGGATTCTTAC	55	400	In this study (MK356272)
Penaeidin (PEN)	F: TGGTGTGGCAGGATTATG R: AAGCAGGAGCTTGTAAAGG	55	400	In this study (MK356273)
Photobdus-like Insect A (PIR A)	F: TTGGACTGTCGAACCAAACG R: GCACCCATTGGTATTGAATG	60	250	Han et al. (2015)
ToxR	F: GAACCAGAAGCGCCAGTAGT R: GCATGGTGCTTAACGTAGCG	60	400	Wang et al. (2013)
*16S rRNA	F: ACAGAGTTGGATCTTGACGTTACCC R: AATCTGTGTTGCTCCCCACGCT	60	400	Daborn et al. (2001)
*β-actin	F: GCCCTTGCTCCTTCCACTATC R: CCGGACTCTTCGTACCATCCT	58	400	Qiu et al., (2008)

*housekeeping gene.

2.5. Absolute quantification of bacterial genes (PIR A, toxR and 16S rRNA) using standard curve methods

Pure cultures of V_{PAHPND} were grown in TSB containing 1.5% NaCl and incubated at 35 °C for 24 h. Total RNA were isolated using TRizol following manufacturer's protocol (Invitrogen, USA). The qualitative control were accessed using Biodrop (BioDrop, UK). Then, the total RNA were reverse transcribed into cDNA with an iScript cDNA Synthesis Kit (Bio-Rad, USA). Ten-fold serial dilution of cDNA samples were used to generate a standard curve Mx3005 P QPCR System (Agilent Technologies, USA). The reaction mixture and thermal profiles were performed as described in Section 2.4.

2.6. Data analysis

The survival data were subjected to One-Way Analysis Of Variance (ANOVA) using SPSS v.25 followed by *t*-test to examine significant differences among control and infected groups. The significant differences were considered at P -value < 0.05. The probability of post-larvae survival was calculated using log-rank test using R program (v 3.6.1) and was illustrated in Kaplan-Meier curve. Expression level of each targeted gene was normalized according to the expression of the β-actin gene (housekeeping gene) for each biological and technical sample. The transcript levels of each targeted gene for control and treatment groups were compared and converted to fold changes by the relative quantification method (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008). Relative fold changes for each gene between control and infected groups, and three PL stages were assessed using *t*-test in SPSS v.25 for statistical significance (P -value < 0.05). ANOVA analysis was carried out using SPSS v.25 (Liu et al., 2013) to observe the significance of expression level for each gene at various time points. Absolute quantification of bacterial genes were conducted based on generated absolute standard curve (Fukui and Sawabe, 2008). The statistical analysis was performed using ANOVA and *t*-test in SPSS v.25.

3. Results

3.1. Survival of *Penaeus monodon* PL in response to V_{PAHPND} infection

To compare the survival among three different PL stages (PL15, PL30 and PL45), each PL stages of *P. monodon* was immersed with 2.7×10^7 cfu ml⁻¹ of V_{PAHPND} and their survival probability were observed until 20 h.p.i (Fig. 1). We observed significant difference in the survival probability for both pairing groups of between the PL15 and the control group, and the PL30 and the control group (P < 0.05,

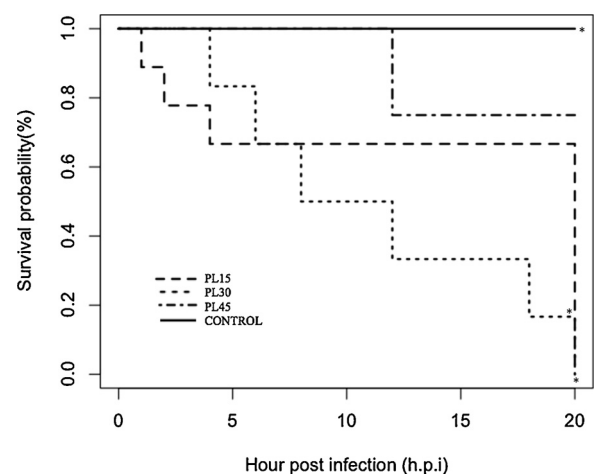


Fig. 1. Kaplan-Meier curves showing the probability of survival for three stages of giant tiger prawn, *Penaeus monodon* post-larvae (PL15, PL30 and PL45) after exposure to *Vibrio parahaemolyticus* (V_{PAHPND}). “*” indicates significant difference of survival probability between infected and control groups [P -value < 0.05]. Groups are compared using log-rank test.

log-rank test), but no significance difference was identified for PL45 and the control group (P > 0.05, log-rank test). The percentage of survival of the infected group at 20 h.p.i was highest at PL45 (98.3%) followed by PL15 (35%) and PL30 (19%), whereas all shrimps in control group survived (Supplementary Fig. 2).

3.2. Expression of TLR, proPO, lyso and PEN genes in different *Penaeus monodon* PL stages upon V_{PAHPND} infection

The expression of TLR, proPO, lyso and PEN genes in each PL stages of *P. monodon* following V_{PAHPND} infection were examined, revealing that all these immune genes were robustly up-regulated in PL30 (Fig. 2). A time-course study of the relative expression of immune genes until 20 h.p.i was determined to understand the potential innate immune responses of three PL stages upon V_{PAHPND} challenge (Fig. 3). Our results demonstrated that TLR and proPO genes expression for both PL30 and PL45 were highest at 16 h.p.i (Fig. 3). However, their expression pattern for PL15 was relatively different, in which the highest expression peaked at 6 h.p.i for TLR gene (23.84-fold) and 12 h.p.i for proPO gene (13.30-fold) (Fig. 3). The gene expression of both lyso and PEN shared similar expression trend where the highest expression of lyso and PEN occurred at 12 h.p.i for PL15 and PL45, and 16 h.p.i for PL30 (Fig. 3).

