Factors Affecting Mortality of Shrimp, *Penaeus monodon*, Experimentally Infected With *Vibrio parahaemolyticus* Causing Acute Hepatopancreatic Necrosis Disease (VP_{AHPND})

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Abstract

One of the most recent diseases affecting the shrimp industry is the early mortality syndrome (EMS). EMS, characterized by observed mortality in shrimp within the first 35 days of culture, is due to several diseases, one of which is the acute hepatopancreatic disease (AHPND). Outbreaks due to AHPND have caused economic losses to many shrimp producing countries globally. This paper investigates factors affecting mortality of shrimp, *Penaeus monodon* experimentally infected with *Vibrio parahaemolyticus* causing AHPND (VP_{AHPND}).

Tank experiments done suggested that exposure to 10^7 cfu/ml VP_{AHPND}, 35 °C, and 10 and 28 ppt increase the risk of shrimp mortality due to AHPND. The VP_{AHPND} concentration in the water that *P. monodon* can overcome is <105 cfu/ml. Observed mortality due to VP_{AHPND} is age related, with higher mortalities in younger infected shrimp.

Introduction

One of the most recent diseases affecting the shrimp industry is the early mortality syndrome (EMS). EMS is characterized by observed mortality in shrimp within the first 35 days of culture. It is due to several diseases, one of which is the acute hepatopancreatic necrosis disease (AHPND) caused by a virulent strain of *Vibrio parahaemolyticus* (Flegel, 2016). AHPND has been reported in China, India. Malaysia, Mexico, Philippines, Thailand and Viet nam (Flegel, 2012; Lightner et. al, 2012; Soto-Rodriguez *et al*., 2015; De la Pena *et al*., 2015). AHPND is caused by a unique strain of *Vibrio parahaemolyticus* (VP_{AHPND}) that has transferrable plasmid carrying the PU:AB-like toxin genes (Tran *et al*., 2013). Variation in *V. parahaemolyticus* isolates from shrimp experiencing AHPND outbreak has been reported (Joshi *et al*., 2014; Kumar *et al*., 2014; Kondo *et al*., 2014).

Outbreaks due to AHPND have caused economic losses to many shrimp producing countries globally. Mortalities due to AHPND have been reported to occur 10–30 days after stocking of post larvae (PL) in ponds (Joshi *et al*., 2014b; Leaño and Mohan, 2012; Soto-Rodriguez *et al*., 2015). The disease can cause up to 100 % cumulative mortality in affected PL within

a week. However, in older penaeids at DOC 46 and 96, lower mortalities of 40–60 % can be observed (de la Peña *et al*., 2015).

Several reports have identified farm level risk factors. Some of these are high salinity or salinity below 5 ppt; high and fluctuating temperature; and pH>7 (FAO, 2013; Bondad-Reantaso and Arthurs, 2018). Salinity of 20 ppt reduces disease incidence (http://www.agriculture.gov.au/pests-diseases-weeds/aquatic).

Infection studies were done to determine and understand the pathogencity of the VP_{AHPND} to healthy shrimp. Shrimp are infected by immersing them in high concentrations of VP_{AHPND} at high stocking density for 15 min and cultured in water with different concentrations of the bacteria (Tran *et al*., 2013; Lai, 2015). This method stresses the shrimp and is far from natural infection. In this study we investigated the VP_{AHPND} concentration in the water that can cause mortality in healthy *P. monodon* without subjecting them to stress, thus simulating natural infection. We also investigated two reported risk factors, salinity and temperature. Age related infection was also verified.

Materials and methods

Experimental shrimp

P. monodon (PL 20) were purchased from a local hatchery and stocked in a concrete tank with seawater and provided with aeration. Shrimp were fed with commercial pellet at 4 % body weight given twice daily until use. Shrimp were starved a day prior to the experiment. Shrimp were analyzed and tested negative using PCR for known shrimp viruses (white spot syndrome virus (WSSV), taura syndrome virus (TSV), infectious hypodermal hemapoietic necrosis virus (IHHNV), yellowhead virus (YHV), infectious myonecrosis virus (IMNV), monodonbaculovirus (MBV) and hepatopancreatic virus (HPV)) and AHPND before the pathogenicity/challenge experiments.

