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Virulence factor gene profiles of *Aeromonas veronii* isolated from diseased Nile tilapia (*Oreochromis niloticus*) in Nakhon Si Thammarat province and its expression towards diurnal water temperature changes

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Abstract *Aeromonas* spp. is the causing agent of motile *Aeromonas* septicemia (MAS) which cause a great loss in Nile tilapia (*Oreochromis niloticus*) farming. More than 200 bacteria were isolated from Nile tilapia exhibiting MAS disease in Nakhon Si Thammarat province, an important tilapia culturing area in Southern Thailand, since 2014–2017. Every collected isolates was Gram-negative and short rod-shaped. Three isolates of bacteria, A2014–1, A2015–8 and A2016–28, were randomly selected. Characterization based on molecular cloning indicated that all 3 isolates were *A. veronii*. This present study aimed to elucidate the appearance of 5 virulence factor genes considerably relevant to pathogenesis including *lipase*, *elastase*, *enolase*, *aerolysin* (*aerA*), and *heat-labile cytotonic enterotoxin* (*alt*) in these 3 isolates. The differences in virulence factor gene profiles were detected; $lipase^{-}/elastase^{+}/enolase^{+}/aerA^{-}/alt^{+}$, $lipase^{+}/elastase^{+}/enolase^{+}/aerA^{+}/alt^{+}$ and $lipase^{-}/elastase^{+}/enolase^{+}/aerA^{+}/alt^{+}$ for A2014–1, A2015–8 and A2016–28, respectively. Moreover, the effects of diurnal water temperature change of 2 different patterns, which were actually recorded from the tilapia culturing pond, on the bacterial growth and the mRNA expression level of the virulence factor genes were determined. The variable paatern of the bacterial growth as well as the expression of virulence factor genes were noticed. These data preliminary suggested the diversity of bacterial genotypes especially that of virulence factor gene profiles in the *Aeromonas* spp. causing MAS in Southern Thailand. However, the relationship between the chage of temperature and the bacteria growth, virulence and pathogenecity will be further studied.

Keywords: motile *Aeromonas* septicemia (MAS), *Aeromonas veronii*, *Oreochromis niloticus*, virulence factor genes, diurnal water temperature change

Introduction

Bacteria in the genus *Aeromonas*, are generally found in aquatic environment including fresh and brackish water. There have been reported that

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the bacteria belonging to this genus are causative agents of significant diseases in fish such as epizootic ulcerative syndrome (*A. hydrophila*), furunculosis (*A. salmonicida*) and motile *Aeromonas* septicemia or MAS (*A. hydrophila, A. caviae* and *A. veronii*) (Vega-Sánchez; 2014; Latif-Eugenin *et al*., 2016; Sarjito *et al*., 2017; Chandrarathna *et al*., 2018). Moreover, *Aeromonas* spp. are increasingly concerned since they have been considered as a human pathogen causing clinical manifestation, for example, gastroenteritis, wound infection, septicemia and pneumonia particularly in immunocompromised patients (Igbinosa *et al*., 2012; Skwor *et al*., 2014; Price *et al*., 2017; Wimalasena *et al*., 2017). Among the *Aeromonas*-causing diseases in aquaculture, MAS has been reported to cause a devastative loss in cultured fish including cyprinid fish in China (Zhang *et al*., 2014) and catfish in West Alabama and East Mississippi (Shoemaker *et al*., 2018).

