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

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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.

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

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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 × 70 × 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.

2.7. PCR detection of AHPND bacteria

All 5 isolates used in this study were tested with an interim PCR detection method (AP2) for AHPND bacteria that was recently released to the public (http://www.enaca.org/modules/library/publication.php?publication_id=1128). The method was based on discovery of unique DNA sequences of *V. parahaemolyticus* AHPND isolates when compared to the DNA of a non-AHPND *V. parahaemolyticus* isolate. The forward (F) and reverse (R) primer sets were AP2F: 5′-TCACCCGAATGCTCGCTTGT GG-3′; and AP2R: 5′-CGTCGCTACTGTCTA GCTGAAG-3′. The cycling conditions were 5 min at 94 °C, followed by 25–30 cycles of 30 s denaturation at 94 °C, 30 s annealing at 60 °C and 60 s extension at 72 °C, plus a final 10 min extension at 72 °C. The amplified PCR products were analyzed in 2% agarose gels, stained with ethidium bromide, and visualized under UV transillumination. AHPND positive samples give a positive band at approximately 700 bp.

2.8. Statistical analysis

In the immersion challenge experiment, mean times to death of shrimp challenged with the test bacteria were compared by ANOVA followed by all pairwise multiple comparisons using the Holm–Sidak method (SigmaPlot 12.0, Systat software). Differences with $p \leq 0.05$ were considered statistically significant. Regression analysis was used to determine the bacterial density that caused 50% lethal dose (LD_{50}) at day 2 post-immersion.

3. Results

3.1. Bacteria from AHPND-positive shrimp identified as V. parahaemolyticus

From 9 bacterial isolates obtained from the HP of individual shrimp specimens from the AHPND outbreak farm by streaking on TSA plates, 4 were arbitrarily selected and named as 1D, 2HP, 3HP and 5HP. They were examined for growth on thiosulfate citrate bile salt sucrose (TCBS) agar and all were able to grow and to produce green colonies of gram-negative, rod-shaped bacteria (data not shown). Analysis of the 16S ssu rRNA gene sequences of these 4 isolates and the 2 reference isolates gave the highest identity (99%) to *V. parahaemolyticus*. This was further supported by positive PCR results obtained with all 6 isolates for the lecithin-dependent hemolysin (*ldh*) gene that is considered to be a species-specific marker for *V. parahaemolyticus* (FAO/WHO, 2011; Shinoda et al., 1991; Taniguchi et al., 1985, 1986). However, all 6 isolates gave negative PCR results for the two human pathogen markers thermostable direct hemolysin (*tdh*) and *tdh*-related hemolysin (*trh*) (Table 1).

3.2. Results of injection challenge with 2 isolates

Only two isolates (1D and 3HP) were arbitrarily selected to examine whether they could induce AHPND in shrimp challenged by injection of 10^3 colony forming unit (cfu) per shrimp into the third abdominal segment. An equal volume of PBS (50 μl) was injected at the same position into the control shrimp group. Mean mortality in two replicates of shrimp injected with isolate 1D was 9.5/10 and with isolate 3HP was 8.5/10 but the difference was not statistically significantly ($p = 0.187$) (Table 2). Most of the mortality occurred within the first 48-hour post-injection (hpi) and there was no mortality up to 144 hpi in the control shrimp injected with PBS. These results indicated high virulence for the injected bacteria. However, histological sections of HP obtained from moribund shrimp injected with 1D or 3HP showed partial collapse of the HP tubule epithelium into undifferentiated cells but did not show massive sloughing of cells characteristic of AHPND (Fig. 1C, D). Thus, 1D and 3HP were highly pathogenic to shrimp but did not cause AHPND by injection challenge.

Table 1
PCR detection results for lecithin-dependent hemolysin (*ldh*), thermostable direct hemolysin (*tdh*), and *tdh*-related hemolysin (*trh*) genes in our bacterial isolates.