Bacterial isolate

Vibrio parahaemolyticus used in the experiment were from AHPND cases in the Philippines and confirmed to be the AHPND bacteria (dela Pena *et al*., 2015). The bacterium was sub-cultured in *Vibrio* Chromogenic Agar (VCA, Pronadisa) and harvested after 18–24 hours' incubation. Harvested bacteria were suspended in tryptic soy broth with 2 % NaCl added (TSB+, Merck). The bacterial count of the inoculum was confirmed by plating onto VCA; presence of AHPND was confirmed by PCR.

Investigation of factors affecting mortality in VP_{AHPND} infected shrimp

Effect of bacterial concentration and exposure method

Glass aquaria, 10 L capacity, were used for the experiment. Clean aquaria (washed with detergent and water) were sterilized by swabbing with cotton soaked in alcohol and covered with sterile-aluminum foil wrapped plywood. Aquaria were filled with 5 L of UV sterilized seawater, and provided with aeration. Plastic tubings, air stones, connectors and other paraphernalia were sterilized by pouring with boiled water prior to use.

Two infection methods were investigated using healthy *P. monodon* post larvae (PL): unimmersed and pre-immersed. In both methods, *P. monodon* PL (ABW=0.18+ 0.04 g) were stocked at 15 ind/aquaria. Three replicates were carried out for each concentration and control group.

Unimmersed. For this method, 21 aquaria were prepared as previously described and stocked with healthy shrimp. One hour after stocking, the prepared bacterial suspension was added into the treated aquaria to obtain the desired concentrations of 10^2, 10^3, 10^4, 10^5, 10^6 and 10^7 cfu/ml. Sterile TSB+ was added onto aquaria that served as the control.

Pre-immersed 1. For this method, healthy *P. monodon* PL were immersed in VP_{AHPND} 10^7 cfu/ml bacterial solution at 1 ind/ml for 15 min prior to stocking in 21 aquaria, prepared as previously described. One hour after stocking, the prepared bacterial suspension was added into the aquaria to obtain the desired concentrations of 10^2, 10^3, 10^4, 10^5, 10^6, 10^7 cfu/ml. Sterile TSB+ was added onto aquaria that served as the positive control.

Pre-immersed 2. This was done to confirm the results obtained in pre-immersed 1, wherein there is no significant difference in the mortality rate observed in all treatments including the control. The same methodology was followed except that two controls were used: positive and negative; and lower final bacterial concentrations of 10^2, $10^{3,}$ 10^4 cfu/ml were used to inoculate the aquaria. A total of 15 aquaria were prepared as previously described. Nine of the aquaria were inoculated with the VP_{AHPND} bacterial suspension to give lower final concentrations of 10^2, 10^3, 10^4 cfu/ml done in triplicates. PL were pre-immersed in 10^7 cfu/ml VP_{AHPND} bacterial solution at 1 ind/ml for 15 min prior and stocked in the 9 VP_{AHPND} inoculated aquaria and in 3 aquaria inoculated with sterile TSB+ which served as the positive control. For the negative control, *P. monodon* PL were immersed in UV sterilized seawater at 1 ind/ml for 15 min prior to stocking in three aquaria with UV sterilized seawater with TSB+ added; no bacteria were added into the aquaria. The experiments were terminated after 120 h.

Monitoring

Shrimp mortality was monitored daily. Samples were taken at 24, 72, 120 hpi for bacteriology and AHPND detection by PCR. For bacteriology, shrimp were rinsed 3x in sterile seawater and suspended in TSB for 1 h. After the 1 h incubation, the shrimp was homogenized and serially diluted. Representative serial dilutions were plated onto *Vibrio* chromogenic agar (VCA) for the presumptive *Vibrio parahaemolyticus* counts. Inoculated NA and TCBS plates were incubated for 24 h; VCA plates for 48 h.