Many studies have focused on the distribution and genetic diversity of *Aeromonas* spp. particularly those relevant to human health, food and environment to provide the information for human health management and protection (Sechi *et al*., 2002; Senderovich *et al*., 2012; Khor *et al*., 2015) while this regard is still scarce in aquaculture. However, the variation of the species isolated and pathogenecity of *Aeromonas* spp. have been noticed. For the latter mentioned point, it has been considered due to the virulence factors produced by the bacteria. There has been reported that the production of virulence factors is essential for bacteria to attack and overcome the host or facilitate the infection process (Khor *et al*., 2015). Several researchers have tried to elucidate the virulence determinants in *Aeromonas* spp. majority based on *A. hydrophila*. Virulence in *Aeromonas* spp. is multifactorial involving in the production and/or secretion of virulence factors, including adhesions, cytotoxins, enterotxins, dedrading enzymes; protease, elastase, lipases, and DNases, as well as the capacity to form biofilms (Thronley *et al*., 1997; Cascon *et al*., 2000; Rasmussen-Ivey *et al*., 2016). However, the growth as well as the expression and production of the virulence factors are affected by several environmental factors including temperature changes. Yu *et al*. (2007) have reported that virulence proteins such as polar and lateral flagellins are under the control of temperature.

As mentioned above that virulence in *Aeromonas* spp. relies on a complex process, the detection of virulence factors is necessary in determining the potential pathogenecity. The work aimed to study the species identification and its virulence factor gene profile of the bacteria isolated from the MAS diseased Nile tilapia collected from the tilapia culturing farm in Nakhon Si Thammarat, a province in Southern Thailand, where tipalia farming is one of the important occupations of local farmers. Even the preliminary study based

on Gram-staining and biochemical characterization, indicated that the dominant isolated bacteria were *Aeromonas* spp., the molecular technique was perfomed in this study in order to identification in the species level. Moreover, 2 different profiles of the diurnal temperature change referred to (1) normal temperature range which mostly recorded and (2) hot-rain temperature range which often occurred throughout the year in Southern Thailand where the weather is tropical climate with high relative humidity. Consequencely, the atmospheric and water temperature in each day varies depending on the weather. It has been noticed that the infectious diseases in fish culture normally spread with high lossing impact when the weather and temperature is fluctuated. The question regarding the involving of the temperature change, pathogen growth as well as the expression of virulence factor genes is addressed. We tried to figure out this question through studying the effects of diurnal water temperature changes on the growth of bacteria and the expression of virulence factor-encoded genes. The findings will elucidate the diversity of the different virulence factors which might be associated with the virulence and pathogenecity in *Aeromonas* spp. as well as the effect of environmental factor on its transcriptional expression. The obtained data lead to subsequently provide the promising bacterial disease control in aquaculture.

Materials and methods

Isolation of the pathogenic bacteria

The fish exhibiting MAS disease was collected from the tilapia culturing farms in Nakhon Si Thammarat between 2014 and 2017. The bacteria were isolated from the target organs of bacterial infection including brain, liver and spleen. Each pured isolate was Gram-staining followed by observation under a light microscope and biochemical test. Three isolates preliminary characterized as *Aeromonas* spp. were randomly selected from the stock for further confirmation of the genus and species identification through molecular characterization. The selected isolates were named A2014–1, A2015–8 and A2016–28.

Molecular characterization and the appearance profile of virulence factor genes

Each selected isolate was cultured in Tryptic Soy Broth (TSB, Difco), incubated at 35℃ and shaken at 180–200 rpm. After 18 h, the bacterial cell was separated for DNA extraction using a Presto^{TM} Mini gDNA Bacteria Kit

(Geneaid). In order to genus and species identification, the PCR was performed in a T100™ Thermal Cycler (Bio-Rad) using the universal primers 20F/1500R (Table 1) to amplify 16S rDNA. The PCR program was set; denaturation at 94 ℃ for 5 min, followed by 35 cycles of [95 ℃/1 min-55 ℃/1 min-72 ℃/1 min] and a 10-min extension at 72 ℃. The amplified product was visualized by agarose gel electrophoresis and the single band with an expected size was purified, ligated into the pGEM®-T Easy Vector (Promega) and transformed into *Escherichia coli* Top10 cells. Purified plasmids were sequenced and the obtained nucleotide sequences were analyzed for genus and species identification according to multiple sequence alignment (ClustalW) (Thompson *et al*., 1994) and phylogenetic tree construction (MEGA X).

