Asian Fisheries Science ***31S*** (2018): 283–292

ISSN 0116-6514
E-ISSN 2071-3720
https://doi.org/10.33997/j.afs.2018.31.S1.021

Asian Fisheries Science
The Journal of the Asian Fisheries Society

Identification and Characterization of *Vibrio* Bacteria Isolated from Shrimp Infected with Early Mortality Syndrome/acute Hepatopancreatic Necrosis Syndrome (EMS/AHPNS) in Viet Nam

DANG THI HOANG OANH[1,*], NGUYEN TRONG NGHIA[1], TRAN VIET TIEN[1] and MELBA G. BONDAD-REANTASO[2]

[1]Department of Aquatic Pathology, College of Aquaculture and Fisheries, Can Tho University, Viet Nam
[2]Aquaculture Branch (FIAA), Fisheries and Aquaculture Resources Use and Conservation Division (FIR), Fisheries and Aquaculture Department, Food and Agriculture Organization of the United Nations (FAO), Rome, Italy

Abstract

The outcomes of the work under FAO project TCP/VIE/3304 on the diagnosis of bacteria isolated from shrimp affected by early mortality syndrome/acute hepatopancreatic necrosis syndrome (EMS/AHPNS) in Viet Nam are presented. Field sampling was conducted in Soc Trang, Bac Lieu and Ca Mau provinces. At each sampling location, the targeted samples included three infected and three non-infected ponds. Disease signs at the pond level included pale hepatopancreas (HP), significant atrophy of the HP, guts with discontinuous contents or no content; onset of clinical signs and mortality starting as early as 10 days post stocking. At the end of the sampling campaign, shrimp from a total of 36 infected and 24 non-infected ponds had been collected. A total of 175 *Vibrio* isolates were isolated from the HP of shrimp specimens. A majority of isolates were identified as *Vibrio parahaemolyticus* by using API 20E kit and 16S rRNA sequencing. Thirty isolates of *V. parahaemolyticus* from each province were subjected to rep PCR analysis and detection of *Tlh*, *Tdh* and *Trh* genes. Rep PCR resulted in at least four different DNA profiles for the tested isolates. The *Tlh* gene was detected from all tested isolates, but neither the *Tdh* nor the *Trh* gene.

Keywords: acute hepatopancreatic necrosis syndrome, early mortality syndrome, *Vibrio parahaemolyticus*

[*]Corresponding author. E-mail address: dthoanh@ctu.edu.vn

Introduction

Early mortality syndrome (EMS)/acute hepatopancreatic necrosis syndrome (AHPNS) first appeared in farmed penaeid shrimp in coastal provinces of the Mekong Delta of Viet Nam in 2010. In 2011 and 2012, EMS/AHPNS continued to occur and caused serious mortality in farmed shrimp in the Mekong Delta, and also appeared on shrimp farms in some northern coastal provinces. The disease occurred all year round, with greatest severity from April to July. It affected farms culturing giant tiger prawns (*Penaeus monodon* Fabricius 1798) and whiteleg shrimp (*P. vannamei* Boone 1931), mainly in areas of intensive and semi-intensive shrimp farming. The incidence of AHPNS seemed to be higher in farms with high salinity and during the dry season when high temperatures occurred.

At the time, the Food and Agriculture Organization of the United Nations (FAO) project TCP/VIE/3304 (E): Emergency Assistance to Control the Spread of an Unknown Disease Affecting Shrimps was being implemented in Viet Nam by the Ministry of Agriculture and Rural Development (MARD). This disease was considered idiopathic, and it was not known whether the cause was an infectious agent or a toxin. Earlier hypotheses suggested a range of possible causes, such as cypermethrin (an insecticide), other pesticides, pollution, contaminated feed, parasites, harmful algae, probiotics and inbreeding.

Field sampling for bacterial isolation from EMS/AHPNS-infected ponds was carried out during the FAO project. The objective of this study was to identify and characterize bacterial strains isolated from EMS/AHPNS-infected shrimp from outbreak and non-outbreak farms in the Mekong Delta to obtain information on the isolated *Vibrio* spp. with regard to their biochemical and physiological characteristics and the presence of selected specific genes.