Water samples were taken before stocking with shrimp, after adding bacteria and daily thereafter. Samples were serially diluted and plated onto VCA.

Effect of salinity and temperature

Fiberglass tanks, 120 L capacity filled with 50 L UV sterilized water were used in the experiment. Tanks were provided with aeration. Plastic tubing, air stones, connectors and other paraphernalia were sterilized by pouring with boiled water prior to use.

Tanks were stocked with *P. monodon* PL (ABW= 0.31+0.06) at 25 ind/aquaria. One day after stocking, salinity and temperature were gradually adjusted to the desired level. Water heater were used to increase water temperature. Ambient UV sterilized seawater was diluted with cartridge filtered freshwater in a flow through system to lower the salinity.

The salinity used were 10, 20 and 28 ppt at ambient temperature. Temperature used were 31 °C and 35 °C at 20 ppt salinity. Two sets of tanks for each treatment in triplicate were prepared. One set for uninfected and the other set, for infected. Desired salinity and temperature levels were attained after 3–4 days. One day

after attaining the desired salinity and temperature, shrimp were removed from each tank, one tank at a time. For the infected group, shrimp were immersed in 10^7cfu/ml VP_{AHPND} bacterial suspension at 1 shrimp/ml for 15 min. Uninfected group were immersed in UV sterilized seawater at 1 shrimp/ml for 15 min. After immersion, shrimp were returned to the tanks were they were originally stocked or were taken. The experiment was terminated after 120 h.

Effect of age

Glass aquaria (10 in x 6 in x 12 in) covered with sterile-aluminum foil wrapped plywood were used in the experiment. The aquaria were filled with 5 L UV sterilized seawater and provided with aeration.

Shrimp used in this study were from the same batch cultured in concrete tanks to attain the desired days of culture. The age/size used were 0.14 g at PL 27 and 1.3 g at PL 150. In this test, shrimp were immersed in 10^7 cfu/ml VP_{AHPND} bacterial suspension for 15 min at 1 shrimp/ml and cultured (15 PL/5L) in UV-sterilized water inoculated with VP_{AHPND} to a final concentration of 10^2 to 10^7 cfu/ml. Control shrimp were immersed in sterile seawater, no bacteria were added in the aquaria. The experiments were terminated after 120 h.

Statistical analysis

Significant difference in the cumulative mortality between treatments was determined using repeated measures in SPSS V 23.

Results

Effect of bacterial concentration and exposure method

In the unimmersed group, significantly higher cumulative mortality was observed in shrimp maintained in aquaria with 107 cfu/ml VP_{AHPND} (**Figure 1**). Cumulative mortality in shrimp maintained in tanks with 10^2 to 10^6 cfu/ml VP_{AHPND} and the control were not significantly different. AHPND was detected in shrimp samples from all treatment except the control at 24 and 72 hour post infection (hpi) (**Table1**). At 120 hpi, AHPND was detected only in shrimp maintained at 10^6 and 10^7 cfu/ml. *V. parahaemolyticus* were recovered only in shrimp maintained in water with 10^7 cfu/ml of the bacteria (**Table 1**). VP count

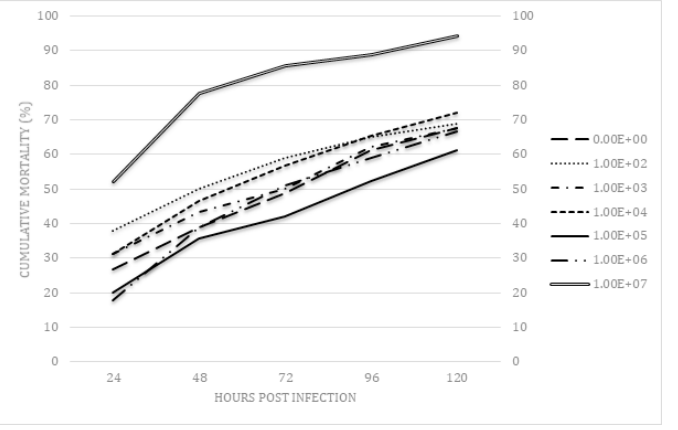

Figure 1. Cumulative mortality in shrimp directly stocked in water with different VP_{AHPND} concentration