The virulence factor genes; *lipase*, *elastase*, *enolase*, *aerolysin* (*aerA*), and *heat-labile cytotonic enterotoxin* (*alt*), were amplified using the specific primers listed in Table 1. The PCR reactions were performed with the program as described above except the annealing temperature was set at 60 ℃. The appearance of the virulence factor genes was analyzed by agarose gel electrophoresis, positive result (+) refers to the visualized band at the desired amplicon sizes.

Table 1. Primers used in this study

Primer name	Primer sequence (5 ´to 3)	Target	PCR product (bp)	References
20F 1500R	5'- AGAGTTTGATCATGGCTCAG -3' 5'- CGGTTACCTTGTTACGACTT -3'	16S rDNA	~1500	Weisburg *et al*., 1991
Lip-F Lip-R	5'- GACTCCCTCAAGGACAGCAG -3' 5'- AGAGGCTTTCAGGGCATTG -3'	Lipase	594	This study
Elas-F Elas-R	5'- GCACATGTACCGCAACTGGTA -3' 5'- GGTGTTGGCCAGCAGGTA -3'	Elastase	466	This study
Enol-F Enol-R	5'- CGACGGTACCGAGAACAAA -3' 5'- CTTGGATGTCGACGTTGTTG-3'	Enolase	212	This study
Aer-F Aer-R	5'- GAGAAGGTGACCACCAAGAACAA -3' 5'- CATAATTGACCTCGGCCTTG -3'	Aerolysin	235	This study
Alt-F1 Alt-R1	5'- AGGATGCCCTCAACACCATC -3' 5'- GCTCTGTTTCAGGTTGTCGC -3'	Enterotoxin	272	This stidy

Effect of diurnal temperature changes on the expression of virulence factor genes

The stock of all 3 tested bacteria were transferred into the freshly prepared media contiaining Tryptic Soy Broth (TSB, Difco) and 0.85% (w/v)

NaCl with a ratio of 1:1 and adjusted to a final concentration of 2 x 10^6 CFU/ml. Every bacterium isolate was cultured under the diurnal temperature profile, which mimicking that of the culturing pond reported in our previous work, using a controlled temperature incubator (RTS-1C Personal bioreactor, Biosan). The water temperature was recorded every hour for 24 h in the tilapia culturing ponds in Nakhon Si Thammarat province in 2017 using an automatic temperature recorder model 175T2 (Testo). Two temperature patterns, representing the normal temperature range (24–30^{o}C) and hot-rain temperature range (22–30^{o}C), were used as the model mimicking the diurnal temperature in the ponds to set the temperature for bacteria culture (Figure 1).

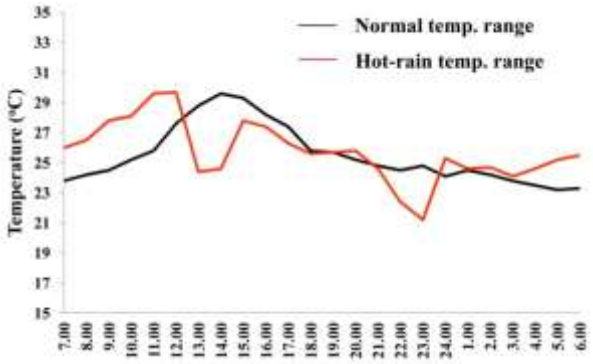

Figure 1. The diurnal water temperature profiles recorded from the tilapia culturing pond

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 24 h, bacterial cells were separated by centrifugation (8,000 × g for 10 min at 4 ℃), collected for RNA isolation and further gene expression analysis. Total RNA was isolated using Presto™ Mini RNA Bacteria Kit (Geneaid) following the manufacturer's instruction. The obtained RNA was checked for the quantity and integrity by spectrophotometric analysis and agarose gel electrophoresis, respectively. The cDNA synthesis using 1 μg of total RNA in a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) following the manufacturing instruction was performed in the T100^{TM} Thermal Cycler (Bio-Rad). The synthesized cDNA of each sample was diluted in DNase/RNase-free water (Promega, US). The amplification of each virulence gene was conducted by PCR (30 cycles) according to the above described procedure. The expression level of genes encoding virulence factors

was analyzed by semi-quantitative PCR. The image densitometry using ImageJ software (Schneider *et al*., 2012) was performed to examine the fold change in gene transcript expression. The band of interest was quantified and normalized against the band of *16S rRNA* as an internal control internal.