Bacterial isolates	LDH	TDH	TRH
1D	+	−	−
2HP	+	−	−
3HP	+	−	−
5HP	+	−	−
China	+	−	−
S02	+	−	−

3.3. Results of immersion challenge with the 6 isolates

All 4 isolates from the AHPND outbreak were used, together with the reference isolates "China" and the reference isolate *V. parahaemolyticus* (S02) isolated from shrimp pond sediment before the reports of AHPND in Thailand. As in the injection challenge tests, the purpose of immersion challenge was to assess whether the isolates would cause histopathology characteristic of AHPND. There was no mortality in the broth negative control or in the test using S02. However, the immersion challenge produced the unique lesions characteristic of AHPND (Tran et al., 2013) with isolates 1D, 3HP and 5HP (Fig. 2C, E and F) and with the reference isolate China, but not with 2HP (Fig. 2D). Despite this difference, mortality was high for all 5 isolates, although the rate differed, in that it reached 100% for 3HP, 5HP and China in 24 h and for 1D in 96 h while mortality reached only 75% for 2HP at 96 h (Fig. 3). In addition to differences in mortality rate, isolate 1D also induced immediate and vigorous jumping of the test shrimp at immersion exposures down to 10^5 cfu/ml, a feature not reported so far for AHPND bacteria, but indicating some effect on the shrimp nervous system. All these results suggested differences in the pathology and virulence among the AHPND isolates (see below) and between them and 2HP. There was no mortality in the control groups, immersed in water containing TSB medium or S02 and the histology of the shrimp in these groups was normal (Fig. 2A, B).

To test for significant differences in the rate of mortality among the 4 AHPND isolates (i.e., 1D, 3HP, 5HP and China), one-way analysis of variance (ANOVA) was used to compare the mean times to death for shrimp exposed to them. There was a statistically significant difference ($p = 0.015$) among the means, but all pairwise multiple comparison using the Holm–Sidak method revealed that there was no significant difference between the means for 3HP, 5HP and China but that all three were significantly different from the mean for 1D. Although 2HP could not be included in the analysis because mortality did not reach 100%, it clearly differed from the other 4 isolates in this respect. Isolate 2HP also differed from 1D, 3HP, 5HP and China in not causing the severe hepatopancreatic necrosis diagnostic for AHPND but instead causing the collapse of HP tubule epithelia (the same as in the injection challenge with 1D and 3HP). It also induced the formation of numerous vacuoles in E-cells (Fig. 2D), in contrast to normal, non-vacuolated E-cells such as those seen in Fig. 2A.

Table 2
Shrimp mortality after injection challenge (n = 10) in 2 replicates with 10^3 cfu of 1D and 2HP. The difference in mean mortality was not significant ($p = 0.184$) by Student's t-test.

HOUR	Control	1D (1)	1D (2)	3HP (1)	3HP(2)
0	0	0	0	0	0
24	0	10	9	7	8
48	0	0	0	1	0
72	0	0	0	0	0
96	0	0	0	0	0
120	0	0	0	0	0
144	0	0	0	1	0
Total dead	0	10	9	9	8
Means	0	9.5		8.5	
SD	–	0.5		0.5	