Materials and Methods

Sampling locations and sampling times

Sampling was conducted in three provinces of the Mekong Delta of Viet Nam, i.e. Soc Trang, Bac Lieu and Ca Mau provinces. At each sampling location, the targeted samples included three infected and three non-infected ponds. Field sampling activities were conducted in three rounds starting on 11 September and finishing on 9 October, 2012. At the end of the campaign, samples had been collected from a total of 36 infected and 24 non-infected ponds. Sampling locations and collection dates are presented in Figure 1 and Table 1.

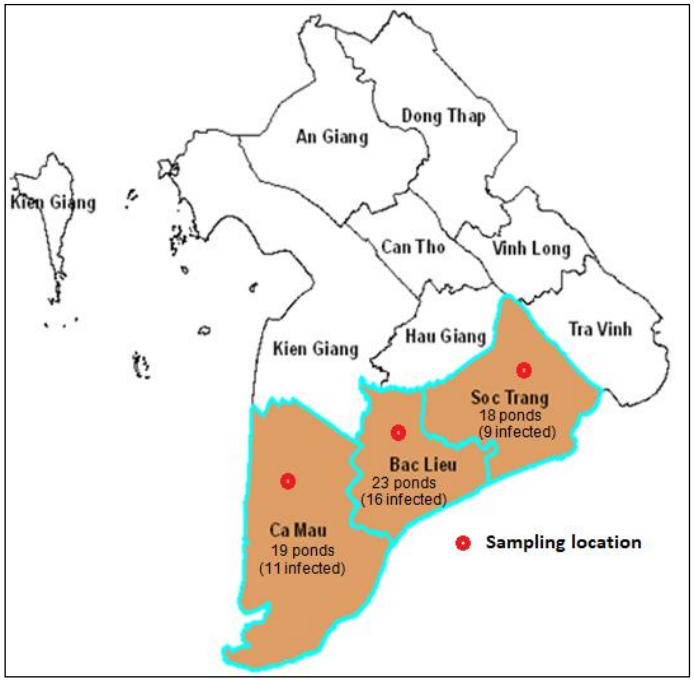


Fig. 1. Sampling locations for samples from existing outbreak and non-outbreak ponds.

Table 1. Sampling dates and locations for collection of samples from existing outbreak and non-outbreak ponds.

Sampling No.	Sampling Date	Location
1	11-12/09/2012	Soc Trang; round 1
2	14-15/09/2012	Bac Lieu; round 1
3	17-18/09/2012	Ca Mau; round 1
4	20-21/09/2012	Bac Lieu; round 2
5	23-24/09/2012	Ca Mau; round 2
6	26-27/09/2012	Soc Trang; round 2
7	29-30/09/2012	Bac Lieu; round 3
8	2-3/10/2012	Ca Mau; round 3
9	8-9/10/2012	Soc Trang; round 3

Sample collection and bacterial isolation from samples from existing outbreak and non-outbreak areas

Live shrimp were collected from each culture pond using a cast-net. Ten individuals from each pond were subjected to fresh smear examination. The external surface of the shrimp was rinsed in 70 % ethanol and an incision made over the head. The hepatopancreas (HP) was then removed, washed briefly with 70 % ethanol, and a small piece taken to make a smear on a glass slide containing a drop of Davidson's fixative (without glacial acetic acid). The preparation was then dried at room temperature, fixed in 1 % acetic acid solution, stained with Gram stain, and observed using a light microscope.

In addition, strains of *Vibrio* spp. were isolated from the HP of five individuals and incubated for 24 h at 28 °C on thiosulfate citrate bile salts sucrose (TCBS, Ovoid) agar and krypton soy agar (TSA, Ovoid) (supplemented with 1.5 % (w/v) sodium chloride) plates. Strains were stored at - 80 °C in trypton soy broth (TSB, Oxoid) containing 25 % glycerol and supplemented with 1.5 % (w/v) sodium chloride.

Table 2. Phenotypic characters of bacterial isolates from EMS/AHPNS-infected shrimp.

