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Research Article

Characterization of *Vibrio parahaemolyticus* strains and evaluation of shrimp cultivation conditions in a farm at the northwestern of Mexico, as risk predictors for its adaptation and dissemination

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ABSTRACT. *Vibrio parahaemolyticus* is recognized as a human pathogen as well as the causative agent of vibriosis in shrimp. This study determined the pathogenic, antimicrobial, and biotic potential of *V. parahaemolyticus* isolated from white shrimp (*Penaeus vannamei*) and seawater on a northwestern Mexico farm. A total of 140 samples were randomly collected, including juvenile organisms (n = 120) and seawater (n = 20). The pH, salinity, and biota of the ponds were used to correlate with bacterium presence. The strains were characterized by virulence genes presence, biofilm formation capacity, antimicrobial sensitivity, and the kinetics growth using PCR, microplates method, minimum inhibitory concentration (MIC), and spectrophotometry, respectively. *V. parahaemolyticus* was detected in 7.1% of the samples with a mean concentration of 3.72 ± 1.24 log CFU mL^{-1}; 6.7% (8/120) in shrimp and 10.0% (2/20) in seawater. Cultivation conditions were not predictive of the specie ($P > 0.05$). *V. parahaemolyticus* showed an adaptation time of 1.0 h, and a growth rate of 0.375 h^{-1} in seawater at 30ºC. The strains were classified into two pathotypes: $tlh^+/tdh^-/trh^-/AP2^-$ (75%) and $tlh^+/tdh^-/trh^-/AP2^+$ (25%) and three resistant profiles to clinical drugs (ampicillin, amikacin, gentamicin, and netilmicin). The MIC values against oxytetracycline (OTC), florfenicol (FFC) and enrofloxacin (ENRO) were >0.50, >0.25 and >0.06 µg mL^{-1}, respectively. Biofilm formation was a property identified in 40% of the strains. The presence of infectious *V. parahaemolyticus* with high adaptative potential justifies integrating integrated aquaculture practices and management to control pathogen growth and shrimp health.

Keywords: *Penaeus vannamei*; *Vibrio parahaemolyticus*; phenotypes; characterization; survival; aquaculture

INTRODUCTION

Shrimp farming is a growing economic activity world-wide, with an annual growth rate of 30% (FAO 2015). Unfortunately, factors such as the exploitation of shrimp production, the emergence of infectious diseases, the unhealthiness of water, and the deficiency in the feeding of organisms have negatively impacted the health of shrimp, generating significant economic losses (USD 15 billion) to this industry (Flegel 2012). The main challenge of shrimp farming is controlling infectious diseases that affect shrimp health and consumer health (FAO 2016).

Vibrio parahaemolyticus is a halophilic bacterium that inhabits marine ecosystems and estuaries, and some marine species (Bauman & Schubert 1984, Liu 2011). The proliferation and distribution of *V. parahaemolyticus* in the aquatic environment are conditioned by parameters such as salinity (0.5-34%), temperature (4-43ºC), pH (5-10), and water biota (Scott et al. 2002, Manjano-Mendoza et al. 2009). When these parameters are favorable, the bacteria can proliferate and persist for prolonged periods in sediment, fauna, and water (Versalovic & Lupski 2002). Some inherent factors, such as the formation of biofilms and the acquisition of antimicrobial resistance, promote their

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persistence in developing strategies to survive and adapt in the environment and consequently hinder their eradication and control (Martínez-Urtaza et al. 2010).

The pathogenic character of *V. parahaemolyticus* is conditioned by genetic factors that modulate adhesion to host cells (Wang et al. 2015). The main virulence factors of the bacteria that affect humans are direct thermostable *(tdh*) and associated (*trh*) hemolysins (Wang et al. 2015). Additionally, the thermolabile hemolysin (*tlh*) is a factor found in *V. parahaemolyticus* regardless of origin. Its expression is a key for the species identification of this species (Broberg et al. 2011). This gene can express two types of activities, the thermolabile hemolysis of human erythrocytes and the activation of the phospholipase enzyme that causes cell wall lysis. Other molecular factors associated with cellular proteolytic capacity (VPA), cell necrosis (Tran et al. 2013), and AHPND marker (AP2) have been described as key elements for the infective cycle of VP (Yáñez et al. 2015, Theethakaew et al. 2017).

The proliferation and physiological properties of *V. parahaemolyticus* in aquatic ecosystems have caused its dissemination and favored the generation of outbreaks of human gastroenteritis (Wang et al. 2015). *V. parahaemolyticus* is currently typified as an emerging human food-borne pathogen worldwide, mainly linked to raw seafood consumption and diarrhea as a clinical manifestation (Liu 2009, Scallan et al. 2011, Newton et al. 2012).

