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# Variation in *Vibrio parahaemolyticus* isolates from a single Thai shrimp farm experiencing an outbreak of acute hepatopancreatic necrosis disease (AHPND)

Jyoti Joshi [a,b], Jiraporn Srisala [a,c], Viet Hong Truong [a,b], I-Tung Chen [d], Bunlung Nuangsaeng [e], Orasa Suthienkul [f], Chu Fang Lo [d], Timothy W. Flegel [a], Kallaya Sritunyalucksana [a,b,c], Siripong Thitamadee [a,b,*]

[a] Centex Shrimp, Faculty of Science, Mahidol University, Rama VI Rd., Bangkok, Thailand
[b] Department of Biotechnology, Faculty of Science, Mahidol University, Rama VI Rd., Bangkok, Thailand
[c] Shrimp-Virus Interaction Laboratory (ASVI), National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Rama VI Rd., Bangkok, Thailand
[d] National College of Bioscience and Biotechnology, National Cheng Kung University, Tainan 70101, Taiwan
[e] Broodstock Multiplication Center (BMC), Faculty of Marine Technology, Burapha University Chanthaburi Campus, Chanthaburi, Thailand
[f] Department of Microbiology, Faculty of Public Health, Mahidol University, Bangkok, Thailand

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ABSTRACT

The Thai Department of Fisheries (DOF), 2013 estimated that outbreaks of acute early mortality (often called early mortality syndrome or EMS) in cultivated shrimp were responsible for a 33% drop in shrimp production during the first quarter of 2013. Similar early mortality in Vietnam was ascribed to specific isolates of *Vibrio parahaemolyticus* that caused acute hepatopancreatic necrosis disease (AHPND) but the status of EMS/AHPND in Thailand was unclear. Here we describe the isolation and characterization of bacteria isolated from the hepatopancreas (HP) of shrimp collected from an early mortality outbreak farm in Thailand. Four independent bacterial isolates were identified as *V. parahaemolyticus* by BLAST analysis and by gene-specific marker detection of a lecithin dependent hemolysin (LDH) considered to be specific for the species. Immersion challenges with 3 of these and a reference isolate, obtained from China in 2010, using a previously published laboratory infection model caused very high mortality accompanied by characteristic AHPND histopathology in the shrimp HP. Tests with one of these isolates (5HP) revealed that rate of mortality was dose dependent. Using the same challenge protocol, the 4th isolate (2HP) also caused high mortality, but it was not accompanied by AHPND histopathology. Instead, it caused a different histopathology of the HP including collapsed epithelia and unique vacuolization of embryonic cells (E-cells). These results revealed the possibility of diversity in isolates of *V. parahaemolyticus* that may cause early mortality in shrimp cultivation ponds. Genomic and episomic DNA of these isolates and isolates of *V. parahaemolyticus* that cause no disease need to be compared to better understand the molecular basis of bacterial virulence in AHPND.

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## 1. Introduction

Early mortality syndrome (EMS) refers to unusual, acute mortality in shrimp approximately within the first 35 days after stocking cultivation ponds. Such outbreaks in farmed pacific white shrimp or whiteleg shrimp (*Penaeus vannamei*) were first reported in Thailand from farms on the eastern coast of the Gulf of Thailand in late 2012 (Flegel, 2012; Leano and Mohan, 2012). In the following year, outbreaks spread to farms on the western coast of the gulf of Thailand (peninsular Thailand). The Thai Department of Fisheries (DOF), 2013 reported that total shrimp production for Thailand in the first quarter of 2013 was 63,500 tons while it was 94,400 tons for the same period in 2012, indicating a production decline of approximately 30,900 tons due to these outbreaks (http://www.fisheries.go.th/ems/).

EMS is a general term that encompasses causes ranging from environmental factors to diseases caused by white spot syndrome virus (WSSV) and yellow head virus (YHV) (NACA, 2012). However, a new disease called acute hepatopancreatic necrosis disease (AHPND) was shown to be a frequent cause of EMS in Vietnam (Tran et al., 2013).