Character	Isolates of *Vibrio parahaemolyticus* obtained from EMS/AHPNS-infected shrimp (n = 90)[1]	ATCC 17802 (Buller 2004)
Gram stain	Negative	-
Shape	Short-rod	Short-rod
Colonies on TCBS agar	Green	Green
0/129 150 µg	+	ND
Haemolysis	+	ND
Swarming	+	ND
Motility	+	+
Catalase	+	+
Oxidase	+	+
O test	+	+
F test	+	+
Nitrate reduction	+	+
Beta-galactosidase production	-	-
Agrinine	-	-
Lysine	+	+
Ornithin	-	+
Citrate utilization	+	+
H_2S production	-	-
Urease	-	+
Tryptophane production	-	-
Indole production	+	+
Voges – Proskauer reaction	+	+
Gelatinase	+	+
Utilization of Glucose	+	+
Manitol	+	+
Inositol	-	-
Sorbitol	-	-
Rhamnose	-	-
Sucrose	-	-
Melibiose	-	-
Amygdalin	+	+
Arabinose	-	-

[1]'+' = positive strain; '-' = negative strain; ND: no data

Morphological and phenotypic characterization

Colony morphology and haemolysis were recorded after incubation for 2 days at 28 °C on blood agar plates (blood agar base (Merck) agar supplemented with 1.5 % (w/v) NaCl and 5% calf blood). Cell morphology was studied in Gram-stained preparations from the same blood agar plates according to Hucker's modification method (Barrow and Feltham 1993). Motility in broth (TSB supplemented with 1.5 % (w/v) NaCl) was studied using a drop of overnight culture on a slide and observed using a light microscope. Selected physiological and biochemical characteristics of isolates are presented in Table 2. Examination of characters was performed according to the principles of the Cowan and Steel's Manual (Barrow and Feltham 1993) using the API 20E system (BioMerieux, France) and 16S rRNA gene sequencing.

PCR analysis

An element palindromic PCR (rep-PCR) analysis, directed by the repetitive primer (GTG)5 (Bartie et al. 2006), was applied to compare isolates collected from EMS/AHPNS-infected shrimp. Detection of *Tlh, Tdh* and *Trh* genes was carried out following the protocol described by Nishibuchi et al. (1986) and Bej et al. (1999).

Results

Gross signs of EMS/AHPNS-infected shrimp

Outbreak ponds were selected as showing disease signs at the pond level, including pale to white hepatopancreas (HP); significant atrophy of HP; soft shells and guts with discontinuous contents or no content (Figs. 2B and 2b); onset of clinical signs and mortality starting as early as 10 days post stocking, and moribund shrimp coming to the pond sides or sinking to the bottom. In contrast, healthy shrimp appeared healthy with good HP and full gut (Figs. 2A and 2a). Gram-staining of fresh smears of HP from affected shrimp clearly showed the presence of Gram-negative, rod-shaped bacteria (Fig. 3B), whereas, fresh smears of HP from healthy shrimp had no bacteria (Fig. 3A).

Phenotypic characteristics of bacterial isolates

A total of 175 isolates of *Vibrio* spp. were collected from the HP of shrimp specimens. They formed large-sized (2–3 mm in diameter) green colonies after two days incubation at 28 °C on TCBS agar plates. The colonies were circular, entire and low convex, and their surface was smooth and shiny (Fig. 4A). In addition, these isolates developed swarming growth on TSA agar plates (Fig. 4B) and revealed haemolysis (beta form) on blood agar plates after 1 day of incubation at 28 °C (Fig. 4C).

The isolated bacteria were Gram-negative, short, rod-shaped, motile, positive for oxidase and catalase, fermented glucose in both aerobic and anaerobic conditions, sensitive to the vibriostatic agent O/129 using 150 μg discs, and reduced nitrate to nitrite.