in aquaria inoculated to 10^7 cfu/ml was significantly high than the other treatments including the control (**Table 1**). VP count in control aquaria was significantly low compared to treated ones.

In the pre-immersed 2 group, cumulative mortality in shrimp in the negative control was lower, but not significantly, compared to those pre-immersed in VP_{AHPND} (**Figure 2**). VP_{AHPND} was detected in shrimp pre-immersed in bacterial solution at 24, 72 and 120 hpi and maintained in water with 0 to 10^4 cfu/ml VP_{AHPND}, but not in those pre-immersed in sterile seawater (**Table 2**). VP count in the shrimp, including the control were not significantly different. VP count in the water was generally high in aquaria with 10^4 cfu/ml wherein the count increased to 10^5 cfu/ml.

Effect of salinity and temperature

Lower survival was observed in infected and uninfected shrimp maintained at 28 ppt and 10 ppt salinities compared to those at 20 ppt (**Figure 3**). Among the infected group, shrimp survival was significantly low (P>0.05) in those maintained at 28 ppt (54.89 %); highest survival was observed in those maintained at 20 ppt (78.70 %), followed by those at 10 ppt (69.45 %). No significant differences were observed in the mortality of uninfected shrimp maintained at different salinities.

In shrimp maintained at different temperatures, survival was significantly high in those maintained at 31 °C (70.29 %) compared to those at 35 °C (42.07 %) (**Figure 4**). No significant difference was observed in the uninfected group, although, survival was higher in those maintained at 31 °C.

Effect of shrimp age

Using repeated measures analysis (SPSS V.18), observed mortality among the older shrimp bathed in different bacterial concentrations including the control were not significantly different (P>0.05) (**Table 3**). In the younger shrimp, mortalities were significantly high in those bathed in 10^6 and 10^7 cfu/ml. Significantly lower mortalities were observed in the control group (P<0.05) (**Table 3**).

Table 1. Average cumulative shrimp mortality, AHPND detection in shrimp and average VP load of samples in the un-immersed group

Final VP concentration in the water (cfu/ml)	Average Cumulative Shrimp Mortality (%)	AHPND detection in shrimp			Average VP load	
		Hours post infection			Shrimp	Water
		24	72	120	(cfu/shrimp)	(cfu/ml)
10^2	57[a]	+	+	-	0[a]	2.68×10^{1b}
10^3	51[a]	+	+	-	0[a]	5.05×10^{1b}
10^4	54[a]	+	+	-	0[a]	8.54×10^{1b}
10^5	42[a]	+	+	-	0[a]	1.77×10^{1b}
10^6	47[a]	+	+	+	0 [a]	4.25×10^{1b}
10^7	80[b]	+	+	+	2.27×10^{0b}	2.7×10^{3c}
Control	49[a]	-	-	-	0[a]	1.99×10^{0a}

Table 2. Average cumulative shrimp, AHPND detection in shrimp, and average VP load of samples from the pre-immersed group

Final VP concentration in the water (cfu/ml)	Average Cumulative Shrimp Mortality (%)	AHPND detection in shrimp			Average VP load	
		Hours post infection			Shrimp	Water
		24	72	120	(cfu/shrimp)	(cfu/ml)
0	61[a]	+	+	+	2.19×10^2	1.37×10^{1a}
10^2	51[a]	+	+	+	1.48×10^2	1.76×10^{1a}
10^3	65[a]	+	+	+	1.81×10^2	3.21×102^{ab}
10^4	62[a]	+	+	+	1.41×10^2	1.37×10^{5b}
Control	31[a]	-	-	-	3×10^2	1.27×10^{1a}