Specifically, shrimp has played an important role as a transmission vehicle for *V. parahaemolyticus* to humans and as a host vulnerable to bacterial infection (Flegel 2012). Notably, the acute hepatopancreatic necrosis syndrome (AHPND) is a shrimp-associated disease due to a strain variant with a 69-70 kb plasmid encoding *Photorhabdus* for *V. parahaemolyticus* (Han et al. 2015). The AHPND has negatively impacted aquaculture farms due to massive shrimp mortality (Anderson et al. 2016), and Mexico is not the exception. In the northwestern Mexico area, diseases of the Pacific white shrimp *Penaeus vannamei* by *V. parahaemolyticus* have affected the aquaculture industry, reducing shrimp production by up to 25% (162.3 thousand ton) and generating closure of shrimp farms (Bermúdez-Almada et al. 2014).

Some descriptions about the pathogenic potential, antimicrobial sensitivity and phylogeny about the pathogen have been made in Mexico (López-Hernández et al. 2015, Rivas-Montaño et al. 2018). *V. parahaemolyticus* strains' resistance to some clinic antibiotics, such as ampicillin and cefotaxime, has increased in recent years (Sudha et al. 2014, Jun et al. 2016).

Among the most widely used antibiotics in Mexican aquaculture are oxytetracycline (OTC), florfenicol (FFC), sarafloxacin (SFX), and enrofloxacin (ENRO) (Soto-Rodríguez et al. 2008). Mainly tetracyclines and quinolones have been used to control diseases such as vibriosis and furunculosis in shrimp. OTC and ENRO are effective due to their broad spectrum of activity against Gram-positive cocci and Gram-negative bacilli (Lara-Espinoza et al. 2015). However, Bermúdez-Almada et al. 2017 described the indiscriminate use of antibiotics in shrimp farms in Mexico's northwest. These authors describe how the antibiotics used in the region have wreaked havoc in the production because the mechanisms of action of commonly used antibiotics are spliced. In addition to antimicrobial resistance, they produce null effects in the organisms to which they were applied.

One strategy in production is the removal of sediment to remove the greatest amount of organic waste. However, the removal of sediments does not guarantee a decrease in the microbial load due to the generation of biofilms characteristic of bacteria that allow them to survive extreme conditions. This activity occurred when ponds are filled with water for the start of the production cycle. The surviving microorganisms can benefit from the high availability of nutrients in the water and sediment. Considering that *V. parahaemolyticus* is a fast-growing and adapting bacterium, it takes advantage of the available nutrients for its proliferation, affecting shrimp larvae in short periods during growth (Schryver et al. 2014).

The necessity of the research and the elucidation of the phenotypic characteristics of *V. parahaemolyticus* represents a threat to public health and shrimp health. It is essential to characterize the properties that favor colonization, persistence, and transmission in the aquaculture industry's environment. In this sense, the knowledge of this bacterium's ability to proliferate into the environment allows tracing the correct eradication strategies, control, and success in shrimp production. Therefore, this research aimed to determine the pathogenic, antimicrobial, and biotic potential of isolates of *Vibrio parahaemolyticus* isolated from white shrimp *P. vannamei* seawater from a farm in northwestern Mexico.

MATERIALS AND METHODS

Penaeus vannamei and seawater sampling from the shrimp farm

This study was conducted in a shrimp farm located in Bahía de Kino, Sonora, Mexico (28°48'57.1"N, 111°54'32.6"W) during the period from June to August

2016. The participating farm was characterized by owning 52 semi-technical ponds located on the ground, which used the Kino Bay as a water source. A regional company provided *P. vannamei* organisms. They were fed through a 4% diet of biomass, administered at different times: 30% in the morning and afternoon, and 40% at night. During shrimp cultivation, a total of 140 samples were randomly taken, including *P. vannamei* juvenile organisms (n = 120) and bay water (n = 20). Shrimp were caught from harvest ponds with nets and transported in sterile plastic bags, and transported immediately to the laboratory. For water samples, a volume of 1 L was collected in sterile polypropylene containers at a depth of 30 cm from the pond. Water samples were transported in refrigeration (4°C) to the laboratory for later microbiological analysis.

Monitoring of physical and chemical parameters of the ponds

pH and salinity values in each of the ponds were considered for evaluating the physical and chemical parameters. A YSI potentiometer (Ecosense pH 10, Yellow Springs, OH, USA) was used for the pH, and an Aquatic Ecosystems refractometer (VitalSine SR-6, Apopka, FL, USA) was used for salinity.

