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Presence of Infectious Hypodermal and Haematopoietic Necrosis Virus (IHHNV) in Native Shrimps from Southern Mexico

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Abstract

A survey for Infectious Hypodermal and Haematopoietic Necrosis Virus (IHHNV) and White Spot Syndrome Virus (WSSV) was performed during two catching seasons of native shrimps in the Yucatan Coast in the Gulf of Mexico. The mtDNA COI barcode identified two endemic species; the southern pink shrimp *Penaeus notialis* (Pérez Fantante, 1967) and the northern pink shrimp *Penaeus brasiliensis* (Latreille, 1987) (previously described as *Farfantepenaeus brasiliensis*). The prevalence of IHHNV was of 18.18% in 2016, and of 8.57% in 2017. All organisms tested negative for WSSV. This is the first identification of wild shrimps in the state of Yucatan Mexico by mtDNA COI barcode as well as the first identification of IHHNV in such species. The presence of IHHNV in wild shrimps populations has a potential of persisting in the coast of Yucatan with putative detrimental effect on local fisheries because once established in natural waters and hosts; such pathogens are almost impossible to eradicate.

Keywords

IHHNV, WSSV, mtDNA COI Barcode, *Penaeus notialis*, *Penaeus brasiliensis*, Yucatan Peninsula

1. Introduction

The small-scale local fishery for shrimps and prawns supports the income of lo-

cal fisheries of the Yucatan Peninsula [1]. In this artisanal fishery, there is little bycatch and limited disruption to the benthic (bottom) habitat. This strategy produces high-quality shrimp with minimal adverse impacts on the local ecosystem [2]. The Mexican government regulates this fishery by way of established fishing seasons and gear restrictions [1]. These restriction protocols help to protect its long-term sustainability. However, the main concern is the sanitary status of native shrimps along the coast of the Yucatan Peninsula.

In Mexico, the shrimp farming industry started in 1983 in the state of Sonora located in northwestern Mexico with the broad culture of the blue shrimp *Penaeus stylirostris* (Stimpson, 1871) [1] [3]. In 1990, this industry suffered severe epizootics being the causative agent the Infectious Hypodermal and Haematopoietic Necrosis Virus (IHHNV). Mexico lost US$25 million due to IHHNV infections in the blue shrimp *P. stylirostris* in the late 1980s/early 1990s [2]. This species was replaced by the Pacific white shrimp *Penaeus vannamei* (Boone, 1931) due to its resistance to IHHNV infections; in this species, IHHNV causes dwarfism and deformities, but no mortality [4] [5]. However, in 2001, another outbreak occurred with the arrival of the White Spot Syndrome Virus (WSSV). From that date onwards, the shrimp industry struggled huge financial losses due to high mortalities related to WSSV, and by 2009 the shrimp production raised up to a steady production of ≈134,000 tons [2]. However, in 2010 a new WSSV outbreak caused mortality in 60% of the national production, and since then, shrimp production is recovering slowly.

IHHNV and WSSV are listed since 1997 as notifiable diseases by the World Organization for Animal Health (OIE) [6]. The Aquatic Animal Health Code recommends the detection of these viral agents by PCR and the IQ2000™ kit has proven to be reliable test. Both IHHNV and WSSV infect tissues of ectodermal and mesodermal origin. Thus the hemolymph represents a suitable tissue for detection of both viruses [7] [8]. In this way, a rapid and accurate diagnosis of the disease should be established either for disease detection, as well as, to monitor wild species co-habiting along the shrimp farms [6]. In Mexico, similar to other countries, the role of aquaculture in harboring and spreading disease in the aquatic environment has become evident. For example, in the Pacific coast of Mexico; WSSV and IHHNV have been detected in wild shrimps from the states of Sonora and Sinaloa [9], as well as in Nayarit with prevalences up to 45.5% [10]. While in the Gulf of Mexico, there is only one report of IHHNV in wild species from the state of Tamaulipas located in northeastern Mexico with prevalences of 4.4% reported in 2005 and 2006 [11]. In all cases, the studies refer that the proximity of the shrimp farms near to the coast could be a risk for the transmission of pathogens [9] [10] [11]. In this sense, the shrimp farming is null or incipient along the shores of the Yucatan Peninsula. Thus, the objectives of this study were first to use the COI DNA-barcoding as a fingerprint to assess the sustainability of the local caching during 2016 and 2017 and second to survey for the presence of IHHNV and WSSV in these wild shrimp populations from southern Mexico.