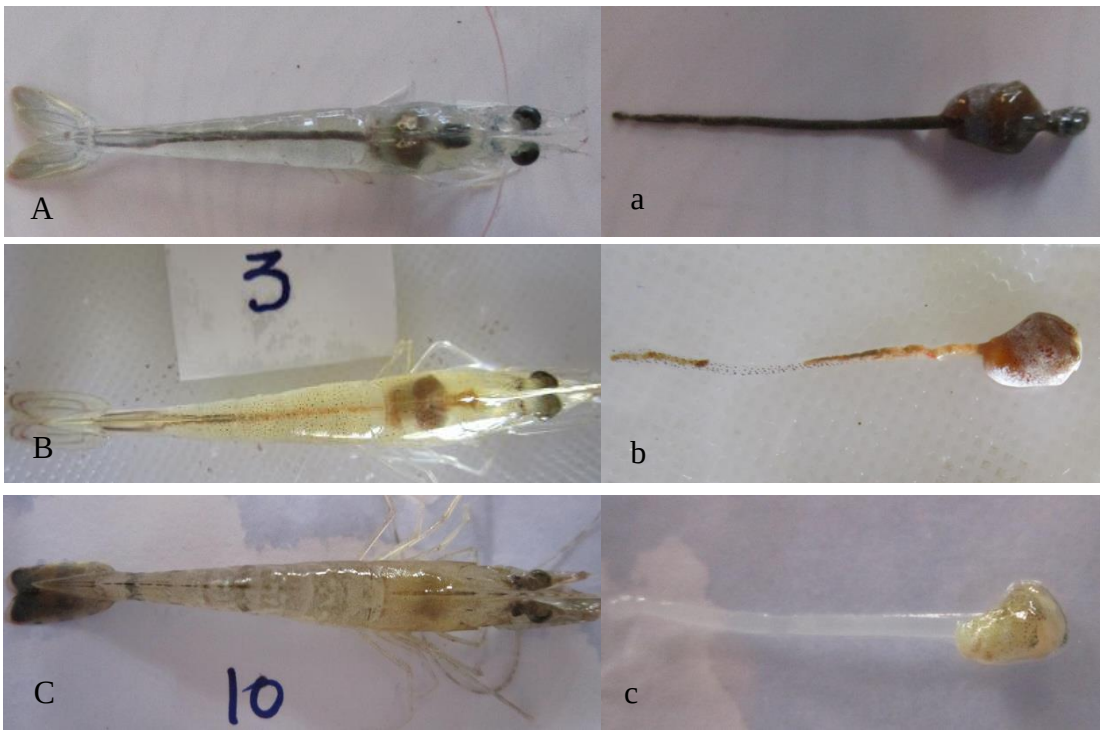


Fig. 2. Gross sign of EMS/AHPNS-infected shrimp. A. Control shrimp appear healthy; a. full gut. B and C: Infected shrimp with pale body colour; b. pale hepatopancreas (HP) and gut with discontinuous contents; c: pale HP and empty gut.

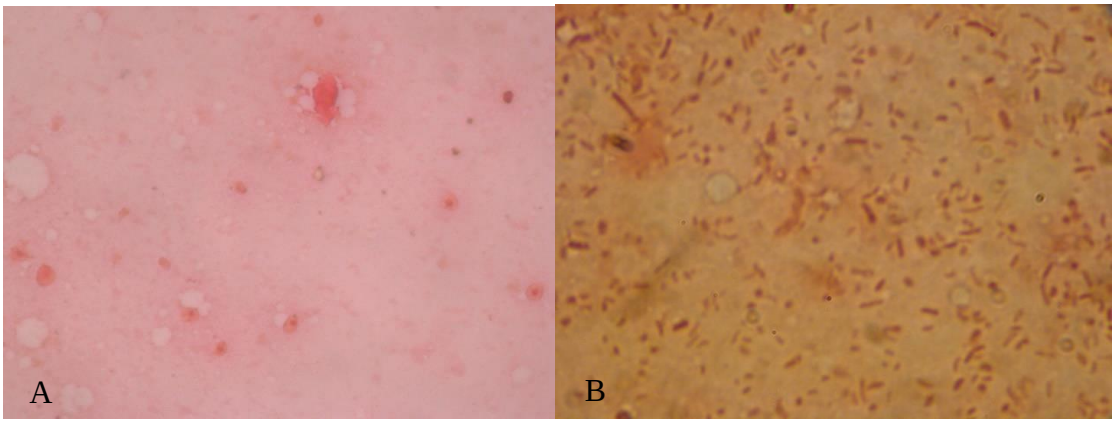
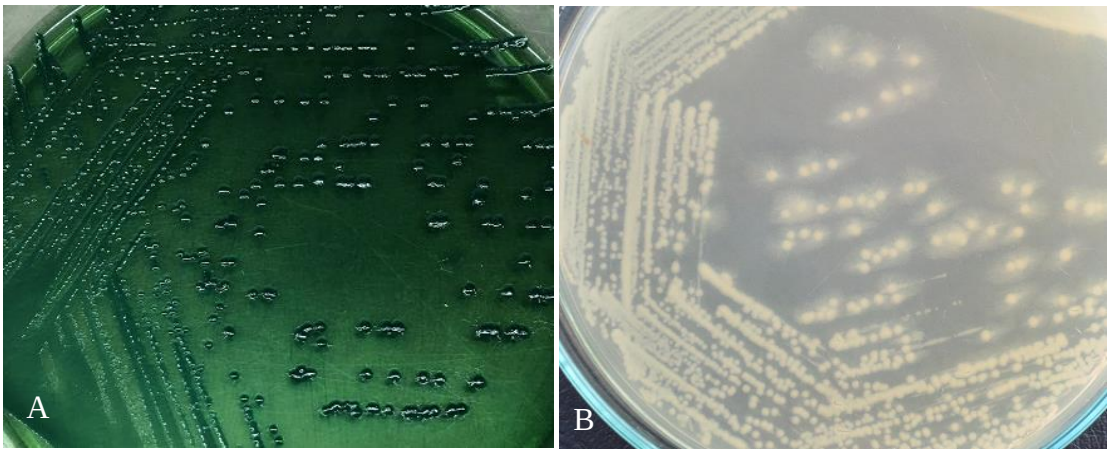


