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Research Article

Isolation and characterization of infectious *Vibrio parahaemolyticus*, the causative agent of AHPND, from the whiteleg shrimp (*Litopenaeus vannamei)*

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ABSTRACT. *Vibrio parahaemolyticus*, the causative agent of acute hepatopancreatic necrosis disease (AHPND), was isolated from the hepatopancreas of moribund whiteleg shrimp of commercial farms from Guasave, Sinaloa, Mexico. The isolates were screened on thiosulfate citrate bile salt sucrose agar plates for the selection of green colonies and further characterized through PCR with AP3 primers, 89F/R primers, hemolysin genes, hemolytic and enzymatic activity, hydrophobicity, autoaggregation, and biofilm formation. Bioassays by immersion challenge were conducted to confirm the pathogenicity of selected bacterial strains. In addition, the LC_{50} was calculated for each isolate. All isolates (35) belonged to *V. parahaemolyticus*, but three isolates did not correspond to strains that cause AHPND since they were negative with 89F/R primers. All isolates were α-hemolytic and showed biofilm formation (from moderate to strong). Isolates were hydrophobic or hydrophilic and showed high autoaggregation capacity. Eight strains did not kill shrimp and eleven were pathogenic, but differences in virulence were found among them perhaps due to α-hemolysis and differences in biofilm formation and hydrophobicity. Therefore, performed characterization may help to understand the pathogenicity of *V. parahaemolyticus*. Finally, results showed that smaller shrimp are less resistant to *V. parahaemolyticus* infection.

Keywords: *Litopenaeus vannamei*, *Vibrio parahaemolyticus*, AHPND, biofilm, aquaculture.

Aislamiento y caracterización de *Vibrio parahaemolyticus* infeccioso, agente causal de AHPND en camarón blanco (*Litopenaeus vannamei*)

RESUMEN. *Vibrio parahaemolyticus*, el agente causante de la enfermedad de la necrosis hepatopancreática aguda (AHPND), fue aislado del hepatopáncreas de camarón blanco moribundo proveniente de granjas comerciales de Guasave, Sinaloa, México. Los aislados (colonias verdes) fueron seleccionados en placas de agar TCBS, purificados y caracterizados mediante PCR con oligos AP3, 89F/R y genes de hemolisinas, actividad hemolítica y enzimática, hidrofobicidad, autoagregación y la formación de biopelículas. Se realizaron infecciones experimentales por inmersión para confirmar la patogenicidad de los aislados bacterianos seleccionados. Además, se calculó la LC_{50} para cada aislado. Todos los aislados (35) pertenecen a *V. parahaemolyticus*, pero tres de ellos no corresponden a las cepas que causan AHPND ya que fueron negativos con los oligos 89F/R. Todos los aislados fueron α-hemolíticos y formaron biopelículas (de moderado a fuerte). Los aislados fueron hidrofóbicos o hidrofílicos y mostraron gran capacidad de autoagregación. Ocho aislados no mataron a los camarones pero once fueron patógenos, aunque se encontraron diferencias en la virulencia entre ellos. Por lo tanto, la caracterización realizada puede ayudar a entender la patogenicidad de *V. parahaemolyticus*. Finalmente, los resultados mostraron que los camarones más pequeños son menos resistentes a la infección por *V. parahaemolyticus*.

Palabras clave: *Litopenaeus vannamei*, *Vibrio parahaemolyticus*, AHPND, biopelícula, acuicultura.

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INTRODUCTION

Shrimp farming has been hampered by diseases caused by potential pathogens such as protozoa, bacteria, fungi, and viruses (Gómez-Gil *et al.*, 2001). However, development of diseases is also influenced by changes in environmental conditions and nutritional imbalances (Kautsky *et al.*, 2000). Worldwide, viruses are blamed for the great losses in shrimp farming. However, there are other pathogens such as *Vibrio* sp. that generate significant production losses (Aguirre-Guzmán, 2004; Goarant *et al*., 2006; Tran *et al.*, 2013).

Vibrios belong to the family Vibrionaceae, are Gram negative, comma-shaped, mobile, salt tolerant, and facultative anaerobes (Thompson *et al.*, 2004). Diseases generated by these bacteria have been described as: vibriosis, bacterial disease, penaeid bacterial septicemia, penaeid luminescent vibriosis, red legs disease (Aguirre-Guzmán, 2004), and acute hepatopancreatic necrosis disease (AHPND), also called early mortality syndrome (EMS) (NACA, 2012). The diseases caused by vibrios generate serious problems in the culture of penaeid shrimp, recording mass mortalities in both larval production labs and farms in many countries. However, little attention has been given to their research; so the knowledge about these diseases and epidemiology of pathogenic vibrios in shrimp farming is limited (Goarant *et al.*, 2006; Jayasree *et al.*, 2006).