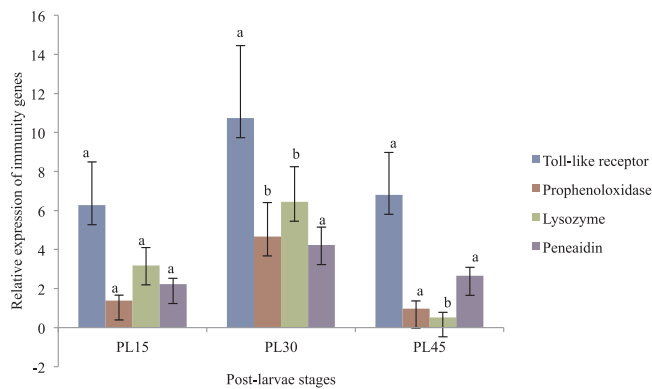


Fig. 2. The average of relative expression of immunity genes [Toll-like receptor (TLR), prophenoloxidase (proPO), lysozyme (lyso), and penaeidin (PEN)] in different stages of giant tiger prawn, *Penaeus monodon* post-larvae (PL15, PL30 and PL45) after exposure to *Vibrio parahaemolyticus* (Vp_{AHPND}). Error bars represent standard error of the means. “alphabet” indicates significant difference in the expression of immunity genes at different stages [P-value < 0.05, N = 5*3 replicate (PL15 and PL30); N = 3*3 replicate (PL45)].

3.3. Absolute quantification of bacterial gene expression (PIR A, toxR and 16S rRNA)

The absolute quantification of PIR A, toxR and 16S rRNA genes in the PL stages of *P. monodon* (PL15, PL30 and PL45) was assessed until 20 h.p.i (Fig. 4). The expression of PIR A gene was highest in PL30 as compared to PL15 and PL45. The highest expression level of PIR A gene at 2, 4 and 16 h.p.i, was 5.25 log cfu ml⁻¹ (PL15), 5.01 log cfu ml⁻¹ (PL30) and 9.21 log cfu ml⁻¹ (PL45) respectively (Fig. 4). On the other hand, toxR gene was detected at 0 h.p.i in all three PL stages of infected

groups. toxR and 16S rRNA gene transcripts was constantly expressed in all three PL stages. Notably, the difference in 16S rRNA gene expression between the three *P. monodon* PL stages was insignificant. In this study, 16S rRNA was used as housekeeping gene for bacteria gene expression assay.

4. Discussion

The survival of shrimp PL15, PL30 and PL45 between control and Vp_{AHPND} groups were significantly different (Fig. 1), with PL30 displaying the lowest survival (19%). This result strongly indicated that PL30 were most susceptible to Vp_{AHPND} infection when compared to other shrimp stages examined. Similar outcome was obtained during *V. harveyi* challenge, but the lowest survival reached 44.0% (Utiswannakul et al., 2011). Approximately 35% of PL15 survived but the differences are statistically insignificant with PL30 (P-value > 0.05). This was further supported with expression profile of on immunity genes (TLRs, proPO, lyso and PEN), in which their highest expression was observed in Vp_{AHPND} infected PL30 (Fig. 2). This might be caused by the feed transitions at different stages which may influence the innate immunity systems in shrimp. In this study, PL15 was fed with a mixture of live feed (*Artemia*) and artificial diet, whereas PL30 and PL45 were fed with 100% artificial feed. The presence of live feed may likely improve the immunity, growth and stress tolerance in PL15, which was also concordant with those of other host organisms (Standen et al., 2013; Liu et al., 2016). On the other hand, PL45 appeared to have the highest survival, partly due to their immune status, which improve as the shrimp grows (Jiravanichpaisal et al., 2007; Martin et al., 2004).

In this present study, TLRs in all *P. monodon* PL stages was up-regulated after exposure to Vp_{AHPND} at 1 h.p.i (Fig. 3 (a)), indicating that TLRs gene act as the first line defence for penaeid shrimp in recognizing the presence of Vp_{AHPND}. However, at 18 h.p.i, the expression