Fig. 3. Fresh smear and Gram staining of hepatopancreas from EMS/AHPNS-infected shrimp. (A) Healthy shrimp and (B) EMS/AHPNS-infected shrimp showing the presence of Gram-negative, rod-shaped bacteria (100X magnification).



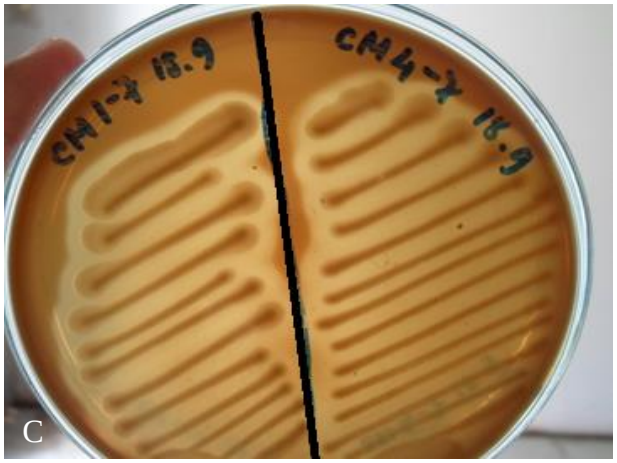


Fig. 4. Phenotypic characters of bacterial isolates from EMS/AHPNS-infected shrimp on solid agar plates. (A) green colonies on TCBS agar. (B) swarming growth on TSA agar. (C) haemolysis (beta form) on blood agar.

Isolates were identified to species level using a combination of conventional biochemical tests and API 20E kit. A majority of them were identified as *V. parahaemolyticus*; their phenotypic characteristics are presented in Table 2. Identification of isolates as *V. parahaemolyticus* was confirmed by 16S rRNA sequencing (99 % identities with GenBank *V. parahaemolyticus* AB680329 strain).

Rep-PCR analysis and detection of Tlh, Tdh and Trh genes

Ninety isolates of *V. parahaemolyticus* were selected from the three sampling locations (30 isolates each from Soc Trang, Bac Lieu and Ca Mau provinces) and subjected to rep-PCR analysis. Rep-PCR analysis resulted in at least four different DNA profiles for the tested isolates, as shown in Figures 5, 6, 7 and 8. Isolates which were isolated from EMS/AHPNS-infected shrimp from Bac Lieu Province displayed two DNA profiles of 11 and 12 bands (Figs. 5 and 6), whereas isolates which were obtained from EMS/AHPNS-infected shrimp from Soc Trang and Ca Mau provinces showed six DNA profiles with 9, 10, 11 and 12 bands (Fig. 7). Thermolabile haemolysin (*Tlh*) encoded by *Tlh* gene was detected from all tested isolates (Fig. 8), but neither the *Tdh* nor the *Trh* gene was detected.

