Molecular detection of acute hepatopancreatic necrosis disease (AHPND) in *Penaeus vannamei* shrimps in Costa Rica

Nelson Peña-Navarro[a,b,*], Ruth Castro-Vásquez[a], Bernardo Vargas-Leitón[c], Gaby Dolz[a,c]

[a] *Maestría en Enfermedades Tropicales, Posgrado Regional en Ciencias Veterinarias Tropicales (PCVET), Universidad Nacional (UNA), Heredia 86-3000, Costa Rica*
[b] *Dirección de Investigación, Universidad Técnica Nacional, Sede del Pacífico, Puntarenas 1902-4050, Costa Rica*
[c] *Programa de Medicina Poblacional, Escuela de Medicina Veterinaria, UNA, Heredia 86-3000, Costa Rica*

A R T I C L E   I N F O

A B S T R A C T

Acute hepatopancreatic necrosis disease (AHPND) is a bacterial disease associated to severe mortality in farmed shrimps, and caused by *Vibrio parahaemolyticus* containing plasmid pVA-1 encoding *pir*A and *pir*B toxins. This study investigates the presence of *Vibrio* spp. carrying plasmid pVA-1 in post larvae and juveniles *Penaeus vannamei* from farms located in Costa Rica. Moreover, a possible corelation between *Vibrio* spp. presence, management parameters, and water quality was also investigated. Between 2017 and 2018, post larvae, the first water pumped into ponds, and juvenile shrimp (6 to 7 weeks after stocking) were collected from 15 farms located in the Gulf of Nicoya and the country's Central Pacific region. On the day when the juvenile shrimp were collected, a survey was applied to farmers to obtain information about management conditions, finally physical-chemical parameters of pond water were measured. Plasmidic pVA-1 *pir*A and *pir*B genes were detected in hepatopancreas of juvenile shrimp in 5 (33.3%) farms, while *Vibrio* spp. were found in 6 (40.0%) farms. Sequencing of pVA-1, *pir*A and *pir*B genes showed 99–100% similarity to pathogenic *Vibrio parahemolyticus* XN89 homologous genes identified in Vietnamese shrimps. Statistically significant differences were found in the water volume ($p < .03$), rate of water replacement ($p < .04$), and farms disease history ($p < .05$). A correlation between presence of *Vibrio* spp. and water quality was not established. The molecular diagnosis of *Vibrio* spp., the plasmid and the genes encoding toxins that are associated with AHPND are reported for the first time in Costa Rica. Further studies aimed to isolate AHPND-causing *Vibrio* spp. from ponds, to generate histopathological data, and to establish economic losses due to AHPND mortalities in *Penaeus vannamei* farms, are needed to clarify the role and pathogenic features of *Vibrio* spp. in AHPND.

1. Introduction

Acute hepatopancreatic necrosis disease (AHPND) is a disease caused by *Vibrio parahaemolyticus*, a Gram-negative bacterium inhabiting marine and estuarine environments (Luan et al., 2007). Pathogenicity of *Vibrio parahaemolyticus* is mediated by pVA-1, a plasmid encoding the proteolytic toxins *pir*A and *pir*B, which destroy host tissue and cause dysfunction of hepatopancreas (Nunan et al., 2014; Han et al., 2015b; Han et al., 2017). Plasmid pVA-1 has been reported in other *Vibrio* species, such as *V. campbellii* (Dong et al., 2017), *V. harveyi* (Liu et al., 2016) and *V. owensii* (Kondo et al., 2015). Clinical signs in affected shrimps include lethargy, paleness, soft exoskeletons and atrophy of the hepatopancreas, while the intestine is empty or discontinuous (Dong et al., 2017). AHPND affects the farmed shrimp

Penaeus vannamei (OIE, 2018), causing mortalities of 100% between 30 and 40 days farming (Ananda et al., 2017; Theethakaew et al., 2017). It was reported for the first time in China in 2009, while in Latin America it was identified for the first time in 2013 in Mexico, and in 2015 in two unspecified countries of Central America, where atypical mortalities occurred during the first 30 days of farming (Han et al., 2015b; Soto-Rodríguez et al., 2015).

Importing infected live shrimps and hatchlings can cause AHPND introduction and spreading into a country (Kim and Kim, 2015). Also, the disease can be introduced in farms through contaminated live food (clams, oysters, polychaetes and fresh squid meat) supplied to shrimp broodstock, as happened in Thailand when the disease was introduced from China (Thitamadee et al., 2016). Finally, temperatures above 30 °C may favor disease by increasing bacterial growth (OIE, 2018).

* Corresponding author at: Maestría en Enfermedades Tropicales, Posgrado Regional en Ciencias Veterinarias Tropicales (PCVET), Universidad Nacional (UNA), Heredia 86-3000, Costa Rica.
E-mail addresses: npena@utn.ac.cr (N. Peña-Navarro), bioruthi@gmail.com (R. Castro-Vásquez), bernardo.vargas.leiton@una.ac.cr (B. Vargas-Leitón), gaby.dolz.wiedner@una.ac.cr (G. Dolz).

Acute and terminal phase of the disease is determined through histopathology, a method with the advantage of availability, utility, and diagnostic specificity and sensitivity. However, cases of AHPND must be confirmed, and two or more of the following criteria are must be satisfied: i) Histopathology consistent with AHPND; ii) Detection of *pir*A and *pir*B genes in *Vibrio parahaemolyticus* pVA-1 plasmid by PCR and sequence analysis, iii) Positive results by bioassay (OIE, 2019).