The AHPND, caused by *Vibrio parahaemolyticus* strains, affects two shrimp species commonly bred in the world, the giant tiger prawn (*Penaeus monodon*) and the whiteleg shrimp (*Litopenaeus vannamei*) (NACA, 2012). The disease often occurs within the first 30 days after stocking shrimp in ponds (NACA, 2012) and the mass mortality can exceed 70% (Kongrueng *et al.*, 2014). The AHPND emerged in China in 2009, causing serious losses in the shrimp industry in Asia (NACA, 2012). In Mexico, AHPND has affected the production of whiteleg shrimp in the northwest of the country (Nayarit, Sinaloa, and Sonora) since 2013 (Nunan *et al.*, 2014; Soto-Rodriguez *et al.*, 2015). The clinical signs of the disease include inactivity, slow growth, empty stomach and midgut, and pale to white atrophied hepatopancreas (Tran *et al.*, 2013).

Information on the virulence factors associated with *V. parahaemolyticus* strains that cause AHPND is still limited (Kongrueng *et al.*, 2014). Hence, research about hemolytic activity and the presence of virulence genes would be useful to clarify their pathogenicity in shrimp (Kongrueng *et al.*, 2014). In addition to the criteria mentioned above, many other criteria have to be met, two of the most important are the ability to adhere to the digestive tract and the capacity of the strains to form biofilms. Several studies have suggested that the ability of vibrios to form biofilms is a survival mechanism associated with their pathogenesis and stress tolerance (Yildiz & Visick, 2009). Biofilms are complex microbial communities associated with biotic and abiotic surfaces and embedded in a matrix of extracellular substances (polymers) produced by them (Hall-Stoodley *et al.*, 2004; Huq *et al.*, 2008). Some bacterial properties, such as increased hydrophobicity and the ability to autoaggregate, are important for colonization and biofilm formation (Decostere *et al.*, 1999; Rickard *et al.*, 2004).

The aim of this work was to isolate and characterize *V. parahaemolyticus* (the causative agent of APHND) from shrimp farms of northwestern Mexico by challenging organisms with experimental infections.

MATERIALS AND METHODS

Animals and hepatopancreas samples

One hundred and ten moribund shrimp (weighing 3-6 g) from four farms (Guasave, Sinaloa, Mexico) with mortality problems were collected from the earthen ponds and transported to the lab facilities in plastic containers with seawater (30-31°C) and constant aeration. Shrimp, presumably infected with AHPND, were alive and showed empty stomach and midgut, inactivity, and pale to white atrophied hepatopancreas (Tran *et al.*, 2013) when they were analyzed visually. Hepatopancreas of shrimp were aseptically removed, placed in Eppendorf tubes with 400 µL of sterile saline solution (2.5% NaCl), and homogenized with a pestle. The volume was then adjusted to 1 mL.

Isolation of presumptive *V. parahaemolyticus*

The homogenate (100 µL) was inoculated into thiosulphate citrate bile sucrose (TCBS, BD DIFCO, Sparks, MD, USA) agar plates supplemented with 2.5% NaCl. The plates were kept at 35°C for 24-48 h. *V. parahaemolyticus* colony would be round in shape, 2-3 mm diameter, opaque, green, or bluish on TCBS agar (Bisha *et al.*, 2012). The initial isolation plates showed green and yellow colonies but the green ones were dominant. Hence, from all colonies (yellow and green), only green colonies were selected and streaked (four times) onto TCBS plates and incubated as above. Each isolate was grown in tryptic soy broth (TSB, BD BBL) supplemented with 2.5% NaCl at 35°C for 24 h, before being stored at -70°C in the same medium supplemented with 15% glycerol.

Pre-screening of isolates with AP3 and 89F/R primers

One hundred and twenty nine isolates (green colonies) were analyzed by PCR with AP3 primers (Sirikharin *et*

al., 2014) that amplify a 336 bp fragment. The primers are specific for *V. parahaemolyticus* strains causing AHPND. Negative isolates were discarded and only positive isolates (35) were further characterized with 89F/R primers that amplify a 470 bp fragment (Nunan *et al.*, 2014).

