IDENTIFICATION OF BACTERIAL AGENT(S) FOR ACUTE HEPATOPANCREATIC NECROSIS SYNDROME, A NEW EMERGING SHRIMP DISEASE

Jyoti Joshi[1,2], Jiraporn Srisala[3], Waraporn Sakaew[2], Anuphap Prachumwat[2,3], Kallaya Sritunyalucksana[1,2,3], Timothy W. Flegel[2] and Siripong Thitamadee[1,2,*]

Received: November 01, 2013; Revised: December 30, 2013; Accepted: January 24, 2014

Abstract

A new emerging shrimp disease known as acute hepatopancreatic necrosis syndrome (AHPNS) has been reported to cause significant losses among shrimp farms in China (2009), Vietnam (2010), and Malaysia (2011). Recently, it has been reported to affect shrimp (*Penaeusmonodon* and *P. vannamei*) in the eastern Gulf of Thailand (2012). The disease is characterized by mass mortalities (reaching up to 100% in some cases) during the first 20-30 days post-stocking in grow-out ponds. The apparent spread of AHPNS throughout the region suggests that infectious or at least biological agent may be involved. Shrimp suffering from AHPNS show significant atrophy of hepatopancreas (HP), pale to white HP due to pigment loss in the connective tissue capsule and guts with discontinuous or no contents. The purpose of this study was to identify if bacteria could be the cause of AHPNS. DNA samples were prepared either directly from hepatopancreatic tissue or from culturable bacteria isolated from hepatopancreas of the diseased shrimp as templates to specifically amplify 770 bp fragment of 16S rRNA gene for bacterial classification. Nucleotide sequences obtained from the direct DNA extraction of the hepatopancreas revealed majority of *Vibrio* species, including *V. parahaemolyticus, V. harveyi, V. vulnificus,* and *V. chaqassi.* Similarly, the consensus sequences obtained from nine culturable bacterial isolates were identified as *V. parahaemolyticus.* The healthy shrimps challenged with the selected *Vibrio* isolate at a dose of 10^3 CFU per shrimp showed high mortality within 6 h after injection, however AHPNS histopathologywas not observed.

Keywords: Penaeid shrimp, acute hepatopancreatic necrosis syndrome (AHPNS), 16S rRNA gene, *Vibrio* species.

[1] *Department of Biotechnology, Faculty of Science, Mahidol University, Rama VI Rd., Bangkok, 10400, Thailand.*

[2] *Center of Excellence for Shrimp Molecular Biology and Biotechnology, Faculty of Science, Mahidol University, Rama VI Rd., Bangkok, 10400, Thailand. Tel: 0-2201-5878; Fax: 0-2354-7344; E-mail: siripong.thi@mahidol.ac.th.*

3 *Shrimp-Virus Interaction Laboratory, National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, Rama VI Rd. Bangkok, 10400, Thailand*

[*] *Corresponding author*

Introduction

The pacific white leg shrimp, *Penaeus* (*Litopenaeus*) *vannamei* is the world's most extensively cultivated species of penaeid shrimp. The rapid expansion of the shrimp farming industry in Thailand has been suffering from many serious infectious diseases such as white spot syndrome virus (WSSV), yellow head virus (YHV), etc. Recently, a new emerging disease known as Early Mortality Syndrome (EMS), descriptively called as Acute Hepatopancreatic Necrosis Syndrome (AHPNS), has been a major issue of concern for economic loss in the shrimp farming industry. EMS was officially reported in Southern China (2009), Vietnam and Malaysia (2011) and reached the eastern coast of Thailand in late 2011. In early 2012, EMS/AHPNS was reported mostly in the coastal provinces of Thailand including Rayong, Chantaburi, Chachoengsao, and Prachuap Khiri Khan. Both *Penaeusmonodon* and *Penaeus vannamei* are susceptible to this disease. As the name suggested, the disease causes high and rapid mortality in 20 to 30 days post-culture shrimp in the grow-out ponds of eastern provinces. Shrimp suffering from EMS/AHPNS show significant atrophy of hepatopancreas (HP), pale to white HP due to pigment loss in the connective tissue capsule, often soft shells and discontinuous or no gut contents. Some pathological features include enlarged hepatopancreatic nuclei, sloughed HP cells–blister-like (B), fibrilla (F), and resorptive (R) cells and frequently followed by secondary bacterial infections (www.enaca.org). However, the potential cause(s) of the disease is still under investigation.

The purpose of this study was to identify if the bacteria could be the causative agent (s) of EMS/AHPNS through microscopic examination, sequence analysis of bacterial isolates, and challenge tests.

