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Frequencies and factors associated with *pirA*, *pirB* and *Vibrio parahaemolyticus* (Vibrionales: Vibrionaceae) in shrimps and farm waters of Costa Rica

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ABSTRACT

Introduction: Acute Hepatopancreatic Necrosis Disease (AHPND) has been reported in cultured shrimp in recent years and is associated with the bacterium *Vibrio parahaemolyticus*, containing a plasmid with the *pirA* and *pirB* genes that confer pathogenicity.

Objective: To establish the presence of *pirA*, *pirB* and *V. parahaemolyticus* genes in postlarvae, juvenile shrimp, and water from farms in the dry and rainy seasons in Costa Rica, as well as to determine associated factors such as season of the year, farm, organ, pond water with the *pirA* gene, with the *pirB* gene or with the *tlh* gene, pond site, and time of shrimp collection.

Methods: Two shrimp farms in Costa Rica were studied in 2021. A total of 72 juvenile shrimp samples, four groups of postlarvae, and 16 water samples were analyzed. The genes of *pirA*, *pirB* and *V. parahaemolyticus* (*tlh* +) were identified by qPCR, and associated factors were determined.

Results: *V. parahaemolyticus* was identified in 33.3 % of the juvenile shrimp samples, in a group of postlarvae, and in 50 % of the water samples. In the shrimp samples 43 and 45 % were positive for the *pirA* and *pirB* gene amplicon, respectively. Of the water samples, 31.2 and 37.5 % were positive for the *pirA* and *pirB* gene amplicon, respectively. Two factors associated with *V. parahaemolyticus* bacteria (rainy season and *tlh*-positive water), and five factors associated with *pirA* and *pirB* genes (rainy season, presence of the *tlh* gene in water, *pirA* gene in water, *pirB* gene in water, and time of collection) were found. Differences in salinity values were found between dry and rainy season in water samples.

Conclusions: The studied genes were successfully detected in shrimp and pond water samples collected during both seasons in Costa Rica. However, detection was more frequent during the rainy season.

Key words: AHPND; *pirA* gene; *Vibrio* spp; diseases in shrimp; *tlh* gene.

RESUMEN

Frecuencias y factores asociados con *pirA* *pirB* y *Vibrio parahaemolyticus* (Vibrionales: Vibrionaceae) en camarones y aguas de granjas en Costa Rica

Introducción: En los últimos años se ha reportado la Enfermedad de la Necrosis Aguda del Hepatopáncreas (AHPND) en camarones de cultivo y se asocia a la bacteria *Vibrio parahaemolyticus* que contiene un plásmido con los genes *pirA* y *pirB* que le confieren la patogenicidad.

Objetivo: Establecer la presencia de los genes *pirA*, *pirB* y de *V. parahaemolyticus* en postlarvas, en camarones juveniles y agua de granjas en la estación seca y lluviosa en Costa Rica, así como determinar factores asociados



como estación del año, finca, órgano, agua de estanque con el gen *pirA*, con el gen *pirB* o con el gen *tlh*, sitio del estanque y tiempo de recolecta de los camarones.

Métodos: Se estudiaron dos fincas camaroneras en Costa Rica en el 2021. En total se analizaron 72 muestras de camarones juveniles, cuatro grupos de postlarvas y 16 muestras de agua. Se identificaron los genes de *pirA*, *pirB* y *V. parahaemolyticus* (*tlh* +) mediante qPCR y se determinaron factores asociados.

Resultados: Se identificó *V. parahaemolyticus* en el 33.3 % de las muestras de camarones juveniles, en un grupo de postlarvas y en el 50.0 % de las muestras de aguas. En las muestras de camarones 43.0 y 45.0 % fueron positivas para el amplicón del gen *pirA* y *pirB*, respectivamente. Con respecto a las muestras de agua 31.2 y 37.5 % resultaron positivas para el amplicón del gen *pirA* y *pirB*, respectivamente. Se encontraron dos factores asociados a la bacteria *V. parahaemolyticus* (época lluviosa y agua positiva al gen *tlh*) y cinco factores asociados a los genes *pirA* y *pirB* (época lluviosa, presencia del gen *tlh* en agua, gen *pirA* en agua, gen *pirB* en agua y tiempo de recolecta). Se encontraron diferencias en los valores de salinidad entre la época seca y lluviosa en muestras de agua.

Conclusiones: Los genes estudiados fueron detectados con éxito en muestras de camarones y aguas de estanques durante ambas estaciones del año. Sin embargo, la detección fue más frecuente durante el invierno.

Palabras clave: AHPND; gen *pirA*; *Vibrio* spp.; enfermedades en camarón; gen *tlh*.

INTRODUCTION

The increase in demand for food, as well as the search for more nutritious, healthy and sustainable diets by the wider public, have led to the expansion of marine product consumption in recent years. Seafood, being an excellent source of minerals and vitamins, opens the opportunity for the production of food under the modality of aquaculture, as is the case of shrimp farming. This activity has shown great potential and growth both in the American continent, as well as in the rest of the world (Varela & Varela-Moraga, 2019).