Recently, the diagnostic use of polymerase chain reaction (PCR) has been increasing, also for its ability to discriminate among species of bacteria causing AHPND (Luan et al., 2007; Han et al., 2015c; Varela et al., 2017). Several PCR protocols for AHPND diagnosis have been developed, most of which based on the detection of the pVA-1 plasmid (Nunan et al., 2014; López-León et al., 2016); and PCR targeting the *pir*A and *pir*B genes carried by pVA-1 plasmid (Lee et al., 2015; Han et al., 2015a; Thitamadee et al., 2016). For *Vibrio* spp. diagnosis the 16S rRNA gene is amplified, since it is highly conserved among bacteria (Vigliotta et al., 2007; Joshi et al., 2014). PCR protocols based on *pir*A and *pir*B genes are considered the most sensitive (OIE, 2018). However, an enrichment step before extracting DNA is recommended for environmental samples, such as sediments and biofilms, where bacteria are present in small quantities (Han et al., 2015a; OIE, 2018).

In 2013, as a preventive measure, the National Animal Health Service of Costa Rica (SENASA) suspended importation of crustaceans and by-products from countries affected by AHPND, and since 2013 the disease has not been confirmed in Costa Rica (SENASA, 2013). However, the high mortalities reported in shrimp farms located around the Gulf of Nicoya since 2016 (Peña and Varela, 2016), and the finding of shrimps with atrophied hepatopancreas, has led producers to suspect the presence of the pathogen in the country. The objective of this study was therefore to investigate the presence of *Vibrio* spp., and of the plasmidic genes encoding *pir*A and *pir*B toxins (associated with AHPND) in water samples, post larvae, and juvenile shrimps from 15 farms using molecular techniques. Also, a possible correlation between presence of *Vibrio* spp. (and genes associated to AHPND) with management conditions and water quality was also explored.

2. Materials and methods

2.1. Study area, type and design

A non-systematic convenience-based, cross-sectional descriptive study was carried out. Fifteen farms were selected from a nationwide total of 89 farms (Dra. Carolina Elizondo, SENASA, personal communication, 2019), which met the following criteria for inclusion: 1. low level of technification of the farms; 2. having only one production pond; and 3. reporting shrimp mortality; since it was assumed that these were the farms in which there would be a higher probability of finding AHPND. The farms were located around the Gulf of Nicoya and the Central Pacific region of Costa Rica. Each of the farms were visited two times during the study: at the beginning of one production cycle (between January 2017 and June 2018) and 6–7 weeks after stocking, which is when the literature reports that the highest mortalities caused by AHPND occur in farms (Ananda et al., 2017; Theethakaew et al., 2017).

2.2. Collection of information

On the day the juvenile shrimp were collected, a survey was applied to each producer, to obtain information about general conditions on the farm (cultivation area, pond area, and use of water), production system (stocking density, post larvae origin, water volume, replacement rate, feeding methods, feed conversion, food type, duration of cultivation cycles, recording of water quality parameters, drying time), and sanitary management (knowledge of diseases, suspicion of presence of diseases, use and application of antibiotics).

2.3. Sampling of water and shrimp

Farms were visited once during the process of filling ponds before stocking, a sample of 10 mL of water was taken from the first water pumped into the ponds, which was stored in vacutainer tubes. Hundred post larvae were collected this day (day 0), before stocking, which were stored in tubes with 70% alcohol. The farms were visited again 6–7 weeks after stocking (Ananda et al., 2017; Theethakaew et al., 2017), and 15 juvenile shrimp (with a weight of 5 to 10 g) were taken in the morning from three different locations using cast nets (five shrimp at the point of entry of water, five in the middle of the pond and five at the point of drainage. The hepatopancreas and stomachs of the three groups of shrimps were removed and stored separately in 70% alcohol. All samples were transported under refrigeration (4–8 °C) to the laboratory and stored at −80 °C until processing (Nunan et al., 2014).

2.4. Obtaining physical-chemical water parameters and geographical coordinates

On the day the juvenile shrimp were collected, the following physical-chemical parameters of the water were measured with a portable multiparameter (Hanna Instruments HI9829): temperature (°C), dissolved oxygen (mg.L^{-1}), pH, and salinity (ppm). In addition, geographic coordinates were obtained for the four corners of the pond using a global positioning system (GPS, Garmin GPSMAP 60CSx), the values were averaged to get a precise centroid.

2.5. Molecular techniques

2.5.1. DNA extraction

DNA was extracted after centrifuging ponds water samples at 16.000 g × 10 min from supernatants and precipitates. DNA was also obtained from postlarvae, hepatopancreas, and stomachs of juvenile shrimp. All DNA extractions were performed by using DNeasy® Blood and Tissue kit (Qiagen), following the instructions provided by the manufacturer. Before DNA extraction from hepatopancreas and stomachs, the following extraction protocol was used (Cardona et al., 2016): 300 μL of PBS solution was added to the sample (to each of the three groups of 5 hepatopancreas or 5 stomachs of each pond), centrifugation at 5000 g × 5 min at 4 °C, addition of 200 μL of a phenol/chloroform/isoamyl alcohol solution (50:48:20 ratio), vortexed for one minute, and centrifuged at 30.000 g × 5 min at 4 °C, removing 150 μL of the aqueous phase. The aqueous phase was subjected to DNA extraction using the Dneasy® Blood and Tissue kit. Finally, the DNA concentration (ng.μL^{-1}) of each extracted sample was measured using a spectrophotometer (Nanodrop™).