Total viable bacterial count (TVBC) from hepatopancreas and seawater samples

Hemolymph and hepatopancreas were extracted from the shrimp, according to the protocol described by Kaysner et al. (2004), to isolate *V. parahaemolyticus*. Shrimps were disinfected with 70% alcohol before microbio-logical analysis. One hundred µL of hemolymph was extracted from each organism by puncturing the ventral hemolymphatic sinus using a sterile syringe containing 10 µL of sodium citrate. For the hepatopancreas analysis, the organs were removed manually under aseptic conditions and macerated in 9 mL of peptonized water (Difco, Diagnostic Systems, USA) supplemented with 3.5% NaCl (PWS), making serial dilutions. Aliquots of 50 µL of hemolymph and 100 µL of the hepatopancreas dilutions were individually extended on marine agar added with 3.5% NaCl (AM) (BD Bioxon, USA) and thiosulfate citrate sucrose bile citrate with 2.5% NaCl (TCBS) (BD, Bioxon USA) for the quantification of the total viable bacterial count (TVBC) and *V. parahaemolyticus*, respectively. They were incubated at 30°C for 24 h.

Emerald green identified the *V. parahaemolyticus* strains on TCBS agar, transparent halo, smooth and round surface staining; TVCB were identified by yellow color. The results were expressed in log CFU mL^{-1}. For the isolation of the bacteria from seawater, the sample was homogenized, and serial dilutions in PWS were prepared. 100 µL of each dilution was inoculated in TCBS and AM agar. Plates were incubated at 30°C for 24 h. The results were expressed as CFU mL^{-1}.

Confirmation of *Vibrio parahaemolyticus* strains

From the TCBS agar, colonies with characteristic morphology were isolated and purified for *V. parahaemolyticus* (emerald green smooth colonies, transparent greenish and blue halo) for subsequent confirmation. The strains of *V. parahaemolyticus* were confirmed by the VITEK automated system (2XL bioMérieux, USA).

Molecular identification of virulence genes

Multiplex PCR was performed based on the protocol described by Kang et al. (2017). Firstly, the GoTaq® PCR Core System commercial kit (Promega Inc., Madison, WI, USA) and a concentration of 100 ng of DNA from all samples were used, adjusting to a final volume 25 µL of reaction. The oligonucleotides used for the detection of *V. parahaemolyticus* genes associated with thermostable and associated hemolysins and the marker associated with AHPND disease are described (Table 1). DNA amplification was carried out using a conventional PCR technique on a C1000 Touch Thermocycler (Thermal Cycler Biorad, USA).

Biofilm formation capacity

The induction and biofilm formation capacity of *V. parahaemolyticus* were evaluated following the methodology proposed by Stepanovic et al. (2004) and Song et al. (2017) with some modifications. A bacterial inoculum was briefly adjusted to an optical density (OD) of 0.1 (λ = 625 nm), corresponding to 1×10^8 CFU mL^{-1}. For the induction of biofilm formation, sterile 96-well microplates were used (Costar, New York, USA). The microplate was filled with 225 µL PWS solution and 25 µL of bacterial inoculum (1:100) and incubated at 30°C for 24 h. Subsequently, the plate was washed three times with 1X phosphate buffer solution (PBS). The biofilm fixation was done with 100 µL of 99% methanol for 15 min, the solvent was removed, and the plate was allowed to dry at room temperature. The biofilm staining was performed with 100 µL of violet crystal dye for 5 min, washed three times with 1X PBS buffer, and 250 µL of 33% (v/v) glacial acetic acid was added. The OD quantification was done using a Multiskan EX spectrophotometer (Thermo Scientific, USA) at 570 nm. Sterile distilled water was included as a negative control due to the absence of microorganisms. The interpretation of results was established according to the criteria described by Stephanovic et al. (2004). The tests were performed per triplicate.

Table 1. Oligonucleotides used for the molecular identification of genes of *Vibrio parahaemolyticus*.

