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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* (Vp_{AHPND}), 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 Vp_{AHPND} challenge, were determined. A 20-hrs challenge test with 2.7×10^7 cfu ml^{-1} of Vp_{AHPND} resulted 81, 65 and 1.7% mortality respectively for PL30, PL15 and PL45, indicating that PL30 was most vulnerable to Vp_{AHPND}. The immune response of shrimp PL at this stage was robust, with Toll-like receptor (TLR), prophenoloxidase (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 Vp_{AHPND} infection. This work also revealed that toxR gene represents a good indicator gene for *Vibrio* detection whereas PIR A, for Vp_{AHPND} pathogenicity determination of *P. monodon*. Overall, these findings provided novel insights into the immune response and Vp_{AHPND} 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 Vp_{AHPND} (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 transmembraneous 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 x *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: CTCCCTAGTCTTCAAGGT R: CATTTCCTGCGAGATACC	54	280	In this study (MK356271)
Lysozyme (lyso)	F: TGGTGTGGCAGCGATTATG R: GATCGAGGTCGCGATTCTTAC	55	400	In this study (MK356272)
Penaeidin (PEN)	F: TGGTCTGCCTGGTCTTCCT R: AAGCACGAGCTTGTAAGGG	55	400	In this study (MK356273)
Photobdus-like Insect A (PIR A)	F: TTGGACTGTCGAACCAAACG R: GCACCCCATTGGTATTGAATG	60	250	Han et al. (2015)
ToxR	F: GAACCAGAAGCGCCAGTAGT R: GCATGGTGCTTAACGTAGCG	60	400	Wang et al. (2013)
*16S rRNA	F: ACAGAGTTGGATCTTGACGTTACCC R: AATCTTGTTTGCTCCCCACGCT	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 Vp_{AHPND} 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 Vp_{AHPND} 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 Vp_{AHPND} 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, 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).

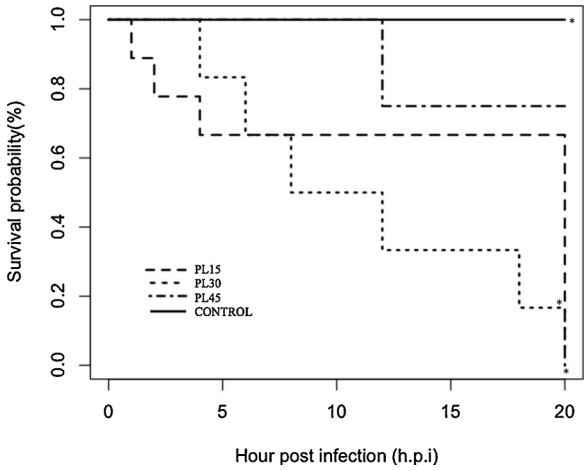

Fig. 1. Kaplan-Meier curves showing the probability of survival for three stages of giant tiger prawn, *Penaeus monodon* post-larvae (PL15, PL30 and PL45) after exposure to *Vibrio parahaemolyticus* (Vp_{APHND}). "*" indicates significant difference of survival probability between infected and control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

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

The expression of TLR, proPO, lyso and PEN genes in each PL stages of *P. monodon* following Vp_{AHPND} 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 Vp_{AHPND} 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).

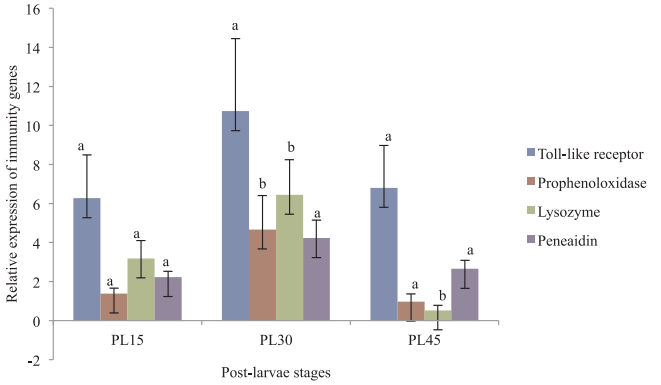

Fig. 2. The average of relative expression of immunity genes [Toll-like receptor (TLR), prophenoloxidase (proPO), lysozyme (lyso), and penaeidin (PEN)] in different stages of giant tiger prawn, *Penaeus monodon* post-larvae (PL15, PL30 and PL45) after exposure to *Vibrio parahaemolyticus* (Vp_{APHND}). 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