2.5.2. PCR analyses

The samples (water sediment, post larvae, hepatopancreas and stomachs of juvenile shrimp) were analyzed to determine the presence of *Vibrio* spp. carrying the plasmid pVA-1 with *pir*A and *pir*B genes using five PCR protocols (Table 1): in the first PCR the β-actin gene of the shrimp samples (post larvae, hepatopancreas and stomachs) was amplified according to the protocol described by Dhar et al. (2001) to confirm the correct extraction of DNA from the samples and discard inhibitions. In the second PCR, a segment of the pVA-1 plasmid gene was amplified following the protocol described by Nunan et al. (2014), and in the third and fourth PCR, segments of the genes of the *pir*A and *pir*B toxins, respectively, were amplified (Han et al., 2015a). The fifth PCR was used to amplify the 16S subunit of the rRNA gene to identify the *Vibrio* species present in the sample (Vigliotta et al., 2007).

The reaction mixture for the PCRs included DNA template (0.8–1.6 μM), primers at 10 μM concentration, and 0.6 μM Master Mix (DreamTaq, Thermofisher®), for a final volume of 25 μL. Shrimp infected with AHPND were acquired from the University of Arizona, USA, and used as a positive control for pVA-1, *pir*A and *pir*B PCR. An isolate

Table 1

Primers used for the amplification of segments of the following genes: β-actin, pVA-1 plasmid, *pir*A toxin, *pir*B toxin and *Vibrio* spp. using conventional PCR.

Gene	Primer	Sequence	Product size (bp)	Reference
β-actin	Actin-F	CCCAGAGCAAGAGAGGTA	339	Dhar et al. (2001)
	Actin-R3	GCGTATCCTTCGTAGATGGG		
Plasmid pVA-1	89F	GTCGCTACTGTCTAGCTGAA	470	Nunan et al. (2014)
	89R	ACGGCAAGACTTAGTGTACC		
*pir*A	VpPirA-284F	TGACTATTCTCACGATTGGACTG	284	Han et al. (2015a)
	VpPirA-284R	CACGACTAGCGCCATTGTTA		
*pir*B	VpPirB-392F	TGATGAAGTGATGGGTGCTC	392	Han et al. (2015a)
	VpPirB-392R	TGTAAGCGCCGTTTAACTCA		
16S rRNA	16SEB20–42-F	TGGCTCAGGAYGAACGCTGGCGG	1488	Vigliotta et al. (2007)
	16SEB1488-R	TACCTTGTTACGACTTCACC		

of *Vibrio parahaemolyticus* from shrimp from Costa Rica, previously confirmed with the Vitek system was used as a positive control in the 16S PCR, whereas nuclease-free water (Fermentas®) was used as a negative control.

2.5.3. Agarose gel electrophoresis

PCR products were visualized using agarose gel electrophoresis (1%) in TBE 1× (Tris base, boric acid, EDTA, pH 8, 0.5 M). The electrophoretic run was performed at 100 V for 45 min. GeneRuler 100 bp DNA Ladder Plus (Fermentas®) was used as a molecular weight marker, and GelRed® Nucleic Acid Gel Stain was used as a fluorescent dye.

2.5.4. DNA sequencing

Amplification fragments with a size of 339 bp (β-actin), 470 bp (pVA-1), 284 bp (*pir*A), 392 bp (*pir*B) and 1488 bp (*Vibrio spp.*) were considered positive and all, but those of actin, were sent to Macrogen (Seoul, Korea) to be sequenced. The partial sequences were aligned with the BioEdit Sequence Alignment Editor® program and compared using the BLASTn algorithm (Joshi et al., 2014; Han et al., 2015a), using the National Center for Biotechnology Information (NCBI) database. The sequences obtained were deposited in the GenBank.

2.6. Statistical analysis

The results of the surveys and PCRs of plasmid, *pir*A, *pir*B and *Vibrio* spp. were entered into a database. A map was created using the QGIS 2.14.0 program to show the distribution of farms analyzed and positive cases identified.

A descriptive analysis was carried out in which the frequency tables (absolute and relative) for categorical variables and measures of central tendency and dispersion for quantitative variables were obtained. Management variables (cultivation area, pond area, place of water extraction, stocking densities, origin of post larvae, volume of water in the pond, rate of pond replacement, feed conversion, application of drying periods, physical-chemical parameters of the water) were statistically associated with the presence of *Vibrio* spp. and genes associated to AHPND (pVA-1, *pir*A, *pir*B) using *t*-test mean comparisons between positive and negative groups to the genes analyzed. Answers obtained from surveys were processed using SAS® statistical software version 9.4. In addition, contingency tables were created, and Chi-square statistics and Fisher's exact test were calculated with $\alpha = 0.05$ (Morales et al., 2011).