Results

Three isolates preliminary characterized as *Aeromonas* sp. through biochemical test, A2014–1, A2015–8 and A2016–28, was determined for its species by molecular characterization. The result based the phylogenetic tree constructed according to the partial sequences of 16S rDNA of all these 3 isolates, *Aeromonas* spp., *Enterobacteriaceae* and out group of *Bacillus* spp. (Figure 2) indicated that they were *A. veronii*. Their nucleotide sequences located in the same clade as other *A. veronii*.

Variation of the appearance of virulence factor gens was observed in the 3 isolates of *A. veronii* examined by PCR in this study. Three virulence factor genes, *elastase*, *enolase* and *alt* were detected in A2014–1 while 5 genes including *lipase, elastase, enolase*, *aerA*, and *alt* were observed in A2015–8. There were 4 genes; *elastase, enolase, aerA,* and *alt* were found in the isolate A2016–28. The information including the fish organs that the bacteria isolated as well as the existence of the virulence factor genes in the bacterial genome and virulence factor gene profiles of these *A. veronii* isolates can be summarized in Table 2.

Table 2. The appearance of virulence factor genes of *A. veronii* 3 isolates in this study

Isolates	Fish organs	Virulence factor genes[1]					Virulence factor gene profiles
		lipase	***elastase***	***enolase***	***aerA***	***alt***	
A2014–1	Brain	–	+	+	–	+	$lipase^{-}$ /$elastase^{+}$/$enolase^{+}$/ $aerA^{-}$/alt^{+}
A2015–8	Brain	+	+	+	+	+	$lipase^{+}$/$elastase^{+}$/ $enolase^{+}$/$aerA^{+}$/alt^{+}
A2016–28	Liver	–	+	+	+	+	$lipase^{-}$ /$elastase^{+}$/$enolase^{+}$/ $aerA^{+}$/alt^{+}

[1/] + indicates the presence of a gene; – indicates the absence of a gene.