AHPND is characterized by severe atrophy of the shrimp hepatopancreas (HP) that exhibits unique histopathology at the acute stage of the disease, consisting of massive sloughing of HP epithelial cells in the absence of bacteria or other pathogens (www.enaca.org). By using a laboratory challenge model, a research group from the University of Arizona satisfied Koch's postulates in identifying unique strains of

* Corresponding author at: Department of Biotechnology, Faculty of Science, Mahidol University, Rama VI Rd., Bangkok, Thailand. Tel.: +66 2 201 5867; fax: +66 2 354 7344.
*E-mail address:* siripong.thi@mahidol.ac.th (S. Thitamadee).

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*Vibrio parahaemolyticus* as the causative agents for AHPND in Vietnam (Tran et al., 2013).

Examination of shrimp specimens from Thai farms exhibiting severe early mortality revealed that not all outbreaks yielded shrimp specimens that showed the massive sloughing of HP cells characteristic of AHPND. Here we describe partial characterization of bacteria isolated from the HP tissue of shrimp collected from one Thai shrimp farm with ponds exhibiting unusually high mortality within the first 35 days after stocking. Histological examination of the collected specimens confirmed the presence of histopathology characteristic of AHPND. The bacterial isolates were subjected to analysis of the small subunit (SSU) ribosomal RNA (16S rRNA) sequences and PCR analysis for standard markers used in identification of *V. parahaemolyticus*. They were also subjected to bioassays using the Arizona laboratory challenge model and to histopathological analysis to assess their ability to cause AHPND (Tran et al., 2013).

## 2. Materials and methods

### 2.1. Collection of AHPND shrimp samples

Since the Ethical Principles and Guidelines for the Use of Animals of the National Research Council of Thailand (1999) apply to vertebrates only and there is no official standard for invertebrates, we adapted its principles to shrimp. We also followed the guidelines of the Australian, New South Wales state government for the humane harvesting of fish and crustaceans (http://www.dpi.nsw.gov.au/agriculture/livestock/animalwelfare/general/fish/shellfish;30March2013) with respect to details regarding the transport of the shrimp and their laboratory maintenance. With respect to processing the shrimp for histological analysis or for killing at the end of an experiment, the salt water/ice slurry method was used as recommended in the Australian guidelines.

*P. vannamei* shrimp were collected from a shrimp farm that was experiencing massive death within 35 days after stocking in Prachuap Khiri Khan province on the western coast of the gulf of Thailand. Approximately 30 live shrimp from the affected ponds were transported to the laboratory in plastic containers with proper aeration and they were temporarily reared indoors using $48 \times 70 \times 41$ cm plastic tanks. Upon arrival at the laboratory, some live shrimp were immediately fixed in Davidson's fixative (Bell and Lightner, 1988) and processed for histological examination of the hepatopancreas for the characteristic histological signs of AHPND.

### 2.2. Bacterial isolation and identification

Aseptically excised tissue of the hepatopancreas (HP) was disaggregated and streaked on tryptic soy agar plates (TSA supplemented with 1.5% NaCl) using sterile loops and incubated at 30 °C for 16 h. Individual colonies obtained from the mixed bacterial isolates were re-streaked to obtain pure isolates before storage as glycerol stocks at −80 °C. For bacterial classification, DNA from bacterial isolates was used as templates for PCR amplification with 16S rRNA gene-specific primers 40F: 5′-GCCT AACACATGCAAGTCGA-3′ and 802R: 5′-GACTACCAGGGTATCTAA TCC-3′ (Horz et al., 2005). The PCR protocol consisted of predenaturation at 95 °C for 5 min followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min with a final elongation step at 72 °C for 10 min. Amplified PCR products were cloned into pGEM-T EASY Vector (Promega) and transformed into *Escherichia coli* JM109. The transformed cells were selected on LB agar containing 50 μg/ml ampicillin. Positive clones were confirmed for inserts by colony PCR and 3–4 clones per bacterial isolate were picked for sequencing analysis (Macrogen, Korea). The nucleotide sequence data were subjected to BLAST sequence analysis against NCBI databases for bacterial identification. One reference isolate named “China” that had previously been confirmed to cause AHPND by the laboratory assay (Tran et al., 2013) was kindly provided by Robins McIntosh from Charoen Pokphand Co. Ltd. This isolate was obtained from a shrimp pond exhibiting early mortality in Hainan Island, China in August 2010. An additional reference isolate of *V. parahaemolyticus* originated from shrimp pond sediment in 2008 from Phang-Nga province, long before reports of AHPND and was obtained from the Faculty of Public Health, Mahidol University.