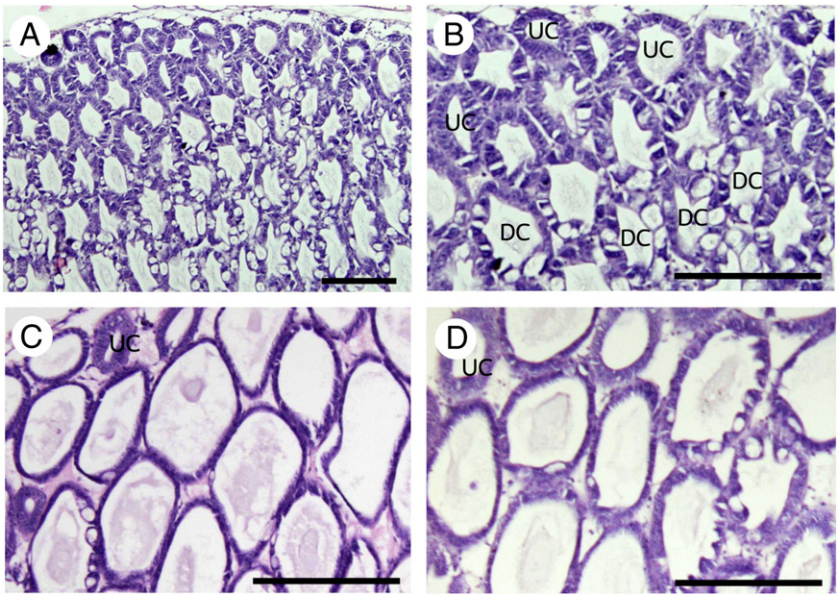

Fig. 1. Hepatopancreas of *P. vannamei* injected with 1D or 3HP bacteria showed non-AHPND histopathology. Histological sections of control shrimp injected with PBS (A–B) or moribund shrimp injected with 1D (C) or 3HP (D) at 6 hpi. Higher magnification shows undifferentiated cells (UC) and differentiated cells (DC) (B). Scale bars, 200 μm.

Overall, the results indicated that a variety of *V. parahaemolyticus* isolates could be obtained from a single Thai shrimp farm exhibiting early mortality, including 100% lethal isolates that caused mortality at different rates and accompanied by typical AHPND pathology, plus a less lethal isolate that did not cause AHPND pathology.

3.4. Dose-dependent manner of the AHPND inducing 5HP isolate

Although AHPND was reported to be induced in healthy shrimp via either cohabitation (27/60) or water (16/60) obtained from AHPND-infected ponds (FAO, 2013), our unpublished work on attempted