However, these farms reach a high density of animals to obtain higher productive yields, which causes a stressful environment for the shrimp, providing ideal circumstances for disease outbreaks. As a result, this industry has been impacted by the consequences of infectious diseases of viral and bacterial origin, effecting significant economic losses (Flegel, 2019; Martínez-Chávez et al., 2022).

On the other hand, environmental factors, both biotic and abiotic, affect the immune system of shrimp, with these effects being more pronounced due to the use of high animal densities. Abiotic factors include temperature, salinity, concentration of nitrogen compounds, pH and dissolved oxygen. However, in recent years, there has been a growing interest in the

study of biotic factors such as the composition of the intestinal microbiota, the presence of biological production compounds by aquatic microorganisms, the use of probiotics, prebiotics and sustainable technologies such as biofloc (Martín-Ríos et al., 2022).

In recent years, a disease affecting farmed shrimp called Acute Hepatopancreatic Necrosis Disease (AHPND) has been presented, which was initially associated with a strain of the bacterium *Vibrio parahaemolyticus* (Tran et al., 2013). *V. parahaemolyticus* is a Gram-negative, halophytic bacterium that is widespread in estuarine, marine and coastal environments (Broberg et al., 2011; Ceccarelli et al., 2013; Letchumanan et al., 2014), this bacterium possesses a plasmid containing two genes coding for the synthesis of PirA and PirB toxins, which confer pathogenicity (Han, Tang & Lightner, 2015). It is now known that other species of the genus *Vibrio* have also been identified as responsible for AHPND (Dong et al., 2017; Kondo et al., 2015; Liu et al., 2018; Restrepo et al., 2018).

In Costa Rica, there is a molecular study whose objective was to investigate the presence of *Vibrio* spp. and the plasmid genes encoding PirA and PirB toxins in shrimp farms. The study detected *pirA* and *pirB* genes in 33.3 % (5 / 15) and *Vibrio* spp. in 40.0 % (6 / 15) of the farms. In two of the six farms where *Vibrio*

spp. was found, the species *V. parahaemolyticus* was identified (Peña-Navarro et al., 2020). However, in Costa Rica there are no periodic studies that evaluate the analyzed genes through sampling at different points in time, nor are there investigations that determine the influence of the two seasons of the year on the prevalence of the disease.

The objective of the present study was to investigate the presence of *pirA*, *pirB* genes and *V. parahaemolyticus* species in postlarvae and juvenile shrimp of the species *Litopenaeus vannamei* and in pond water samples, in two farms located in the Gulf of Nicoya, Costa Rica, in both dry and rainy seasons, as well as to establish factors associated with *pirA*, *pirB* genes and *V. parahaemolyticus* bacteria.

MATERIALS AND METHODS

Study design, origin and sample size: A longitudinal non-experimental research design of trend was carried out, given that the independent variables were not intentionally altered, but rather the phenomena were observed as they occurred in the natural context. In addition, the studies collected data at different points in time, using a series of samples that covered different participants at each moment, which were part of the same population (Hernández-Sampieri et al., 2014). Two private shrimp farms (Farm A and Farm B) using the conventional production method, located in the Gulf of Nicoya on the Pacific coast of Costa Rica, were studied.

In Costa Rica, the aquaculture of white shrimp of the genus *Litopenaeus* is primarily developed in the Gulf of Nicoya, and the Central and South Pacific areas (Instituto Costarricense de Pesca y Acuicultura 2020). However, the Gulf of Nicoya is the area of Costa Rica with the greatest potential for the cultivation of aquaculture species (Quesada-Céspedes et al., 2019). It is characterized by a rainy period from May to November (rainy season) and a period where rainfall is scarce between December and April (dry season) (Herrera, 2016).

During the harvest months, both farms sampling was carried out approximately every

three to four weeks, starting with sampling one (time 0, day of stocking), sampling two (three to four weeks after stocking), sampling three (six to seven weeks after stocking) and sampling four (nine to ten weeks after stocking). Dry season samples were collected between February and April and rainy season samples between July and September 2021. On each farm, a pond of approximately four hectares was sampled.

Both shrimp of the species *Litopenaeus vannamei* (postlarvae and juveniles), and pond water were studied. The postlarvae studied were of national origin and came from a laboratory near the area, supplier of the two farms. A total of 72 samples (36 hepatopancreas and 36 intestines) from 36 groups of juvenile shrimps and four groups of postlarvae were analyzed. Sampling of juvenile shrimp was done at three points in the pond using cast nets: inlet, center and outlet of the water, five shrimp were sampled at each point, for a total of three groups per farm per sampling, the three groups were placed in different sterile containers with water from the same pond.

Water sampling in the ponds was done at only one point in the pond; in the central part, one sample was taken per farm per sampling, for a total of 16 water samples from the two farms and from the two seasons of the year. Approximately 250 ml of water were sampled at 10 cm from the surface in sterile bags. In each sampling, *in situ* physical chemical parameters of the water were measured in the center of the pond: temperature, pH, dissolved oxygen and salinity, using a LabQuest 2 interface and temperature, pH, dissolved oxygen and salinity probes.

All samples were placed in a cooler and transported to the Biotechnology Laboratory of the Greece Campus of the University of Costa Rica, where they were stored at 4 °C until analysis, no more than 24 hours after collection.

