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Pathological, Genomic and Phenotypical Characterization of *Vibrio parahaemolyticus,* Causative Agent of Acute Hepatopancreatic Necrosis Disease (AHPND) in Mexico

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Abstract

Moribund whiteleg shrimp (*Penaeus vannamei*) affected by acute hepatopancreatic necrosis disease (AHPND) from farms in northwestern Mexico were sampled for pathological analysis. Bacterial isolates were molecularly identified as *Vibrio parahaemolyticus* (VP) by the presence of the *tlh* gene. The *tdh*-negative, *trh*-negative and *tlh*-positive VP strains were further characterized by enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR). The VP pure strains were used in immersion challenges with shrimp, and farmed and challenged shrimp presented the same clinical and pathological signs: lethargy, empty gut, pale and aqueous hepatopancreas and expanded chromatophores. Using fresh mount, histological analysis and bacterial density count, three stages of AHNPD (initial, acute and terminal) were identified in the affected shrimp. Pathognomonic lesions indicated severe desquamation of tubular epithelial cells of the hepatopancreas. VP had different virulence and was dose dependent. VP strains showed wide tolerance to different environment conditions of temperature, salinity and pH, and pathogenic and non-pathogenic VP strains from Mexico had similar morphological and physiological characteristics but pathogenic VP strains were most sensitive to nalidixic acid and showed resistance to penicillin. Whole genomic sequence of 22 VP strains from Asia, Mexico and South America shows the presence of similar chromosomic pathogenic mechanisms, and comparative analysis by multilocus sequence analysis (MLSA) of seven genes clearly showed that most of the isolates are independent strains.

Keywords: AHPND, antimicrobial sensitivity, genomic analysis, histopathology, shrimp, *Vibrio parahaemolyticus*, virulence

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Introduction

Production of cultured shrimp in Mexico is supported mainly from the northwest states of Sonora, Sinaloa and Nayarit. In 2013, shrimp farms from these states were affected by atypical mortalities that primarily occurred in the first days after stocking. During the first culture cycle of Nayarit State, shrimp farms presented atypical mortalities during the first 30 days of culture. Mortality events with the same characteristics as Nayarit's were subsequently observed in the states of Sinaloa and Sonora, affecting regional production and subsequently producing economic losses of over 2.5 million pesos (Julio Cabanillas, CEO of the Aquatic Animal Health Sinaloa State Committee personal communication, May 2013).

The Mexican shrimp farming industry was deeply affected, because of the large scale of the mortalities and the lack of effectivity of the antibiotics commonly used in shrimp farming (Soto-Rodriguez et al. 2006), such as enrofloxacin, oxytetracycline and florfenicol. Despite the economic losses of 2013, a slight recovery of the Mexican production of cultured shrimp was observed in 2014 and 2015. Studies conducted by our research team showed no evidence of VP_{AHPND+} colonization in the shrimp's digestive tract (unpublished data). Acute hepatopancreatic necrosis disease (AHPND) isn´t a typical vibriosis infection, rather it is an acute intoxication caused by PirA and PirB toxins delivered directly into the culture water.

Gross Signs and Histopathology of Affected Shrimp

Moribund shrimp, between 0.2 and 2.5 g, affected by AHPND from farms in northwestern Mexico revealed clinical signs that typically included slightly expanded chromatophores, lethargy and erratic swimming and were characterized by presenting macroscopic changes distinguishable in the external appearance of the digestive tract and hepatopancreas (HP) and changes to the microanatomy associated with the development of lesions in the tissue. Based on histopathological evidence and bacterial density counts for shrimp affected under natural and experimental conditions (Soto-Rodriguez et al. 2015), we identified three phases of the disease:

Initial Phase

Macroscopically, at this phase, organisms affected by AHPND showed altered HP with partial or total absence of food in the stomach and midgut. These changes can be better appreciated by dissecting the digestive tract and removing the membrane covering the HP (Fig. 1b). Microscopically, tubules of the HP presented modification and elongation of their epithelial cells (appearing like drops) towards the tubular lumen (Fig. 2a), causing the subsequent cellular desquamation. A reduction in size of the vacuoles in R and B cells was observed, which increased as the disease progressed (Fig. 2b); meanwhile other tubules were affected.