3. Results

3.1. Descriptive analysis of production

Results from 15 farms are shown in Table 2; ten farms were located in the province of Guanacaste and five in Puntarenas (Fig. 1). More than half of the producers (53.3%) cultivated in ponds with areas between 30.000 and 45.000 m², using mainly estuary water (73.3%), and post

larvae stocking densities between 100.000 and 300.000 units per hectare (60.0% of the farms). Of these larvae, 73.3% came from Guatemala and the rest from Nicaragua (Table 2). The volumes of water used in the ponds and replacement rates varied; however, 40.0% of the producers used volumes between 20.000 and 35.000 m³ of water and had daily replacement rates between 10 and 20% (Table 2). Feeders were the most frequently used feeding technique (66.6%), with feed conversion factors between 1.0 and 1.2 (40.0%). Most producers used commercial pelleted food (30–35% protein) (93.3%); 66.6% of them preferred production cycles of three months, with three annual harvests (60.0%). A total of 40.0% of the producers measured the most important water quality parameters (dissolved oxygen, temperature, salinity, pH, turbidity), whereas 20.0% never measured parameters (Table 2).

With respect to farms' sanitary management, 93.3% of the producers were aware of infectious diseases and thought that they were present in their farms. The use of antibiotics in farms was common (100.0% Guanacaste, 80.0% Puntarenas), especially oxytetracyclines (46.7%, 7/15) or combinations of oxytetracyclines and enrofloxacin's (33.3%, 5/15), and most (73.3%, 11/15) used them one time per production cycle, at the moment when mortality was recorded in the pond, which they associated with bacterial infections (Table 3). Only one out of 15 farms did not apply antibiotics. Producers were aware of AHPND, but only three (18.2% Guanacaste, 25.0% Puntarenas) indicated that the disease might be present in their farms. On the other hand, only one producer (6.7%) from a farm located in Puntarenas was not aware of infectious diseases that could affect shrimp or diseases that could be present on his farm (Table 3).

3.2. Water quality parameters

None of the 15 farms showed optimal physical-chemical parameters of the water in their ponds (Table 4). In four farms (26.6%) only one altered parameter (temperature) was recorded; in nine farms (60.0%) two altered parameters (temperature/oxygen or temperature/salinity), while 3 and 4 altered parameters were determined on two farms, respectively (Table 4).

3.3. Detection of pVA-1, pirA, pirB, and Vibrio spp. using PCR

The presence of the pVA-1 plasmid and of the *pir*A and *pir*B genes were detected in five (33.3%) of 15 farms using PCR, while the presence of *Vibrio* spp. was found in the same five farms and one other farm (40%).

The presence of plasmid pVA-1, *pir*A, *pir*B, and *Vibrio* spp. was detected only in one location in each of the five farms in hepatopancreas of shrimp, but not in their stomachs. Two producers reported suspicions of the presence of AHPND in their farms (farms 1 and 4), while the use of antibiotics was reported in all six positive farms. Water samples (sediment) from the 15 farms and post larvae from 14 farms were negative for plasmid pVA-1, *pir*A, *pir*B and *Vibrio* spp. Only post larvae from one farm (farm 3) were positive for *Vibrio spp.*, which had been

Table 2

Distribution of farms according to management characteristics of 15 shrimp farms in the provinces of Guanacaste and Puntarenas, Costa Rica.

Characteristics	Guanacaste	Puntarenas	Total
	$n = 10$ (%)	$n = 5$ (%)	$n = 15$ (%)
Pond area (m^2)			
10.000–30.000	3 (30.0)	2 (40.0)	5 (33.3)
30.000–45.000	6 (60.0)	2 (40.0)	8 (53.3)
45.000–55.000	1 (10.0)	1 (20.0)	2 (13.3)
Origin of water			
Sea	3 (30.0)	1 (20.0)	4 (26.7)
Estuary	7 (70.0)	4 (80.0)	11 (73.3)
Stocking density (post larvae.ha^{-1})			
< 100.000	1 (10.0)	1 (20.0)	2 (13.3)
100.000–300.000	6 (60.0)	3 (60.0)	9 (60.0)
300.000–500.000	3 (30.0)	1 (20.0)	4 (26.7)
Origin of post larvae			
Nicaragua	4 (40.0)	0 (0.0)	4 (26.7)
Guatemala	6 (60.0)	5 (100.0)	11 (73.3)
Water volume (m^3)			
10.000–20.000	1 (10.0)	3 (60.0)	4 (26.7)
20.000–35.000	4 (40.0)	2 (400)	6 (40.0)
35.000–50.000	5 (50.0)	0 (0.0)	5 (33.3)
Replacement rate (%)			
< 10	2 (20.0)	2 (40.0)	4 (26.7)
10–20	4 (40.0)	2 (40.0)	6 (40.0)
20–40	4 (40.0)	1 (20.0)	5 (33.3)
Feeding method			
Feeders	7 (70.0)	3 (60.0)	10 (66.6)
Mixed (Blind feeding + Feeder)	3 (30.0)	2 (40.0)	5 (33.3)
Feed conversion			
< 1	3 (30.0)	1 (20.0)	4 (26.7)
1–1.2	5 (50.0)	1 (20.0)	6 (40.0)
1.2–1.4	1 (10.0)	3 (60.0)	4 (26.7)
> 1.4	1 (10.0)	0 (0.0)	1 (6.7)
Type of food			
Organic	0 (0.0)	1 (20.0)	1 (6.7)
Conventional	10 (100.0)	4 (80.0)	14 (93.3)
Duration of production cycle (months)			
≤3	2 (20.0)	3 (60.0)	5 (33.3)
≥3–4	8 (80.0)	2 (40.0)	10 (66.6)
Cultivation cycles (annual)			
2	5 (50.0)	1 (20.0)	6 (40.0)
≥3	5 (50.0)	4 (80.0)	9 (60.0)
Measurement of water quality parameters			
Turbidity	0 (0.0)	1 (20.0)	1 (6.7)
Oxygen + Temperature	1 (10.0)	0 (0.0)	1 (6.7)
Oxygen + Temperature + Salinity	3 (30.0)	1 (20.0)	4 (26.7)
Oxygen + Temperature + Salinity + pH	4 (40.0)	2 (40.0)	6 (40.0)
None	2 (20.0)	1 (20.0)	3 (20.0)