Bacterial strains and culture conditions: The following strains were used as positive controls: *V. parahaemolyticus* (*pirA*+ and *pirB*+, donated by the Universidad Técnica



Nacional, Sede Regional de Guanacaste, Costa Rica) and *V. parahaemolyticus* ATCC 17802 (*tlh*⁺). Nuclease-free water was used as a control without template and commercially acquired *Escherichia coli* K-12 and a strain of *Vibrio alginolyticus*, donated by the Faculty of Microbiology of the University of Costa Rica, were used as negative controls for the genes studied.

The shrimp groups collected at each sampling point were necropsied aseptically, the hepatopancreas and intestine were extracted, and then these organs were macerated separately. In the case of postlarvae, hundreds of them were macerated. The macerates were enriched in APW (alkaline peptonized water) with 2 % sodium chloride.

The water samples were homogenized, and 25 ml of each sample was taken and enriched separately in 225 ml of APW with 2 % sodium chloride (Kaysner et al., 2004). The water and shrimp samples in APW were then incubated for six hours at 37 °C. Then they were inoculated on TCBS agar (thiosulfate citrate bile sucrose agar) with 1 µl, 10 µl and 100 µl of each of the enriched samples, using sterile micropipettes and Drigalsky loops. The inoculated plates were placed in the incubator at a temperature of 37 °C for 24 hours.

Extraction and verification of DNA quality and quantity: DNA was extracted from colonies grown on TCBS plates, compatible with morphological characteristics reported for the species *V. parahaemolyticus*: round, blue-green, opaque colonies, 2 to 3 mm in diameter (Kaysner et al., 2004; Nelapati et al., 2012). The extraction kit used was Pure Link Genomic DNA Mini Kit Invitrogen (K1820-01), the manufacturer's protocol was followed, with some modifications. Once the DNA was extracted from the samples, it was stored at -20 °C.

The quantity and quality of DNA in the samples was verified by measuring absorbance at 260 nm in a microvolume spectrophotometer (NanoDrop One Thermo Fisher Scientific) and by fluorescence (Qubit® 4 fluorimeter, Thermo

Fisher Scientific-Invitrogen). All samples had the required amount of total DNA (on average 23 ng/µl) to be analyzed by qPCR. Samples were run on 2 % agarose gels to confirm their integrity.

Molecular identification: Identification of *V. parahaemolyticus* bacteria species was carried out by qPCR amplification of a partial sequence of approximately 248 bp of the *tlh* (thermolysin thermolabile hemolysin) gene following the protocol described by Micky et al. (2014), with the following modifications: each 25 µL reaction was carried out with a final concentration of 1X Maxima SYBR Green/ROX qPCR Master Mix (2X) Thermo Scientific and, as for the qPCR conditions an initial denaturation for 7 min at 95 °C was used, and a final extension of 72 °C for 7 min was added (Table 1).

Identification of the amplicons of the *pirA* and *pirB* genes was carried out by implementing two different qPCR protocols, the primers used were designed by Han, Tang, Pantoja et al. (2015); Han, Tang, Tran et al. (2015), respectively. A partial sequence of approximately 135 bp of the *pirA* gene was amplified, a reaction was prepared with 10 µl of Maxima SYBR Green/ROX qPCR Master Mix (2X) Thermo Scientific, 2 µl of each primer (15 µM), 2 µl of the extracted DNA and 4 µl molecular biology grade water (Thermo Scientific), for a final volume of 20 µl. The qPCR conditions used are specified in Table 1.

For the partial sequence of the *pirB* gene, a fragment of approximately 102 bp was amplified, a reaction was prepared with 10 µl of Maxima SYBR Green/ROX qPCR Master Mix (2X) Thermo Scientific, 2 µl of each primer (3 000 nM), 1 µl of the extracted DNA and 5 µl of molecular biology grade water (Thermo Scientific), for a final volume of 20 µl. The qPCR conditions were the same as those used for the amplification of the *pirA* gene amplicon (Table 1).

The efficiency of the three protocols implemented in qPCR was determined by standard curves of 5 dilutions, each dilution in triplicate.

Table 1
Conditions used in the qPCR protocols for molecular identification of *tlh*, *pirA* and *pirB* genes.

qPCR Protocol	PCR Stages	Temp (°C)	Cycles	Time	Primer	Sequence	Amplicon size
<i>tlh</i>	Initial denaturation	95	1	7 min	Forward	5'-GTT GCA CTC	248 bp
	Denaturation	95	50	5 s	MV2B-TLF	GGT GAC AGC TTG-3'	
	Annealing	60	50	10 s	Reverse MV2B-TLR	5'- AGT TTT GCG TAG GTT AAG TAC-3'	
	Extending	72	50	25 s			
	Final extending	72	1	7 min			
<i>pirA</i>	Initial denaturation	95	1	3 min	Forward VpPirA-F	5'-TTG GAC TGT CGA ACC AAA CG-3'	135 bp
	Denaturation	95	40	5 s	Reverse	5'-GCA CCC CAT	
	Annealing and extending	60	40	30 s	VpPirA-R	TGG TAT TGA ATG3'	
<i>pirB</i>	Initial denaturation	95	1	3 min	Forward VpPirB-F	5'-ACT AGG CAA GGC TCA TAA ATA TGA CG-3'	102 bp
	Denaturation	95	40	5 s	Reverse	5'-ATT GCT CAG GTC	
	Annealing and extending	60	40	30 s	VpPirB-R	CAT TGG CA ATA A-3'	

Temp: temperature, bp: base pair.