Fig. 1. Macroscopic observation of the digestive tract of *Penaeus vannamei* affected by acute hepatopancreatic necrosis disease (AHPND). (a) Healthy organism; (b) Initial phase; (c and d) Acute phase; (e) Terminal phase.

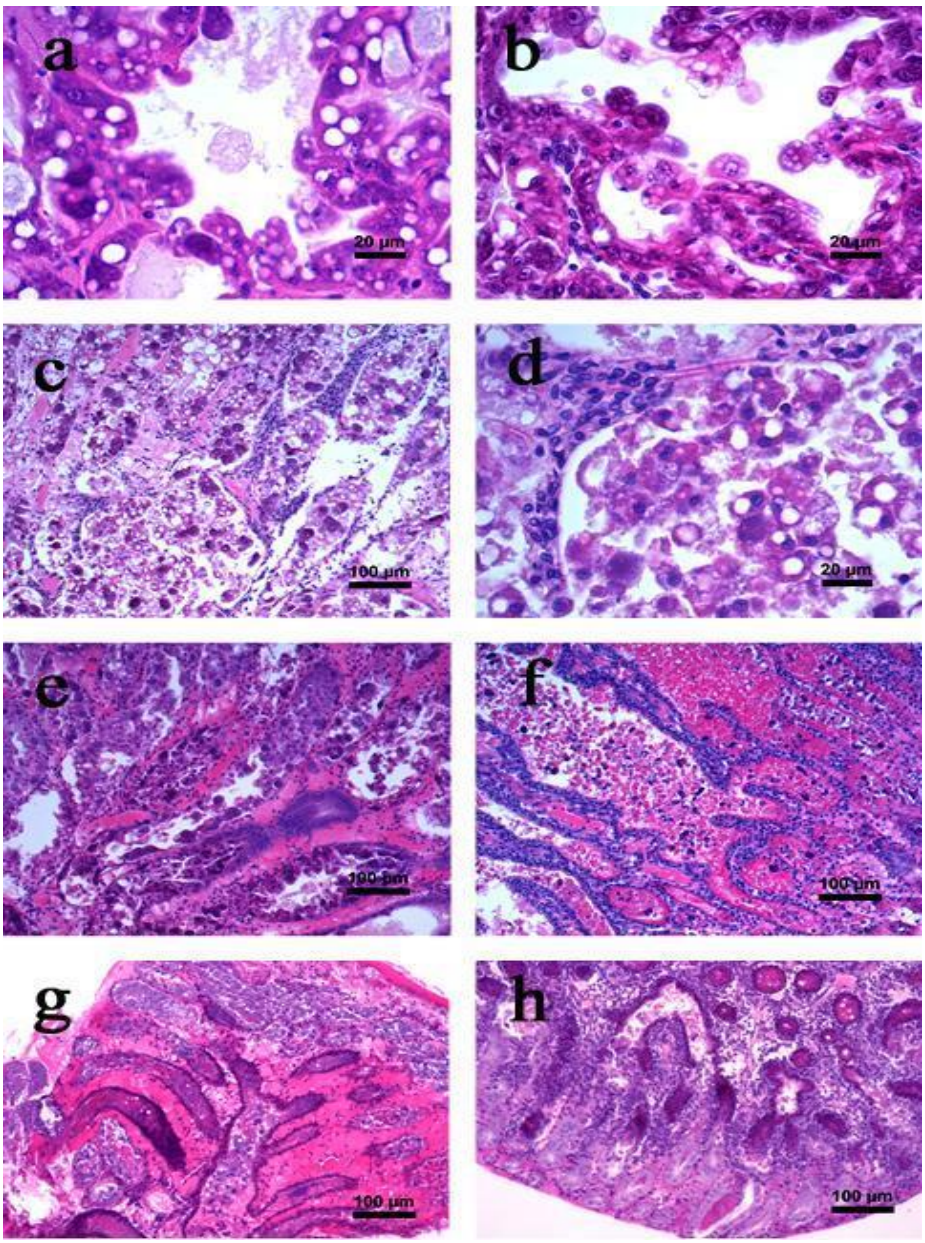

Fig. 2. Photomicrograph of the hepatopancreas of *Penaeus vannamei* with lesions associated with acute hepatopancreatic necrosis disease (AHPND). (a-b) Initial phase; (c-f) Acute phase; (g-h) Terminal phase of disease. Haematoxylin-eosin-floxin staining.