acquired in Guatemala. However, the presence of the pVA-1 plasmid, *pir*A and *pir*B was not detected in that farm. In two farms (6 and 11) the presence of the agent was determined only in juvenile shrimp collected at the point of entry of water to the pond, in one case (farm 12) in the middle of the pond, and in two cases (farm 1 and 4) the location was not recorded.

3.4. Sequencing

The *pir*A gene sequences of the Costa Rican samples (farms 1, 4, 11: 284/284 bp, [GenBank MH685878]), (farm 6: 284/287 bp [GenBank MH685877] and farm 12: 281/284 bp, [GenBank MH700244]) were 100% similar to several *pir*A sequences of *Vibrio* genus. Likewise, *pir*B sequences (392/392 bp, GenBank MH714298) and plasmid pVA-1 (490 bp/490 bp, GenBank MH822862) from the five farms, were identical between them and with other *pir*B and pVA-1 sequences of different *Vibrio* species. Samples from all positive farms also amplified the 16S subunit of the rRNA gene. Sequencing identified the presence of *V. parahaemolyticus* on farm 4 and 12 (GenBank MH819479), while on the remaining farms (1, 3, 6, 11) the *Vibrio* species could not be determined (GenBank MH819492).

3.5. Geographical distribution of AHPND in Costa Rican shrimp farms

The geographical distribution of AHPND-positive and -negative farms is shown in Fig. 1. Most of AHPND-positive farms (farms 1, 4, 11, 12) were found in the county of Abangares, in the Guanacaste province, while only one (6.7%) was positive in the county of Puntarenas, in the province of Puntarenas (farm 6). The presence of an undetermined species of *Vibrio* was detected in post larvae from farm 3 (orange spot), but the presence of genes associated with the plasmid or the toxins causing AHPND were not detected.

3.6. Management conditions related with Vibrio spp. and genes associated to AHPND (pVA-1, pirA, pirB)

Statistically significant differences were found between farms that were positive and negative for *Vibrio* spp. with respect to water volume ($p < .03$), water replacement rates ($p < .04$) and farms with a history of the presence of diseases ($p < .05$). Farms that used water volumes between 20.000 and 25.000 m^3 or 35.000–40.000 m^3, water replacement rates of 35–40%, and farms with a greater historical presence of infectious diseases were more likely to be *Vibrio* spp. positive (Table 5).
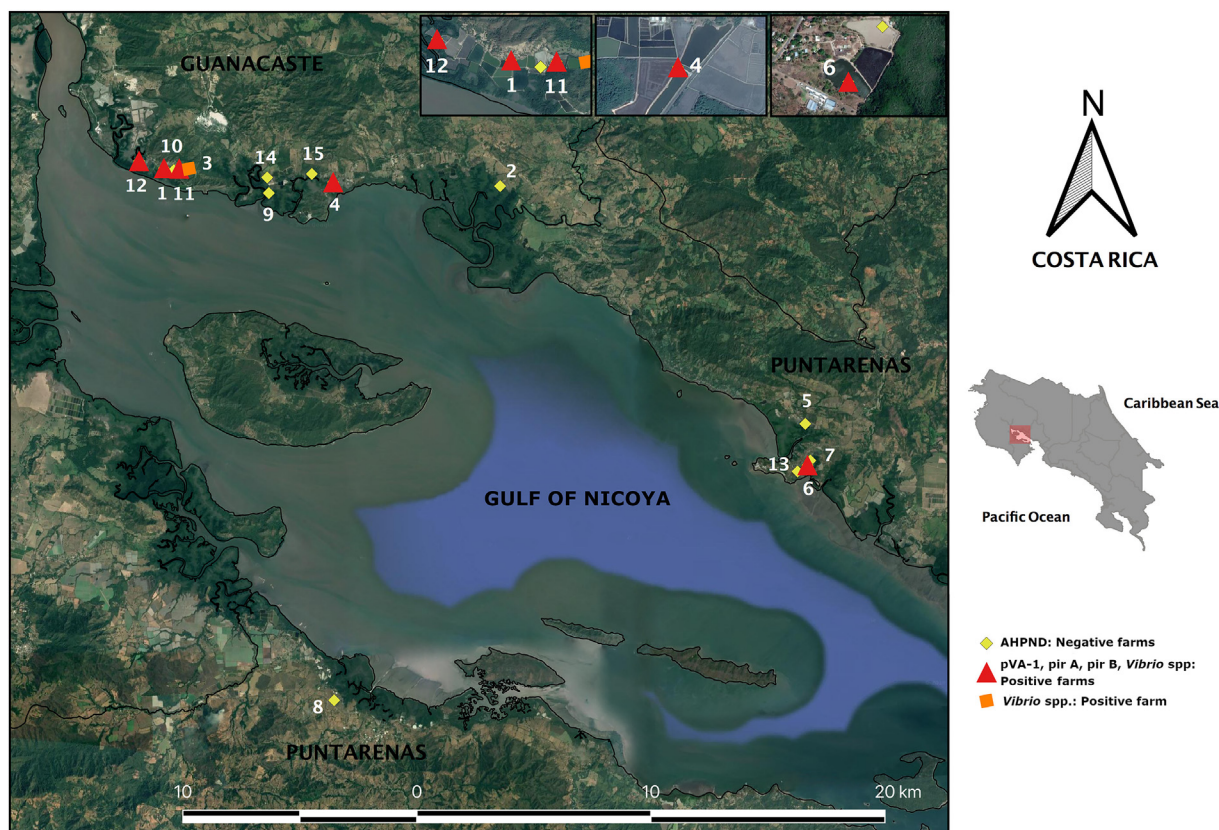
Fig. 1. Geographical distribution of shrimp farms negative and positive to genes associated with the plasmid and the toxins that cause AHPND and genes associated with *Vibrio* spp. in Costa Rica, 2017–2018 (Insets show enlargements of the areas were positive farms were found).