The curves showed efficiencies of 100, 110 and 106 % for the partial sequence of the *tlh*, *pirA* and *pirB* genes, respectively. The specificity of the protocols was evaluated in two ways: 1) qPCR products from the three standard curves were run on a 1.5 % agarose gel to corroborate the existence of a single product with the expected size, 2) dissociation curve analyses were performed at the end of each PCR run, which always showed a single amplified product. Samples with Ct (threshold cycle) values between 14 and 35 were considered positive.

Positive samples for *pirA*, *pirB* genes were subjected to conventional PCR protocols for confirmation and the protocol and primers designed by Sirikharin et al. (2015) were used. Samples that were successfully amplified by conventional PCR for the *pirA* genes and some samples amplified by qPCR for the *tlh* gene were sent for sequencing to CIBCM (Centro de Investigación en Biología Celular y Molecular) at the University of Costa Rica. The sequences of the samples were aligned with the BioEdit Sequence Alligment Editor®2.2.28 program and compared using the BLASTn® 2.2.28 algorithm with the NCBI database.

Statistical analysis: Factors associated with the *pirA*, *pirB* and *tlh* genes of

qPCR-positive shrimp were studied using a non-conditional logistic regression model. The risk was estimated using the odds ratio (OR) and those factors with a $p < 0.05$ were considered associated factors. The factors analyzed were season of the year, farm, organ, water with the *pirA* gene, water with the *pirB* gene, water with the *tlh* gene, pond site and time of shrimp collection.

Depending on the distribution of the data, an independent sample t-test was performed for pH and dissolved oxygen and a nonparametric Mann Whitney U test for temperature and salinity to compare the averages or medians of the physicochemical parameters between the positive and negative water samples for the *tlh*, *pirA* and *pirB* genes, in order to determine if there are differences between the two groups.

Likewise, the averages or medians of the physicochemical parameters obtained during dry season and rainy season were obtained and compared between them to determine any existing differences. The Mann Whitney U-test was used for samples with non-normal distribution (temperature and oxygen) and the T-test for samples with normal distribution (salinity and pH).

Statistically significant differences were considered for those values with a $p < 0.05$. All

statistical analyses were conducted using SPSS Statistics version 25 (IBM Corp., 2017).

RESULTS

Molecular analysis in shrimp: Of the shrimp groups studied, 44.4 % (16 / 36) were positive for the presence of *V. parahaemolyticus*, in five of them the bacterium was detected only in the hepatopancreas, in three exclusively in the intestine, and in eight in both intestine and hepatopancreas. Fig. 1 shows the results of the frequencies of detection of *V. parahaemolyticus* in individual samples of juvenile shrimp organs and postlarvae groups.

Of the groups of juvenile shrimps analyzed, 55.5 % (20 / 36) were determined to be positive for the *pirA* gene amplicon, of which six groups presented the amplicon only in the hepatopancreas, three only in the intestine, and 11 in both the intestine and hepatopancreas.

The results of the detection frequencies of the *pirA* gene amplicon in individual samples of juvenile shrimp organs and postlarvae groups are shown in Fig. 2. Of the 31 samples positive for this gene in juvenile shrimp organs, 30 were

also positive for the *pirB* gene amplicon and 18 were identified as *V. parahaemolyticus*, the latter suggesting that other species of the genus *Vibrio* present the *pirA* gene amplicon.

Of the groups of juvenile shrimps analyzed, 55.5 % (20 / 36) were determined to be positive for the *pirB* gene amplicon, of which, four showed the amplicon only in the hepatopancreas, three only in the intestine, and 13 in both the intestine and hepatopancreas.

The results of the detection frequencies of the *pirB* gene amplicon in individual samples of juvenile shrimp organs and postlarvae groups are represented in Fig. 3. Of the 33 *pirB*-positive samples in juvenile shrimp organs, 30 were also positive for the *pirA* gene amplicon and 19 were identified as *V. parahaemolyticus*, the latter suggesting that other *Vibrio* species have the *pirB* gene amplicon.

Molecular analysis in waters: The frequencies of detection of *V. parahaemolyticus* and the *pirA* and *pirB* gene amplicon in the pond water samples are presented in Fig. 1, Fig. 2 and Fig. 3. Of the five water samples that were positive for the *pirA* gene amplicon,