Primer	Sequence	Molecular size (pb)	Reference
tlF	*tl*/F 5´- AAAGCGGATTATGCAGAAGCACTG -3´	450	Bej et al. (1999)
tlR	*tl*/R 5´- GCTACTTTCTAGCATTTTCTCTGC -3´	450	Bej et al. (1999)
*tdh*F	*tdh*F 5´- GTAAAGGTCTCTGACTTTTGGAC -3´	259	Bej et al. (1999)
tdhR	*tdh*R 5´- TGGAATAGAACCTTCATCTTCACC -3´	259	Bej et al. (1999)
trhF	*trh*F 5´TTGGCTTCGATATTTTCAGTATCT -3´	500	Bej et al. (1999)
*trh*R	*trh*R 5´- CATAACAAACATATGCCCATTTCCG -3´	500	Bej et al. (1999)
AP2F	AP2F-5'TCACCCGAATGCTCGCTTGTGG-3'	700	Dangtip et al. (2015)
AP2R	AP2R-5'CGTCGCTCTGTCTAGTGAAG-3'	700	Dangtip et al. (2015)

Antimicrobial sensitivity test

The antibiotic sensitivity of the strains of *V. parahaemolyticus* was determined by the Kirby-Bauer diffusion method and the Institute of Clinical Standards guidelines (CLSI, 2008). Aliquots of 1 mL of a standardized bacterial suspension (MacFarland 0.5 scale) were taken and spread with a sterile swab on Müeller-Hinton agar plates added with 3.5% NaCl prepared. Subsequently, the antibiotic discs were placed on the inoculated agar and incubated at 30°C for 24 h. The antimicrobial resistance profile evaluated included 11 antibiotics used in clinical for humane treatment: ampicillin (10 µg), amikacin (30 µg), gentamicin (10 µg), netilmicin (30 µg), levofloxacin (5 µg), cefepime (30 µg), cefotaxime (30 µg), nitrofurantoin (30 µg), ceftriaxone (30 µg), chloramphenicol (10 µg), trimethoprim-sulfamethoxazole (25 µg). For the interpretation of results, the inhibition halos (mm) diameter was measured with a vernier. They were categorized as sensitive, intermediate, and resistant according to the criteria established by the CLSI (2008). *Escherichia coli* ATCC 5327 was included as a control to evaluate the antibiotic inhibition. These analyses were performed per triplicate. The antibiotic multiple resistance index (MAR) was calculated using the formula described by Krumperman (1983): MAR: X/Y, where X represents the antibiotics to which the strain was resistant and Y the antibiotics to which the strain was exposed.

The minimum inhibitory concentration of oxytetracycline, enrofloxacin, and florfenicol

The minimum inhibitory concentration (MIC) was determined for antibiotics used in aquaculture, including oxytetracycline (OTC) (Sigma-Aldrich, USA), enrofloxacin (ENRO) (Avimex, Mexico) and florfenicol (FFC) (Avimex, Mexico) at concentrations of 512, 256, 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.12, 0.06 µg mL^{-1} in PWS broth. Each treatment was inoculated with 40 µL of an adjusted bacterial suspension (1×10^8 CFU mL^{-1}) at a ratio of 1:100 and incubated at 30°C for 48 h. The MIC was defined as the antibiotic's minimum concentration that inhibited the bacteria's visible growth as a cloudy broth in the culture after the incubation period (Alderman & Smith 2001). The absorbance (λ = 415 nm) of each treatment before inoculation and after the incubation period was determined. In the analysis's interpretation, a positive control as a cloudy broth (without antibiotic) and negative control (PWS broth) showing the microorganism's absence was included. The analyses were performed in triplicate.

Growth kinetics

An isolated strain of shrimp hepatopancreas cultivated in the aquaculture farm studied was selected to determine the growth kinetics of *V. parahaemolyticus*, according to Shi et al. 2017. Briefly, a standardized suspension of *V. parahaemolyticus* (1×10^8 CFU mL^{-1}) in previously sterilized seawater obtained from the water effluent from Kino Bay, which fed the shrimp farm ponds, were inoculated in a 1:1000 ratio. Seawater conditions were pH 8.1, 36‰ NaCl, and 30ºC. Five serial 10-fold dilutions (10^{-1}-10^{-5}) determined the bacterial concentration in the marine microenvironment at times 0, 1, 3, 6, 12, 24, 48, and 72 h in TCBS agar. The analyses were performed in duplicate, and the results were expressed in CFU mL^{-1}. The modified Gompertz model (Wang et al. 2014) was used to describe the growth of *V. parahaemolyticus* in seawater at 30°C utilizing the equation:

$$Y_0 = N_0 + A\exp\,[-\exp(-B \times (t - M))]$$

Y_0 is the *V. parahaemolyticus* (CFU mL^{-1}) concentration at time t; N_0 is the initial concentration of bacteria ($\log_{10}$ CFU mL^{-1}).

$$A = \log_{10} (N_{max} / N_0),$$

where N_{max} represents the growth from the inoculum to the stationary phase; and the parameters exp, B, and M represent the constant e, the maximum specific growth rate (h^{-1}), and the time at which the maximum concentration (h) is reached, respectively. Latency

time, specific growth rate, doubling time, and maximum density was determined with the Statistica 7.0 program.

