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Temperature-dependent expression of virulence genes in *Vibrio parahaemolyticus* AHPND strain (Vp_{AHPND})

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Abstract The risk of acute hepatopancreatic necrosis disease (AHPND) outbreaks in the shrimp farms are governed by interactions between host, pathogen, and environmental factors. Among the environmental factors, temperature plays distinct role in triggering diseases. Temperature fluctuation is not only an acceleration–deceleration modulation of the whole cell's enzymatic activity but also affects membrane-associated functions and leads to changes in bacterial virulence gene expression. Moreover, temperature is one of abiotic stresses which arouse pathogen infection by changing host susceptibility. In the present study, we investigated the effect of different diurnal temperature fluctuation on the growth and expression of virulence genes of *Vibrio parahaemolyticus* AHPND strain (Vp_{AHPND}) cultured in a bioreactor. The pathogen, which had evolved under 3 different temperature patterns for 4 days, and an ancestor of the evolved strains (AAHMRU04), were observed for their virulence gene expressions by using quantitative RT-PCR. The mRNA expression levels for 3 virulence factors including Photorhabdus insect-related (Pir) A toxin and Pir B toxin and membrane-associated transcriptional factor (ToxR) were measured. The transcriptional analysis demonstrated that all evolved strains exhibited the expression levels of virulence genes different from those of the ancestor, which was further supporting its pathogenicity. In conclusion, the temperature fluctuation is an important environmental factor that influences the virulence of Vp_{AHPND} and these results would contribute to further study on the pathogenesis and host defensive system in the diurnal temperature fluctuation.

Keywords: Acute hepatopancreatic necrosis disease, Temperature, Virulence gene expression, *Vibrio parahaemolyticus*

Introduction

Acute hepatopancreatic necrosis disease (AHPND) is an emerging shrimp disease that has severely damaged the production of Pacific white shrimp (*Litopenaeus vannamei*) worldwide. It is also known as early mortality syndrome (EMS). The causative agent of AHPND is originally reported to be a unique strain of *Vibrio parahaemolyticus* (Vp_{AHPND}) containing a 70-kbp

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plasmid (pVA1) that carrys the genes encoding the Photorhabdus insect-related (Pir) A and Pir B toxins (Tran *et al*., 2013; Dangtip *et al*., 2015; Sirikharin *et al*., 2015; Xiao *et al*., 2017). Recently, there have been studies documented AHPND-causing strains belonging to the other closely related species such as *Vibrio owensii* (Liu *et al*., 2015), *Vibrio harveyi* (Kondo *et al*., 2015), *Vibrio campbellii* (Dong *et al*., 2017), and *Vibrio punensis* (Restrepo *et al*., 2018). However, all the newly reported AHPND-causing *Vibrio* spp. contain the plasmid encoding the binary $PirAB^{vp}$ (Muthukrishnan *et al*., 2019).

The risk and virulence of this emerging disease is considerably dependent to the changes of environmental factors. Among the environmental factors, temperature has been report to play a distinct role in triggering diseases and influence the spread of disease. Our previous work has demonstrated diurnal termperature changes influence the growth and the production of protein and Pir toxin of Vp_{AHPND} (Chirapongsatonkul *et al*., 2018a). Moreover, we have shown the effect of temperature on the expression of virulence factor genes including *lipase*, *elastase*, *enolase*, *aerolysin* (*aerA*), and *heat-labile cytotonic enterotoxin* (*alt*) of the pathogenic *Aeromonas veronii* strains causing the motile *Aeromonas* septicaemia (MAS) (Chirapongsatonkul *et al*., 2018b). There has been reported that the production of virulence factors contribute to the successful attack and overcome the host or facilitate the infection process of bacteria (Khor *et al*., 2015).

To uncover and understand the effect of temperature, the candidate Vp_{AHPND}, AAHMRU04 which was isolated from the AHPND-exhibiting shrimp and collected in our laboratory, was continuously cultured under different temperature patterns for 4 days. The information for setting temperature pattern trials were mimicked from those collected from the environment in the shrimp rearing farm in Nakhon Si Thammarat, Thailand. The bacterial growth, growth curve and specific growth rate, as well as the expression of the genes associated with the AHPND; *Pir A* and *Pir B* genes, as well as other virulence gene such as *membrane-associated transcriptional factor* (*ToxR*) were determined in this study.