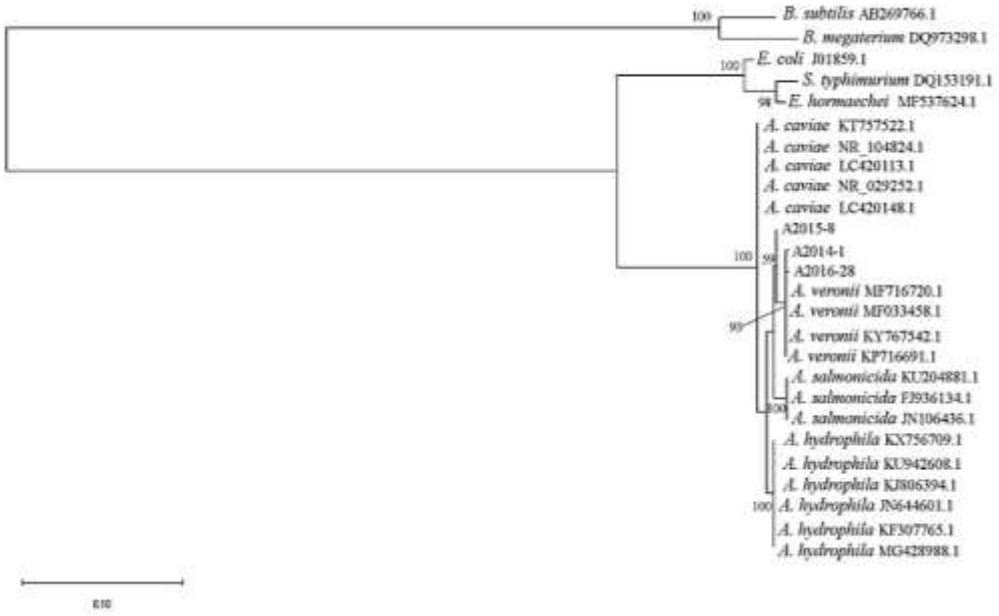

Figure 2. Phylogenentic analysis based on partial nucleotide sequences of 16S rDNA from the 3 tested isolate of *Aeromonas* sp. in this study (A2014–1, A2015–8 and A2016–28), other *Aeromonas* spp. and *Enterobacteriaceae* while *Bacillus* spp. were used as outgroup. The phylogenetic tree was constructed via maximum likelihood (ML) with 1000 bootstraps, K2P and BioNJ algorithm (MEGA X)

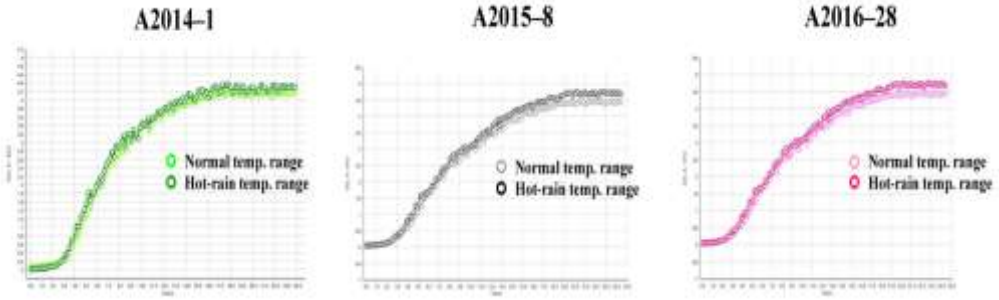

Figure 3. Growth curve of *A. veronii* isolate A2014–1, A2015–8 and A2016–28 cultured under 2 different diurnal temperature patterns, normal temperature range (24–30^{o}C) and hot-rain temperature range (22–30^{o}C)

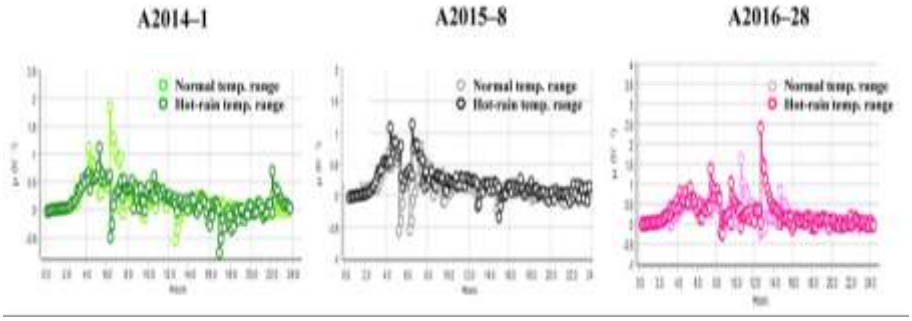

Figure 4. Specific growth rate of *A. veronii* isolates A2014–1, A2015–8 and A2016–28 cultured under 2 different diurnal temperature patterns, normal temperature range (24–30^{o}C) and hot-rain temperature range (22–30^{o}C)