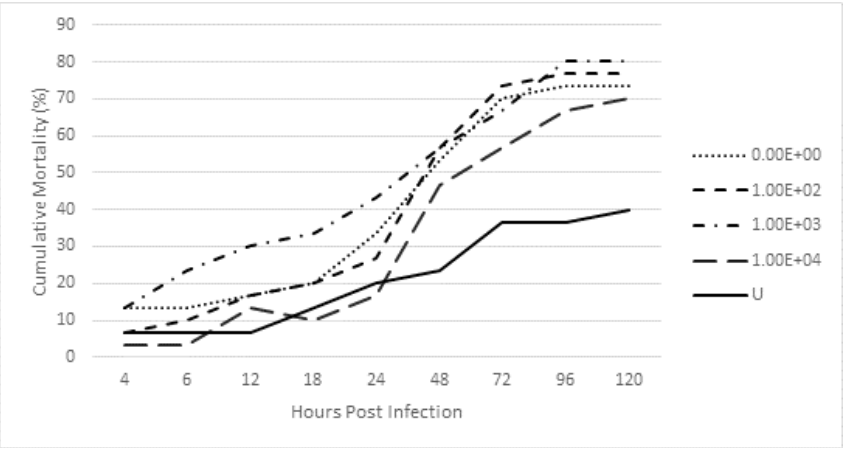

Figure 2. Cumulative mortality in shrimp pre-immersed in VP_{AHPND} prior to stocking in water with different VP_{AHPND} concentration

Discussion

Exposure to 10^7 cfu/ml VP_{AHPND} even for a few minutes is an important risk factor that can result in high mortality in healthy *P. monodon* PL as shown in the results of the infection method studies. The bacteria could have entered the shrimp system, multiply, produce the toxin which increased with the increase in bacterial number and overwhelmed shrimp immune response. This could have led to the irrevocable infection leading to high mortality even if shrimp are cultured in a clean water or water with low bacterial load after exposure. This is validated by the recovery of VP in shrimp cultured in 10^7 cfu/ml VP_{AHPND}, in this study, but not in those cultured in lower concentrations. Results also suggest that VP can multiply inside the shrimp and are excreted into the culture water. Furthermore, shrimp are able to survive VP_{AHPND} infection if exposed to concentrations lower than 10^7 cfu/ml. Results are in consonance with the report that the VP_{AHPND} concentration that the shrimp are exposed to is directly correlated with the mortality rate (Choi *et al*., 2017).

Table 3. Observed cumulative mortality in 2 age groups of *P. monodon* maintained in different concentrations of VP_{AHPND} at 120 hours post infection

Bacterial concentration (cfu/ml)	PL 27 (ABW=0.14)	DOC 150 (ABW=1.3 g)
Control	49[a]	18[a]
10^2	57[a]	35[a]
10^3	51[a]	40[a]
10^4	54[a]	38[a]
10^5	42[a]	29[a]
10^6	47[a]	34[a]
10^7	80[b]	41[a]

The threshold level for VP_{AHPND} bacteria, isolated from the Philippines, in the water that shrimp may overcome is $<10^5$ cfu/ml. This may be attributed to the decrease in the toxin present in the shrimp as implied in the detection of VP_{AHPND} at 24hpc and at 72hpc and not at 120 hpc in those cultured at 10^2-10^5 cfu/ml. The threshold level of the VP_{AHPND} from the Philippines is higher than the reported infectious level of the bacteria isolated from Mexico which is 10^4 cfu/ml (Soto-Rodriguez *et al*., 2015). Furthermore, they noted that different *V. parahaemolyticus* strains have different virulence.