Materials and methods

AHPND−causing V. parahaemolyticus (Vp_{AHPND})

The test strain of Vp_{AHPND} AAHMRU04, used throughout this study was isolated from white shrimp exhibiting clinical signs of AHPND under sterile condition. The pured Vp_{AHPND} AAHMRU04 was stored in 20% (v/v) glycerol

broth at−80°C. For all experiments, Vp_{AHPND} AAHMRU04 was cultured in Tryptic Soy Broth (TSB, Difco) containing 1.5% (w/v) NaCl or TSB^{+}.

The pathogenic bacteria were confirmed for the species and their existence of the specific plasmid (pVA1) and gene encoding Pir toxin by multiplex PCR following the method described by Chirapongsatonkul *et al*. (2018a). The PCR program was set; initial denaturation at 94℃ for 3 min, followed by 29 cycles of [94℃/30 s-60℃/30 s-72℃/30 s] and a final extension at 72℃ for10 min. The PCR reaction was performed in $T100^{TM}$ Thermal cycler (Bio-Rad). The details of primers and reagents for multiplex PCR reaction are listed in Table 1 and Table 2, respectively. PCR products were checked trough 1.5% (w/v) agarose gel electrophoresis.

Table 1. Primers used for multiplex PCR to verify the Vp_{AHPND} strain

Primer name	Primer sequence (5´to 3´)	Target	PCR product (bp)	References
Vp.flaE-79F	5'-GCAGCTGATCAAAACGTTGAGT-3'	*V. parahaemolyticus*	897	Tarr *et al*., 2007
Vp.flaE-934R	5'-ATTATCGATCGTGCCACTCAC-3'			
TUMSAT-Vp1F	5'- CGCAGATTTGCTTTTGTGAA-3'	Plasmid (pVA1)	500	Tinwongger *et al*., 2014
TUMSAT-Vp1R	5'-AGAAGCTGGCCGAAGTGATA-3'			
TUMSAT-Vp3F	5'-GTGTTGCATAATTTTGTGCA-3'	Pir toxin	360	Tinwongger *et al*., 2014
TUMSAT-Vp3R	5'-TTGTACAGAAACCACGACTA-3'			

Table 2. Reagents used in multiplex PCR reaction

PCR components	Volume (µl)/Reaction	Final concentration
DNA free water	5.60	
2X KAPA2G Fast Multiplex Mix (Sigma-Aldrich)	10.00	1X (contains 3 mM $MgCl_2$) 0.2 µM each
10 µM F/R Mix Primer (Vp.flaE, Vp1, Vp3)	2.40	
DNA template	2.00	
Total	20.00	

Determination of the bacterial growth and the expression of virulence factor genes

The stock of Vp_{AHPND} isolate AAHMRU04 weas transferred into the freshly prepared media contiaining Tryptic Soy Broth (TSB, Difco) and 1.5% (w/v) NaCl with a ratio of 1:1 and adjusted to a final concentration of 2 x 10^6 CFU/ml. The temperature profiles including normal temperature range or N (24–30^{o}C), high temperature range or H (26–32^{o}C) and low temperature range or L (22–28^{o}C) were used as the model mimicking the diurnal temperature in the ponds to set the temperature for bacteria culture (Figure 1).