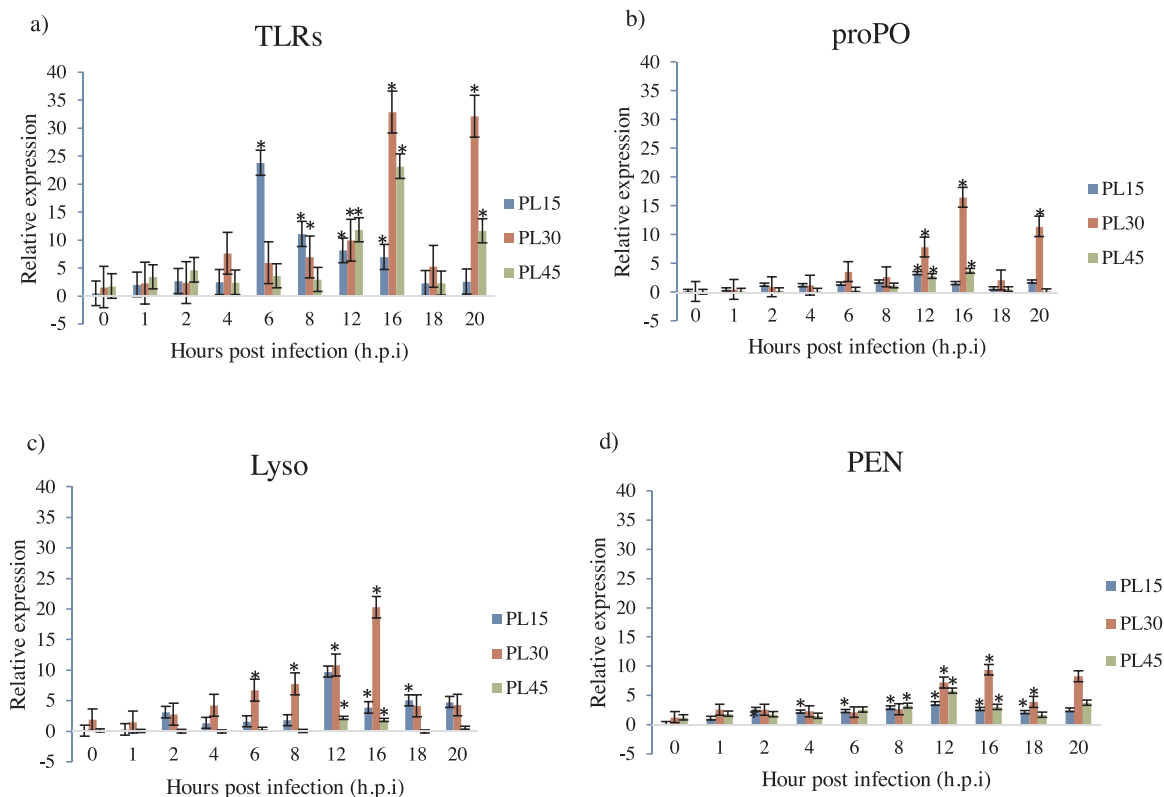


Fig. 3. Expression profiles of immunity genes at different time point : [a] Toll-like receptor (TLR), b) prophenoloxidase (proPO), c) lysozyme (lyso), and d) penaeidin (PEN)] in the post-larvae stages of giant tiger prawn, *Penaeus monodon* (PL15, PL30 and PL45) in response to *Vibrio parahaemolyticus* (Vp_{AHPND}) infection are presented as the relative expression ratios of the targeted genes when normalized by β-actin. Error bars represent standard error of the means. “*” indicates significant difference between different time points when compared to 0 h.p.i [P-value < 0.05, N = 5*3 replicate (PL15 and PL30); N = 3*3 replicate (PL45)].

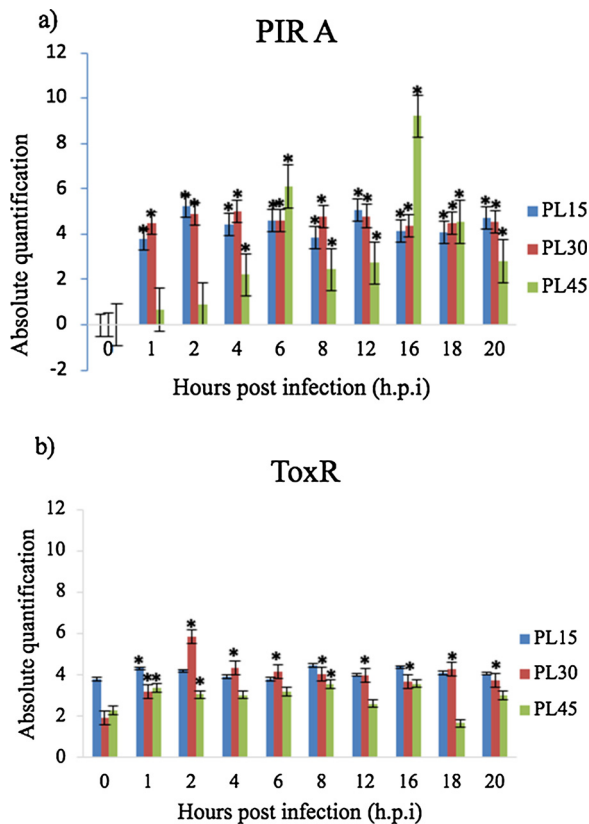


Fig. 4. Absolute quantification of bacterial genes at different time-point, bacterial toxic genes: a) PIR A and b) *toxR* in three stages (PL15, PL30 and PL45) of giant tiger prawn, *Penaeus monodon* post-larvae infected by *Vibrio parahaemolyticus* (V_{PAHPND}). [P-value < 0.05, N = 5*3 replicate (PL15 and PL30); N = 3*3 replicate (PL45)].

of TLRs gene was markedly reduced after reaching the plateau, which is congruent with those observed in the hepatopancreas of freshwater crayfish, *Procambarus clarkii* infected with *V. anguillarum* (Wang et al., 2015). With this, we hypothesized that TLRs likely possess two phases in combating V_{PAHPND} infection, namely the defence and recovering phases. Notably, as one of the main Pattern Recognition Receptor (PPRs), TLRs is able to recognize a wide range of pathogen-associated molecules patterns (PAMPs), before triggering the innate immunity of the host organisms (Akira, 2006; Fink et al., 2016). Lipopolysaccharides (LPS) component in pathogenic *Vibrio* spp. were recognized by TLRs gene which have initiated the signalling of TLRs pathway (TLR-MYD88-TNFR) (Krieg, 2002; Akira, 2006; Kumar et al., 2009; Rauta et al., 2014; Wang et al., 2015). Likewise, TLRs in *P. monodon* PL stages may have recognized the LPS component on the cell membrane of V_{PAHPND}, which further activates the proPO cascade and AMP.