Molecular identification of isolates (detection of total and hemolysin-producing *V. parahaemolyticus*)

To corroborate the previous analysis with AP3 and 89F/R primers, multiplex PCR amplifications were done for thermolabile hemolysin (*tlh*), thermostable direct hemolysin (*tdh*), and thermostable direct hemolysin-related (*trh*) genes according to Bej *et al.* (1999). PCR amplification was optimized in a 25-µL reaction consisting of 1000 ng of purified genomic DNA from each isolate, 1.0 µM of each of the oligonucleotide primers for *tlh*, *tdh*, *trh* (1.0 µL of each of the primers from a 10 µM stock suspension), 10 × reaction buffer (2.5 µL), 50 mM $MgCl_2$ (0.7 µL), 10 mM dNTPs (0.5 µL), 0.25 µL (5 units μL^{-1}) Biolase Taq DNA polymerase (Bioline USA Inc., Tauton, MA, USA), and 5.05 µL of ultrapure water (Invitrogen, Grand Island, NY, USA).

Multiplex PCR amplifications were performed in a DNA thermal cycler (Bioer, Model LifePro, Hangzhou Bioer Technology Co. Ltd., Hangzhou, Binjiang, China) as follows: initial denaturation at 94°C for 3 min followed by 30 cycles of amplification; each cycle consisted of denaturation at 94°C for 1 min, primer annealing at 58°C for 1 min, and primer extension at 72°C for 1 min. Following the amplification cycles, samples were kept at 72°C for 5 min to allow final extension of the incompletely synthesized DNA. Expected amplicon size was 450 bp (*tlh*) (Taniguchi *et al.*, 1985, 1986), 269 bp (*tdh*) (Nishibuchi & Kaper, 1985), and 500 bp (*trh*) (Honda *et al.*, 1991; Honda & Iida, 1993).

Hemolytic activity of isolates

Hemolytic activity was determined twice for each isolate (Cowan & Steel, 2004). The 24-h culture of each isolate in TSB (BD BBL) was obtained and tested for its hemolytic activity on blood agar (BA, BD Bioxon) plates prepared with 1 mL of heparinized human blood. Two wells of 6-mm diameter were made on BA plates for each isolate. Each well was filled with 40 µL of bacterial culture or TSB (negative controls) and plates were incubated at 30-33°C for 24 h. Three types of hemolytic activity were expected: α-hemolysis (incomplete hemolysis) when the agar around the well is dark and greenish, β-hemolysis (complete hemolysis) the agar around the well appears lightened and transparent, and γ-hemolysis (lack of hemolysis) when the agar around the well shows no changes. To observe the halo, bacteria grown around the well were eliminated with an inoculation loop.

Biofilm formation

Isolates (35) were tested for biofilm formation according to Mandhi *et al.* (2010). Briefly, following overnight incubation at 30-33ºC in TSB, 200 µL of bacteria were transferred in triplicate to a U-bottom 96-well microtiter plate and the optical density was measured at 595 nm using a spectrophotometer (Multiskan™ GO Microplate, Thermo Scientific, NC, USA). The overnight culture was diluted 1:100 in TSB with NaCl (2.5%) and glucose (2%). The diluted sample (100 µL) was transferred to a U-bottom 96-well microtiter plate. Each isolate was tested in triplicate. The plates were incubated aerobically at 30-33ºC for 24 h. The cultures were removed and the microtiter wells were washed twice (inverted plate) with 200 µL of PBS (7 mmol L^{-1} Na_2HPO_4, 3 mmol L^{-1} NaH_2HPO_4, and 130 mmol L^{-1} NaCl at pH 7.4) to remove non-adherent cells, and plates were dried in an inverted position. Adherent bacteria were fixed with 200 µL of ethanol (95%), decanted, dried at room temperature, and stained with 100 µL of crystal violet (Golden Bell, Jalisco, Mexico) for 10 min. The excess stain was rinsed and poured off and the wells were washed three times with sterile distilled water (300 µL). The water was then cleared and the microplates were air dried. The absorbance was read at 595 nm in a spectrophotometer (Multiskan™ GO). Each value represents the mean of three bioassays. Adhesion ability can be strong ($OD \geq 1$), moderate ($0.1 \leq OD_{595} < 1$) or weak ($OD_{595} < 0.1$).