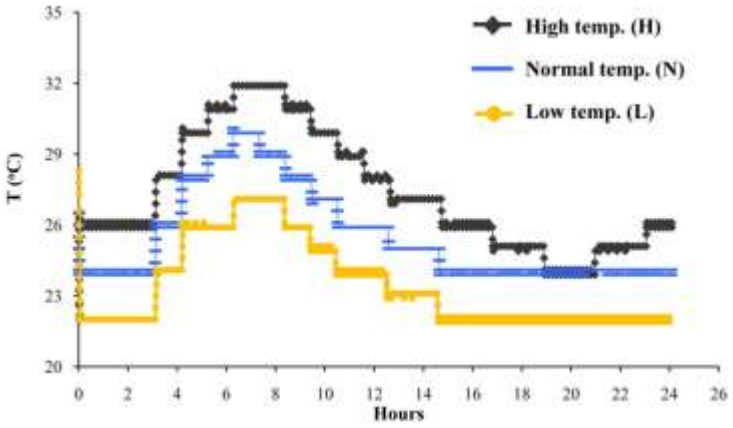

Figure 1. Three different diurnal water temperature profiles used in this study

All test bacteria were grown in a controlled temperature incubator (RTS-1C Personal bioreactor, Biosan) at 30^{o}C for 24 h (as starter) prior to be cultured under 3 set of different temperature trials which was changed every 24 h representing diurnal temperature continually for 4 days; Trial 1 (T1): Normal temp. for 4 days (N−N−N−N), Trial 2 (T2): Normal temp. to Low temp. to High temp. to Normal (N−L−H−N), Trial 3 (T3): Normal temp. to High temp. to Low temp. to Normal (N−H−L−N). The optical density (OD) was automatically detected every 10 min for 24 h. The growth curve and specific growth rate was constructed according to the recorded OD.

After incubation for 1, 2, 3, and 4 days, cell-containing media were 1-ml aliquoted. Bacterial cells were separated by centrifugation (8,000 × g for 10 min at 4 ℃), collected for RNA isolation and analysis for the gene expression. Total RNA was isolated using Presto™ Mini RNA

Bacteria Kit (Geneaid) following the manufacturer's instruction. Prior to being used in further analysis, the obtained RNA was checked for the quantity and integrity by spectrophotometric measurement and agarose gel electrophoresis, respectively. One µg of total RNA was used for cDNA synthesis using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) following the manufacturing instruction in the T100^{TM} Thermal Cycler (Bio-Rad). The synthesized cDNA of each sample was diluted in DNase/RNase-free water (Promega). The mRNA expression level of the virulence genes was quantitatively analyzed through a qPCR technique using *16S rRNA* as a reference gene. The specific primers used for qPCR analysis are shown Table 3. Each qPCR reaction mixture (total 20 µl) contained cDNA, HOT FIREPol® EvaGreen® (Solis Biodyne), primers and the desired final volume was adjusted with DEPC-treated water. The reactions were performed in a CFX96 Touch™ Real-Time PCR (Bio-Rad) in triplicate in a 96-well plate. Cycling conditions were as follows: 95 ℃ for 12 min followed by 40 cycles of 15 s at 95 ℃, 20 s at 58 ℃ (depending on the primer pairs) and 20 s at 72 ℃. Melting curves were also analyzed for all amplification products. The fold change of the relative expression of all virulence genes was calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Table 3. Primers used for quantitative RT-PCR (qRT-PCR) analysis of the expression of virulence genes

Primer name	Primer sequence (5´to 3´)	Target	References
16S-Vibrio_F567	5'-GGCGTAAAGCGCATGCAGGT-3'	*16S rRNA*	Thompson *et al*., 2004
16S-Vibrio_R680	5'-GAAATTCTACCCCCCTCTACAG-3'		
PirA-1_F143	5'-GTGGGGAGCTTACCATTCAA-3'	*Pir A toxin*	This study
PirA-1_R310	5'-CACGACTAGCGCCATTGTTA-3'		
PirB-11_F514	5'-TACATGGCTTGTGGTCTGGA-3'	*Pir B toxin*	This study
PirB-1_R715	5'-ACCAACTACGAGCACCCATC-3'		
ToxR2VP-F105	5'-AGGAAGCAACGAAAGCCGTA-3'	*membrane-associated transcriptional factor* (*ToxR*)	This study
ToxR2VP – R314	5'-TAGCCTCGTTTTGGAACGGT-3'		

Statistical analysis

The statistical difference of the expression levels of virulence genes were examined by one-way analysis of variance (ANOVA) via the SPSS Statistics software version 16.0 (SPSS Inc.). The significant differences among treatments were analyzed using a multiple comparison by Duncan's Multiple Range Test (DMRT). P <0.05 was considered statistically significant.

Results

Effect of temperature changes on the growth of Vp_{AHPND} AAHMRU04

The growths of the starter of pathogenic Vp_{AHPND} isolate AAHMRU04 which were cultered at 30^{o}C for 24 h are shown in Figure 2. This culture was performed to ensure the homogeneity of the bacterial seed or starters that were further used in later experiment. Growth curve (Figure 2A) and specific growth rate (Figure 2B) of all trail starters was almost the same. Thereafter, Vp_{AHPND} AAHMRU04 was grown under each temperature trail and the bacterial growth curve and specific growth rate are present in Figure 3 and Figure 4, respectively. Considering the growth curve, the results showed that bacteria grew similarly with the same pattern and number throughout the experimental period with the different temperature trials (N−N−N−N, N−L−H−N and N−H−L−N) suggesting that temperature had no effect on the growth or total number of bacteria (Figure 3). The specific growth rate present in Figure 4 showed that temperature had some influence on the bacterial growth and multiplication.