Materials and Methods

Sample Collection

P. vannameisuffering from EMS were collected from KO shrimp farm (KO represents name code for the particular farm) at Sam RoiYod, PrachuapKhiri Khan province. Shrimps were transported to the laboratory in plastic containers with proper aeration and temporarily reared indoor using 48×70×41 cm plastic tanks. Upon arrival to the laboratory, some shrimp specimens were immediately fixed in Davidson's fixative(32% EtOH, 22% formalin and 11% acetic acid in distilled water) and processed for histological examination.

Bacterial Identification by 16S rDNA Analysis

The HP of each individual lived EMS shrimps were removed by using sterile scissors and subsequently excised into two pieces. One piece of the tissue was ground in lysis buffer for DNA extraction. The second piece of hepatopancreatic tissue was used for bacterial isolation on the agar plate (section 2.3). DNA was extracted from the frozen HP tissue and used as templates for PCR amplification with16S rRNA gene-specific primers 40F: gCCTAACACATg CAAgTCgA and 802R: gACTACCAggg TATCTAATCC. The amplification condition was performed by pre-denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 30 sec, 55°C for 30 sec, 72°C for 1 min, and a final elongation step at 72°C for 10 min. Amplified PCR products were cloned into pGEM-T EASY Vector (Promega) and transformed into *E. coli* JM109. The transformed cells were plated on LB agar containing 50 µg/ml ampicillin. The positive clones were confirmed by colony PCR and 50 clones were picked for sequencinganalysis (Macrogen, Korea). The nucleotide sequences data were subjected to BLAST sequence analysis against NCBI databases for bacterial identification.

Culturable Bacterial Identification by 16S rDNA Analysis

The excised HP was disaggregated and streaked on tryptic soy agar plates (TSA supplemented with 1.5% NaCl) using sterile

loop. The streaked plates were incubated at 30°C for 16 h to allow the culturable bacteria to grow. Each individual colony obtained were re-streaked to obtain the pure isolate before storing in glycerol stocks at -80°C for later use. The culturable bacteria obtained were identified by 16S rDNA analysis. DNA was extracted from the pure bacterial isolates using phenol-chloroform extraction method. The DNA template was amplified by PCR reaction according to the conditions mentioned elsewhere. The amplified PCR products were analysed for their nucleotide sequences by Macrogen, Korea and subjecting to BLAST sequence analysis against NCBI databases for bacterial identification.

Bacterial Challenge Test

For bacterial preparation, the selected *V. parahaemolyticus* isolate, KO HP03 (HP03) was cultured in tryptic soy broth (TSB) containing 1.5% NaCl at 30°C with vigorous shaking until reaching OD600 of 0.6-0.8. The bacterial cells were collected at 3000 rpm for 10 min prior to diluting with 0.1 M PBS (pH 7.4) at the concentration of 10^3 CFU per 50 µl.

The specific pathogen free (SPF) *P. vannamei* shrimps with body weight of 12-15 g (Syaqua, Thailand) were transported to the lab and reared temporarily in plastic containers for 4 days before challenge test. For challenge trials, 20 shrimps each were used for either injection with 1) HP03 bacteria or 2) phosphate buffer saline (PBS) control, with two replicates of 10 shrimps in each tank. Shrimps were intramuscularly injected with HP03 into the third abdominal segment at concentration of 10^3 CFU per shrimp. The equal volume of PBS (50 µl) was injected into control shrimps at the same position. The shrimps were observed for the histopathological signs of EMS/AHPNS and recorded for mortality rate after injection.

Construction of Phylogenetic Trees

Multiple sequences alignment by MUSCLE was used for phylogenetic tree construction in MEGA 5 with Neighbor-Joining method (5000 bootstrap replicas). For the culturable bacteria samples, consensus sequences used for multiple sequences alignment were generated by CAP3. The average read-length of 16S rRNA amplified gene product was 770 bp.

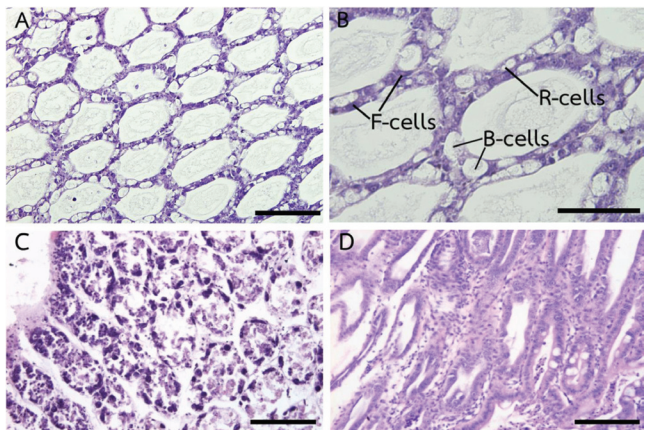

Figure 1. Hepatopancreatic tissues obtained from *P. vannamei* showing an intact morphology of B, F, and R cells in hepatopancreatic lobes (A and B). Histological changes in the hepatopancreas of shrimp affected by EMS/AHPNS (C and D). Hepatopancreatic defects showing sloughed B, F and R cells (C). Haemocytic infiltration (D). Hepatopancreatic sections in A-B and C-D were obtained from SPF and EMS/AHPNS shrimps harvested from KO farm, respectively. Scale bars, 200 µm (A, C and D), 100 µm (B)