2. Material and Methods

2.1. Sample Collection

Wild shrimps were collected from Progreso, Yucatan (Latitude: 21.261196˚N; Longitude: 89.704264˚W) in March 2016 and 2017. This area is used by local fishermen for a small-scale fishery and local trading. Shrimps were collected during the routine fishing of local fishermen, using the fishing gear known as passive mesh net. The number of collected organisms (≈30) was according to the OIE recommendations implemented for surveillance of exotic pathogens [12]. Thirty-three organisms were collected in 2016, and 35 organisms were collected in 2017. Once collected, the organisms were separated and maintained alive in 30 L tanks. Juveniles of 9.4 g (±1.27) from the catching season of 2016 and juveniles of 8.7 g (±2.14) from the catching season of 2017 were sampled. 300 µL of hemolymph were collected individually from its ventral sinus, with 600 µL of anticoagulant solution (NaCl 450 mM, KCl 10 mM, HEPES 10 mM, EDTA 10 mM). The samples were fixed with absolute alcohol in 1:1 ratio and transported to the CINVESTAV facilities. In the laboratory, the samples were centrifuged for 5 min at 2,500 ×*g* and at 4˚C. The supernatant was discarded, and the cell pellet was used to subsequent gDNA isolation.

2.2. Molecular Analysis

2.2.1. Species Identification

Genomic DNA was extracted using the Quick-DNA™ Universal Kit by the manufacturer's protocols. A partial mitochondrial COI sequence (barcoding fragment) was amplified using the primers LCO1490: 5'-ggtcaacaaatcataaagatattgg-3' and HC02198: 5'-taaacttcagggtgaccaaaaaatca-3' [13]. Each 15 µL reaction volume contained 1X DreamTaq Green PCR Master Mix (Thermo Scientific®), 0.25 mM of each primer, and 0.5 µL of gDNA (5 - 60 ng). PCRs were conducted as follows: an initial denaturation step of 94˚C for 3 min, followed by 34 amplification cycles (94˚C for 30 s, 50˚C for 30 s, and 72˚C for 1 min) and a final extension of 72˚C for 5 min. Amplicons were resolved in 1.5% agarose gel, stained with GelRed®. Confirmed amplicons were diluted 1:1 with nuclease-free water and sequenced in the forward direction by the Sanger method. Sequences were edited, trimmed, and aligned manually in MEGA version 6.0 [14] and blasted against the NCBI data bank (GenBank™) as well as the BOLD system repository. All sequences were placed in the (GenBank™). See **Table 1** for the reference numbers.

2.2.2. Detection of IHHNV and WSSV

The molecular detection of IHHNV and WSSV was done in each shrimp using a final point PCR-IQ2000™ kit following the manufacturer´s instructions. The WSSV was also screened by real-time qPCR using the IQ SYBR® green supermix kit (Biorad) and the primers Ie1-126 F (5'-tgaaacggtgtgctgttagc-3') and Ie1-R (5'-aagttcctccatcgtcatcg-3'), for the immediately early WSSV gene amplification, which produce an amplicon of 126 bp, according to the following protocol: 60 s

Table 1. Species identification and IHHNV and WSSV detection for 2016 and 2017 in Yucatan coast. ND = not detected; D = detected.