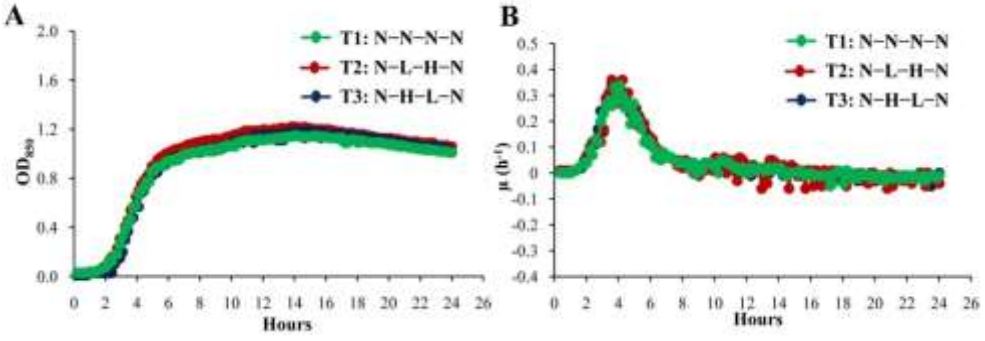

Figure 2. The real-time growth of Vp_{AHPND} isolate AAHMRU04, (A) Growth curve and (B) Specific growth rate, grown 30^{o}C for 24 h in TSB medium and 1.5% (w/v) NaCl with the ratio of 1:1