Acute Phase

Organisms affected by this phase of the disease showed anorexia, lethargy and empty digestive tract, with loss of tissue pigmentation (Fig. 1c) until the HP became whitish and atrophied (Fig. 1d). The HP tissue was friable with an aqueous consistency during the first hours post-infection, and as the disease progressed, developed a hard consistency, becoming difficult to disintegrate. Microscopically, a generalized HP disorganization was observed due to severe necrosis of the epithelium. Intertubular tissue showed haemocytic infiltration, and most of the tubules had a necrotic epithelium with a massive accumulation of dead cells into the tubular lumen (Fig. 2c and d), a pathognomonic lesion reported for AHPND (Tran et al. 2013, Soto-Rodriguez et al. 2015).

In the first hours post-infection, R and B cells may have some cytoplasmic vacuoles; however, as the disease progresses, there was a vacuole reduction (Fig. 2e) until these disappeared; meanwhile mitosis was interrupted in E cells. In this phase, there weren't bacteria in the affected tissues, as reported by Tran et al. (2013) and Soto-Rodriguez et al. (2015). Possibly at this stage, PirA and PirB toxins, responsible for AHPND (Han et al. 2015), caused the greatest damage in the epithelial cells of the HP tubules, which has not been observed in any organ or tissue of affected shrimp (Soto-Rodriguez et al. 2015). At the end of the acute phase, a large haemocytic infiltration in the intertubular tissue is observed, and the tubular epithelium is completely necrotic with loss of continuity of the epithelium or, in some instances, its absence. The tubular lumen is filled with dead cells, increasing their size due to accumulation of necrotic tissue (Fig. 2f).

Terminal Phase

Organisms at this phase showed an empty digestive tract, anorexia, lethargy, expanded chromatophores and large atrophy of the HP with whitish coloration (Fig. 1e). In addition, the HP feels fibrous when it is squashed. Histology showed black streaks, indicating focal tubular melanization, and an increase of haemocytic infiltration in the intertubular connective tissue with haemocytic capsules surrounding the affected tubules as a response to bacterial load and necrotic tissue (Fig. 2g). At this phase, the bacterial proliferation is caused by a secondary infection, possibly a vibriosis (Fig. 2h).

Identification and Characterization of Isolates From Shrimp Farms Affected by AHPND

Microbiological analyses indicated a poor presence of bacteria in the haemolymph (HL) and HP. In contrast, a high load of *Vibrio* bacteria was found in the stomach (ST). By Mann-Whitney test no significant differences were observed between bacterial densities among shrimp on thiosulfate-citrate-bile salts-sucrose agar (TCBS) and marine agar: ST (TCBS, $p = 0.109$; marine agar, $p = 0.113$) and HP (TCBS, $p = 0.131$; marine agar, $p = 0.074$) in any of the three stages of the disease. An increase in the density of *Vibrio* spp. was observed as the infection progressed from healthy shrimp to shrimp at the terminal stage, with dominance of yellow colonies (YCs) and a presence of green colonies (GCs) on TCBS that went from 20 to 38 % for the HP and from 13 to 42 % for the ST.

Field and laboratory observations have been similar to reports from Asia (Tran et al. 2013; NACA 2014) and Mexico as AHPND (Nunan et al. 2014; Soto-Rodriguez et al. 2015). Bacterial isolates were molecularly identified as *Vibrio parahaemolyticus* (VP) by the tlh gene (Bej et al. 1999). The VP strains tdh-, trh- and tlh+ were further characterized by enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR) (Gomez-Gil et al. 2004); primers AP, AP1, AP2, AP3, AP4, ems2 IQ2000™ and IQ REAL™ AHPND/EMS (GeneReach, Taiwan POC) were used in the diagnostic tests for AHPND. Molecular characterization of tlh+, tdh- and trh- isolates was fingerprinted using rep-PCR, and several clones were detected. All other isolates produced different results and were considered strains; in total, 31 strains were obtained (Fig. 3). All primers and kits used as diagnostic tests have shown inconsistency to detect AHPND, except for AP4.