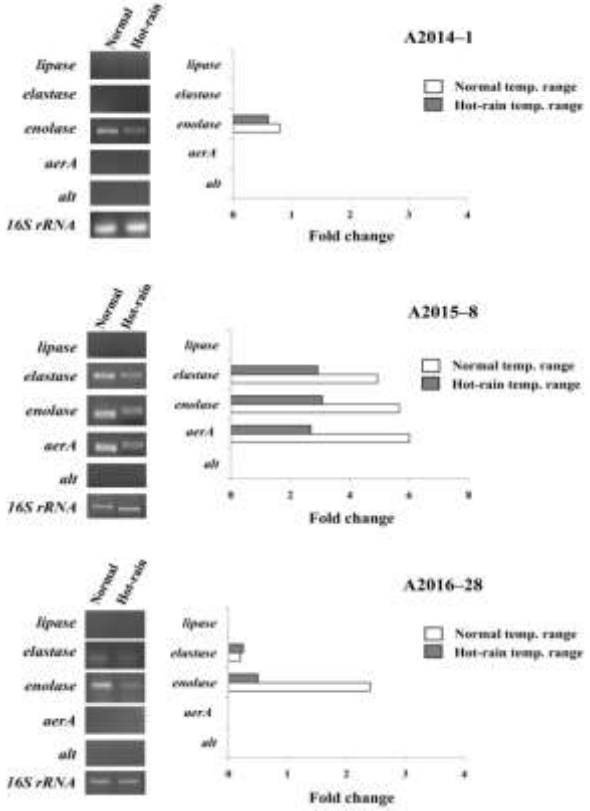

Figure 5. Semi-quantitative RT-PCR analysis of virulence factor gene expression in *A. veronii* 3 isolates, A2014–1, A2015–8 and A2016–28 cultured under the different diurnal temperature patterns, normal temperature range (24–30^{o}C) and hot-rain temperature range (22–30^{o}C). The amplican bands were visualized after agarose gel electrophoresis. Each column represents the relative fold change of virulence factor gene against *16S rRNA* as the internal marker

All 3 *A. veronii* isolates were cultured under 2 different patterns of diurnal water temperature change those mimick the water temperature in the tilapia culturing pond, normal temperature range (24–30^{o}C) and hot-rain temperature range (22–30^{o}C) (Figure 1). The growth curves of all *A. veronii* isolates were similar in pattern but slight difference was observed between the bacteria cultured under normal and hot-rain temperature rage; the latter condition seemed to give higher growth propotion (Figure 3). Conversely, the specific growth rates were varied among the bacteria isolates even they were cultured in the same temperature profile. Normal temperature range caused higher fluctuating pattern in A2014–1 while this phenomenon occurred in hot-raintemperature range for A2015–8 and A2016–28 (Figure 4).

The effect of the different diurnal temperature patterns on the transcription level of virulence factor mRNA was comparatively studied. The results showed that the temperature affected the gene expressions (Figure 5). Even, there were the genes appeared in the bacterial genomic DNAs, there were some virulence factor genes expressed *in vitro* under the culture condition. Only *elastase* was transcriptional expressed in A2014–1. Two genes, *elastase* and *enolase*, were expressed in A2016–28 while the transcriptional expressions of *elastase* and *enolase* and *aerA* were detected in A2015–8. In all expressed genes, hot-rain temperature range seemed to down-regulate the virulence factor genes compared to did normal temperature range.

Discussion

In this present study, we selected 3 isolates of the Gram-negative bacteria, A2014–1, A2015–8 and A2016–28, wich cuased MAS in Nile tilapia in culturing ponds in Nakhon Si Thammarat, the province in Southern Thailand, for elucidation the virulence factor gene profile. According to the molecular characterization through phylogenetic tree constructed based on the partial 16S rDNA sequence, the results indicated that all selected isolates were *A. veronii*. The 16S rDNA gene sequence is typically molecular identification tool for phylogenetic tree construction (Sun *et al*., 2016). However, several researchers have recently suggested that other housekeeping genes such as gyraseB (*gyrB*) and RNA polymerase sigma-factor (*rpoD*) are more suitable for disguishing *Aeromnas* at the species level (Khor *et al*., 2015; Sun *et al*., 2016).