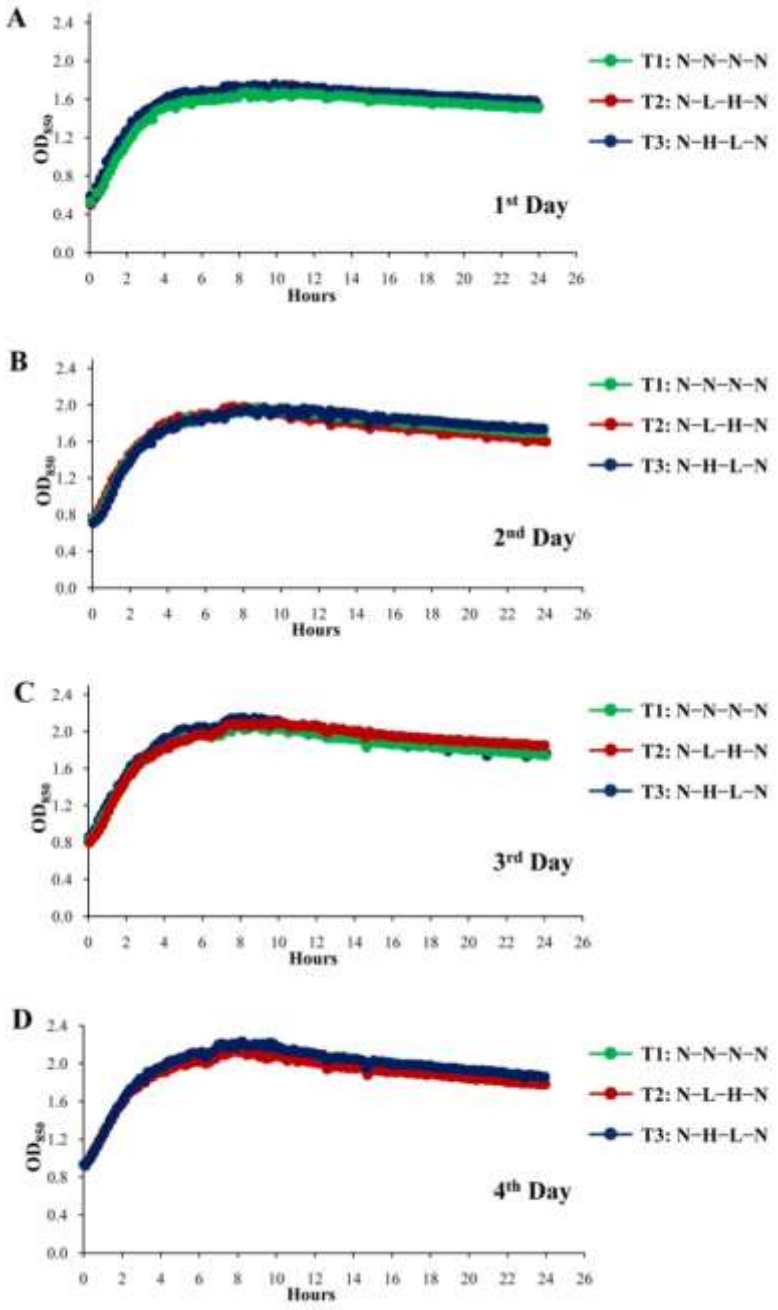

Figure 3. Growth curve of *Vp*$_{AHPND}$ isolate AAHMRU04 cultured under 3 different temperature trials, T1; N−N−N−N, T2; N−L−H−N and T3; N−H−L−N on (A) 1st day, (B) 2nd day, (C) 3rd day, and (D) 4th day

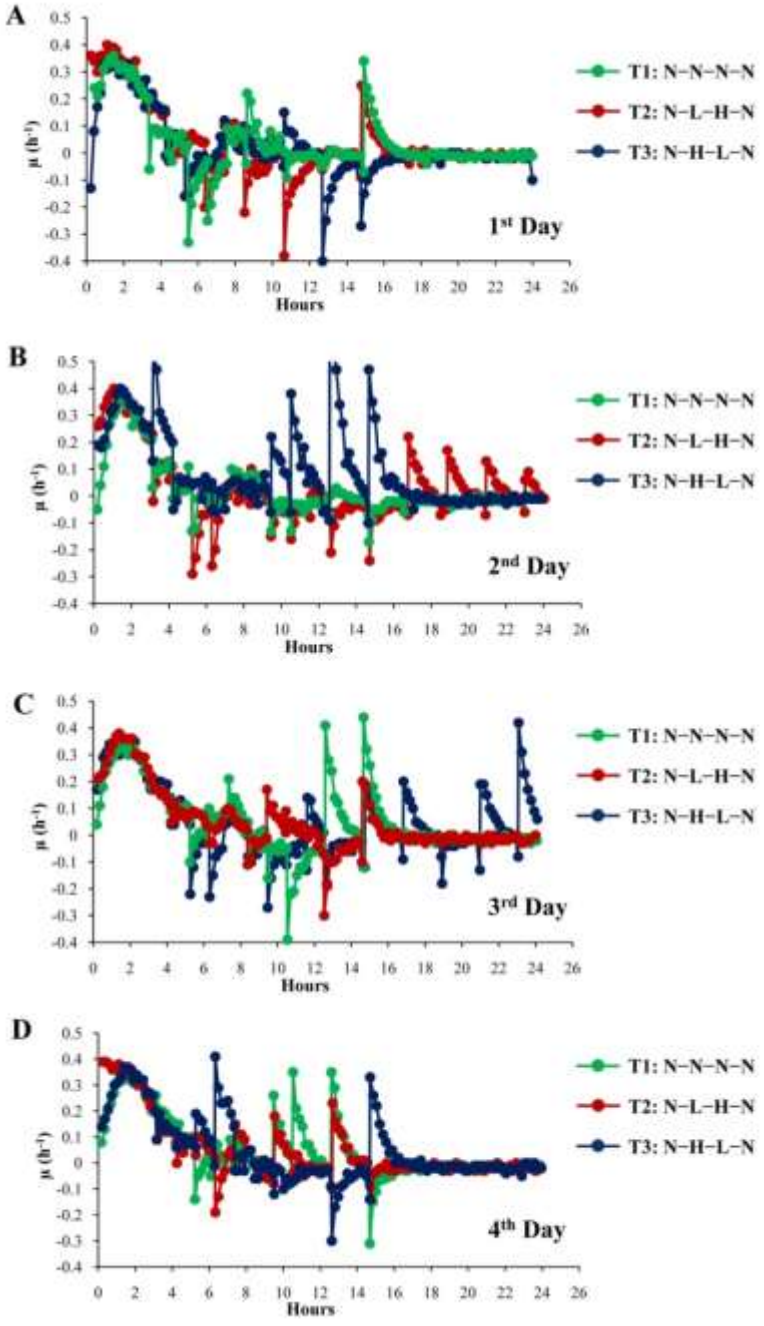

Figure 4. Specific growth rate of *Vp*$_{AHPND}$ isolate AAHMRU04 cultured under 3 different temperature trials, T1; N−N−N−N, T2; N−L−H−N and T3; N−H−L−N on (A) 1st day, (B) 2nd day, (C) 3rd day, and (D) 4th day