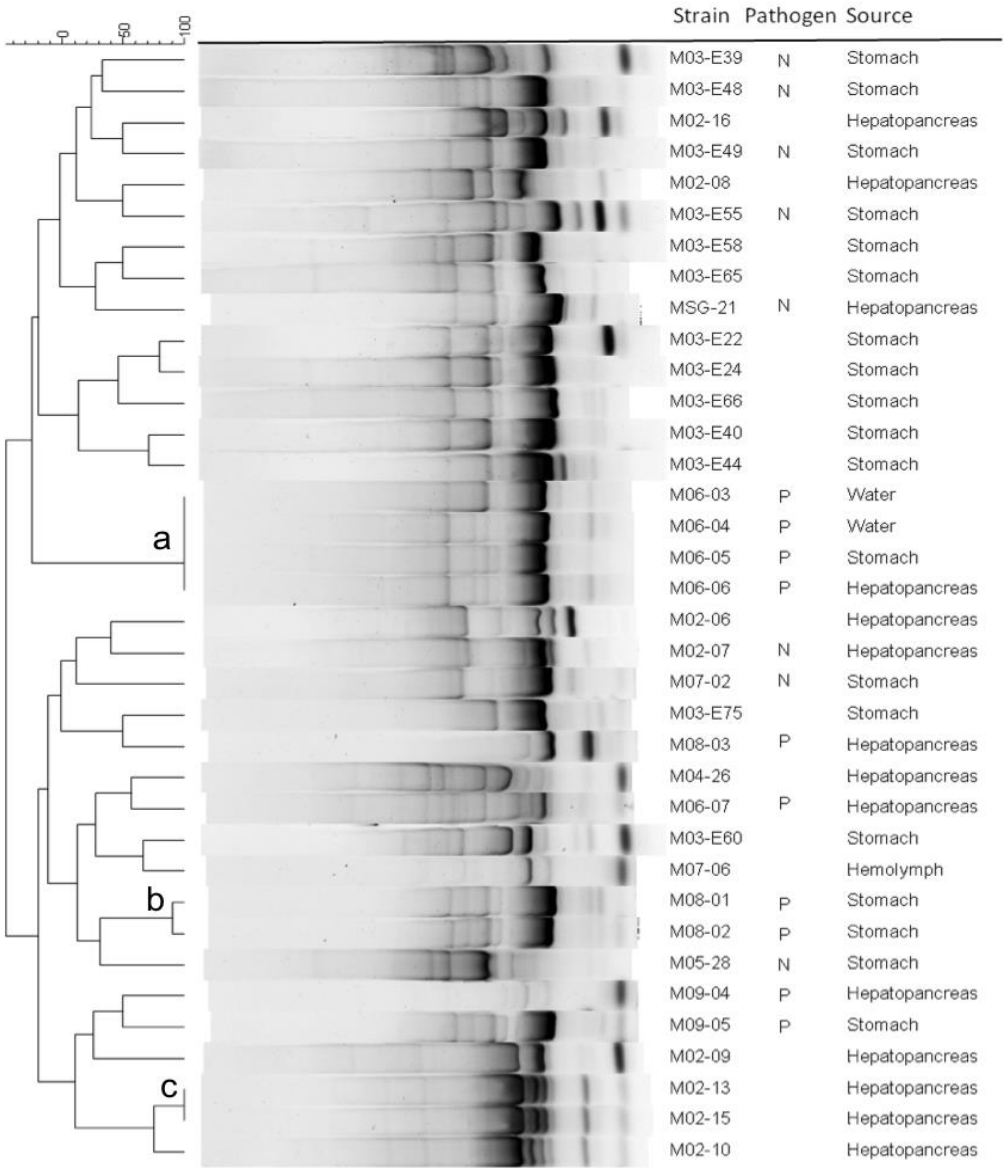

Fig. 3. ERIC-PCR fingerprinting of *Vibrio parahaemolyticus* isolated from shrimp (*Penaeus vannamei*) and water from ponds affected by acute hepatopancreatic necrosis disease (AHPND) in Mexico. N, nonpathogenic; P, pathogenic. Letters in the dendrogram denote clonal groups (95 %). Band position tolerance, 1 %; optimization, 0.2 %.