2016					2017				
Species	GenBank Accesion number	WSDV		IHHNV	Species	Genebank Accesion number	WSDV		IHHNV
		Ie1-126	IQ2000™	IQ2000™			Ie1-126	IQ2000™	IQ2000™
Penaeus notialis	MF287097	ND	ND	ND	*Penaeus notialis*	MF287130	ND	ND	ND
Penaeus notialis	MF287098	ND	ND	ND	*Penaeus notialis*	MF287131	ND	ND	ND
Penaeus notialis	MF287099	ND	ND	ND	*Penaeus notialis*	MF287132	ND	ND	ND
Penaeus notialis	MF287100	ND	ND	ND	*Farfantepenaeus brasiliensis*	MF287133	ND	ND	ND
Farfantepenaeus brasiliensis	MF287101	ND	ND	ND	*Farfantepenaeus brasiliensis*	MF287134	ND	ND	ND
Penaeus notialis	MF287102	ND	ND	D	*Farfantepenaeus brasiliensis*	MF287135	ND	ND	ND
Penaeus notialis	MF287103	ND	ND	ND	*Farfantepenaeus brasiliensis*	MF287136	ND	ND	ND
Penaeus notialis	MF287104	ND	ND	ND	*Farfantepenaeus brasiliensis*	MF287137	ND	ND	D
Farfantepenaeus brasiliensis	MF287105	ND	ND	ND	*Penaeus notialis*	MF287138	ND	ND	ND
Penaeus notialis	MF287106	ND	ND	ND	*Penaeus notialis*	MF287139	ND	ND	ND
Penaeus notialis	MF287107	ND	ND	ND	*Penaeus notialis*	MF287140	ND	ND	ND
Penaeus notialis	MF287108	ND	ND	ND	*Penaeus notialis*	MF287141	ND	ND	ND
Farfantepenaeus brasiliensis	MF287109	ND	ND	D	*Farfantepenaeus brasiliensis*	MF287142	ND	ND	ND
Penaeus notialis	MF287110	ND	ND	D	*Farfantepenaeus brasiliensis*	MF287143	ND	ND	ND
Farfantepenaeus brasiliensis	MF287111	ND	ND	D	*Farfantepenaeus brasiliensis*	MF287144	ND	ND	ND
Penaeus notialis	MF287112	ND	ND	ND	*Farfantepenaeus brasiliensis*	MF287145	ND	ND	ND
Penaeus notialis	MF287113	ND	ND	ND	*Penaeus notialis*	MF287146	ND	ND	ND
Farfantepenaeus brasiliensis	MF287114	ND	ND	ND	*Penaeus notialis*	MF287147	ND	ND	ND
Penaeus notialis	MF287115	ND	ND	ND	*Penaeus notialis*	MF287148	ND	ND	ND
Farfantepenaeus brasiliensis	MF287116	ND	ND	ND	*Farfantepenaeus brasiliensis*	MF287149	ND	ND	ND
Penaeus notialis	MF287117	ND	ND	ND	*Penaeus notialis*	MF287150	ND	ND	ND
Farfantepenaeus brasiliensis	MF287118	ND	ND	ND	*Farfantepenaeus brasiliensis*	MF287151	ND	ND	ND
Penaeus notialis	MF287119	ND	ND	ND	*Penaeus notialis*	MF287152	ND	ND	ND
Penaeus notialis	MF287120	ND	ND	ND	*Penaeus notialis*	MF287153	ND	ND	ND
Farfantepenaeus brasiliensis	MF287121	ND	ND	ND	*Penaeus notialis*	MF287154	ND	ND	ND
Farfantepenaeus brasiliensis	MF287122	ND	ND	D	*Penaeus notialis*	MF287155	ND	ND	ND
Penaeus notialis	MF287123	ND	ND	ND	*Penaeus notialis*	MF287156	ND	ND	D
Penaeus notialis	MF287124	ND	ND	ND	*Farfantepenaeus brasiliensis*	MF287157	ND	ND	ND
Penaeus notialis	MF287125	ND	ND	ND	*Farfantepenaeus brasiliensis*	MF287158	ND	ND	ND
Penaeus notialis	MF287126	ND	ND	ND	*Farfantepenaeus brasiliensis*	MF287159	ND	ND	ND
Farfantepenaeus brasiliensis	MF287127	ND	ND	ND	*Penaeus notialis*	MF287160	ND	ND	D
Penaeus notialis	MF287128	ND	ND	ND	*Farfantepenaeus brasiliensis*	MF287161	ND	ND	ND
					Penaeus notialis	MF287162	ND	ND	ND
Penaeus notialis	MF287129	ND	ND	D	*Farfantepenaeus brasiliensis*	MF287163	ND	ND	ND
					Penaeus notialis	MF287164	ND	ND	ND