Microbial adhesion to solvents (MATS)

Microbial adhesion to solvents (*p*-xylene) was performed according to Crow *et al.* (1995). Bacterial isolates (35) were grown as in biofilm formation, harvested at the stationary phase by centrifugation at 2379x *g* (Sigma 2-6E, Germany), for 20 min, and suspended in PBS as above, to obtain 10^8 CFU mL^{-1}. Absorbance of the cell suspension was measured at 600 nm (A_0) to obtain an optical density of 1. One milliliter of the solvent was added to 3 mL of the cell suspension. The sample in triplicate was incubated for 10 min at room temperature and then the two phase system was mixed in a vortex (2 min). The aqueous phase was removed after 20 min of incubation at room temperature and the absorbance at 600 nm (A_1) was measured in a spectrophotometer (Thermo Spectronic Genesys 2, Thermo Scientific, Waltham, MA, USA). PBS was used as a negative control. The percentage of bacterial adhesion to solvent was calculated as follows: $A= [(1-A_1/A_0)] \times 100$. Each value represents the mean

of three bioassays. Strains were considered strongly hydrophobic when values were >50%, moderately hydrophobic when values were in the range of 20-50%, and hydrophilic when values were <20% (Mattos-Guaraldi *et al.*, 1999).

Autoaggregation assays

The 35 isolates were tested for autoaggregation capacity according to Kos *et al.* (2003). Bacteria were grown for 18 h at 30-33ºC in TSB. The cells were harvested by centrifugation at 2379x *g* (Sigma 2-6E) for 20 min and suspended in PBS as above, to obtain 10^8 CFU mL^{-1}. Cell suspensions (4 mL) were mixed in a vortex for 10 s and then incubated at room temperature for 5 h. Every hour, 0.1 mL of the upper suspension was transferred to another tube with 3.9 mL of PBS and the absorbance at 600 nm (A) was read in a spectrophotometer (Thermo Spectronic Genesys 2). PBS was used as blank. Each value represents the mean of three bioassays. The autoaggregation percentage was expressed as: 1- (At/A0) x 100, where At represents the absorbance at time t = 1, 2, 3, 4, or 5 h and A0 the absorbance at t = 0.

Extracellular enzymatic activity

Production of extracellular enzymes was determined qualitatively (León *et al.*, 2000). Isolates (35) were grown in TSB supplemented with 2.5% NaCl and incubated at 30-33°C for 24 h. The cultures were centrifuged at 12 000x *g* (Sigma 1-15PK, Germany) for 10 min to obtain the supernatant.

Petri plates were prepared with basal medium (1.5% agar and 0.5% yeast extract) supplemented with 2% skim milk or 1% gelatin for proteases and 1% Tween 80 for lipases. Wells of 6-mm diameter were made on plates and filled with 50 µL of supernatant of bacteria grown in TSB with 2.5% NaCl. TSB was used as negative control. The plates were incubated at 30-33°C for 24 h. For casein degradation (skim milk), isolates with a clear halo around the well were considered as positive. For gelatin and Tween 80 hydrolysis, isolates with a cloudy halo around the well were considered as positive.

Growth kinetics

Growth curves were determined for each isolate (35) to identify growth phases, especially the log phase because it was used for the challenging bioassays with viable cells. Isolates were grown in 50 mL TSB supplemented with 2.5% NaCl and incubated at 30-33°C for 24 h. Absorbance (580 nm) was determined at 3, 6, 9, 12, 24, 48, 72, 96 h in a Thermo Spectronic Genesys 2 spectrophotometer.

Bacterial count

The bacterial count was done twice on isolates selected for challenging bioassays in *L. vannamei*. Isolates were grown in TSB supplemented with 2.5% NaCl and incubated at 30-33°C for 18 h. Samples were centrifuged at 2379x *g* (Sigma 2-6E) for 20 min and cell pellet was suspended in 1 mL of sterile saline solution (2.5% NaCl). The bacterial solution was adjusted spectrophotometrically to an optical density of 1 at 580 nm. Bacterial count was done with the serial dilution method using plates with TS agar supplemented with 2.5% NaCl and incubated at 30-33°C for 24 h.