Results

Histopathological Analysis

Histological analysis of the samples obtained from the KO farm at Sam Roi Yot, Prachuap Khiri Khan province showed obvious histological lesions of typical AHPNS symptoms including sloughed hepatopancreatic cells (Figure 1(c)) and followed by haemocytic infiltration (Figure 1(d)) as compared to the normal HP (Figure 1(a, b). In addition to the histological analysis, the diseased shrimps harvested from KO farm were parallelly processed for further bacterial isolation and identification.

Identification of the Bacterial Species in Diseased HP by Sequence Analysis of 16S rRNA Amplified PCR Products

Among 50 clones, 37 clones were identified as *V. parahaemolyticus*, while 3 clones were as *V. vulnificus, V. harveyi,* and *V. chaqassi*. The remaining 10 clones were excluded from BLAST analysis due to non-specific sequences and short-read length. Phylogenetic tree was generated and shown in Figure 2(a).

Identification of Culturable Bacteria Isolated from Diseased HP

After 16 h of incubation at 30°C, the bacterial colonies with white and opaque appearance appeared on non-selective TSA plates were randomly picked and cultured on thiosulfate citrate bile salt sucrose (TCBS) for selective screen of the Vibrio species. All tested bacterial colonies were able to grow on TCBS and identified as gram-negative rod-shaped bacteria (data not shown). A consensus sequence of nine bacterial isolates was produced from the sequence information of 3-4 clones per each isolate. The sequence homology of each isolate was identified as *V. parahaemolyticus*. The phylogenetic construction revealed a possible sub-grouping of *V. parahaemolyticus* among the nine isolates (Figure 2(b)).

Confirmation of the Causative Pathogen

In order to identify whether the *V. parahaemolyticus* isolates obtained form KO farm was the causative pathogen, SPF shrimps were collected and divided into two groups. The SPF shrimps were randomly

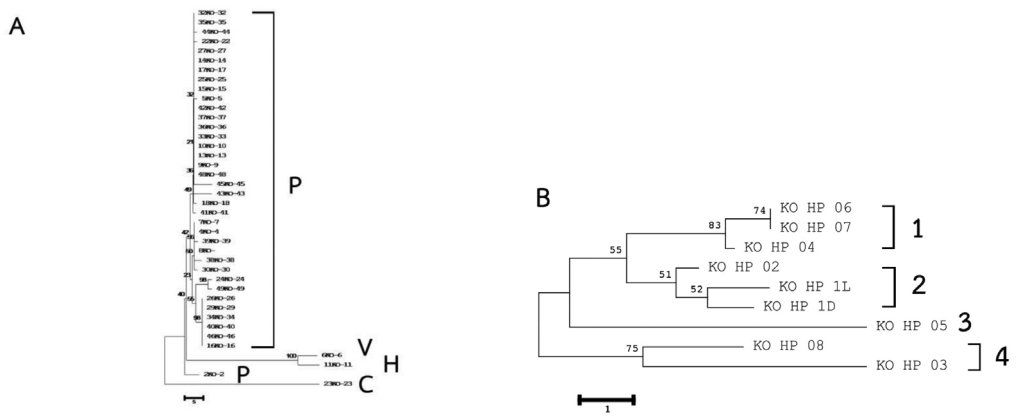

Figure 2. Phylogenetic tree of bacterial species in diseased HP by sequence analysis of 16S rRNA gene amplified PCR products (A). Phylogenetic tree of culturable bacteria isolated from diseased HP (B). Numbers at the branching point indicate percent bootstrap value. Numbers in bold indicate arbitrary bacterial grouping. The capital letters V, H, C, and P represent *V. vulnificus, V. harveyi, V. Chaqassi,* and *V. parahaemolyticus,* respectively. Scale bars denote number of nucleotide differences

injected by *V. parahaemolyticus* isolate HP03 (Figure 2(b)) and PBS for the HP03 treatment and the control, respectively. The shrimps injected with HP03 were died within 6 h after injection (19/20), whereas no mortality was observed in the shrimps injected with PBS. The HP of moribund (dying) shrimps was prepared for histological analysis. Pathological lesions found in the shrimp injected with *V. parahaemolyticus* showed partial collapse of the cell types which normally appeared in hepatopancreatic lobes (Figure 3(b)) as compared to control (Figure 3(a)). The histopathological results suggested that, by intramuscular injection, the selected isolate of *V. parahaemolyticus* is pathogenic to shrimp but unlikely to be the direct cause of EMS/AHPNS since there was no typical cellular defects unique to the disease.