Bacterial Challenges with Pure VP Strains

In addition, experimental challenges with juvenile shrimp showed VP strains had different virulence: some of the less-virulent strains do not induce 100 % mortality, and mortality rates also rise more slowly than they do for the more virulent strains (Fig. 4).

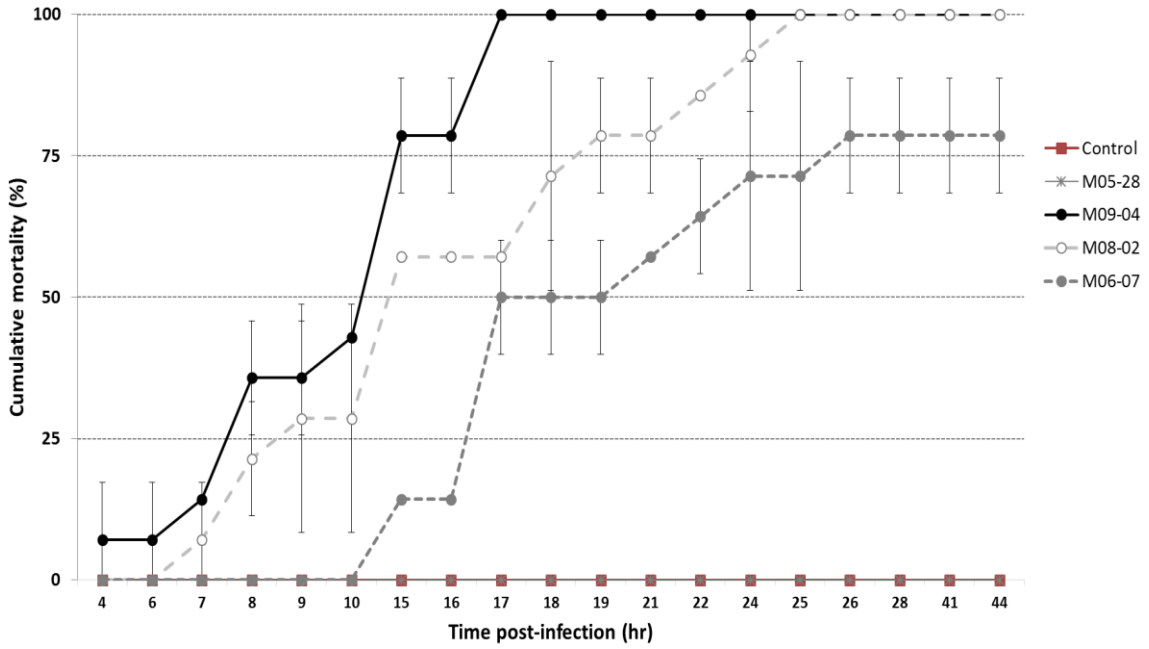

Fig. 4. Example of cumulative mortality of juvenile shrimp challenged with *Vibrio parahaemolyticus* strains. M05-28: 1.20 x 10^7, M09-04: 2.20 x 10^6, M08-02: 3.30 x 10^6, M06-07: 7.82 x 10^6 cfu.mL^{-1}. Control: tryptic soy broth+2.0 % NaCl. Bars indicate standard deviations.

The virulence of VP strains was dose dependent, where the threshold infective density was 10^4cfu.mL^{-1}; below that density, no mortality was observed. Field and experimental results showed that the VP strain that causes AHPND acts as a primary pathogen for shrimp compared with the VP strains reported to date (Soto-Rodriguez et al. 2015).

All VP strains were used in immersion challenges with shrimp, showing that farmed shrimp present the same pathological damage in the HP as shrimp coming from controlled environments. Organisms exposed to pathogenic strains (VP_{AHPND+}) systematically showed lesions associated with each disease phase previously described, but in addition, histopathological analysis of survival organisms from challenges showed lesions, considered as an additional phase of the disease (unpublished data) resulting from the evolution of the disease. In preliminary studies, survival organisms of AHPND showed filled gut and normal appearance of HP. Histology showed damage in the HP ranging from low necrotic lesions (similar to the terminal phase of AHPND) to a flattened epithelium without vacuoles and with a few elongated cells. Future studies are needed to obtain a deeper knowledge of the pathological progress of survival organisms of AHPND and to know the repair mechanisms, including the homeostatic state used by survival organisms to overcome the disease.