Immersion challenge tests

Bioassays were performed during 3-4 days. Shrimp (Ecuadorian strain resistant to WSSV) weighing 10-60 mg (pathogen free) were obtained from Hatcheries of Sinaloa (Proveedora de Larvas, S.A. de C.V. and Acuacultura Integral, S.A. de C.V.) and grown in the Laboratorio de Acuacultura del CIIDIR-IPN, Unidad Sinaloa in plastic tanks (120 L) with 80 L of filtered seawater (salinity of 30) and constant aeration. Animals were fed twice daily (09:00 to 17:00) with a commercial feed (Camaronina 35% Purina®, Mexico). Challenge bioassays with animals (weighing 30-716 mg) from the stock were done in glass aquariums (5 L) with 3 L of filtered seawater (salinity of 30) and constant aeration without water exchange. Ten organisms were placed in each glass tank. Animals were fed as above. After two days of acclimation, uneaten food and waste material were removed. Treatments were evaluated in triplicate. *V. parahaemolyticus* isolates were centrifuged at 2,379x *g* (Sigma 2-6E) for 20 min, the cell pellet was resuspended in 1 mL of sterile saline solution (2.5% NaCl), and then adjusted spectrophotometrically to an optical density of 1 at 580 nm for experimental challenge bioassays. The experimental doses were done according to the previous bacterial count. Values of pH (HI 98127 pHep, Hanna Instruments, Woonsocket, RI, USA), salinity (Refractometer W/ATC 300011, Sper Scientific, Scottsdale, AZ, USA), dissolved oxygen and temperature (YSI model 55 oxygen meter, Yellow Spring Instruments, Yellow Springs, OH, USA) were monitored daily. Animals were cultured under natural photoperiod. During each bioassay, mortality was recorded two times a day and dead shrimp were removed. Shrimp was grown in optimal conditions (Brock & Main, 1994); however, no cleaning of the tanks was made during the challenging period and temperature was maintained between 28-30°C to promote vibrio infection. Only in one bioassay with isolate 16, moribund shrimp were tested (72 h) after challenge for *V. parahaemolyticus* according with the

method published in NACA (2012). The analyzed shrimp were positive for *V. parahaemolyticus*.

LC_{50} determination

Mortality results from each bioassay were used to calculate the median lethal concentration (LC_{50}) by using Probit analysis (Finney, 1971) with StatPlus® 2009 professional 5.8.4.

RESULTS

Isolation and characterization of presumptive *V. parahaemolyticus* isolates

One hundred and twenty nine presumptive *V. parahaemolyticus* isolates were obtained from the hepatopancreas of diseased shrimp. All the selected isolates formed green colonies in TCBS. Thirty-five isolates were positive with AP3 primers, showing a fragment of 336 bp. Isolates were also positive (with exception of isolates 1, 8, and 33) with primers 89F/R, showing a fragment of 470 pb. Further characterization was done only on selected isolates (35) (Table 1). All isolates were positive for *tlh* but negative for *tdh* and *trh* (Table 1). All isolates showed α-hemolysis with halo diameters between 1.43 and 2.18 cm (Table 1).

Isolates 10, 16, 19, 29, and 33 showed strong capacity to form biofilms (1.04-1.13 OD). The rest of isolates showed moderate adherence (0.10-0.88 OD) (Table 1). Results showed that 8 isolates were strongly hydrophobic (51.46-58.24%). Twenty two isolates were moderately hydrophobic (22.7-49.52%), and five isolates were hydrophilic (11.6-19.9%) (Table 1).

Results of autoaggregation showed high values in all isolates ranging from 96.0 to 98.9% (Table 1). Results showed that isolates 11 and 12 had protease activity (casein degradation) with a diameter of lysis halo of 13 and 16 mm, respectively. No lipase activity was found.

The lag phase of 35 isolates was found between 6 and 12 h, whereas the log phase was found between 12 and 96 h. Bacterial count was done only to isolates (19) of the challenging bioassays. The CFU mL^{-1} of the isolates ranged from 10.6×10^6 to 94×10^6.

Immersion challenge tests

The isolates 1, 8, 10, 13, 14, 16, 17, 18, 19, 20, and 29 were pathogenic to *L. vannamei*; however, isolate 16 seems to be more virulent. Isolates 9, 15, 25, 28, 32, 33, 34 and 35 were not pathogenic for white shrimp (Table 2).

LC_{50} determination

Probit analysis of pathogenic isolates (11) yielded LC_{50} values from 6×10^4 to 353×10^3 CFU mL^{-1} (Table 2).