Discussion

Vibrio species are the most frequent bacterial pathogens detected in the cultivated shrimp (Zhou *et al*., 2012). Our results showed that *V. parahaemolyticus* was the major bacterial population found in the diseased shrimps with the signs of EMS/AHPNS. However, due to highly conserved sequences of 16S rRNA gene among the *Vibrio* species, to gain a precise classification of the isolated bacteria at species level would be problematic. According to the phylogenetic construction of the culturable *Vibrio* isolates, we are currently examining the correlation between each phylogenetic sub-group and its ability to cause EMS/AHPNS in challenged shrimps. We initially examined the virulence of the selected HP03, which was able to cause high mortality at a dose of 10^3 CFU per shrimp within 6 h post injection, indicating that the isolates were highly virulent and lethal. Although these Vibrioswere isolated from shrimp with AHPNS, the injection of the selected bacteria did not induce EMS/AHPNS in the challenged shrimps.

Based on the histological analysis of EMS/AHPNS pathology, a series of infectivity studies were designed to identify the source and mode of infection (Tran *et al*., 2013). Since the challenge test by intramuscular injection could not reproduce EMS/AHPNS, natural transmission routes might be crucial for induction of the typical EMS/AHPNS pathology in hepatopancreas. Recently, Lightner *et al*. (2012) has identified the EMS/AHPNS pathogen as a unique strain of a relatively common bacterium, *V. parahaemolyticus,* by immersion route. Therefore, immersion assay could be a promising way to reproduce EMS/AHPNS with our *Vibrio* isolates as well.

Conclusions

The causative bacterial agent (s) of EMS/AHPNS is still under investigation. Sequencing results of 16S rRNA gene from the diseased shrimp revealed majority of *V. parahaemolyticus*. Intramuscularly injection of the selected

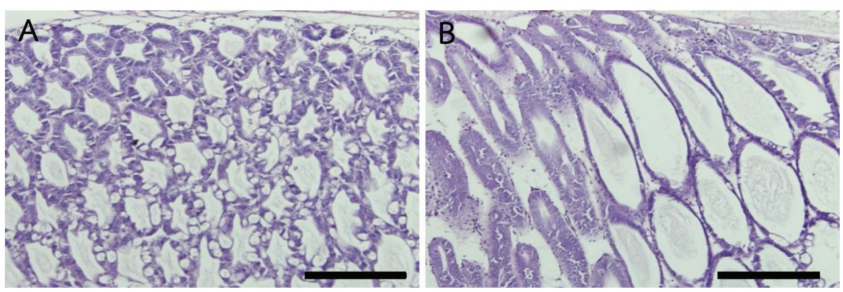

Figure 3. A typical histology of shrimp HP injected with PBS, the control (A) and with selected *V. parahaemolyticus,* the HP03 treatment (B). Scale bars, 200 µm

V. parahaemolyticus isolate could not induce EMS/AHPNS in naïve shrimp.

Acknowledgements

This work was supported by grants from National Research Council of Thailand and Department of Biotechnology, Mahidol University. We also thank Syaqua Thailand for the experimental materials.The authors would like to convey special appreciation to the academic committee of 25th Annual Meeting of Thai Society for Biotechnology and International Conference (TSB2013) for providing opportunity for this work to be published in this journal.

Referances

Flegel, T.W. (2012). Historic emergence, Impact and current status of shrimp pathogens in Asia. J. Invertebr. Pathol.110:166-173.

Lightner, D.V., Redman, R.M., Pantoja, C.R., Noble, B.I., and Tran, L. (2012). Early mortality syndrome affects shrimp in Asia. Global Aquaculture Advocate. 15:40.

Tran, L., Nunan, L., Redman, R.M., Mohney, L.L., Pantoja, C.R., Fitzsimmons,K., and Lightner, D.V. (2013). Determination of the infectious nature of the agent of acute hepatopancreatic necrosis syndrome affecting penaeid shrimp. Dis Aquat Organ, 105:45-55.

Zhou, J., Fang, W., Yang, X., Zhou, S., Hu, L., Li, X., Qi, X., Su, H., and Xie, L.(2012). A Nonluminescent and Highly Virulent Vibrio harveyi Strain Is Associated with "Bacterial White Tail Disease" of *Litopenaeus vannamei* Shrimp. PLOS ONE. 7(2): e29961.