When PRRs bind to PAMPs of specific microbes, this initiated the serine proteinase (SerPs) cascade that results in the activation of the proPO activating system (Cerenius and Söderhäll (2004); Liu et al., 2007). proPO hydrolysed into its active form, phenoloxidase (PO) which oxidize tyrosine into toxic quinone and other transitional molecules to form melanin (Fagutao et al., 2011). As a precursor of melanization in the host cell, melanin does not only kill the pathogenic bacteria prior to phagocytosis process (Cerenius and Söderhäll (2004); Dechamma et al., 2015), but could also cause damage to the host cell (Diamond et al., 2008). The expression of proPO gene peaked at the later stages of V_{PAHPND} infection (16 and 20 h.p.i), which have also been reported in freshwater crayfish, *Cherax quadricarinatus*, whiteleg shrimp, *P. vannamei* and mud crab, *Scylla paramamosain* following *Aeromonas hydrophilla* infection at 24 h.p.i, *V. harveyi* at 36 h.p.i and *V. parahaemolyticus* at 72 h.p.i respectively (Wang et al., 2010; Liu et al.,

2013; Zhang et al., 2019). Although the expression of proPO gene increased significantly again at 20 h.p.i, the sharp decline of its expression at 18 h.p.i was needed to control the production of excessive melanin reaction products (highly reactive and toxic quinone intermediates), so that the damaging effects of melanisation to the host (infected PLs) can be reduced while allowing the recovery process of the host cell to occur (Amparyup et al., 2013).

Lysozyme (lyso) is an important antimicrobial peptide (AMP) which is involved in the host defence system of invading microorganisms (Sotelo-Mundo et al., 2003; Xing et al., 2009; Liu et al., 2017). Lyso hydrolyse the β -1,4-glycosidic linkage of peptidoglycans of bacteria, and disrupts their cell wall (Laible and Germaine Greg, 1985; Ko et al., 2017). Congruent with the up-regulation of lyso genes following the infection of gram negative *Vibrio* spp. in several invertebrates (Burge et al., 2007; Wang et al., 2010; Yang et al., 2017), lyso gene was also significantly up-regulated in our V_{PAHPND} infected PL (Fig. 3 (c)). Lyso gene was drastically reduced at 18 h.p.i, supporting by the fact that AMPs gene expression is highly associated with the expression of proPO gene (Tassanakajon et al., 2018).

Penaeidin (PEN) is another AMP specifically found in penaeid shrimp (de Lorgeril et al., 2008). In the present study, we observed that PEN gene in all *P. monodon* PL stages was highly expressed at 16 h.p.i after V_{PAHPND} administration (Fig. 3 (d)), but its expression was drastically reduced at 18 h.p.i. This differential pattern of expression suggest that the transcription of PEN gene might be likely caused by two phases of defence mechanism, namely the bacterial killing and wound healing phases (Kawabata et al., 1996; Bacher et al., 2000; Munoz et al., 2002; Li et al., 2010; Song and Li, 2014). PEN has a C-terminal cysteine-rich domain (C-terminal CRD) which contains amphipathic structure that might act as pathogen binding domain involved in bactericidal processes (Li et al., 2010; Song and Li, 2014). The C-terminal CRD in the PLs of this present study might bind to the polysaccharides of V_{PAHPND}, which further initiated the bactericidal process within the host cell.

PIR A proteins were only detected in virulent bacteria strain, in which their virulence is dependent on the expression level of toxin genes which increases in parallel with the amount of cytotoxic produced (Waterfield et al., 2005; Tinwongger et al., 2016; Maralit et al., 2018). Given that PIR A gene was expressed highest in V_{PAHPND} infected (Fig. 4 (a)), which was also supported by significant inverse correlation of the PL's survival rate with the presence of PIR A genes, we proposed that PIR A gene in V_{PAHPND} might be capable to induce the lethal effects of AHPND in PL. On other aspect, ToxR gene was used to detect the presence of *Vibrio* spp. in a wide range of samples such as cultured water and host organisms, while its expression level measured in pure bacteria culture isolated from infected host (Pang et al., 2005; Zulkifli et al., 2009; Okuda et al., 2010). ToxR, a chromosomally encoded gene in *Vibrio* spp., triggers other virulent factors of the pathogenic bacteria (Li et al., 2000; Provenzano et al., 2001; Krukoniš et al., 2000). With the observed correlation between the expression of PIR A and *toxR* gene, we hypothesized that the expression of PIR A gene might be initiated by the existing *toxR* gene in infected PL. Considering this context, *toxR* is also a good candidate gene which could exclusively detect the presence of all *Vibrio* species.

Unexpectedly, V_{PAHPND} infected PL30 showed the highest expression of PIR A gene and lowest survival rate among all the PL stages. Our findings strongly indicated that toxic PIR A gene was highly involved in AHPND infection in *P. monodon*. Knowing that PL30 represents the most critical stage which is susceptible to AHPND infection, and the damaging role of PIR A gene in V_{PAHPND} in the host infection mechanism, a more biosecured aquaculture practices protruding to the rearing of transitional stages of *P. monodon* post-larvae and the development of new health therapy maybe necessary. Given that this study only used a small range of targeted host immunity and bacterial genes, it is still early to provide conclusive assumption about the complete defence mechanism of the *P. monodon* PL stages against V_{PAHPND}.