Table 3

Survey of infectious diseases: awareness and perception of the presence of infectious diseases and use of antibiotics in 15 shrimp farms in the provinces of Guanacaste and Puntarenas, Costa Rica.

Characteristics	Guanacaste	Puntarenas	Total
	n = 10 (%)	n = 5 (%)	n = 15 (%)
Knowledge of infectious diseases			
WSSV	10 (100.0)	4 (80.0)	14 (93.3)
AHPND	10 (100.0)	4 (80.0)	14 (93.3)
NHP	2 (20.0)	3 (60.0)	5 (33.3)
IHHNV	5 (50.0)	2 (40.0)	7 (46.7)
Parasites	6 (60.0)	2 (40.0)	8 (53.3)
None	0 (0.0)	1 (25.0)	1 (6.7)
Perception of the presence of infectious diseases in the farm			
WSSV	10 (100.0)	4 (80.0)	14 (93.3)
AHPND	1 (10.0)	2 (40.0)	3 (20.0)
NHP	9 (90.0)	4 (80.0)	13 (86.7)
IHHNV	6 (60.0)	3 (60.0)	9 (60.0)
Parasites	9 (90.0)	4 (80.0)	13 (86.7)
Does not know	0 (0.0)	1 (25.0)	1 (6.7)
Use of antibiotics			
Oxytetracyclines	5 (50.0)	2 (13.3)	7 (46.7)
Enrofloxacin's	0 (0.0)	2 (13.3)	2 (13.3)
Both	5 (50.0)	0 (0.0)	5 (33.3)
Does not apply	0 (0.0)	1 (20.0)	1 (6.7)
Application of antibiotics per production cycle			
One time	7 (70.0)	4 (80.0)	11 (73.3)
Two times	3 (30.0)	0 (0.0)	3 (20.0)
Does not apply	0 (0.0)	1 (20.0)	1 (6.7)

WSSV: white spot syndrome virus; AHPND: acute hepatopancreatic necrosis disease; NHP: hepatopancreatic necrosis; IHHNV: infectious hypodermic and hematopoietic necrosis virus.

However, it was not possible to determine any statistical association between the presence of *Vibrio* spp. carrying the plasmid pVA-1 with *pir*A and *pir*B genes with environmental factors.

3.7. Association between water quality and presence of Vibrio spp.

The presence of *Vibrio* spp. was not related to the amount of dissolved oxygen, temperature, pH and salinity of the water from the shrimp farms at the sampling day; however, positive farms showed low average levels of dissolved oxygen (2.82 ± 1.55 mg.L^{-1}) (Table 6).

4. Discussion

The presence of *Vibrio parahaemolyticus* carrying plasmid pVA-1 encoding *pir*A and *pir*B genes associated with AHPND is reported for the first time in shrimp farms affected by mortality, disease and clinical signs; providing evidence for AHPND in Costa Rica, and complementing previous reports from Mexico (Gómez-Gil et al., 2014; Nunan et al., 2014), and unspecified Central American countries (Han et al., 2015b). Although, in order to confirm AHPND cases, histopathological analysis of samples is recommended (OIE, 2019).

In this study the AHPND-associated plasmidic *pir*A and *pir*B genes were not found in post larvae imported from Guatemala and Nicaragua; only post larvae from a single farm were found to be positive for *Vibrio* spp., possibly due to contamination in the maturation laboratory, because of infection that occurred in the very farm during the process of acclimatization before stocking. Alternatively, post larvae were already contaminated when they arrived from the country from which they were imported. However, in this farm, *Vibrio* spp. carrying pVA-1 plasmid and *pir*A/*pir*B genes were not detected in post larvae and juveniles. According to Aguirre-Guzmán et al. (2001), the critical phase for post larvae to contract the bacteria is the last stage of

Table 4
Physical-chemical parameters of the water in shrimp farms from Costa Rica during 2017 and 2018.