Statistical analysis

The data obtained on the frequency, quantification, and typing of *V. parahaemolyticus* were analyzed using ANOVA if the data showed normality or Kruskal-Wallis statistical tests if the data do not show statistical continuity criteria. A regression analysis was conducted to determine the relationship of water parameters with bacterial survival. A $P \leq 0.05$ value was considered statistically significant.

RESULTS

Vibrio parahaemolyticus and total viable bacteria count (TVBC)

V. parahaemolyticus was detected in 7.1% (10/140) of the analyzed samples. Isolates were recovered from 6.6% (8/120) and 10.0% (2/20) of the *Penaeus vannamei* and seawater samples, respectively (Table 2). The average concentration of *V. parahaemolyticus* in the shrimp samples was 2.84 ± 1.34 log CFU mL^{-1} and in water 4.60 ± 0.13 log CFU mL^{-1}; pH (7.89 ± 0.14) and salinity (36.64 ± 0.84) of the pond water were not predictors of the presence and concentration of *V. parahaemolyticus* according to the Pearson test. However, the total viable bacterial count in water (4.60 ± 0.13 log CFU mL^{-1}) was associated as a positive indicator of the concentration of *V. parahaemolyticus* in the shrimp ($P = 0.000$, $r = 0.590$) (Table 3).

The isolates of *V. parahaemolyticus* recovered from the shrimp samples (n = 8) were obtained mainly from hepatopancreas (75%), showing characteristic manifestations of vibriosis, such as red coloration, ampoules in the uropods, and rough surface antigens and some organ injuries (Fig. 1).

Identification of virulence genes of strains of *V. parahaemolyticus*

Table 4 shows the strain pathotypes of *V. parahaemolyticus* isolated from shrimp and seawater samples. The *tlh* and AP2 genes were detected in 100 and 20% of the strains, respectively. The 10 strains were classified into two groups: *tlh*+/*tdh*-/*trh*-/*AP2*- (75%) and *tlh*+/*tdh*-/*trh*-/*AP2*+ (25%), the latter being an exclusive group of shrimp isolates (hepatopancreas).

Biofilm formation capacity

Forty percent (4/10) of the *V. parahaemolyticus* strains isolated from shrimp showed the capacity of biofilm formation with low expression (n = 1), moderate (n = 2), and high (n = 1) (Table 4). The biofilm formation capacity expression was a characteristic property of the *V. parahaemolyticus* strains ($P = 0.000$).

Antibiotic sensitivity

All strains of *V. parahaemolyticus* showed resistance to at least one of the 11 antibiotics tested with a MAR ranged from 0.2 to 0.4. The primary resistance phenotype was ampicillin (100%), followed by amikacin (75%), gentamicin (50%), and netilmicin (50%). The resistant strains were classified into three resistance profiles (Pr): ampicillin (Pr1), ampicillin and amikacin (Pr2), and ampicillin, amikacin, gentamicin, and netilmicin (Pr3), without showing association with the origin of the strains ($P = 0.665$). The MIC for the strains against OTC, FFC, and ENRO was >0.50, >0.25, and >0.06 µg mL^{-1}, respectively.

Growth kinetics

The results showed that seawater at 30ºC provides a favorable environment (Scott et al. 2002, Manjano-Mendoza et al. 2009) to promote the growth of the strain of *V. parahaemolyticus* isolated from shrimp, presenting an adaptation time of 1.0 h and reaching a maximum concentration of 7.6 log CFU mL^{-1} before 24 h of exposure. The average growth rate and doubling time of the isolate was 0.375 log CFU h^{-1} and 9.5 h, respectively (Fig. 2).

DISCUSSION

The results showed that *Vibrio parahaemolyticus* strains spread in the different ponds of the farm when they are detected interchangeably in shrimp and seawater samples, which infers a health risk of *Penaeus vannamei* shrimp. The detection rate of *V. parahaemolyticus* reported in shrimp and culture water has varied from 20.0 to 66.7% (Zhao et al. 2018) and from 5.9 to 36.4% (Park et al. 2018), respectively. In Mexico, infection with *P. vannamei* and water contamination cultivated by *V. parahaemolyticus* (Bermúdez-Almada et al. 2017) have also been reported. However, the strategies used by *V. parahaemolyticus* for environmental colonization and the shrimp infection cycle have not been widely described.