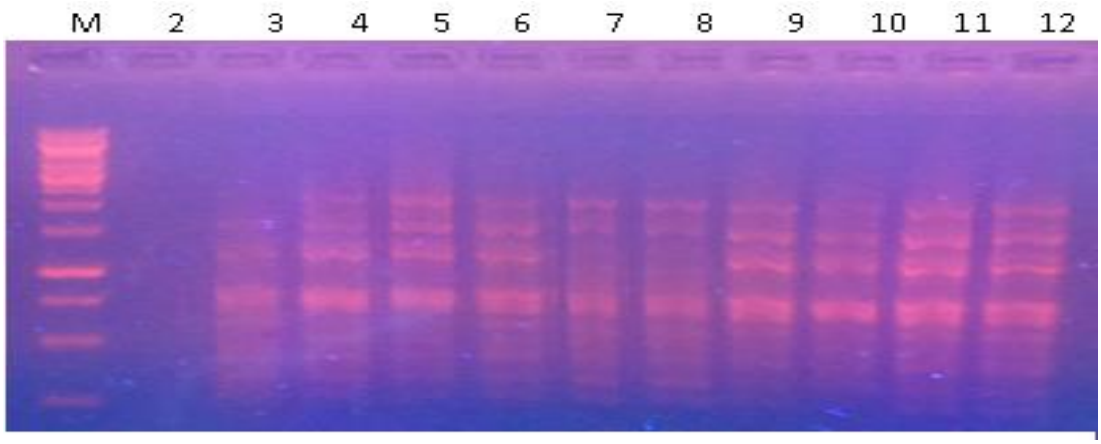


Fig. 5. Rep-PCR profiles (11 bands) of isolates of *Vibrio parahaemolyticus* obtained from EMS/AHPNS-infected shrimp from Bac Lieu Province. (M) DNA ladder 1 kb plus (Invitrogen), (2) water, (3–12) *V. parahaemolyticus* isolates.

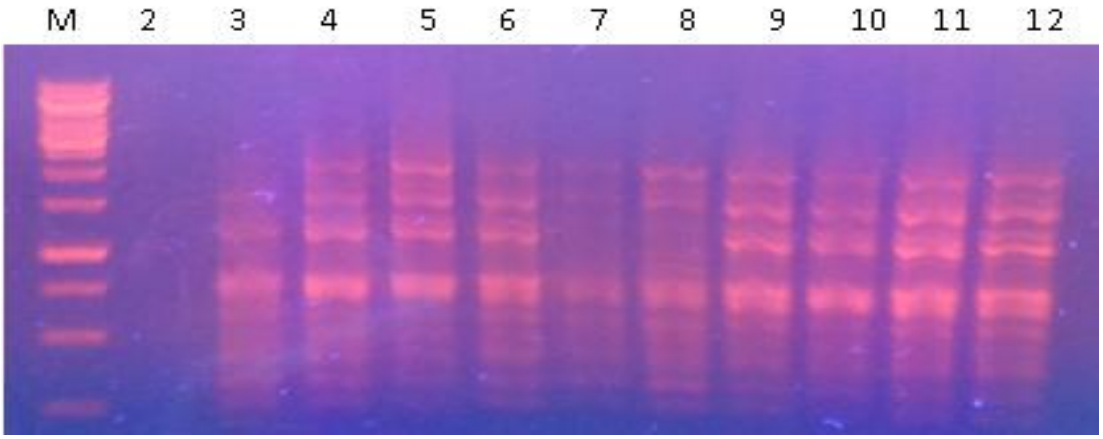


Fig. 6. Rep-PCR profiles (12 bands) of isolates of *Vibrio parahaemolyticus* obtained from EMS/AHPNS-infected shrimp from Bac Lieu Province. (M) DNA ladder 1 kb plus (Invitrogen), (2) water, (3–12) *V. parahaemolyticus* isolates.