The studies relevant to virulence factors, both in transcriptional and translational levels, are increased interesting since virulence factors have been reported to contribute and manifest the pathogenicity of pathogenic bacteria (Senderovich *et al*., 2012; Rasmussen-Ivey *et al*., 2016; Sun *et al*., 2016; Chandrarathna *et al.*, 2018). It is well known that *Aeromonas* infections rely on

multiple virulence factors including biologically active substances, secreted extracellular toxins and enzymes (Janda and Abbott, 2010). Five virulence factor genes including *lipase*, *elastase*, *enolase*, *aerA* and, *alt*, were determined for their apperarance and transcription level change influenced by the diurnal water temperature changes. All 3 *A. veronii* isolates used in this study contained *elastase*, *enolase* and *alt* genes in their genomic DNAs while *lipase* and *aerA* were occationally found in some isolates. Lipase, elastase and enolase are degrading enzymes that contribute to host cell damage resulting in facilitating the pathogen invading and infection (Rodriguez *et al*., 1992). Even enolase is one of the enzyme function in glycolysis pathway which involved in metabolism and energy conversion in the cell; it has been identified as a virulence factor in *Aeromonas* spp. since it can bind to human plasminogen and leads to production of plasmin which can degrade blood plasma proteins) (Sha *et al*., 2009). Heat-labile enterotoxin encoded by *alt* gene is one of the considerably important cytotonic enteroxin in psthogenensis of *Aeromonas* spp. because of its ability to cause significant fluid secretion (Sha *et al*., 2002; Li *et al*., 2011). Aerolysin is a multifunctional enzyme possesses cytotoxic and hemolytic activities which have been reported to correlate with the pathogenecity of *Aeromonas* spp. (Janda and Abbott 2010; Rasmussen-Ivey *et al*., 2016). Our results exhibiting the variation in virulence factor gene appearance even they were all *A. veronii*. There is variation in the genotypic profile of virulence-encoded genes reported in *A. veronii* studied in diseased gibel carp (*Carassius gibelio*) (Sun *et al*., 2016), *A. hydrophila* from clinical and environmental samples reported by Aguilera-Arreola *et al*. (2005) and *Aeromonas* spp. isolated from fresh water lakes in Malaysia (Khor *et al*., 2015). Moreover, it is interesting that the difference in virulence factor gene profile may result in different pathogenicity (Sun *et al*., 2016).

However, the transcriptional expression level of the virulence factor genes are scarce especially those affected by environmental factors. Temperature is one of the crucial environmental factors that have to be concerned in aquaculture because of global warming and climate change effect. In this study, 2 different profiles of the diurnal temperature change referred to the normal temperature range and hot-rain temperature range mimicking that recorded in Southern Thailand was used for studying the effect of diurnal temperature changes on the growth and the expression profile of the genes encoding virulence factors of these *A. veronii*. Based on our obtained results, we could not conclude the effect on how the temperature involves in the growth and transcriptional expression of virulence factor genes. To our knowledge, most of the researches have focused on the growth of bacteria towards the constant temperature (Zwietering *et al*., 1994; Van Derlinden *et al*., 2008; Zotta

et al., 2013) while our study aimed to verify the effect of the dynamic changes of temperature. We found that more fluctuating temperature increased the growth or total number of bacteria but the correlate between the temperature pattern and specific growth rate is still unclear. Similarly, the temperature change probably affected the expression level of virulence factor genes but the correlation was still in doubt. The results showed that the normal temperature range caused the higher expression level than did the hot-rain temperature range. There has been reported that the expression of virulence factors depends on the ability of the pathogen to sense of multiple environmental cues, for example, temperature, pH, oxygen, amount of certain anion such as phosphate and bicarbonate, and osmorlaity (Thomas and Wigneshweraraj, 2014). In addition, the virulence gene expression also be employed in response to a single environmental parameter fluctuation and single regulatory mechanism especially some quorum sensing regulation (Antunes *et al*., 2010).

This study demonstrated the first time of a variety of virulence factor gene profiles in *A. veronii* isolated from MAS–exhibiting Nile tilapia. In addition, the effects of environmental factor, diurnal temperature changes, on the growth and expression of the genes encoding virulence factors were also examined. However, the correlation of virulence factor gene appearance and pathogenicity degree has to be further evaluated. The findings will be beneficial data for development of vaccine applied in aquaculture to reduce the losses caused by virulent *A. veronii* and *Aeromonas* spp.

Acknowledgement

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