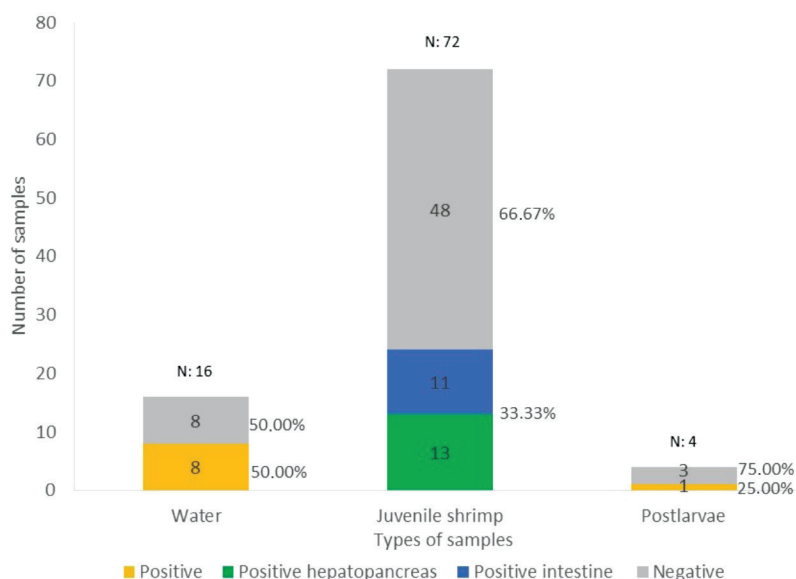


Fig. 1. Presence of the *tllh* amplicon in individual organ samples from juvenile shrimp, postlarvae groups and pond water. N: total number of samples analyzed.

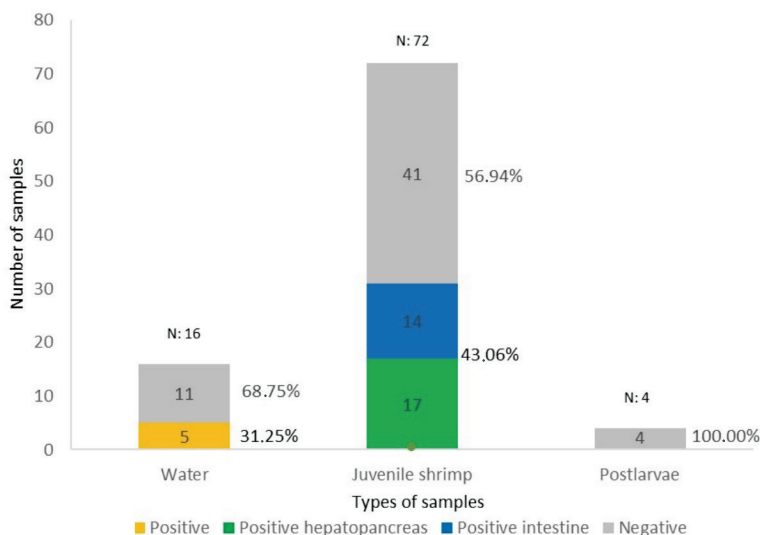


Fig. 2. Presence of the *pirA* amplicon in individual organ samples from juvenile shrimp, postlarvae groups and pond water. N: total number of samples analyzed.

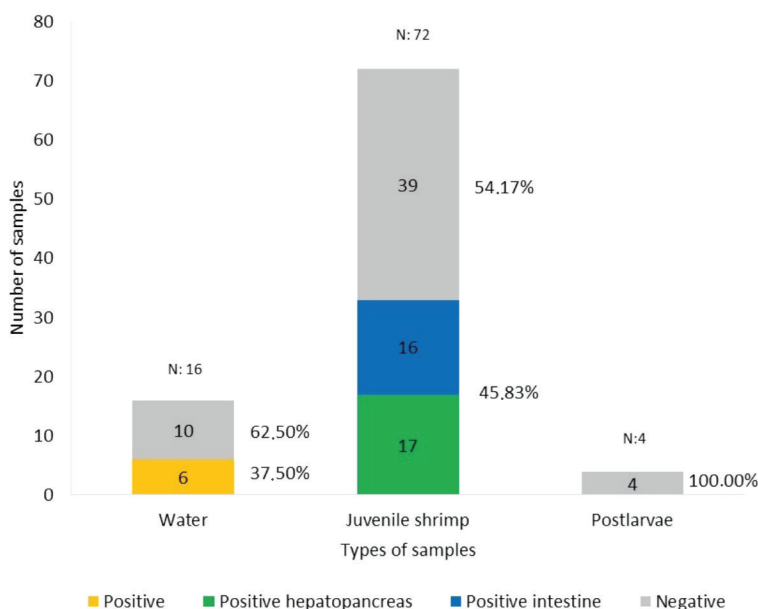


Fig. 3. Presence of the *pirB* amplicon in individual organ samples from juvenile shrimp, postlarvae groups and pond water. N: total number of samples analyzed.

three were identified as *V. parahaemolyticus* and all five were also positive for the *pirB* gene amplicon. Of the six water samples that were positive for the *pirB* gene amplicon, four were identified as *V. parahaemolyticus* and five were positive for the *pirA* gene amplicon.

Sequencing: Sequencing of three PCR products from hepatopancreas (248 / 248 bp), intestine (247 / 247 bp) and water (248 / 248 bp) samples amplified with the qPCR protocol for the *tlh* gene [GenBank PP591942, PP591943 and PP591944, respectively]



confirmed the presence of *V. parahaemolyticus* species and were found to be 100 % identical to other sequences of this same species deposited in GenBank. Of all the samples positive for the *pirA* gene in conventional PCR, we were able to sequence a product from a 322 / 323 bp hepatopancreas sample, [PP795453], this sequence was 99.7 % identical to other sequences of the *pirA* gene of the genus *Vibrio* deposited in GenBank. With the conventional PCR protocol used for the *pirB* gene, positive control was amplified, but no sample from the present study.