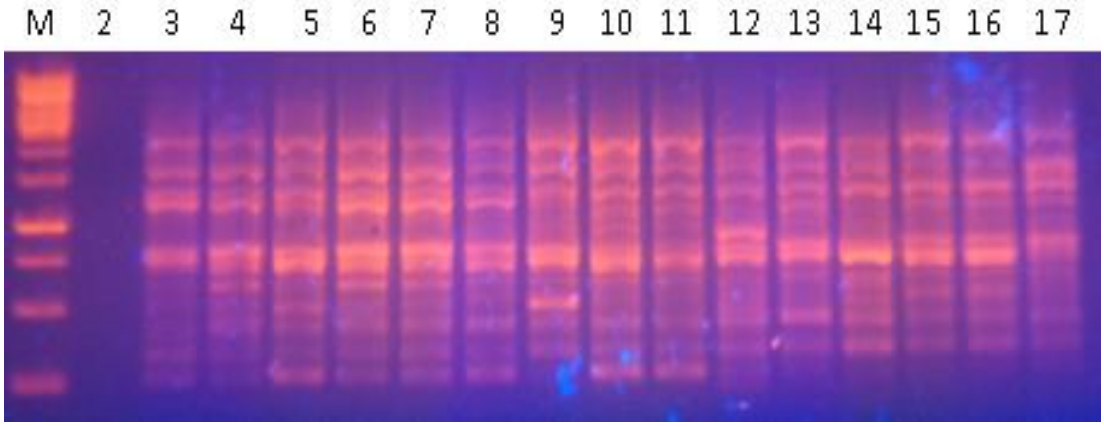


Fig. 7. Rep-PCR profiles of isolates of *Vibrio parahaemolyticus* obtained from EMS/AHPNS-infected shrimp from Soc Trang and Ca Mau provinces. (M) DNA ladder 1 kb plus (Invitrogen), (2) water, (3–17) *V. parahaemolyticus* isolates.

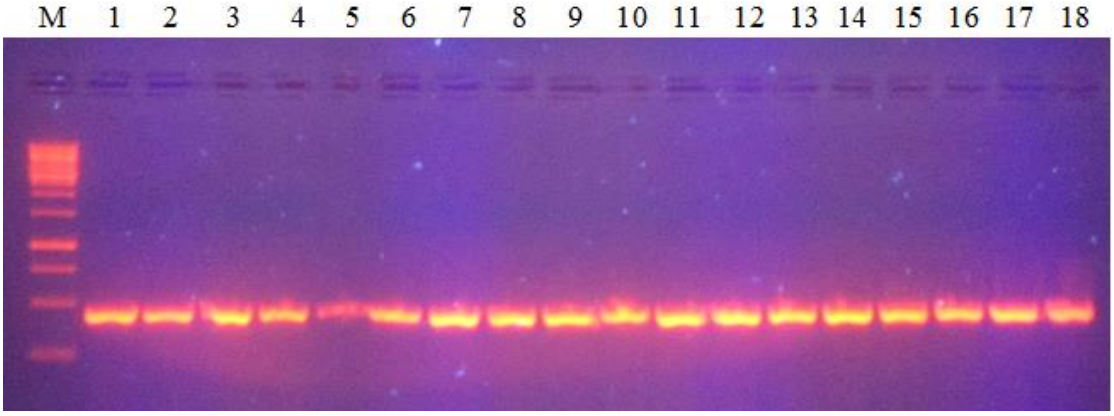


Fig. 8. Detection of *tlh* gene from isolates of *Vibrio parahaemolyticus* obtained from EMS/APHNS-infected shrimp collected during September to October 2012 field sampling activities. (M) DNA ladder 1 kb plus (Invitrogen). (1–18) representative isolates from EMS/AHPNS-infected shrimp.

Discussion

Vibrio species occur widely in aquatic environments as part of the normal flora of coastal seawater. They also exist as normal flora in fish and shellfish but have also been recognized as opportunistic pathogens in many marine animals (Austin and Austin, 1993). All bacterial isolates obtained from EMS/AHPNS-infected shrimp displayed the key phenotypical features of bacteria belonging to the genus *Vibrio*. Thus, all were motile, oxidase and catalase positive, Gram-negative rods which degraded D-glucose fermentatively, reduced nitrate to nitrite, grew on a *Vibrio* selective medium (TCBS), were sensitive to the vibriostatic agent O/129 using 150 μg discs (West et al. 1986) and gave positive results for indole production.

A majority of *Vibrio* isolates collected from EMS/AHPNS-infected shrimp were identified *V. parahaemolyticus*. The biochemical and physiological data were supported by 16S rRNA gene sequencing, while BLASTn analysis of 16S rRNA sequences from these isolates gave 99 percent identity to *V. parahaemolyticus*. *Vibrio parahaemolyticus* is a common inhabitant of coastal and estuarine environments all over the world. Therefore they are often found naturally associated with shrimp aquaculture systems. Certain environmental conditions may be more favourable for the establishment, survival and growth of these organisms, such as high pH, high temperature and high salinity, as well as tidal flushing. *Vibrio parahaemolyticus* is closely related to shrimp-pathogenic luminous bacteria such as *V. harveyi*, *V. campbelli* and *V. owensii*. These, along with other closely related *Vibrio* spp. form a "*V. harveyi* clade" (Cano-Gomez et al. 2009). Bacteria within this clade have a very high degree of similarity at both the phenotypic and genotypic levels.