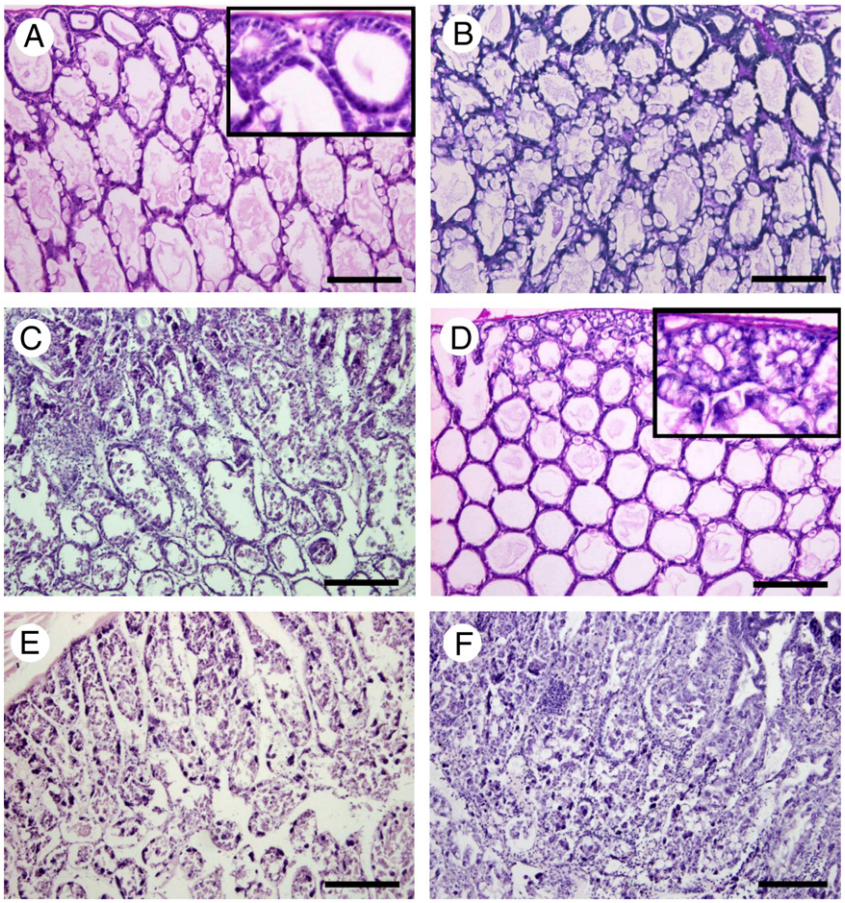

Fig. 2. Hepatopancreatic morphology of *P. vannamei* in 5-day-immersion challenges with the specific strains of *V. parahaemolyticus*. The sections obtained from shrimp immersed with 1% TSB control (A) or 10^6 cells/ml of S02 (B), 1D (C), 2HP (D), 3HP (E) and 5HP (F) bacteria. The insets show undifferentiated cells. Scale bars, 200 μm.

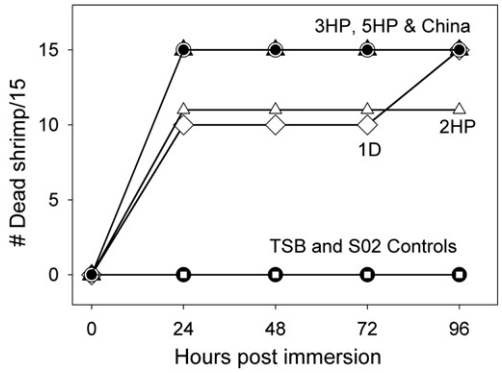

Fig. 3. Mortality after immersion challenge of 4 isolates from an AHPND outbreak farm (1D, 2HP, 3HP and 5HP), a reference AHPND isolate (China) and a control isolate of *V. parahaemolyticus* (S02) obtained from Thai shrimp pond sediment in 2010 and 2008, respectively.

transmission of AHPND by feeding healthy shrimp with HP or midgut tissue of AHPND shrimp was inconclusive. Thus, we reasoned that the threshold concentration of the challenge bacteria might be crucial for causing the disease in shrimp. To test the hypothesis, isolate 5HP was used in direct immersion challenges with bacterial concentrations of 10^6, 10^5, 10^4 and 10^3 cfu/ml (i.e., without a pre-challenge for 15 min at 10^8 cfu/ml). The cumulative mortality in the first 2 days after challenge was proportional to the concentration of bacteria (Table 3 and Fig. 4A) and regression analysis revealed that the median lethal dose (LD_{50}) of 5HP isolate was 10^5 cfu/ml ($R^2 = 0.99$) (Fig. 4B). The moribund shrimp challenged with 10^6, 10^5 and 10^4 of 5HP showed clear signs of AHPND histopathology (Fig. 5A–C) similar to those shown in Fig. 2C, E and F. On day 3 after challenge, there was 20% mortality in the group treated with 10^3 cfu/ml but this was not associated with AHPND pathology. Instead, the moribund shrimp showed collapsed HP tubule epithelia without vacuolization in the E-cell region (Fig. 5D).

3.5. PCR detection of AHPND bacteria

Using the AP2 primer set, all 4 of our isolates from the shrimp farm experiencing an AHPND outbreak (including 2HP) and the reference isolate China gave positive PCR test results (bands at approximately 700 bp) while the non-AHPND *V. parahaemolyticus* obtained from shrimp pond sediment in 2008 gave a negative result (not shown).