DISCUSSION

Shrimp farming has been affected by viral and bacterial diseases. Among bacterial diseases, those caused principally by *Vibrio alginolyticus*, *V. parahaemolyticus*, and *V. harveyi* are of primary concern (Rajasekar *et al.*, 2011; Wei & Wendy, 2012; Zhou *et al.*, 2012; Tran *et al.*, 2013; Nunan *et al.*, 2014). EMS or AHPND caused by *V. parahaemolyticus* appeared in Asia in 2009 (Tran *et al.*, 2013) causing significant economic losses. In Mexico, it was reported since 2013 (Nunan *et al.*, 2014; Soto-Rodriguez *et al.*, 2014), causing the collapse of this activity in the farms of the northwest. In this study, 35 isolates of *V. parahaemolyticus* with potential to cause AHPND in *L. vannamei* were obtained. Pre-screening and identification was performed by PCR with primers AP3 (Sirikharin *et al.*, 2014), primers 89F/R (Nunan *et al.*, 2014), and primers for hemolysin genes (*tlh*, *tdh*, and *trh*) (Bej *et al.*, 1999). All isolates were positive using primers AP3. However, isolates 1, 8, and 33 were negative using primers 89F/R. Results agree with Soto-Rodriguez *et al.* (2014), who found a false positive strain (M0604) with primers AP3. They mentioned that primers AP3 produced a predictive positive value of 90%. Bacterial identification was confirmed with the gene coding for the thermolabile hemolysin (*tlh*) that is specific for *V. parahaemolyticus* (Bej *et al.*, 1999; Gutiérrez-West *et al.*, 2013). The 35 isolates were positive for *tlh* and negative for human toxigenic genes *tdh* and *trh*. The *tlh* hemolysin of *V. parahaemolyticus* does not cause lysis in vertebrate erythrocytes (Bej *et al.*, 1999) as occurs with the *tdh* and *trh* hemolysins of the same species that are considered virulence factors in humans (Shirai *et al.*, 1990) and animal pathogens (Zhang & Austin, 2005). We concluded that all the isolates belong to *V. parahaemolyticus* but isolates 1, 8, and 33 do not belong to AHPND-causing isolates.

In this work, several tests were performed to select isolates with pathogenic potential since Joshi *et al.* (2014) and Soto-Rodriguez *et al.* (2015) mention that strains causing AHPND may differ in virulence among them despite the fact that two proteins have been identified in the supernatant of a culture of pathogenic *V. parahaemolyticus* (AHPND) strains from Asia (Sirikharin *et al.*, 2014), which have a high identity to delta endotoxins (PirA and PirB) of the bacterium *Photorhabdus luminescens* that are active against insects (Waterfield *et al.*, 2005). Also, the secretion systems type III (T3SS1) and VI (T6SS1 and T6SS2) have been demonstrated in *V. parahaemolyticus*, causing AHPND (Kongrueng *et al.*, 2014). The T3SS system is a structure that enables, in Gram-negative bacteria, the secretion and injection of bacterial effector

Table 1. Characterization of isolates of presumptive *V. parahaemolyticus* obtained from the hepatopancreas of cultured shrimp (*L. vannamei*). AP3 = AP3 primers. 89F/R = 89F/R primers. Tlh: thermolabile hemolysin, tdh: thermostable direct hemolysin, trh: thermostable direct hemolysin-related. Hemolysis = lysis halo. Biofilm = strong adherence (OD ≥1), moderate ($0.1 \leq OD_{595} < 1$) or weak ($OD_{595} < 0.1$). Hydrophobicity = strong (>50%), moderate (20-50%), hydrophilic (<20%).