The role of bacteria in EMS/AHPNS was suggested to be secondary infection, as bacterial colonization was prominent at the latter stage of the disease (Lightner et al. 2012). However, *V. parahaemolyticus* was consistently isolated from EMS/AHPNS-infected shrimp during the present study. Moreover, based on work done in Guangxi Province, P.R. China in 2010, Zhang et al. (2012) reported on a virulent strain of *V. parahaemolyticus* (strain 20100612001) which they isolated from *P. vannamei* suffering from EMS/AHPNS. The strain produced green colonies on TCBS agar, did not utilize sucrose and showed high antibiotic resistance. The results of this study suggested that further research should be focused on *V. parahaemolyticus*. Certain strains of *V. parahaemolyticus* can cause gastroenteritis in humans, and such clinical strains are characterized by the ability to produce a thermostable direct haemolysin (*Tdh*) or a *Tdh*-related haemolysin (*Trh*). The genes encoding these haemolysins (*tdh* and *trh* genes) are generally used as markers for human pathogenic strains of *V. parahaemolyticus* (FAO/WHO 2011). Human pathogenic strains possessing these markers account for 1–2 percent of environmental strains of *V. parahaemolyticus*. All strains (both clinical and environmental) produce a thermolabile haemolysin (*Tlh*) encoded by the *Tlh* gene, and this is generally used as a marker for *V. parahaemolyticus* in diagnostic tests (Keysner and Depaola 2004). Preliminary investigations of isolates of *V. parahaemolyticus* from EMS/AHPNS-infected shrimp detected the *Tlh* gene, but not the *Tdh* nor the *Trh* gene. Since the emergence of EMS/AHPNS, there has been no report of human-related disease (e.g. gastroenteritis) linked to the consumption of affected shrimp from any of the affected countries.

Acknowledgements

This study was carried out within the framework of FAO project TCP/VIE/3304 (E) Emergency Assistance to Control the Spread of an Unknown Disease Affecting Shrimps, which was implemented by Viet Nam's Ministry of Agriculture and Rural Development.

References

Austin, B. and D.A. Austin. 1993. Bacterial fish pathogens. Diseases in farmed and wild fish, 2nd edn. Ellis Horwood Ltd., Chichester. pp. 265–307.

Barrow, G.I. and R.K.A. Feltham. 1993. Covan and Steel's manual for the identification of medical bacteria, 3rd edn. Cambridge University Press, Cambridge. pp. 15–150.

Bartie, K., D.T.H. Oanh, G. Huys, C. Dickson, M. Cnockaert, J. Swings, N.T. Phương and A. Teale. 2006. Application of rep-PCR and pulsed-field gel electrophoresis for typing chloramphenicol resistant bacterial isolates from aquaculture sites in the Mekong River Delta, Vietnam. Journal of Biotechnology 4:31–40.

Bej, A.K., D.P. Patterson, C.W. Brasher, M.C.L. Vickery, D.D. Jones and C.A. Kaysner. 1999. Detection of total and hemolysin-producing *Vibrio parahaemolyticus* in shellfish using multiplex PCR amplification of tlh, tdh and trh. Journal of Microbiological Method 36:215–225.

Buller, B.N. 2004. Bacteria from fish and other aquatic animals: a practical identification manual, CABI Publishing, London. pp. 83–116.

Cano-Gomez, A., D.G. Bourne, M.R. Hall, L. Owens and L. Hoj. 2009. Molecular identification, typing and tracking of *Vibrio harveyi* in aquaculture systems: current methods and future prospects. Aquaculture 287:1–10.

FAO/WHO. 2011. Risk assessment of *Vibrio parahaemolyticus* in seafood: interpretative summary and technical report. Microbiological Risk Assessment Series No. 16. Rome, 193 pp.

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

Nishibuchi, M., W.E. Hill, G. Zon, W.L. Payne and J.B. Kaper. 1986. Synthetic oligodeoxyribonucleotide probes to detect Kanagawa phenomenon-positive *Vibrio parahaemolyticus*. Journal of Clinical Microbiology 23:1091–1095.

West, P.A., P.R. Brayton, T.N. Bryant and R.R. Colwell. 1986. Numerical taxonomy of vibrios isolated from aquatic environments. International Journal of Systematic Bacteriology 36:531–543.

Zhang, B., F. Liu, H. Bian, J. Liu, L. Pan and J. Huang. 2012. Isolation, identification, and pathogenicity analysis of a *Vibrio parahaemolyticus* strain from *Litopenaeus vannamei*. Progress in Fishery Science 33:56–62. (in Chinese).