Extensive investigation of the immunity system of *P. monodon* PLs, infection mechanism of Vp_{AHPND} and strategies to control AHPND infections are warranted via multiparametric omics levels, from transcriptomic to metabolomic analyses to understand the response of dynamic biological systems towards pathophysiological stimuli.

Declaration of Competing Interest

There is no conflict interests in this study

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.aqrep.2019.100248>.

References

- Akira, S., 2006. TLR signaling. *Current Top Microbiology Immunology* 311, 1–16.
- Amparyup, P., Charoensapsri, W., Tassanakajon, A., 2013. Prophenoloxidase system and its role in shrimp immune responses against major pathogens. *Fish Shellfish Immunol.* 34, 990–1001.
- Bachere, E., Destoumieux, D., Bulet, P., 2000. Penaeidins, antimicrobial peptides of shrimp: a comparison with other effectors of innate immunity. *Aquaculture* 19, 71–88.
- Burge, E.J., Madigan, D.J., Burnett, L.E., Burnett, K.G., 2007. Lysozyme gene expression by hemocytes of pacific white shrimp, *Penaeus vannamei*, after injection with *Vibrio*. *Fish Shellfish Immunol.* 22, 327–339.
- Cerenius, L., Lee, B.L., Kenneth, Söderhäll, 2008. The proPO-system: Pro and Cons for its Role in Invertebrate Immunity. *Trends in Immunology*. Cell Press 29, 263–271.
- Cerenius, L., Söderhäll, K., 2004. The prophenoloxidase-activating system in invertebrates. *Immunol. Rev.* 198, 116–126.
- Chu, K.B., Ahmad, I., Siti Zahrah, A., Irene, J., Norazila, J., Nik Haiha, N., Teoh, T.P., 2016. In: Paking Jr.R.V., de Jesus-Ayson, E.G.T., Acosta, B.O. (Eds.), *Current Status of Acute Hepatopancreatic Necrosis Disease (AHPND) of Farmed Shrimp in Malaysia*.
- Dabu, I.M., Lim, J.J., Arabit, P.M.T., Joi Ann, S., Orense, B., Tabardillo Jr, J.A., Corre Mary Beth Jr., V.L., Maningas, B., 2015. The first record of acute hepatopancreatic necrosis disease in the Philippines. *Aquac. Res.* 1–8. <https://doi.org/10.1111/are.12923>.
- Daborn, P.J., Waterfield, N., Blight, M.A., French-Constant, R.H., 2001. Measuring virulence factor expression by the pathogenic bacterium *Photobacterium lumibaculum* in culture and during insect infection. *J. Bacteriol.* 5834–5839. <https://doi.org/10.1128/JB.183.20.5834-5839.2001>.
- de la Vega, E., Degnan, B.M., Hall, M.R., Wilson, K.J., 2007d. Differential expression of immune-related genes and transposable elements in black tiger shrimp (*Penaeus monodon*) exposed to a range of environmental stressors. *Fish Shellfish Immunol.* 23, 1072–1088.
- De Schryver, P., Defoirdt, T., Sorgeloos, P., 2014. Early Mortality Syndrome Outbreaks: A Microbial Management Issue in Shrimp Farming? *PLoS Pathology* 10 (4), e1003919. <https://doi.org/10.1371/journal.ppat.1003919>.
- Dechamma, M.M., Rajeisha, M., Maitib, B., Mania, M.K., Karunasagar, I., 2015. Expression of Toll-like-receptors (TLR) in Lymphoid Organ of Black Tiger Shrimp (*Penaeus monodon*) in Response to *Vibrio harveyi* Infection. *Aquac. Rep.* 1, 1–4.
- Destoumieux, G.D., Munoz, M., Cosseau, C., Rodriguez, J., Bulet, P., Comps, M., Bachere, E., 2000. Penaeidins, antimicrobial peptides with chitin-binding activity, are produced and stored in shrimp granulocytes and release after microbial challenge. *J. Cell. Sci.* 113, 461–469.
- Diamond, S., Powell, A., Shields, R.J., Rowley, A.F., 2008. Is spermatophore melanisation in captive shrimp (*Penaeus vannamei*) a result of an auto-immune response? *Aquaculture* 285, 14–18.
- Fagutao, F.F., Kondo, H., Aoki, T., Hirono, I., 2011. Prophenoloxidase has a role in innate immunity in penaeid shrimp. In: Bondad-Reantaso, M.G., Jones, J.B., Corsin, F., Aoki, T. (Eds.), *Diseases in Asian Aquaculture VII*. Fish Health Section. Asian Fisheries Society, Selangor, Malaysia, pp. 171–176 pp : 385.
- FAO, 2012. Cultured aquatic species information programme. *penaeus monodon*. Cultured Aquatic Species. Information Programme. Text by Kongkeo, H. FAO Fisheries and Aquaculture Department [online], Rome Updated 29 July 2005. [Cited 16 January 2015].
- FAO, 2013. FAO/MARD Technical Workshop on Early Mortality Syndrome (EMS) or Acute Hepatopancreatic Necrosis Syndrome (AHPNS) of Cultured Shrimp (under TCP/VIE/3304). FAO Fisheries and Aquaculture Report No. 1053. .
- Fink, I.R., Pietretti, D., Voogdt, C.G.P., Westphal, A.H., Savelkoul, H.F.J., Forlenza, M., Wiegertjes, G.F., 2016. Molecular and Functional Characterization of Toll-like receptor (TLr) 1 and TLr2 in Common carp (*Cyprinus carpio*). *Fish Shellfish Immunol.* 56, 70–83.
- Fukui, Y., Sawabe, T., 2008. Rapid detection of *Vibrio harveyi* in seawater by real-time PCR. *Microbes Environment*, vol. 23 (2), 172–176.
- GAA (Global Aquaculture Alliance), 2017. Shrimp Production Review. <https://www.aquaculturealliance.org>.
- Han, J.E., Tang, Kathy F.J., Pantoja, Carlos R., Brenda, WhiteL., Lightner, Donald V., 2015. qPCR assay for detecting and quantifying a virulence plasmid in acute hepatopancreatic necrosis disease (AHPND) due to pathogenic *Vibrio parahaemolyticus*. *Aquaculture* 442, 12–15.