Isolates	AP3	89F/R	Hemolysins			Hemolysis (cm)	Biofilm (OD)	Hydrophobicity (%)	Autoaggregation (%)
			tl	tdh	trh				
1	+	-	+	-	-	1.65 ± 0.33	0.18 ± 0.01	19.35 ± 2.41	98.73 ± 0.01
2	+	+	+	-	-	1.66 ± 0.26	0.17 ± 0.02	55.95 ± 0.77	97.89 ± 1.13
3	+	+	+	-	-	1.93 ± 0.33	0.34 ± 0.02	36.29 ± 2.29	96.98 ± 1.06
4	+	+	+	-	-	2.18 ± 0.33	0.33 ± 0.01	35.71 ± 1.79	97.11 ± 0.41
5	+	+	+	-	-	1.80 ± 0.20	0.15 ± 0.01	51.46 ± 2.97	97.88 ± 0.18
6	+	+	+	-	-	1.70 ± 0.06	0.10 ± 0.01	47.31 ± 0.33	97.29 ± 0.22
7	+	+	+	-	-	1.85 ± 0.05	0.10 ± 0.01	53.86 ± 0.24	98.19±0.13
8	+	-	+	-	-	1.75 ± 0.15	0.13 ± 0.03	37.58 ± 0.74	98.80 ± 0.35
9	+	+	+	-	-	1.75 ± 0.05	0.12 ± 0.02	21.77 ± 0.50	96.97 ± 0.17
10	+	+	+	-	-	1.95 ± 0.05	1.04 ± 0.06	55.26 ± 0.33	97.65 ± 0.21
11	+	+	+	-	-	1.70 ± 0.00	0.11 ± 0.04	26.37 ± 0.78	97.94 ± 0.12
12	+	+	+	-	-	1.85 ± 0.05	0.19 ± 0.06	40.24 ± 1.64	97.30 ± 2.26
13	+	+	+	-	-	1.50 ± 0.00	0.13 ± 0.03	48.66 ± 2.21	97.48 ± 0.27
14	+	+	+	-	-	1.53 ± 0.48	0.88 ± 0.05	29.63 ± 1.09	96.46 ± 1.64
15	+	+	+	-	-	1.60 ± 0.31	0.28 ± 0.01	30.88 ± 1.90	97.02 ± 1.97
16	+	+	+	-	-	1.57 ± 0.12	1.10 ± 0.07	55.92 ± 0.08	97.82 ± 0.44
17	+	+	+	-	-	1.43 ± 0.22	0.21 ± 0.08	27.12 ± 1.18	96.96 ± 1.87
18	+	+	+	-	-	1.93 ± 0.38	0.26 ± 0.02	43.58 ± 0.69	97.58 ± 1.84
19	+	+	+	-	-	1.50 ± 0.06	1.09 ± 0.01	55.00 ± 1.33	98.01 ± 0.83
20	+	+	+	-	-	1.67 ± 0.13	0.12 ± 0.06	37.85 ± 1.58	98.94 ± 0.16
21	+	+	+	-	-	1.67 ± 0.09	0.21 ± 0.02	35.06 ± 2.12	97.69 ± 0.14
22	+	+	+	-	-	2.03 ± 0.09	0.14 ± 0.04	43.97 ± 2.81	97.86 ± 0.17
23	+	+	+	-	-	1.90 ± 0.06	0.18 ± 0.06	56.01 ± 2.70	98.40 ± 0.05
24	+	+	+	-	-	2.0 ± 0.25	0.21 ± 0.04	19.90 ± 0.07	96.00 ± 0.29
25	+	+	+	-	-	1.55 ± 0.05	0.17 ± 0.05	13.30 ± 0.80	97.61 ± 0.17
26	+	+	+	-	-	1.78 ± 0.11	0.17 ± 0.03	35.53 ± 1.41	97.78 ± 0.04
27	+	+	+	-	-	1.63 ± 0.05	0.10 ± 0.02	39.16 ± 1.02	96.69 ± 0.48
28	+	+	+	-	-	1.68 ± 0.08	0.28 ± 0.01	49.52 ± 0.68	96.42 ± 0.86
29	+	+	+	-	-	1.55± 0.05	1.13 ± 0.03	29.01 ± 0.34	98.11 ± 0.19
30	+	+	+	-	-	1.45 ± 0.05	0.27 ± 0.08	44.36 ± 1.16	96.68 ± 0.21
31	+	+	+	-	-	2.43 ± 0.23	0.24 ± 0.01	22.94 ± 1.41	97.19 ± 0.08
32	+	+	+	-	-	2.17 ± 0.12	0.27 ± 0.02	18.00 ± 1.97	97.71 ± 0.25
33	+	-	+	-	-	2.18 ± 0.12	1.06 ± 0.06	58.24 ± 0.35	97.79 ± 0.41
34	+	+	+	-	-	1.63 ± 0.19	0.10 ± 0.01	11.62 ± 0.25	97.66 ± 0.01
35	+	+	+	-	-	1.33 ± 0.33	0.44 ± 0.01	32.55 ± 0.91	97.35 ± 0.04

proteins in the cytoplasm of eukaryotic cells, whereas the T6SS system is common in Gram-negative bacteria and is associated with adhesion, cytotoxicity, invasion, and intracellular growth, or survival and persistence within the host (Cascales, 2008).

Hemolytic activity is one of the virulence factors of pathogenic vibrios (*V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, *V. anguillarum*, and *V. mimicus*) (Zhang & Austin, 2005). Hemolysis may result from the enzymatic activities demonstrated in some bacterial

Table 2. Experimental infections of *L. vannamei* challenged with *V. parahaemolyticus* isolates. Weight values are mean ± SD. LC_{50}: lethal concentration (colony forming units per milliliter [CFU mL^{-1}]). ND: not determined.

Isolates	Bioassays	Weight (mg)	LC_{50}	Isolates	Bioassays	Weight (mg)	LC_{50}
1	1	174 ± 6	270×10^3	17	1	100 ± 7	145×10^3
8	1	100 ± 7	158×10^3	18	1	114 ± 8	100×10^3
9	1	716 ± 30	ND	19	1	100 ± 7	227×10^3
	2	40 ± 15	ND	20	1	174 ± 6	162×10^3
	3	174 ± 6	ND	25	1	187 ± 9	ND
10	1	174 ± 6	115×10^3	28	1	200 ± 29	ND
13	1	100 ± 7	353×10^3	29	1	174 ± 6	198×10^3
14	1	174 ± 6	198×10^3	32	1	114 ± 8	ND
15	1	187 ± 9	ND	33	1	200 ± 29	ND
16	1	200 ± 19	62×10^3		2	187 ± 9	ND
	2	520 ± 26	144×10^3	34	1	187 ± 9	ND
	3	30 ± 9	60×10^3	35	1	187 ± 9	ND
	4	440 ± 15	175×10^3				
	5	80 ± 15	68×10^3				
	6	79 ± 21	71×10^3				
	7	114 ± 8	81×10^3				
	8	318 ± 25	96×10^3				