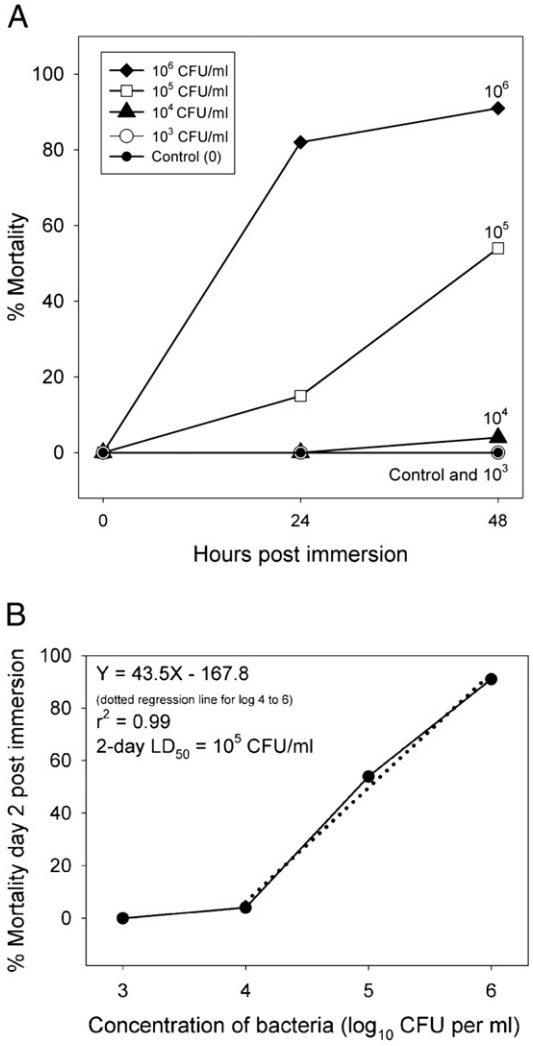

Fig. 4. (A) Cumulative mortality within 2 days after direct immersion challenge of shrimp with a single bacterial isolate (5HP) at different concentrations. (B) Determination of 2-day LD_{50} concentration of bacteria using the data from (A) and calculated from the regression line (dotted) for the interval of the first concentration to give mortality (10^4 cfu/ml) to the highest concentration used (10^6 cfu/ml).

4. Discussion

The results from this research revealed that isolates of *V. parahaemolyticus* that cause AHPND may differ in virulence and must also be present at a sufficient threshold dose to cause rapid, high mortality accompanied by histopathological signs of acute hepatopancreatic necrosis consistent with the case definition of AHPND (i.e., massive sloughing of HP tubule epithelial cells). At doses lower than the threshold, mortality may be low or absent, and histology of the HP may show collapsed tubule epithelia comprised of undifferentiated cells without signs of sloughing. In some cases, this may be accompanied by abnormal, highly vacuolated E-cells. Although it was not tested, it is possible that shrimp at this stage of pathology might be able to recover under suitable management conditions.

Table 3
Dose dependence for mortality in immersion challenges with isolate 5HP.

Bacterial density (cells/ml)	% cumulative mortality		
	Day 1	Day 2	Day 3
10^6	82	91	100
10^5	15	54	54
10^4	4	4	13
10^3	0	0	20
Control	0	0	0

Although our 3 bacterial isolates (1D, 3HP and 5HP) and one reference isolate (China) that caused AHPND in this study were identified as *V. parahaemolyticus* by commonly recognized methods, differences in the rate of mortality and shrimp behavior caused by them not only suggests that they differed in virulence but also reflects the variation among these bacterial isolates. Unknown variables such as shrimp health status, gut microflora of individual shrimp, etc., may increase or decrease the virulence of AHPND isolates. LD_{50} comparisons from experiments using isolates under different environmental conditions and in combination with other bacterial isolates would be needed to measure such parameters. If combination with other bacterial isolates has a significant effect on virulence, variation in the microflora of the stomach and midgut of various batches of test shrimp might affect the results of experiments done at different times. In any case, determining the basis for any differences in virulence among isolates will probably depend on comparison of their proteomic and genetic profiles.