- Hultmark, D., 2003. Drosophila immunity: paths and patterns. *Curr. Opin. Immunol.* 15, 12–19.
- Jiravanichpaisal, P., Puanglarp, N., Petkon, S., Donnuea, S., Söderhäll, I., Söderhäll, K., 2007. Expression of immune-related genes in larval stages of the giant tiger shrimp, *Penaeus monodon*. *Fish Shellfish Immunol.* 23, 815–824.
- Joshi, J., Jiraporn, S., Viet, H.T., Chen, I.-T., Bunlung, N., Suthienkul, O., Lo, C.F., Flegel, T.W., Sritunyaluksana, K., Thitamadee, S., 2014. Variation in *Vibrio parahaemolyticus* isolates from a single thai shrimp farm experiencing an outbreak of acute hepatopancreatic necrosis disease (AHPND). *Aquaculture* 428–429, 297–302.
- Kawabata, S.I., Nagayama, R., Hirata, M., Shigenaga, T., Agarwala, K.L., Saito, T., Cho, J., Nakajima, H., Takagi, T., Iwanaga, S., 1996. Tachycitin, a Small Granular Component in Horseshoe Crab Hemocytes is An Antimicrobial Protein with Chitin-binding Activity. *J. Biochem.* 120, 1253–1260.
- Ko, J.Y., Wan, Q., Bathige, S.D.N.K., Lee, J.H., 2017. Molecular characterization, transcriptional profiling, and antibacterial potential of G-type lysozyme from Seahorse (*Hippocampus abdominalis*). *Fish Shellfish Immunol.* 58, 622–630.
- Krieg, Arthur M., 2002. Cpg motifs in bacterial DNA and their immune effects. *Annu. Rev. Immunol.* 20, 709–760.
- Krukonis, E.S., Yu, R.R., DiRita, V.J., 2000. The *Vibrio cholerae* ToxR/TcpP/ToxT virulence cascade: distinct roles for two membrane-localized transcriptional activators on a single promoter. *Mol. Microbiol.* 38 (1), 67–84.
- Kumar, H., Kawai, T., Akira, S., 2009. Pathogen Recognition in The Innate Immune Response. *J. Biochem.* 420, 1–16.
- Lai, H.C., Ng, T.H., Ando, M., Lee, C.T., Chen, I.T., Chuang, J.C., Mavichak, R., Chang, S.H., Yeh, M.D., Chiang, Y.A., Takeyama, H., Hamaguchi, H., Lo, C.F., Aoki, T., Wang, H.C., 2015. Pathogenesis of acute hepatopancreatic necrosis disease (AHPND) in shrimp. *Fish Shellfish Immunol.* 47, 1006–1014.
- Laible, Nancy J., Germaine Greg, R., 1985. Bactericidal activity of human lysozyme, Muramidase-Inactive Lysozyme, and cationic polypeptides against *Streptococcus sanguis* and *Streptococcus faecalis*: inhibition by chitin oligosaccharides. *Infection and Immunity*, Vol. 48, No. 3, 720–728.
- Lee, C.T., Chen, I.T., Yang, Y.T., Ko, T.P., Huang, Y.Tzu., Huang, J.Y., Huang, M.F., Lin, S.J., Chen, C.Y., Lin, S.S., Lightner, D.V., Wang, H.C., Wang, A.H.J., Wang, H.C., Hor, L.I., Lo, C.F., 2015. The opportunistic marine pathogen *Vibrio parahaemolyticus* becomes virulent by acquiring a plasmid that expresses a deadly toxin. *PNAS* 112 (34), 10798–10803.
- León, P.L., González, A.L., Montes, R.E., Miranda, M., del, C.F., Coronado, J.A.F., Ruiz, P.A., Plata, G.D., 2016. Isolation and characterization of infectious *Vibrio parahaemolyticus*, the causative agent of AHPND, from the whiteleg shrimp (*Litopenaeus vannamei*). *Lat. Am. J. Aquat. Res.* 44 (3), 470–479.
- Li, C.Y., Yan, H.Y., Son, Y.L., 2010. Tiger shrimp (*Penaeus monodon*) penaeidin possesses cytokine features to promote integrin-mediated granulocyte and semi-granulocyte adhesion. *Fish Shellfish Immunol.* 28, 1–9.
- Li, C.C., Crawford, J.A., Di Rita, V.J., Kaper, J.B., 2000. Molecular cloning and transcriptional regulation of ompT, a ToxR-repressed gene in *Vibrio cholerae*. *Mol. Microbiol.* 35, 189–203.
- Lightner, D.V., 2012. Early mortality syndrome affects shrimp in Asia. *Global aquaculture Advocate*.
- Liu, H., Jiravanichpaisal, P., Cerenius, L., Lee, B.L., Söderhäll, I., Söderhäll, K., 2007. Phenoloxidase is an important component of the defense against *Aeromonas hydrophila* infection in a crustacean, *Pacifastacus leniusculus*. *J. Biol. Chem.*, vol. 282 (46), 33593–33598.
- Liu, H.T., Wang, J., Mao, Y., Liu, M., Niu, S.F., Qiao, Y., Su, Y.Q., Wang, C.Z., Zheng, Z.H., 2016. Identification and expression analysis of a new invertebrate lysozyme in Kuruma Shrimp (*Marsupenaeus japonicus*). *Fish Shellfish Immunol.* 49, 336–343.
- Liu, J.X., Zhou, N., Fu, R., Cao, D., Si, Y., Li, A., Zhao, H., Zhang, Q., Yu, H., 2017. The polymorphism of chicken-type lysozyme gene in Japanese flounder (*Paralichthys olivaceus*) and its association with resistance/susceptibility to *Listonella anguillarum*. *Fish Shellfish Immunol.* 66, 43–49.
- Liu, Y.Y., Chang, C.I., Hseu, J.R., Liu, K.F., Tsai, J.M., 2013. Immune responses of prophenoloxidase and cytosolic manganese superoxide dismutase in the freshwater crayfish *Cherax quadricarinatus* against a virus and bacterium. *Mol. Immunol.* 56, 72–80.
- Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2 (Delta Delta C (T)) method. *Methods* 25, 402–408.
- Maralit, B.A., Jareea, P., Boonchuena, P., Tassanakajona, A., Sombonniwita, K., 2018. Differentially Expressed Genes in Hemocytes of *Penaeus vannamei* Challenged