Phenotypic Characterization

VP_{AHPND+} and VP_{AHPND-} strains from Mexico have similar morphological and physiological characteristics. All strains are motile Gram-negative rods; non-luminescent; grow as green colonies on TCBS and as mauve, blue and beige colonies on CRHOMagar ™ Vibrio; show OF metabolism; do not produce hydrogen sulfide; and do not utilize either melibiose, L-rhamnose, sorbitol, sucrose or tryptophan deaminase. Bacterial cells of VP had an exponential growth starting at 2 hr at 30 °C; strains showed a wide tolerance to environmental conditions (Table 1), e.g. pH (5–10), temperature (20, 30, 37 and 44 °C) and salinity (from 0.5 to 10.0 % NaCl).

Table 1. Growth of *Vibrio parahaemolyticus* pathogenic (VP_{AHPND+}) and nonpathogenic (VP_{AHPND-}) strains at different temperature, salinity and pH.

Condition		**VP_{AHPND+} strains**					**VP_{AHPND-} strains**				
Temp. (°C)		**M0603**	**M0605**	**M0802**	**M0904**	**M0905**	**M0528**	**M0604**	**M0702**	**M0903**	**SMG21**
	4	-	+	-	-	-	-	-	-	-	-
	20	+	+	+	+	+	+	+	+	+	+
	30	+	+	+	+	+	+	+	+	+	+
	37	+	+	+	+	+	+	+	+	+	+
	44	+	+	+	+	-	-	-	-	-	-
NaCl (%)	0.0	-	-	-	-	-	-	-	-	-	-
	0.5	+	+	+	+	+	+	+	+	+	+
	1.0	+	+	+	+	+	+	+	+	+	+
	2.0	+	+	+	+	+	+	+	+	+	+
	3.0	+	+	+	+	+	+	+	+	+	+
	4.0	+	+	+	+	+	+	+	+	+	+
	5.0	+	+	+	+	+	+	+	+	+	+
	6.0	+	+	+	+	+	+	+	+	+	+
	7.0	+	+	+	+	+	+	+	+	-	+
	8.0	+	+	+	+	+	+	+	+	-	+
	9.0	+	+	+	+	+	-	+	-	-	+
	10.0	-	+	-	-	-	-	+	-	-	+
pH	4	-	-	-	-	-	-	-	-	-	-
	5	+	+	+	+	+	-	+	-	+	+
	6	+	+	+	+	+	+	+	+	+	+
	7	+	+	+	+	+	+	+	+	+	+
	8	+	+	+	+	+	+	+	+	+	+
	9	+	+	+	+	+	+	+	+	+	+
	10	+	+	+	+	+	+	+	+	+	+

Antimicrobial Sensitivity

Pathogenic VP strains were most sensitive to nalidixic acid and showed resistance to penicillin (Fig. 5). Some alternatives used by shrimp farmers to control AHNPD in Mexico include the use of controlled production systems, prebiotics, probiotics, biofloc technology (BFT) and the use of genetic lines more tolerant to pathogens.