Farm	Province	Date	Oxygen (mg.L^{-1})	Temperature (°C)	pH	Salinity (ppt)
Optimal			≥4.0	28–30	7.5–8.5	15–30
1	Guanacaste	May (2017)	**2.96**	**31.16**	7.54	25.44
2	Guanacaste	November (2017)	4.60	**31.46**	7.90	26.80
3	Guanacaste	May (2017)	4.50	**31.50**	8.09	23.20
4	Guanacaste	June (2017)	4.10	30.09	**4.51**	**42.32**
5	Puntarenas	July (2017)	4.30	**29.34**	**7.31**	**63.03**
6	Puntarenas	August (2017)	**3.40**	**32.16**	7.90	**43.47**
7	Puntarenas	October (2017)	6.50	**27.20**	7.88	26.73
8	Puntarenas	October (2017)	**3.50**	**31.40**	**7.18**	**57.60**
9	Guanacaste	November (2017)	**3.00**	**34.53**	8.24	22.39
10	Guanacaste	October (2017)	**0.60**	**33.17**	7.75	22.01
11	Guanacaste	October (2017)	**0.13**	**34.03**	7.67	21.57
12	Guanacaste	March (2018)	**3.51**	**33.10**	7.73	30.47
13	Puntarenas	March (2018)	**3.80**	29.38	**6.75**	**37.82**
14	Guanacaste	March (2018)	**2.28**	**31.25**	7.72	28.81
15	Guanacaste	March (2018)	5.13	**32.17**	7.65	29.12
Average			3.49	31.46	7.45	33.40
S.D.			1.62	1.92	0.89	12.98
Max.			6.50	34.53	8.24	63.03
Min.			0.13	27.20	4.51	21.57

S.D. = Standard deviation, Max. = maximum value, Min. = minimum value. Bold numbers indicate values below or above optimal.

metamorphosis, which takes place in the maturation laboratory.

The presence of plasmid pVA-1, *pir*A and *pir*B was detected only in the hepatopancreas of juvenile shrimp, a finding that is consistent with that reported by OIE (2018). However, since only two tissues were analyzed (hepatopancreas and stomach), the presence of AHPND in other tissues (e.g. intestines) cannot be ruled out (OIE, 2018). Three of the positive farms (1,11 and 12) were very close to each other, making it possible that *Vibrio* spp. spread through water. In the present study, the presence of *Vibrio* spp. and genes associated to AHPND was not detected in water, probably because cultures in selective agar (Chonsin et al., 2016), or enrichment of the samples (Sirikharin et al., 2015) were not performed. This should be investigated in future studies, including an analysis of the presence of bacteria in sediments (Chonsin et al., 2016).

Positive samples were found primarily at places of entry of water and in the middle of the pond, and this is not consistent with previous reports in the literature (Morales and Cuéllar-Anjel, 2014), in which the strongest and healthiest shrimps are found at the places where fresh, oxygenated water enters the pond, while the weakest and sickest ones are concentrated in areas where water exits the pond; however, our results may be explained by the fact that this study was not systematic (Morales and Cuéllar-Anjel, 2014).

It is necessary to determine whether plasmid sequences detected in Costa Rica are different from those reported in Mexico and Central America (Han et al., 2015b), and if they are similar to the Asian genotype (Phiwsaiya et al., 2017). This would play for an introduction of the agent from Asia either through frozen animals or by imported larvae. Since the sequenced regions of *pir*A and *pir*B were however very small and identical among variants available in the GenBank, future studies aimed to amplify larger segments of the plasmid (or the complete plasmid sequence) are recommended. For instance, similarly to what was done by Han et al. (2015b), which identified variable regions of the *V. parahaemolyticus* genome associated with geographical variation among Mexican and Central America isolates. Also, in Texas, United States, positive samples were identified, which were similar to the Mexican genotype rather than to the Asian genotype (Dhar, 2019).

Vibrio parahaemolyticus has been identified as the main causal agent of AHPND (Han et al., 2015a, 2015b), which agrees with the findings of the present investigation, although it is not excluded that AHPND is caused by other bacteria of the *Vibrio* genus (Dong et al., 2017; Han et al., 2017). It is possible that the farm with *Vibrio* spp.-positive post larvae, that were negative to the plasmid, *pir*A and *pir*B, showed only

bacterial contamination, or some other disease (septic necrosis of the hepatopancreas or necrosis of the hepatopancreas), which was not analyzed in the present study (Morales and Goméz-Gil, 2014).

The shrimp farms studied have large areas under cultivation, feed conversions greater than 1.2, and stocking densities greater than 100.000 post larvae.ha^{-1}, which, compared to the level of technical sophistication of the farms, could be causing low production and the emergence of diseases (Valverde and Alfaro, 2013). All farms analyzed showed differences in the management, however farms in the province of Guanacaste showed better management practices, mainly in volume of water, feed conversions and water replacement rates, possibly because the producers are better trained and have less extensive production systems. However, non-optimal water quality parameters were found in all farms, which could be causing alterations in the normal development of shrimp (Lavilla-Pitogo et al., 2000; Lazur, 2007). Fluctuations in water quality can be associated with the presence of AHPND, specifically when oxygen is below 3 mg.L^{-1} (Putth and Polchana, 2016), which coincides with the results obtained in this investigation.