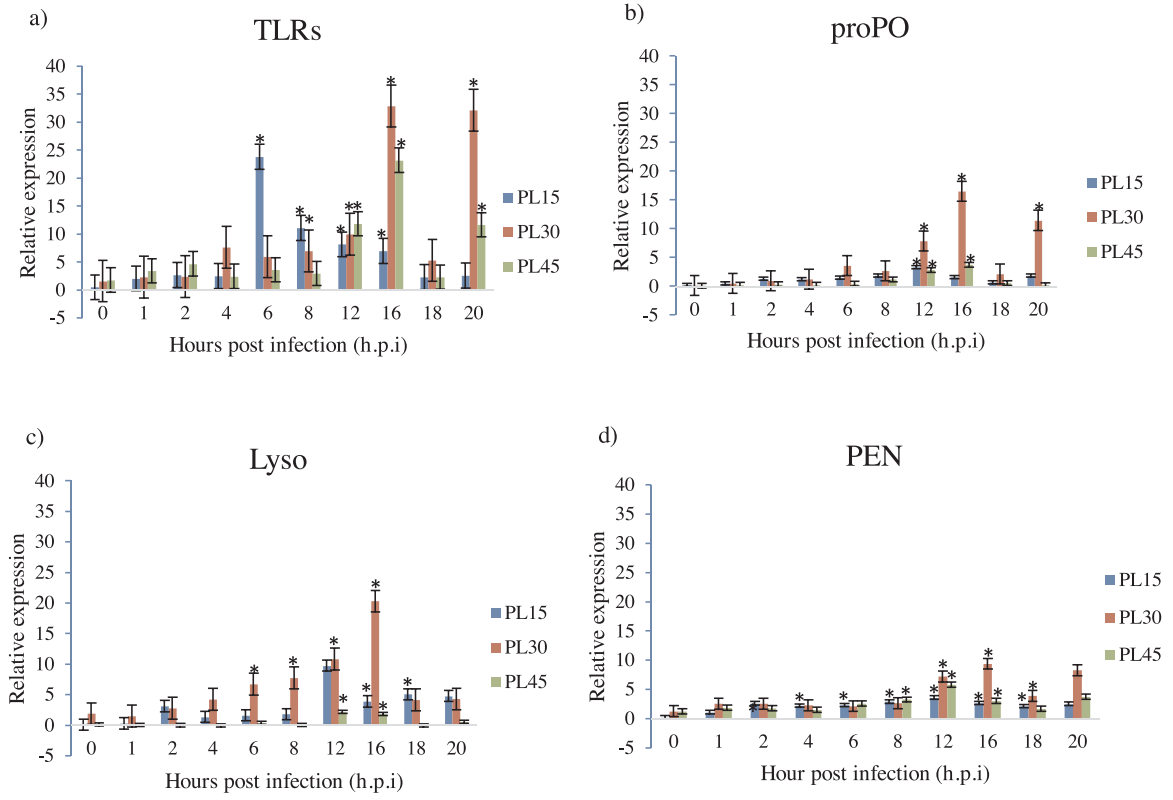

Fig. 3. Expression profiles of immunity genes at different time point : [a) Toll-like receptor (TLR), b) prophenoloxidase (proPO), c) lysozyme (lyso), and d) penaeidin (PEN)] in the post-larvae stages of giant tiger prawn, *Penaeus monodon* (PL15, PL30 and PL45) in response to *Vibrio parahaemolyticus* (Vp_{APHND}) 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)].

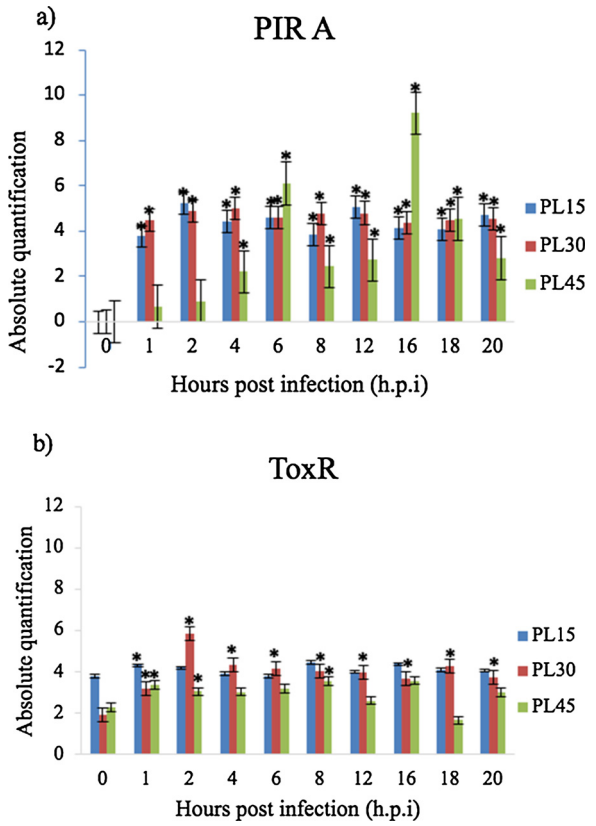

Fig. 4. Absolute quantification of bacterial genes at different time-point, bacterial toxic genes: a) PIR A and b) toxR) in three stages (PL15, PL30 and PL45) of giant tiger prawn, *Penaeus monodon* post-larvae infected by *Vibrio parahaemolyticus* (Vp_{APHND}). [P-value < 0.05, N = 5*3 replicate (PL15 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 Vp_{AHPND} 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). Lipopolysaccarides (LPS) component in pathogenic *Vibrio* spp. were recognized by TLRs gene which have initiated the signalling of TLRs pathway (TLR-MYD88-TNRF) (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 Vp_{AHPND}, 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 thyrosine 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 Vp_{AHPND} 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 Vp_{AHPND} 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 Vp_{AHPND} 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; Bachere 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 Vp_{AHPND}, 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 Vp_{AHPND} 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 Vp_{AHPND} 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; Krukonis 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, Vp_{AHPND} 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 Vp_{AHPND} 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 Vp_{AHPND}.

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.

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