Factors associated with the *pirA*, *pirB* and *tlh* genes of positive shrimp in qPCR:

Five factors were found to be associated with the presence of the *pirA* and *pirB* gene in shrimp ($p < 0.05$): rainy season (*pirA*: OR = 3.64, 95 % CI (confidence interval) = 1.36-9.75; *pirB*: OR = 2.8, 95 % CI = 1.07-7.30), presence of *pirA* in water (*pirA*: OR = 4.32, 95 % CI = 1.59-11.74; *pirB*: OR = 3.45, 95 % CI = 1.29-9.22), presence of *pirB* in water (*pirA*: OR = 11.36, 95 % CI = 3.68-35.09; *pirB*: OR = 7.95, 95 % CI = 2.76-22.92), presence of *tlh* in water (*pirA*: OR = 3.34, 95 % CI = 1.25-8.92; *pirB*: OR = 3.45, 95 % CI = 1.29-9.21) and collection time: 42-49 days after stocking (*pirA*: OR = 4.20, 95 % CI = 1.23-14.36; *pirB*: OR = 3.4, 95 % CI = 1.03-11.26). Two factors were found to be associated with the presence of *V. parahaemolyticus* bacteria: rainy season (OR = 15.40, 95 % CI = 3.97-59.70) and presence of the *tlh* gene in water (OR = 9.00, 95 % CI = 2.90-27.90) (SMT1, SMT2, SMT3).

Physicochemical parameters of the water in the ponds studied: No significant differences were found in terms of the values of physicochemical parameters between the groups of positive and negative waters for the amplicons of the *pirA*, *pirB*, and *tlh* genes. Table 2 shows the physicochemical parameters of the water in the ponds of the two farms studied; the averages of each parameter were calculated according to the season of the year. Significant differences ($p < 0.05$) were only

found in the salinity parameter of pond water between the dry and rainy seasons.

The qPCR protocols implemented in the present investigation proved to be efficient and economical and allowed the identification of *V. parahaemolyticus* and the pathogenic genes *pirA* and *pirB* in shrimp and pond water samples, as well as in samples that had not been analyzed (shrimp intestines) in Costa Rica. In addition, factors associated with the presence of *V. parahaemolyticus* bacteria (rainy season and water positive for the *tlh* gene) and pathogenic genes (rainy season, water positive for the *tlh*, *pirA* and *pirB* genes and time of collection: 42 to 49 days after stocking) were identified.

DISCUSSION

The three protocols implemented presented good efficiencies and specificity, which agrees with what has been reported by other authors (Han, Tang, Pantoja et al., 2015; Han, Tang, Tran et al., 2015; Micky et al., 2014). It is important to mention that the primers used in the amplification protocol of the partial sequence of the *pirA* gene were previously used with TaqMan probes (Han, Tang, Pantoja et al., 2015), however, in the present work a much more economical technique, the SYBR Green I, was used. Our study shows this technique provides reliable results.

The presence of plasmidic genes *pirA*, *pirB* and *V. parahaemolyticus* in shrimp samples and pond waters is reported high frequencies. It was also possible to determine the genes in the intestines of juvenile shrimp, an organ that had not been studied for genes in the country so far.

In relation to the groups of shrimp analyzed, high frequencies of the *pirA* and *pirB* genes (55.5 %) and *V. parahaemolyticus* (44.4 %) were determined; these data are consistent with those reported by other authors when finding high prevalences of AHNPD in shrimp (Morales-Covarrubias et al., 2019; Peña-Navarro et al., 2020).

Sirikharin et al. (2015) point out that when working with field samples, including shrimp tissues such as hepatopancreas and stomach,

Table 2

Physicochemical parameters of the water quality of two ponds belonging to two shrimp farms (farm A and farm B) producing *L. vannamei* shrimp in the Gulf of Nicoya, Costa Rica, during the year 2021.

Parameters		Temperature (°C)	pH	Dissolved oxygen (mg/L)	Salinity (ppt)
Optimal growth <i>V. parahaemolyticus</i> ¹		35-37	7.5-8.6	Facultative anaerobic	5-25
Optimal from shrimp farming ²		28-30	7.5-8.5	5.0-10.0	15-25
Farm	Sampling time	Temperature (°C)	pH	Dissolved oxygen (mg/L)	Salinity (ppt)
A	Sampling one	28.50	7.05	10.90	27.00
A	Sampling two	34.20	8.65	8.80	28.90
A	Sampling three	33.10	8.38	10.30	30.30
A	Sampling four	33.50	8.52	6.84	29.40
B	Sampling one	26.00	8.15	8.00	25.50
B	Sampling two	31.60	8.07	7.40	32.00
B	Sampling three	33.40	7.86	10.00	30.70
B	Sampling four	33.90	8.09	8.92	31.60
Average dry season		31.80	8.09	8.89	29.42
Max.		34.20	8.65	10.90	32.00
Min.		26.00	7.05	6.84	25.50
A	Sampling one	32.10	8.42	7.80	17.00
A	Sampling two	21.60	8.93	11.75	19.60
A	Sampling three	32.20	8.34	8.36	17.30
A	Sampling four	33.40	7.63	8.50	19.70
B	Sampling one	32.10	8.50	9.71	17.90
B	Sampling two	34.10	9.07	11.80	16.40
B	Sampling three	26.00	8.78	8.53	16.60
B	Sampling four	32.00	8.43	7.83	17.50
Average rainy season		30.44	8.51	9.28	17.75
Max.		34.10	9.07	11.80	19.70
Min.		21.60	7.63	7.80	16.40