The pH and salinity values determined in this study (Table 2) correspond to optimal values for aquaculture activities according to Mexico's ecological criteria guidelines (CE-CCA-001/89). Although these parameters failed to predict the presence of *V. parahaemolyticus*, it has been described that these physicochemical and other factors (dissolved oxygen, NO_2 concentration, total ammoniacal nitrogen, and alkalinity expressed in $CaCO_3$) can promote the develop-

Table 2. Detection and quantification of *Vibrio parahaemolyticus* at a shrimp farm. *Shrimp samples represent a sample consisting of three shrimp. ND: undetermined, n: number of samples, ps: positive samples.

Category	Water (n = 20)	Shrimp* (n = 120)
V. parahaemolyticus % (ps)	20.0 (2)	6.7 (8)
V. parahaemolyticus (log UFC mL^{-1})	4.60 ± 0.13	2.84 ± 1.34
Marine biota (log UFC mL^{-1})	4.60 ± 0.13	3.76 ± 4.20
pH	7.89 ± 0.14	ND
Salinity (mg L^{-1})	36.64 ± 0.84	ND

Table 3. *Vibrio parahaemolyticus* presence predictors evaluated at the shrimp farm. *(*P*-value <0.05)

	Water (log UFC mL^{-1})	Shrimp (log UFC mL^{-1})	Biota (log UFC mL^{-1})	pH
Shrimp (log UFC mL^{-1})	0.590 *P* = 0.000*			
Biota (log UFC mL^{-1})	0.0874 *P* = 0.000*	0.503 *P* = 0.000*		
pH	-0.011 *P* = 0.896	-0.053 *P* = 0.531	-0.024 *P* = 0.783	
Salinity (mg L^{-1})	-0.074 *P* = 0.384	-0.034 *P* = 0.688	-0.100 *P* = 0.238	-0.080 *P* = 0.348

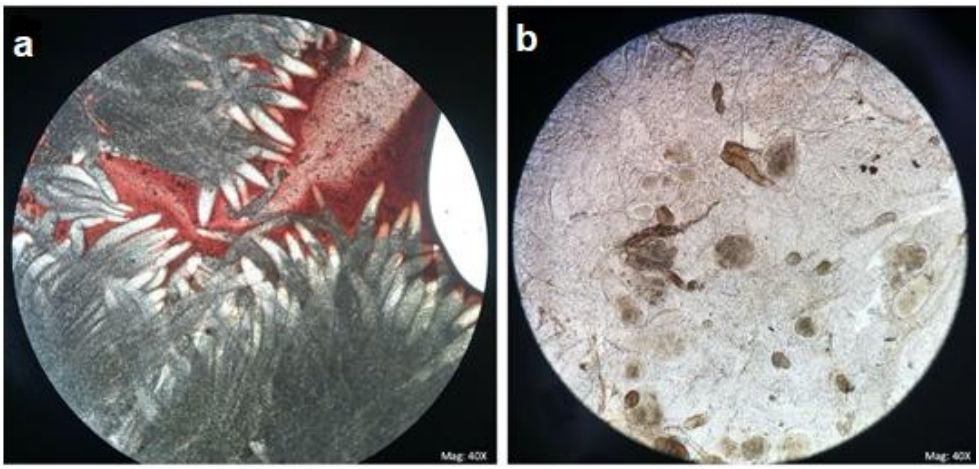

Figure 1. Hepatopancreas was seen at the optical microscope (40x). a) Hepatopancreas of a healthy organism presenting tubules with normal morphological characteristics, b) Hepatopancreas of an organism with manifestations of acute hepatopancreatic necrosis syndrome (AHPND) presenting tubules without lipid content, lysed, and necrotic.

ment and survival of bacteria in the aquatic ecosystem (Karlburge et al. 2014, Mizan et al. 2015).

The concentration of *Vibrio* and its response to a new environment determines the time to colonize and the risk of infection in the host (Cannon & Garner 1999). Quiroz-Guzmán et al. (2013) estimated that the growth rate of *V. parahaemolyticus* in simulated conditions of a marine environment is 0.248 h^{-1}. Our results showed an adaptation time of 1.0 h with a specific growth rate of 0.375 log CFU h^{-1} that infer the adaptability and growth of the strain of *V. parahaemolyticus* from shrimp and justifies the water-shrimp transmission cycle. The viability time of *V. parahaemolyticus* in seawater has been estimated at 1.4 weeks when it has a growth rate of 0.66 log CFU h^{-1} (Chung et al. 2018). In this way, the evaluated strain could remain in the marine environment for long periods, at high concentrations (7.5 log CFU mL^{-1}), and favor its dissemination.