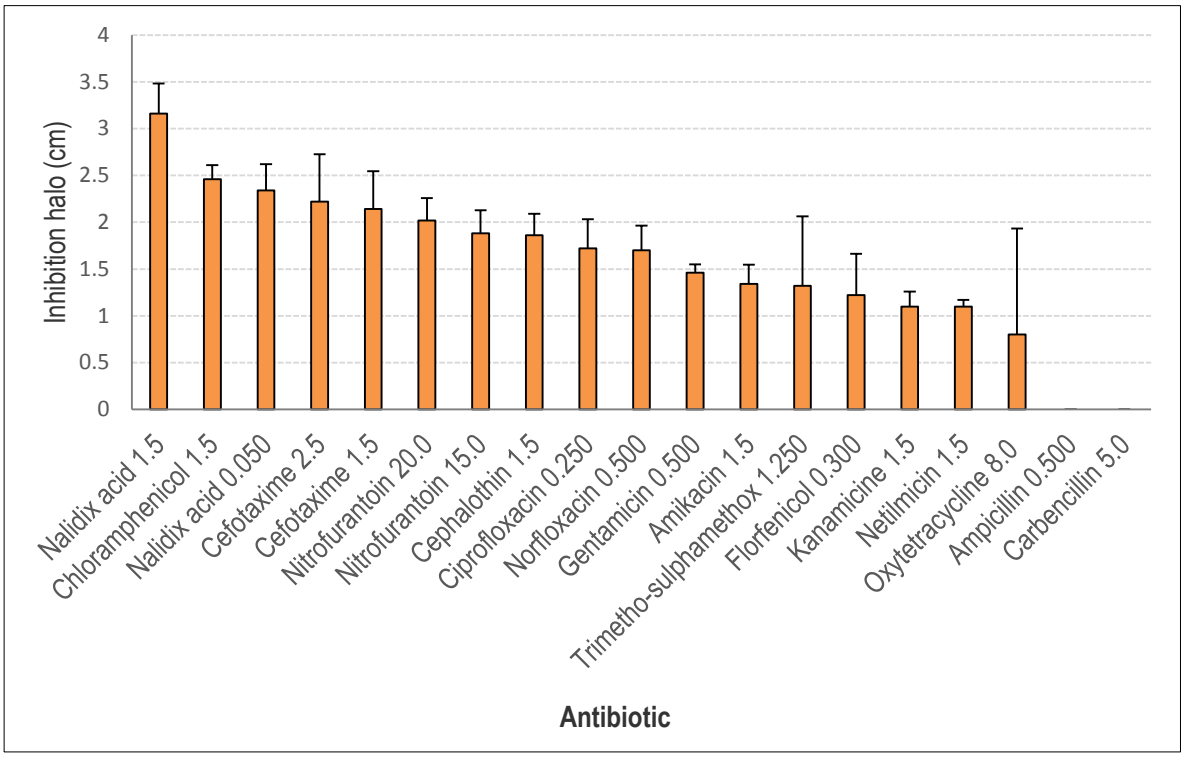

Fig. 5. Antibiotic sensitivity of pathogenic *Vibrio parahaemolyticus* (n=5) isolated from Mexico. Antibiotic concentration is in mg mL^{-1}. Bars indicate standard deviations.

Genomic Comparison

Whole genomic sequence of 22 VP_{AHPND+} and VP_{AHPND-} strains from Asia, Mexico and South America shows the presence of similar chromosomic pathogenic mechanisms. Comparative analysis by multilocus sequence analysis (MLSA) of seven genes clearly showed that most of the isolates are independent strains, although some could not be differentiated at this level and thus considered MLSA clones because they have similarity values equal or above 99.9 % (Fig. 6). By comparison of the 73.5 bp conjugative plasmid (pVp-AP) of pathogenic VP from Asia and Mexico, we observed that all VP strains have one transposon (that includes the PirA and PirB-toxin like genes), but only strains from Mexico and Viet Nam have a second transposon. Genomic characterization of pathogenic VP strains found the PirA- and PirB-like toxins as the main virulence factor in a conjugative plasmid flanked by transposases (Han et al. 2015).