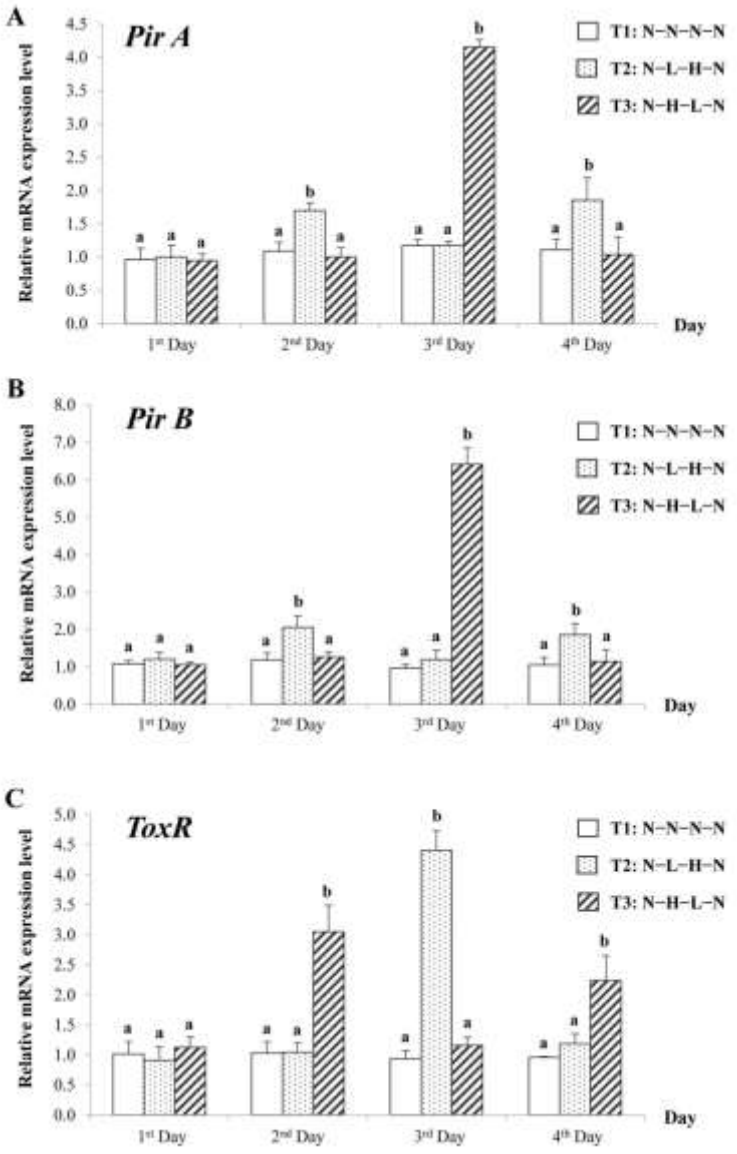

Figure 5. The expression profile of virulence gactor genes of Vp_{AHPND} isolate AAHMRU04 cultured under 3 different temperature trials, T1; N−N−N−N, T2; N−L−H−N and T3; N−H−L−N on 1st day, 2nd day, 3rd day, and 4th day. (A) *Pir A*, (B) *Pir B* and (C) *ToxR*. The results were expressed as the mean ± SD (n=6). Bars and different letters stand for statistically significant differences (P<0.05) between groups

Effect of temperature changes on the expression of virulence genes of Vp_{AHPND} AAHMRU04

The expression profiles of the genes encoding virulence factors, *Pir A*, *Pir B* and *ToxR* measured in Vp_{AHPND} isolate AAHMRU04 are shown in Figure 5. The candidate bacteria were grown under 3 different temperature patterns then total RNA was isolated for analysis of the virulence gene expression. Comparison among the trails analysed at the same time, the results showed that the levels of *Pir A* was significantly higher ($P<0.05$) in the sample grown under the temperature shifted from high to lower temperature range than those of the normal. Significantly greater level ($P<0.05$) of *Pir A* was observed in T2, T3 and T4 on the 2nd day, 3rd day, and 4th day, respectively (Figure 5A). Similar pattern and statistical differences were obtained for the expression of *Pir B* gene (Figure 5B). Conversely, the expression level of *ToxR* was significantly induced in the bacteria grown under the temperature shifted from low to higher temperature range which was observed in T3, T2 and T3 on the 2nd day, 3rd day, and 4th day, respectively (Figure 5C).