- with *Vibrio parahaemolyticus* AHPND (VPAHPND) and VPAHPND Toxin. *Fish Shellfish Immunol.* 81, 284–296.
- Martin, G.G., Rubin, N., Swanson, E., 2004. *Vibrio parahaemolyticus* and *V. Harveyi* cause detachment of the epithelium from the midgut trunk of the penaeid shrimp *Sicyonia ingentis*. *Disease of Aquatic Organisms* Vol.60 21–29.
- Mekata, T., Kono, T., Yoshida, T., Sakai, M., Itami, T., 2008. Identification of cDNA encoding toll receptor, MjToll gene from Kuruma Shrimp, *Marsupenaeus japonicus*. *Fish Shellfish Immunol.* 24, 122–133.
- Munoz, M., Vandenbulcke, F., Saulnier, D., Bachere, E., 2002. Expression and distribution of penaeidin antimicrobial peptides are regulated by haemocyte reactions in microbial challenged shrimp. *Eur. J. Biochem.* 269, 2678–2689.
- Nunan, L., Lightner, D., Pantoja, C., Gomez-Jimenez, S., 2014. Detection of acute hepatopancreatic necrosis disease (AHPND) in Mexico. *Disease of Aquatic Organisms* 111, 81–86.
- Okuda, J., Nakai, T., Park, S.C., Oh, T., Takeshi, N., Koitabashi, T., Nishibuchi, M., 2010. The toxR gene of *Vibrio (Listonella) anguillarum* controls expression of the major outer membrane proteins but not virulence in a natural host model. *Infection and Immunity*, Vol.69, No 10, 6091–6101.
- Pang, L., Zhang, X.H., Zhong, Y., Chen, J., Li, Y., Austin, B., 2005. Identification of *Vibrio harveyi* using PCR Amplification of the toxR Gene. *Letter in Applied Microbiology*, ISSN 0266-8254.
- Peña, D., de la, L., Cabillon, N.A.R., Catedral, D.D., Amar, E.C., Roselyn, C., Usero Monotilla, W.D., Adelaida, T., Calpe, F., Dalisay, D.G., Saloma, P.C., 2015. Acute hepatopancreatic necrosis disease (AHPND) outbreaks in *Penaeus vannamei* and *P. Monodon* cultured in the Philippines. *Diseases of Aquatic organisms*, Vol. 116, 251–254.
- Provenzano, D., Lauriano, C.M., Klose, K.E., 2001. Characterization of the role of the ToxR modulated outer membrane porins OmpU and OmpT in *Vibrio cholerae* virulence. *J. Bacteriol.* 183, 3652–3662.
- Rauta, P.R., Samanta, M., Dasha, H.R., Nayaka, B., Das, S., 2014. Toll-like receptors (TLRs) in aquatic animals: signalling pathways, expressions and immune responses. *Immunol. Lett.* 158, 14–24.
- Rowley, A.F., Powell, A., 2007. Invertebrate immune system-specific, quasi specific, or nonspecific? *J. Immunol.* 179, 7209–7214.
- Schmittgen, T.D., Livak, K.J., 2008. Analyzing real-time PCR data by the comparative C (T) method. *Nature Protocol* 3 (6), 1101–1108.
- Söderhäll, K., Cerenius, L., 1998. Role of the prophenoloxidase-activating system in invertebrate immunity. *Curr. Opin. Immunol.* 10, 23–28.
- Song, Y.L., Li, C.Y., 2014. Shrimp immune system-special focus on penaeidin. *J. Mar. Sci. Technol.* 22, 1–8.
- Sotelo-Mundo, R.R., Islas-Osuna, Maria A., de-la-Re-Vegaa, Enrique, Jorge, Herna'ndez Lo'pez, Vargas-Albores, Francisco, Yepiz-Plascencia, Gloria, 2003. cDNA cloning of the lysozyme of the white shrimp *Penaeus vannamei*. *Fish Shellfish Immunol.* 15, 325–331.
- Sritunyaluksana, K., Söderhäll, K., 2000. The proPO and clotting system in crustaceans. *Aquaculture* 19, 53–69.
- Standen, B.T., Rawling, M.D., Davies, S.J., Castex, M., Foey, A., Gioacchini, G., Carnevali, O., Merrifield, D.L., 2013. Probiotic *Pediococcus acidilactici* modulates both localised intestinal and peripheral-immunity in *Tilapia (Oreochromis niloticus)*. *Fish Shellfish Immunol.* 35, 1097–1104.
- Tassanakajon, A., Somboonwiwat, K., Supungul, P., Tang, S., 2013. Discovery of immune molecules and their crucial functions in shrimp immunity. *Fish Shellfish Immunol.* 34, 954–967.