Sampling one: day of stocking, sampling two: three to four weeks after stocking, sampling three: six to seven weeks after stocking, and sampling four: nine to ten weeks after stocking. Values of parameters found to be outside the optimal range for shrimp culture are highlighted in bold. / ¹ Source taken from Urquhart et al. (2016) and Zamora-Pantoja et al. (2005). / ² Source taken from Valverde (2020).

whole postlarvae and environmental samples such as pond water in which the levels of AHPND bacteria are low, a preliminary enrichment step should be conducted. This is why all the samples in this study were pre-enriched in APW with NaCl. This culture medium is recommended as enrichment broth for *Vibrio*, so perhaps this contributed to the high detection of the genes of interest in addition to the fact that the qPCR technique has a high detection sensitivity when compared to conventional PCR (Valasek & Repa, 2005).

The difference observed in the number of cases identified as positive for the *pirA* gene with respect to *pirB* could be explained by

one of the amplicon detection methods being more sensitive than the other or by the presence of strains having only one of the genes (Aranguren-Caro et al., 2020), however, further studies are recommended to corroborate this.

With respect to the bacterial species isolated from shrimp and water samples, not all were identified as *V. parahaemolyticus*. Although *V. parahaemolyticus* was initially identified as the causative agent of AHPND in shrimp, more recent studies have reported the plasmid with the genes of interest in other *Vibrio* species, which caused AHPND in shrimp, these species include *Vibrio harveyi* (Kondo et al., 2015), *Vibrio owensii* (Liu et al., 2018),



Vibrio campbellii (Dong et al., 2017) and *Vibrio punensis* (Restrepo et al., 2018). Lee et al. (2015) indicate that the pVA1 plasmid possessed by *V. parahaemolyticus* strains contain a group of genes related to conjunctival transfer, so it can potentially transfer not only between *V. parahaemolyticus* strains but also to different *Vibrio* species. Therefore, it is recommended in future research to determine which species the strains carrying the *pirA* and *pirB* genes that are negative for *V. parahaemolyticus* belong to. Thus, determining which other species in Costa Rica present the pathogenic genes.

The detection of *V. parahaemolyticus* bacteria in a group of shrimp postlarvae is consistent with reports by other authors that indicate that infection of postlarvae with *V. parahaemolyticus* bacteria is possible in the laboratory, when they are in the last stage of metamorphosis (Aguirre-Guzmán et al., 2001). On the contrary, the non-detection of the *pirA* and *pirB* genes in the shrimp postlarvae indicates that they were seeded free of pathogenicity genes, which tends to think that the bacteria with pathogenic genes could have been acquired from water, food or sediments in the ponds and not from the seed (Alonzo et al., 2017; Chonsin et al., 2016; Thitamadee et al., 2016).

Alonzo et al. (2017) found in their study that *V. parahaemolyticus* in shrimp rearing water adhered to added feed, which facilitated its entry into the shrimp gut; thereby, showing that the oral cavity serves as an important portal of entry for pathogens from the shrimp rearing environment. In the farms studied, feed was provided to the shrimp in troughs located at different points in the ponds, at scheduled times so that the shrimp in the water had access to it, which could also provide a possible explanation for the presence of this bacterium and the genes of interest in the shrimp studied.

Another source of AHNPD infection could also be sediments, as pathogenic *V. parahaemolyticus* has been detected in shrimp pond sediments (Thitamadee et al., 2016). It is for this reason that it is recommended to always implement proper preparation of the culture area before stocking and properly manage the

pond during culture to exclude pathogenic *V. parahaemolyticus* strains from the shrimp culture system. Because of the above, it is recommended in future research to also analyze other types of samples such as sediments or feed in search, for the bacteria and pathogenic genes (de Souza-Valente & Wan, 2021; Thitamadee et al., 2016).

Zamora-Pantoja et al. (2005) indicate that *V. parahaemolyticus* grows at temperatures between 10 and 44 °C, but with an optimum growth temperature of 35 °C and 37 °C. Therefore, in this study, a higher probability of finding qPCR-positive shrimp in summer, when temperatures are higher, was expected. In contrast, qPCR positive shrimp samples for *pirA*, *pirB* and *tlh* were 4 (OR = 3.64), 3 (OR = 2.8) and 15 (OR = 15.4) times more likely to occur in the rainy season than qPCR negative samples, respectively.