species, including phospholipase C and D or by forming pores (*tdh*) in the cytoplasmic membrane of erythrocytes (Iida & Honda, 1997; Parker & Feil, 2005). In this study, all isolates showed α-hemolysis (incomplete hemolysis). Results agree with those obtained with the genetic analysis because *tdh* and *trh* (related with β-hemolysis [complete hemolysis]) genes were not found.

The studied isolates showed autoaggregation, biofilm formation, extracellular enzymatic activity, and hydrophilic or hydrophobic nature. The ability of *Vibrio* spp. to form biofilms is a mechanism of survival, pathogenesis, and stress tolerance (Yildiz & Visick, 2009). Biofilms are complex microbial communities attached to living and nonliving surfaces and embedded in a matrix of extracellular material (polymers) produced by them (Hall-Stoodley *et al.*, 2004; Huq *et al.*, 2008). Some properties of bacterial cells as increased hydrophobicity and the ability of coaggregation and autoaggregation are important for colonization and biofilm formation (Decostere *et al.*, 1999; Rickard *et al.*, 2004). Authors like Yuehuei & Friedman (2000) and Rickard *et al.* (2004) mention that the cell surface properties, compounds secreted by microorganisms, the hydrodynamics of the aquatic environment, the surface roughness, nutrient availability, and speed differences in colonization affect biofilm formation. Additionally, bacteria in biofilms can be up to 1,000 times less susceptible to most antibiotics and biocides (Mah *et al.*, 2003).

Extracellular lipolytic activity of isolates (35) was negative. These results are in agreement with Meyers *et al.* (1996), who found negative results in lipolytic activity of lactic acid bacteria. Regarding the proteolytic activity, only isolates 11 and 12 were positive. Some authors claim that the overproduction of these enzymes is a virulence factor, since pathogenic strains have high proteolytic, lipolytic, and hemolytic activity (Quesada-Herrera & Rosa-Placencia, 2004). However, the role of extracellular enzymatic activity as virulence factor is not clear since most isolates studied in challenge bioassays are pathogenic.

According to previous studies, of the 35 isolates, 19 were selected to be tested in *L. vannamei* challenges based on their hemolytic activity, hydrophobicity, autoaggregation, and ability to form biofilm. Eleven isolates were pathogenic for white shrimp (*L. vannamei*) and eight isolates were not, at least at the concentrations tested. The clinical signs observed included empty stomach and midgut, inactivity, and pale to white atrophied hepatopancreas (Tran *et al.*, 2013). Among pathogenic isolates, those numbered as 1 and 8 did not belong to AHPND-causing strains. Therefore, more research is needed to clarify this and the differences in virulence found among pathogenic strains. Regarding differences in virulence, it seems that isolate 16 is the most virulent according to LC_{50}. The mentioned isolate showed α-hemolysis, strong biofilm formation, strong hydrophobicity, and high autoaggregation capacity. Results of virulence agree

with those reported by Soto-Rodríguez *et al.* (2015) and Joshi *et al.* (2014), who found that strains causing AHPND may differ in virulence. Mortality in immersion challenges of isolate 16 began in treatments with 1×10^4 CFU mL^{-1} (5-72 h) in the smaller shrimp (30-200 mg) as has been reported by Joshi *et al.* (2014) and Soto-Rodriguez *et al.* (2015). In shrimp weighing 318, 440, and 520 mg, higher concentration of CFU mL^{-1} of strain 16 were necessary to kill 50% shrimp (LC_{50}). The results show that the smaller shrimp are less resistant to *V. parahaemolyticus* because fewer bacteria per milliliter of water were required to kill 50% of the shrimp (LC_{50}).

In this work, it is important to note that not all the isolates belong to *V. parahaemolyticus* strains that cause AHPND, but the isolates that can cause the disease in shrimp from Guasave farms exhibited variable mortality percentages maybe due to α-hemolysis and differences in biofilm formation and hydrophobicity. Therefore, performed characterization may help to understand the pathogenicity of *V. parahaemolyticus*. Finally, results showed that smaller shrimp are less resistant to *V. parahaemolyticus* infection.

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