- Tassanakajon, A., Rimphanitchayakit, V., Visetnan, S., Amparyup, P., Somboonwiwat, K., Charoensapsri, W., Tang, S., 2018. Shrimp humoral responses against pathogens: antimicrobial peptides and melanization. *Dev. Comp. Immunol.* 8, 81–93.
- Tinwongger, S., Nochiria, Y., Thawonsuwan, J., Nozaki, R., Kondo, H., Awasthi, S.P., Hinenoya, A., Yamasaki, S., Hirono, I., 2016. Virulence of Acute Hepatopancreatic Necrosis Disease PirAB-like Relies on Secreted Proteins Not on Gene Copy Number. <https://doi.org/10.1111/jam.13256>.
- Tran, L., Linda, N., Redman, Rita M., Mohny, Leone L., Pantoja, Carlos R., Fitzsimmons, K., Lightner, D.V., 2013. Determination of the infectious nature of the agent of acute hepatopancreatic necrosis syndrome affecting penaeid shrimp. *Disease of Aquatic Organisms*, Vol. 105, 45–55.
- Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B.C., Remm, M., Rozen, S.G., 2012. Primer3- new capabilities and interfaces. *Nucleic Acids Res.* 40 (15), 115.
- Utiswannakul, P., Sangchai, S., Rengpipat, S., 2011. Enhanced growth of black tiger shrimp *Penaeus monodon* by dietary supplementation with *Bacillus* (BP11) as a probiotics. *Journal Aquaculture Research Development*, S1:006. <https://doi.org/10.4172/2155-9546.S1-006>.
- Wang, D., Fang, Z., Xie, C., Liu, Y., 2013. Construction of method for rapid detection of *Vibrio parahaemolyticus* using the quantitative real-time PCR based on the ToxR gene. *Adv. J. Food Sci. Technol.* 5 (8), 1022–1030.
- Wang, K.C., Han-Ching, C.-W., Tseng, H.-Y., Lin, I.-T., Chen, Y.-H., Chen, Y.-M., Chen, T.Y., Yang, H.L., 2010. RNAi knock-down of the *Penaeus vannamei* toll gene (LvToll) significantly increases mortality and reduces bacterial clearance after challenge with *Vibrio harveyi*. *Dev. Comp. Immunol.* 34, 49–58.
- Wang, Z., Chen, Y.-H., Dai, Y.-J., Tan, J.M., Huang, Y., Ren, J.F.L.Q., 2015. A novel vertebrates toll-like receptor counterpart regulating the antimicrobial peptides expression in the freshwater crayfish, *Procambarus clarkia*. *Fish Shellfish Immunol.* 43, 219–229.
- Waterfield, N., Kamita, S.G., Hammock, B.D., Constant, R.F., 2005. The Potorhabdus pir toxins are similar to a developmentally regulated insect protein but show No juvenile hormone esterase activity. *FEMS Microbiol. Lett.* 245, 47–52.
- Xing, Y., Gao, F.Y., Mei, Z.Q., Jie, B.J., Wang, H., Lao, H.-H., Jian, Q., 2009. Cloning and characterization of the tiger shrimp lysozyme. *Molecule Biology Rep* 36, 1239–1246. <https://doi.org/10.1007/s11033-008-9303-7>.
- Yang, D.L., Wang, Q., Cao, R., Chen, L., Liu, Y.L., Cong, M., Wu, H., Li, F., Ji, C., Zhao, J., 2017. Molecular characterization, expression and antimicrobial activities of two c-type lysozymes from Manila clam *Venerupis philippinarum*. *Dev. Comp. Immunol.* 73, 109–118.
- Zhang, X.S., Tang, X.X., Tran, N.T., Huang, Y., Gong, Y., Zhang, Y.L., Zheng, H.P., Ma, H.G., Li, S.K., 2019. Innate immune responses and metabolic alterations of mud crab (*Scylla paramamosain*) in response to *Vibrio parahaemolyticus* infection. *Fish Shellfish Immunol.* 87, 166–177.
- Zorriehzahra, M.J., Banaederakhshan, R., 2015. Early mortality syndrome (EMS) as new emerging threat in shrimp industry. *Advance Animal Veterinal Science* 3 (2s), 64–72.
- Zulkifli, Y., Alitheen, N.B., Son, R., Yeap, S.K., Lesley, M.B., Raha, A.R., 2009. Identification of *Vibrio parahaemolyticus* isolates by PCR targeted to the toxR gene and detection of virulence genes. *Int. Food Res. J.* 16, 289–296.