It is curious that isolate 2HP did not cause AHPND pathology, even though it gave a positive result with the AP2 method designed for detection of AHPND bacteria. Since this isolate did cause mortality using the immersion challenge at 10^8 cfu/ml followed by 10^6 cfu/ml (Tran et al.,

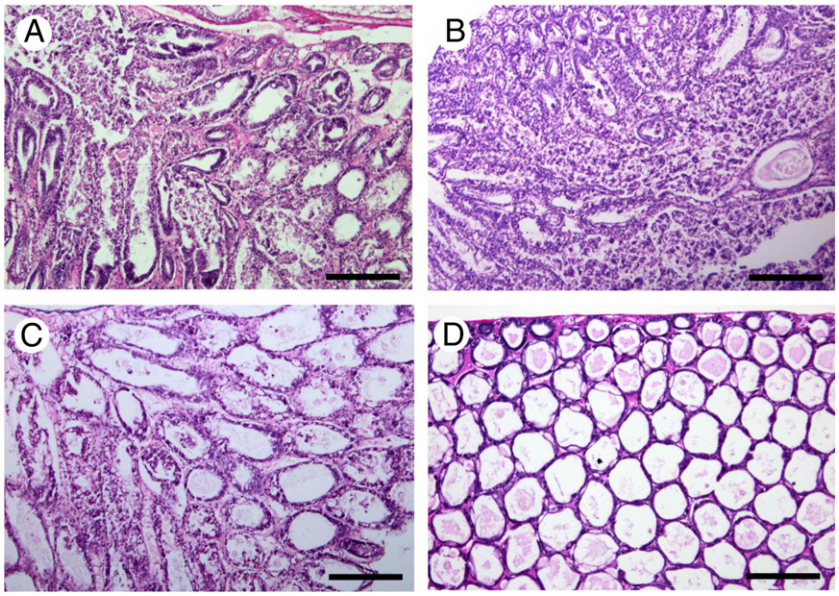

Fig. 5. Dose-dependent induction of AHPND in hepatopancreatic cells of *P. vannamei* by 5HP strain. The sections were obtained from shrimp immersed with 10^6 (A), 10^5 (B), 10^4 (C), and 10^3 (D) cells/ml of 5HP strain, respectively. Scale bars, 200 μm.

2013) and since the moribund shrimp showed flatten HP tubule epithelia similar to that of moribund shrimp subjected to immersion challenge by isolate 5HP at 10^3 cfu/ml, it is possible that 2HP is related to AHPND isolates but has lower virulence than the isolates that caused characteristic AHPND histopathology. Again, molecular analysis will be required to verify this possibility and explain any differences in virulence. A feature that counters the previous argument is the appearance of highly vacuolated E-cells in the HP of shrimp challenged with 2HP, since these were not seen in shrimp that received the low dose challenges of 5HP. This phenomenon may be a unique feature of 2HP and may indicate that it possesses different virulence factors from AHPND bacteria. If so, the positive test result with AP2 would need to be explained. We could find no other reports of the vacuolated E-cell anomaly in shrimp or crabs, so it is difficult to speculate on its meaning and significance, except to state that it is associated with immersion challenge with 2HP.

In conclusion, we found bacterial isolates that can cause AHPND pathology in specimens from a single Thai shrimp farm exhibiting high, early mortality, and we have shown that these isolates resemble *V. parahaemolyticus* isolates that were first reported to cause AHPND from Vietnam (Tran et al., 2013). However, there are indications that our isolates differ in virulence, and we suspect that isolate 2HP may be an extreme example of a variant with relatively low virulence, since it did not cause AHPND histopathology but did give a positive result with a recently introduced PCR method designed for detection of AHPND bacteria. If this turns out to be the case after detailed genetic and molecular analysis, the case definition of AHPND may have to be modified somewhat to accommodate such isolates lest they be overlooked or their importance underrated. Study of such isolates may also be useful in better understanding the basis of virulence in AHPND bacteria.

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