The evaluation of the bacterial concentration is of utmost importance in the expression of the pathogenic potential since, under optimal conditions, the bacteria can cause a greater impact the health of the shrimp and the consumer (Zhao et al. 2018). Quiroz-Guzmán et al. (2013) indicate that the infective dose of *V. parahaemolyticus* (1×10^9 CFU mL^{-1}) together with the

Table 4. Phenotypic characterization of the strains of *Vibrio parahaemolyticus*. HP: hepatopancreas, HF: hemolymph, AM: sea water, Pr1: AMP, Pr2: (AK, AMP), Pr3: (NET, AK, CN, AMP). AK: Amikacin, AMP: ampicillin, NET: netilmicin, CN: gentamicin. OTC: oxytetracycline, FFC: florfenicol, ENRO: enrofloxacin.

Strain	Origin	Isolate date	Expressed gen				Biofilm	Antimicrobial sensitivity		MIC (µg mL^{-1})		
			tlh	*tdh*	*trh*	AP2		Profile	MAR Index	OTC	FFC	ENRO
1	HP	20/07/2016	+	-	-	-	Moderate	Pr2	0.2	1.0	0.50	0.12
2	HP	20/07/2016	+	-	-	+	Negative	Pr2	0.2	1.0	0.50	0.50
3	HP	20/07/2016	+	-	-	-	Negative	Pr2	0.2	1.0	4.00	0.12
4	HP	03/08/2016	+	-	-	-	Moderate	Pr3	0.4	1.0	4.00	1.00
5	HP	03/08/2016	+	-	-	+	Negative	Pr1	0.1	0.5	0.50	0.06
6	HF	10/08/2016	+	-	-	-	Negative	Pr2	0.2	1.0	0.50	0.50
7	HP	17/08/2016	+	-	-	-	Moderate	Pr3	0.4	1.0	0.25	0.06
8	HF	17/08/2016	+	-	-	-	High	Pr3	0.4	0.5	0.12	0.12
9	AM	17/08/2016	+	-	-	-	Negative	Pr3	0.4	0.5	2.00	0.12
10	AM	17/08/2016	+	-	-	-	Negative	Pr2	0.2	1.0	0.25	0.20

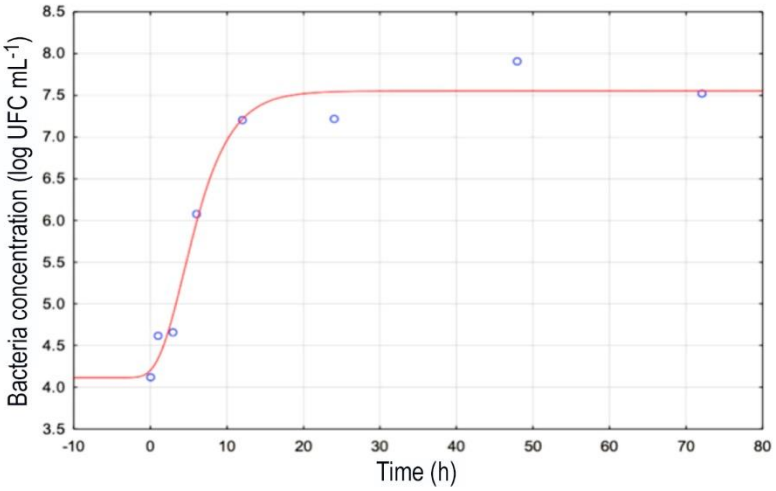

Figure 2. Growth kinetics of *Vibrio parahaemolyticus* in seawater with controlled conditions at 30°C.

virulence genes that the bacterium possesses determines the clinical signs of the disease in its host and the mortality rate in the shrimp during its farm cultivation. The concentrations of *V. parahaemolyticus* determined in shrimp samples are below those previously reported in shrimp samples (500 CFU mL^{-1}) obtained from La Paz, Baja California (Quiroz-Guzmán et al. 2013), and within acceptance criteria for consumption (10^4 NMP g^{-1}) according to NOM-242-SSA1-2009. However, preventive measures are recommended for food preparation and shrimp cooking before consumption (FAO/WHO, 2006).

Several authors have evidenced differences in the frequency of detection of virulence genes associated with the pathogenic cycle of *V. parahaemolyticus*, which can define the environmental and clinical behavior of the strains (Zhang & Orth 2013). Identifying the *tlh* gene in *V. parahaemolyticus* isolates represents a species' marker gene (Yáñez et al. 2015). The *tdh* and *trh* genes' presence is related by conferring the ability to lyse erythrocytes to *V. parahaemolyticus* and associated with the Kanawaga phenomenon gastrointestinal diseases (Liu 2011). The inclusion of these genes to detect this pathogen represents a rapid and reproducible health emergency technique (Wang et al. 2015). In our study, strains of *V. parahaemolyticus* lacking these virulence factors were observed mainly in those of marine origin.