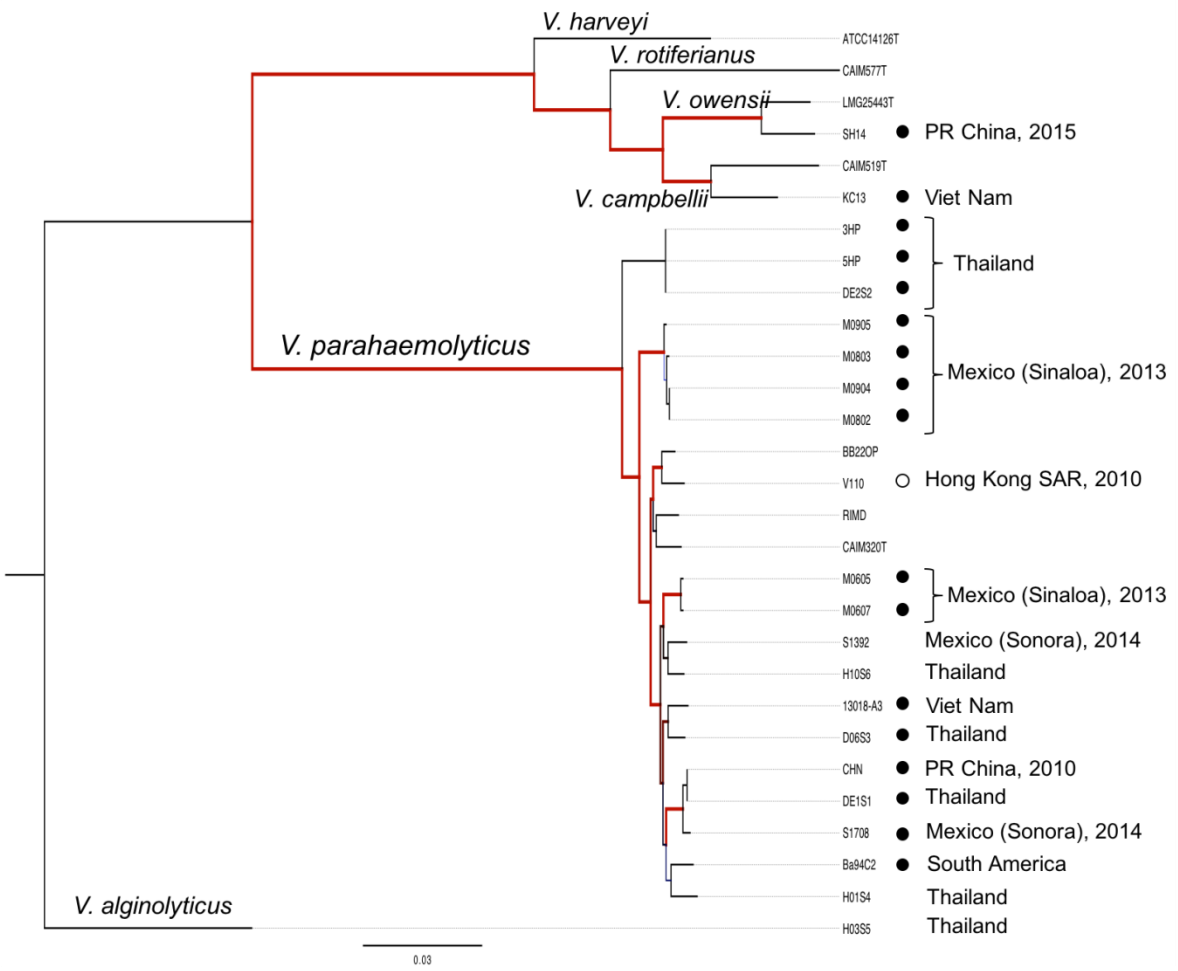

Fig. 6. Comparative analysis by multilocus sequence analysis (MLSA) of seven genes from 22 VP_{AHPND+} and VP_{AHPND-} strains from Asia, Mexico and South America.

Conclusion

The VP pure strains used in challenges with shrimp, and farmed and challenged shrimp presented the same clinical and pathological signs. AHPND had three phases (initial, acute and terminal) while challenged survival organisms showed an additional phase. VP had different virulence and was dose dependent. Our research team will do a further study on the toxin dynamics during the infectious process in P. vannamei. Pathogenic and non-pathogenic VP strains showed a wide tolerance to temperature, salinity and pH due to high metabolic diversity. This enabled them to adapt and survive in almost all marine-estuarine environments where shrimp farms are located, which implies a big risk of outbreaks and fast disease dispersion to free zones. The phenotypic profile (biochemical, morphological and physiological) was similar between pathogenic and non-pathogenic VP strains, but pathogenic VP were most sensitive to antibiotics and showed moderate resistance to oxytetracycline, one of the antibiotics most used in Mexican shrimp farms to control vibriosis.

Since 2013, some alternatives used by shrimp farmers to control AHNPD in Mexico include the use of controlled production systems, prebiotics, probiotics, biofloc technology (BFT) and the use of genetic lines more tolerant to pathogens. VP strains from Asia, Mexico and South America showed that most of the isolates are independent strains by genomic and molecular analysis, so that new pathogenic strains could be detected.

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