The routine uses of antibiotics on farms, which can cause bacterial resistance, was surprising (Hien et al., 2016). Only one of the fifteen farms analyzed did not apply antibiotics in the production cycle, and in 35.7% of the farms that used antibiotics, the presence of *Vibrio* spp. carrying the plasmid pVA-1 with the *pir*A and *pir*B genes was detected. Furthermore, another of these farms was positive to *Vibrio* spp. but *pir*A and *pir*B genes were not detected. In two of the three farms, in which the producers suspected the presence of AHPND, the presence of *Vibrio* spp. carrying the *pir*A and *pir*B genes was confirmed. No relationship between antibiotics usage and *Vibrio* presence was established.

It was not possible to determine association between the presence of pVA-1, *pir*A, and *pir*B and the variables analyzed. However, some associations were determined between parameters and the presence of *Vibrio* spp., mainly when water volume and water replacement rates were high, and farms did report diseases. It is possible that replacement of water associated to water volume of the pond may affect biogeochemical parameters and bacterial communities, affecting the stabilization and competition between beneficial and pathogenic bacteria (Alfiansah et al., 2018). More research is needed, performing a dynamic study in which repeated sampling is carried out on farms, or experimental studies manipulating the factors, to provide robust results about factors associated with this bacterial disease.

Although the presence of *Vibrio* spp. carrying the plasmid pVA-1

Table 5
Distribution of *Vibrio* spp.-positive and -negative samples and variables associated to *Vibrio* spp. in shrimp farms in Costa Rica, 2017–2018.

Factor	Category	Vibrio spp.			
		N	Positive (%)	Negative (%)	Pr ≤ P
Stocking density (post larvae.ha^{-1})	< 100.000	2	50	50	1
	100.000–300.000	9	33.33	66.67	
	300.000–500.000	4	50	50	
Cultivation area (ha)	< 2	4	50	50	0.57
	2–3	3	66.67	33.33	
	3–4	3	0	100	
	> 4	5	40	60	
Origin of larvae	Guatemala	11	36.36	63.64	1
	Nicaragua	4	50	50	
Origin of water	Sea	4	75	25	0.24
	Estuary	11	27.27	72.73	
Water volume (m^3)	10.000–20.000	4	50	50	**0.03**
	20.000–25.000	3	100	0	
	30.000–35.000	3	0	100	
	35.000–40.000	1	100	0	
	40.000–45.000	1	0	100	
	45.000–50.000	3	0	100	
Water replacement rate	< 10%	4	0	100	**0.04**
	10–20%	6	50	50	
	20–30%	2	0	100	
	30–40%	3	100	0	
Feed conversion	< 1	4	0	100	0.13
	1–1.2	6	50	50	
	1.2–1.4	4	75	25	
	> 1.4	1	0	100	
Drying period (days)	8	2	0	100	0.55
	15	1	0	100	
	30	6	66.67	33.33	
	45	3	66.67	33.33	
	60	1	0	100	
	> 60	1	0	100	
	No	1	0	100	
History of diseases[a]	1.2	1	0	100	**0.05**
	1.4	1	0	100	
	1.3.4	2	0	100	
	1.4.5	2	50	50	
	1.2.3.4	1	0	100	
	1.2.4.5	1	100	0	
	1.3.4.5	4	100	0	
	1.2.3.5	2	0	100	
	Does not apply	1	0	100	

Bold numbers indicate significant differences ($p < .05$).
[a] 1: White spot syndrome 2: Vibriosis 3: Hepatopancreatic necrosis 4: Parasites 5: Infectious hypodermic and hematopoietic necrosis.

with *pir*A and *pir*B genes was detected for the first time in Costa Rica, future research should include isolation of *Vibrio* causing AHPND from ponds, PCR analysis of diverse shrimp tissues, confirmation of tissue lesions using histopathological techniques, genotyping the virulence plasmid and determine mortality and economic losses caused by AHPND.

5. Conclusions

The presence of *Vibrio* spp. carrying the plasmid pVA-1 with *pir*A and *pir*B genes that cause AHPND was determined for the first time on shrimp farms in Costa Rica, based on data obtained from sequencing, mortality, and clinical signs recorded on farms. It is recommended, to establish mortalities and economic losses caused by AHPND in *Penaeus vannamei* farms in Costa Rica, and that health authorities provide farmers training on good management practices, encouraging producers to control physical-chemical water parameters and to reduce the use of prophylactic antibiotics.

Table 6
Association between physical-chemical parameters of water with or without *Vibrio* spp. in shrimp farms from Costa Rica, 2017–2018.

Quantitative variables		Vibrio spp.	
		Positive	Negative
Oxygen (mg.L^{-1})	Average	3.10	3.75
	S.D.	1.55	1.70
	C.I. (95%)	1.47–4.73	2.43–5.05
	Pr < l t l	0.47	
Temperature (°C)	Average	32.00	31.10
	S.D.	1.41	2.20
	C.I. (95%)	30.53–33.49	29.41–32.80
	Pr < l t l	0.39	
pH	Average	7.79	7.60
	S.D.	0.096	0.15
	C.I. (95%)	7.52–8.05	7.25–7.94
	Pr < l t l	0.40	
Salinity (ppt)	Average	31.08	31.41
	S.D.	3.93	4.13
	C.I. (95%)	20.96–41.19	21.66–41.15
	Pr < l t l	0.96	

S.D.: Standard deviation; C.I.: Confidence interval; Pr < l t l: Statistical differences ($p < .05$).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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