Results confirm anecdotal reports of the effect of salinity and temperature on VP_{AHPND}. The higher mortalities observed in infected and uninfected shrimp maintained at 35°C is consistent with Selven and Philip (2012) who observed higher mortality in *V. harveyi* infected *Fenneropenaeus indicus* maintained at 35 ppt. Higher mortality in infected shrimp maintained at higher temperature confirms that high temperature is a risk factor for AHPND. This may be due to the synergistic effect of high temperature to both the shrimp and the pathogen. As earlier mentioned, higher mortality is observed at higher temperature in penaeids; at the same time, an increase in temperature increases the virulence of the toxic gene present in most bacteria (Guijarro *et al*., 2015). The same mechanism could explain for the effect of salinity on VP_{AHPND} infection in *P. monodon*. *V. parahaemolyticus* toxic genes are better expressed at higher salinity (Alamelu *et al*., 2019) at the same time that shrimp are stressed. The immune ability and disease resistance of *P. monodon* are reduced if transferred to high or low salinity levels (Wang and Chen, 2006). This confirms reports that high salinity is a VP_{AHPND} risk factor.

Mortalities due to VP_{AHPND} seems to be age related as shown in the observe mortalities in the present study. Exposure to as high as 10^7 cfu/ml did not cause mortality in shrimp at DOC 150, but high mortalities were observed in younger shrimp exposed to 10^6 and 10^7 cfu/ml. As shrimp gets older, in the absence of stressor, their susceptibility to VP_{AHPND} decreases as shown in the survival of infected shrimp in the two age groups. Results were in consonance with dela Peña *et al* (2015) who reported 40–60 % mortality in penaeids at DOC 46 and 96, respectively.

To summarize, exposure to 10^7cfu/ml VP_{AHPND}, high temperature (35 °C), and high salinity (28 ppt) increase the risk of shrimp mortality due to VP_{AHPND}. The VP_{AHPND} concentration in the water that *P. monodon* can overcome is $<10^5$ cfu/ml. Mortality due to VP_{AHPND} is age related, higher mortalities observed in younger infected shrimp.

It is recommended that stressors/risk factors that may enhance/stimulate the increase in bacterial population in environments with low bacterial load or toxin production be investigated. Possible preventive measures and treatments should be identified taking into consideration the synergistic effects of the identified stressors/risk factors and bacterial load of the environment.

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References

Alamelu V, Sony DMM, Santhosh K, Krishnakumar B, Venugopal MN. 2019. Effect of salinity on the expression of virulent T3SS Gene of *Vibrio parahaemolyticus*. Book of Abstracts: Asian -Pacific Aquaculture 2019. Chennai Tamil Nadu - India.

Bondad-Reantaso MG, Arthur JR. 2018. FAO Technical Assistance Efforts to Deal with Acute Hepatopancreatic Necrosis Disease (AHPND) of Cultured Shrimp. Asian Fisheries Science 31: 01–14

Choi M, Stevens AM, Smith SA, Taylor DP, Kuhn DD.. 2017. Strain and dose infectivity of *Vibrio parahaemolyticus*: the causative agent of early mortality syndrome in shrimp. Aqua Res 4 (7): 3719-3727.

D.V. Lightner, R.M. Redman, C.R. Pantoja, B.L. Noble, L.H. Tran. 2012. Early mortality syndrome affects shrimp in Asia Glob. Aquacult. Advocate, 40.

Daniels NA, Mackiman L, Bishop R, Altekruse S, Ray B, Hammond RM, Thompson S, Wilson, S., Bean, N.H., Griffin, P.M. and Slutsker, L. 2000. *Vibrio parahaemolyticus* Infections in the United States, 1973–1998. The Journal of Infectious Diseases 2000;181:1661–6

Dela Pena LD, Cabillon NAR , Catedral DD , Amar EC , Usero RC , Monotilla WD , Calpe AT, Fernandez DDG , Saloma CP. 2015. Acute hepatopancreatic necrosis disease (AHPND) outbreaks in Penaeus vannamei and *P. monodon* cultured in the Philippines. Diseases in Aquatic Organism 116: 251–254.