Discussion

V. parahaemolyticus is one of the known pathogenic species belonging to the genus *Vibrio* that associated with vibriosis in finfish and shellfish (Khouadja *et al*., 2013) causing economic losses in aquaculture industries (Kumaran and Citarasu, 2016). AHPND is the emerging disease which originately reported to be caused by *V. parahaemolyticus* encoding Pir A (ToxA) and Pir B (ToxB) toxins (Lee *et al*., 2015; Sirikharin *et al*., 2015). The presence of 2 homologous genes related to the insecticidal toxin genes *Pir A* and *Pir B* (Pir^{vp}) which is described the first time in *Photorhabdus* sp. has been reported to contribute to the pathogenicity of AHPND-causing bacteria. These toxin coding genes are located on the plasmid pVA1 or pV_{AHPND} (Tran *et al*., 2013; Han *et al*., 2015). Lee *et al*. (2015) have proposed the cytotoxicity-related function of Pir A and Pir B toxins according to their crystal structures which correspond to the domains of the *Bacillus thuringiensis* Cry toxin. This toxin causes cell death by undergoing a series of processes including receptor binding, oligomeriztion and pore forming (Soberón *et al*., 2010; Xu *et al*., 2014). Accordingly, the transcriptional and translational expression of Pir A and Pir B toxins might be an indicator suggesting the possibility of AHPND pathogenicity. Moreover, virulence factors have been reported to associate with the virulence and pathogenecity of the pathogenic bacteria. Li *et al*. (2019) have reviewed diverse virulence factors and the molecular mechanisms underlying

the pathogenisis of *V. parahaemolyticus*. Type III secretion system or T3SS has been mostly reported to be involved in the pathogenesis and can be used as a genetic marker of *V. parahaemolyticus* (Osei-Adjei *et al*., 2017). ToxR, a member of the membrane-associated transcriptional factor, has been shown to involve on the regulation of T3SS, play a role in the pathogenecity and contribute to the colonization in the mammalian intestine (Hubbard *et al*., 2016).

There have been reported that pathogens belonging to the genus *Vibrio* are associated with temperature-related diseases as well as increased in abundance and pathogenicity (Igbinosa and Okoh, 2008; Oh *et al*., 2009; Iwamoto *et al*., 2010). Temperature is one of the crucial environmental factors that have increasingly been concerned in aquaculture because of global warming and climate change effect. The results obtained in this study indicated that temperature affects independently on the growth and abundance of the test Vp_{AHPND} which was similar to the results shown in *V. coralliilyticus* cultured in different temperatures (Kimes *et al*., 2012). However, our results demonstrated that temperature shift, either from low to higher or high to lower temperature range, affected the transcriptional expression of all studied virulence factor genes. Kimes *et al*. (2012) have demonstrated that temperature has influence on the expression of the genes involving in virulence and quorum sensing of *V. coralliilyticus Vc*450. They have shown that bacterial genome contains the thermosensors and regualtors, known as regualtors of downstream virulence signalling in other *Vibrios*, which are differently expressed between 24^{o}C and 27^{o}C.

In conclusion, this present work demonstrated for the first time the effect of diurnal temperature and temperature shift daily on the growth and virulence factor gene expression of the pathogenic strain of AHPND-causing *V. parahaemolyticus*. We found that more fluctuating temperature seemed to affect the growth or total number of bacteria but the correlation between the temperature pattern and specific growth rate is still unclear. Interestingly, the temperature shift dependently influence the expression level of virulence factor genes. However, the pattern of induced expression level was still in doubt, *Pir* genes were incuded when the temperature shifted from high to lower temperature range while the expression level of *ToxR* was increased when the temperature shifted from low to higher temperature range. The correlation of virulence factor gene appearance and pathogenicity degree has to be further evaluated. The findings will be beneficial data for development of disease management strategy against AHPND to reduce the loss of shrimp culture.

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