The gene-specific primers for the detection of *V. parahaemolyticus* hemolysin genes were the following: 1) lecithin dependent hemolysin (*ldh*) *ldh*-F: 5′-AAAGCGGATTATGCAGAAGCACTG-3′ and *ldh*-R: 5′-GCTACTTTCTAGCAT TTTCTCTGC-3′ (Taniguchi et al., 1985) 2) thermostable direct hemolysin (*tdh*) *tdh*-F: 5′-GTACCGATATTTTGCAAA-3′ and *tdh*-R: 5′-ATGTTGAAGCTGTAC TTGA-3′ (Nishibuchi et al., 1985) and 3) *tdh*-related hemolysin (*trh*) *trh*-F: 5′-CTC TACTTTGCTTTCAGT-3′ and *trh*-R: 5′-TACCGTTATATAGGCGCTTA-3′ (Nishibuchi et al., 1989). The PCR conditions were performed according to Taniguchi et al. (1985) and Nishibuchi et al. (1985). The PCR products were analyzed by agarose gel electrophoresis followed by ethidium bromide staining and viewing with an ultraviolet (UV) transilluminator.

### 2.3. Preparation of bacteria for challenge tests

The bacterial isolates from glycerol stocks were re-streaked on the TSA plate prior to culture of a single colony in 2 ml TSB containing 1.5% NaCl at 30 °C. The bacterial inocula were subsequently transferred to 150 ml TSB with vigorous shaking at 30 °C until $OD_{600} = 0.6–0.8$ which was equivalent to a bacterial density of approximately $10^8$ colony forming units (cfu)/ml.

### 2.4. Preparation of shrimp for bacterial challenge tests

Stocks of specific-pathogen free (SPF) *P. vannamei* shrimp (2–5 g body weight) were purchased from local hatcheries and maintained in aerated tanks containing artificial seawater (Marinium) at 20 ppt salinity.

### 2.5. Intramuscular injection challenge tests

The bacterial cultures 1D and 3HP at $OD_{600} = 0.6–0.8$ were collected at 3000 rpm for 10 min prior to dilution with 0.1 M PBS (pH 7.4) to a concentration of $10^3$ cfu/50 μl. The SPF *P. vannamei* shrimp (5 g body weight) were injected intramuscularly with these individual bacterial isolates into the third abdominal segment at a concentration of $10^3$ cfu per shrimp with two replicates of 10 shrimp/tank. An equal volume of PBS (50 μl) was injected into shrimp in the control group. The moribund and surviving shrimp were fixed with Davidson's fixative (Bell and Lightner, 1988) and further processed for histopathological analysis.

### 2.6. Immersion challenge tests

The bacterial isolates were further tested directly using the Arizona laboratory challenge model described by Tran et al. (2013) or with some modifications as indicated. Briefly, 150 ml of fresh bacterial culture containing approximately $10^8$ cells/ml was used for immersion of 15 individual shrimp (2 g body weight) for 15 min before transferring to 15 l of 20 ppt artificial seawater (Marinium) to obtain a final bacterial density of approximately $10^6$ cfu/ml with proper aeration. Shrimp in the control group were immersed in 1/100 dilution of sterile TSB supplemented with 1.5% NaCl in 15 l of 20 ppt artificial seawater (Marinium). Cumulative mortality (dead and moribund shrimp) was recorded and mean time to death was calculated for those isolates that gave 100% mortality within 5 days. Isolate 5HP was used to determine the relationship between challenge dose and mortality using immersion doses ranging from $10^3$ to $10^6$ in steps of 10 over 3 days and regression analysis was used with the results to calculate the 2-day $LD_{50}$ dose.

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