However, when comparing the water temperature in the ponds during the dry and rainy seasons, no significant differences were observed; moreover, the water temperatures recorded in both seasons were within the values considered optimal for the growth of *V. parahaemolyticus*. Therefore, temperature in this study was not a determining factor in the probability of finding qPCR-positive shrimp for the genes studied. The only factor analyzed that was significantly different between the two seasons was salinity, which could be related to a higher probability of the presence of the genes studied in the rainy season.

Studies have shown that in tropical zones, salinity is an important factor that regulates the dynamics of *Vibrio* and that a moderate decrease in this parameter can promote the abundance of *Vibrio* spp. On the other hand, in some research works, a negative correlation between salinity and *V. parahaemolyticus* abundance has been observed (Machado & Bordalo, 2016; Reyes-Velásquez et al., 2010). In our study, it was determined that during rainy season salinity presented on average a significantly lower value (17.75 ppt) compared to those from dry season (29.43 ppt), probably due to the amount of rainfall that occurs during

rainy season in Costa Rica, which produces an increase in the water levels of the shrimp ponds and thus a decrease in the concentration of salinity.

If the optimal environmental range of *V. parahaemolyticus* is considered, it generally increases in abundance with salinity between 5 and 25 ppt (Urquhart et al., 2016). According to this, the average salinity that was presented during rainy season was more favorable for the growth of *V. parahaemolyticus* than that determined during dry season, which was presented above the optimal range for this bacterium, which could explain the higher probability that the samples of shrimp positive for *pirA*, *pirB* and *V. parahaemolyticus* were presented in the rainy season than the negative ones.

Shrimp qPCR positive for *pirA*, *pirB* and *tlh* were identified to be 3 (OR = 3.35), 3 (OR = 3.45) and 9 (OR = 9.00) times more likely to occur in *tlh* gene positive pond water than qPCR negative for these genes, respectively. On the other hand, qPCR positive shrimp for *pirA* and *pirB* were identified to be 4 (OR = 4.32) and 3 (OR = 3.45) times more likely to occur in pond water positive for the *pirA* gene than qPCR negative shrimp, respectively. In addition, they were 11 (OR = 11.36) and 8 (OR = 7.95) times more likely to occur in pond water positive for the *pirB* gene than negative qPCRs, respectively. *V. parahaemolyticus* is known to be a ubiquitous bacterial pathogen naturally found in marine environments (Baker-Austin et al., 2010; World Organization for Animal Health, 2023). Therefore, this pathogenic bacterium could be expected to enter through the seawater used to fill and replace the water in the ponds. Under favorable environmental conditions, the bacteria would have proliferated, thus facilitating its detection.

Such results are in agreement with those found in a previous study that reported an association of the presence of *Vibrio* spp bacteria with high replacement rates and water volume in the ponds (Peña-Navarro et al., 2020), which could lead to an imbalance in the abundance of the different bacterial communities and a proliferation of pathogenic *Vibrio*. Accordingly,

it has been reported that AHNPD can cause a dysbiosis in the microbiota of the hepatopancreas of shrimp *Litopenaus vannamei* (Ávila-Villa et al., 2023).

On the other hand, in our study, the *pirA* and *pirB* genes began to be detected in both shrimp and pond water between sampling two and three, which corresponds to the period between 21 and 49 days after restocking the ponds. Authors such as Ananda-Raja et al. (2017), Han, Tang, Tran et al. (2015), and Zorichzahra and Banaederakhshan (2015), note that *V. parahaemolyticus* appears between 20 and 30 days after stocking ponds with post-larvae. Thitamadee et al. (2016) point out that mortalities occur between 35 and 45 days after postlarvae stocking. These studies support our results by presenting the first detection in the second sampling (21 and 30 days). However, it was not until the third sampling (42-49 days) when an association with the presence of the genes was observed.

It was not possible to find differences between the averages or medians of the salinity, temperature, dissolved oxygen and pH factors between positive and negative detection of the gene of interest in the water. This could be due to the fact that the samples came from a single point in each sampling. Therefore, it is recommended that more pond water sampling, which better represents the distribution of the data, be conducted in future research.

With respect to the optimal values of the pond water for shrimp culture, the averages of two variables were found to be above the optimal values during dry season: temperature and salinity. Higher temperature values could generate stress conditions in shrimp and higher salinity values could represent a higher energy expenditure in osmoregulation and thus a lower growth rate. These alterations can be exploited by opportunistic pathogens such as *V. parahaemolyticus*, increasing their population and colonizing their hosts (Carbajal-Hernández & Sánchez-Fernández, 2013; Lazur, 2007). Therefore, adequate management of the physicochemical parameters of the water in shrimp culture ponds is recommended.



Our results showed that *Vibrio parahaemolyticus* bacteria and the pathogenic genes *pirA* and *pirB* were successfully detected in shrimp and pond water samples collected during both seasons of the year in Costa Rica. However, detection was more frequent during the rainy season, probably due to a significantly lower concentration of dissolved salts in pond water compared to the concentrations found during the summer season. This suggests that salinity could be an important environmental variable to consider in the ecology of pathogenic *Vibrio parahaemolyticus* bacteria and the incidence of AHNPD. In addition, high frequencies of the genes studied were found, so it is recommended that animal health authorities in Costa Rica pay special attention to AHNPD, which has been declared notifiable by the World Organization for Animal Health (WOAH).

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See supplementary material
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