FAO. 2013. Report of the FAO/MARD Technical Workshop on Early Mortality Syndrome (EMS) or Acute Hepatopancreatic Necrosis Syndrome (AHPNS) of Cultured Shrimp (under TCP/VIE/3304). Hanoi, Viet Nam, on 25–27 June 2013. FAO Fisheries and Aquaculture Report No. 1053. Rome. 54 pp.

Flegel TW. 2012. Historic emergence, impact and current status of shrimp pathogens in Asia. J Invertebr Pathol.110(2):166-73

Flegel TW. 2016. Update June 2016 on AHPND and EHP research in Thailand. FAO Second International Technical Seminar/Workshop on Acute hepatopancreatic necrosis disease (AHPND) There is a way forward! FAO Technical Cooperation Programme: TCP/INT/3501 and TCP/INT/3502 Sukosol HotelBangkok, Thailand23-25 June 2016 pp. 45-46.

Guijarro JA, Cascales D, García-Torrico AI, García-Domínguez M, Méndez J. 2015. Temperature-dependent expression of virulence genes in fish-pathogenic bacteria. Front. Microbiol. 6:700.

Joshi J, Srisala J, Truong VH, Chen I-T, Nuangsaeng B, Suthienkul O, Lo CF, Flegel TW, Sritunyalucksana K, Thitamadee S. 2014. Variation in *Vibrio parahaemolyticus* isolates from a single Thai shrimp farm experiencing an outbreak of acute hepatopancreatic necrosis disease (AHPND). Aquaculture 428-429: 297- 302.

Kondo H, Tinwongger S, Proespraiwong P, Mavichak R, Unajak S, Nozaki R, Hirono I. 2014. Draft genome sequences of six strains of *Vibrio parahaemolyticus* isolated from early mortality syndrome/acute hepatopancreatic necrosis disease shrimp in Thailand, Genome Announc. 2.

Kumar BK, Deekshit VK, Raj JRM, Rai P, Shivanagowda BM, Karunasagar I, Karunasagar I. 2014. Diversity of *Vibrio parahaemolyticus* associated with disease outbreak among cultured *Litopenaeus vannamei* (Pacific white shrimp) in India. Aquaculture 433 (20): 247–251.

Lai HC, Ng TH, Ando M, Lee CT, Chen IT, Chuang JC, Mavichak R, Chang SH, Yeh MD, Chiang YA, Takeyama H, Hamaguchi HO, Lo CF, Aoki T, Wang HC. 2015. Pathogenesis of acute hepatopancreatic necrosis disease (AHPND) in shrimp. Fish Shellfish Immunol. 47(2):1006-14.

Leano EM, Mohan CV. 2012. Early mortality syndrome threatens Asia's shrimp farms. Global. Aquacult. Advocate (2012), pp. 38-39 (July/Aug 2012).

Selven S, Philip R. 2013. Salinity a significant environmental factor for *Vibrio* harveyi virulence in Fenneropenaeus indicus Aquaculture Research 44(5): 747-759.

Tran L, Nunan L, Redman RM, Mohney LL, Pantoja CR, *et al*. .2013. Determination of the infectious nature of the agent of acute hepatopancreatic necrosis syndrome affecting penaeid shrimp. Dis Aquat Organ 105: 45–55.

Wang FI, Chen JC. 2006. Effect of salinity on the immune response of tiger shrimp *Penaeus monodon* and its susceptibility to Photobacterium damselae subsp. damselae. Fish Shellfish Immunol. 20(5):671-81.

Soto-Rodriguez SA, Gomez-Gil B, Lozano-Olvera R, Betancourt-Lozano M, Morales-Covarrubias MS. 2015. Field and experimental evidence of *Vibrio parahaemolyticus* as the causative agent of acute hepatopancreatic necrosis disease of cultured shrimp (*Litopenaeus vannamei*) in Northwestern Mexico. Appl Environ Microbiol. 81(5):1689-99.