Previously in Mexico, Han et al. (2015) reported that 11.5% (9/78) of *V. parahaemolyticus* isolates carried the molecular marker AP2. Theethakaew et al. (2017) denote that this marker in *V. parahaemolyticus* can be correlated with AHPND manifestation capacity. Therefore, monitoring of the AP2 gene can be a useful

indicator for producers in the aquaculture sector, which allows taking measures in the presence of *V. parahaemolyticus* with the potential to cause AHPND.

Survival phenotypes exhibiting strains of *V. parahaemolyticus* isolated from shrimp and seawater (Table 4) evidenced their potential to establish free life in the aquatic environment or as a pathogen of shrimp. *V. parahaemolyticus* can form biofilms for its preservation and survival in the shrimp farms due to the indiscriminate use of antibiotics and their residue in the culture ponds as a polluting agent. This expression depends on the environment, nutrients, temperature, and salinity of the environment in which it is found (Turner et al. 2014, Han et al. 2016). Song et al. (2017) argue that the biofilm formation of *V. parahaemolyticus* is temperature-dependent. Mizan et al. (2016) indicate that a temperature value of 30ºC favors the degree of biofilm formation of *V. parahaemolyticus*. Meanwhile, the formation capacity is reduced to values of 4 to 10°C (Gharechahi et al. 2012). In this context, most of the strains evaluated have a weak phenotype of biofilm formation, presumably influenced by other factors such as the presence and expression of genes (toxR), some transcriptional regulators (OxyR, VPA0606, and CpsR), and mobility by flagella. However, *V. parahaemolyticus* biofilm formation regulation's full details are unknown (Long et al. 2018).

The emergence of antimicrobial resistance is a major public and environmental health problem. Our study shows a predominant phenotype of resistance to ampicillin, amikacin, netilmicin, and gentamicin shared between *V. parahaemolyticus* isolates of both origins (Table 4). Previously, several studies have reported the RA of *V. parahaemolyticus* strains isolated from seawater to ampicillin, streptomycin, tetracycline, gentamicin, chloramphenicol, and trimethoprim-sulfamethoxazole (Pazhani et al. 2014, Wang et al. 2015). Additionally, the quantified MAR value (0.2 to 0.6) demonstrates the effect of environmental contamination by antibiotics used to eradicate the pathogen. Kang et al. (2018) suggest that a value greater than 0.2 found in food samples of marine origin is considered as high risk for human health. In our study, the average MAR value suggests a high exposure of the evaluated strain to antibiotics.

Oxytetracycline, enrofloxacin and florfenicol are widely used antibiotics in shrimp culture, which has favored the emergence of *V. parahaemolyticus* strains with antimicrobial resistance against these antibiotics (Bermúdez-Almada et al. 2014). Gracia-Valenzuela et al. (2012) describe the RA of different *Vibrio* species showing a MIC of >100, >10, and >5 µg mL^{-1} for OTC, ENRO, and FFC, respectively. Mainly, Bermúdez-Almada et al. (2014) evaluated the MIC of strains of *V. parahaemolyticus* obtained from *P. vannamei*, water and soil of an aquaculture farm in the same region, finding values of MIC for OTC (50 µg mL^{-1}) FFC (5 µg mL^{-1}) and ENRO (5 µg mL^{-1}) similar between the origins of the strains. This test demonstrates the importance of using adequate doses in the presence of bacterial outbreaks during shrimp production. Additionally, identifying antimicrobial resistance to the most widely used antibiotics in aquaculture allows the design of new strategies with new treatments different from those already established. This scenario shows the challenge for the control of the use of antimicrobials in shrimp farming, and in the same way, find new alternatives for outbreak prevention such as strengthening the immune system through supplementation through the shrimp diet and nutrition of the organism for optimal development (Li et al. 2019).

CONCLUSIONS

The presence of *Vibrio parahaemolyticus* with pathogenic potential and antimicrobial resistance highlights the importance of continuously monitoring this bacterium in the environment of aquaculture. The ability that *V. parahaemolyticus* showed to grow in saline aquatic ecosystems rapidly manifests the risk of proliferation in the areas of production and dissemination to the commercial destinations of shrimp that occurs in northwestern Mexico. The implementation of control measures during aquaculture production and minimization of antibiotics use, and the strengthening of the shrimp's immune system, are strategies to regulate *V. parahaemolyticus* presence and ensure the health of the shrimp and consumer health.

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