at 60˚C, 2 min at 94˚C, 40 cycles of 45 s at 94˚C, 45 s at 60˚C, and finally 7 min at 72˚C.

The prevalence was reported as the proportion of infected individuals among the total number of sampled organisms per year.

3. Results

A total of 68 organisms were collected during the catching seasons of 2016 and 2017. None of the sampled organisms showed physical signs of the disease RDS "runt deformity syndrome," in the rostrum and cuticular deformities [15]. The shrimp's DNA sequences caught in 2016 and 2017 showed the predominance of two species in the port of Progreso; the southern pink shrimp *P. notialis* and the northern pink shrimp *P. brasiliensis*. The mtCOI DNA sequences were submitted to the GenBank™ and the results are shown in **Table 1**, observing a proportion of 69.6% for *P. notialis* and 30.3% for *P. brasiliensis* in 2016. Moreover, in 2017 a proportion of 54.2% of *P. notialis* and 45.7% for *P. brasiliensis* were obtained.

The shrimps sampled during both years showed infection with IHHNV but not with WSSV. A prevalence of 13% was observed for IHHNV in *P. notialis*, while 30% was detected in *F. brasiliensis* in 2016. In 2017 the prevalences were 10.5% and 6.2% in *P. notialis* and *F. brasiliensis* respectively.

In the case of the WSSV two PCR systems were used; the IQ2000™ as well as a a real-time PCR using primers designed to amplify a fragment of the immediately early gene of the virus (Ie1). All organisms were negative to WSSV.

4. Discussion

The artisanal shrimp fishery has a social and economic impact in the state of Yucatan [16]. Sustainability of wild species in a given area is always a priority. In this study, both catching seasons were identified by mtDNA COI barcode; the southern pink shrimp *P. notialis* and the northern pink shrimp *P. brasiliensis*. Both species are native in the Gulf of Mexico and inhabit naturally in lagoons and coasts of Yucatan. They are of commercial importance for the local fisheries [17] [18]. In this study, both species were positive by PCR for IHHNV with prevalences of 18.18% and 8.57% for 2016 and 2017 respectively, as well as the absence of WSSV. No physical signs of the disease like RDS "runt deformity syndrome", rostrum and cuticular deformities or dwarfism [15] were found in the sampled organisms. The sample size was in accordance to the OIE for monitoring viral infections as well as the use of the IQ2000™ [12]. The IQ2000 test has been validated all around the world and includes an internal DNA control. Thus, the risk of having a false positive result is remote. In this case to confirm IHHNV infection, DNA from suspected native shrimps was injected to uninfected *L. vannamei* shrimps following the Koch's postulate (Data not shown).

Shrimp farming in Yucatan is incipient differing to the shrimp industry from the northwest of Mexico were the shrimp farm's fringe is well established and commonly operate at intensive or hiper-intensive levels. In the Mexican Pacific

Ocean, IHHNV and WSSV have been reported in wild species [9]. IHHNV had up to 45.5% of prevalence in wild species in the state of Nayarit [10]. And, in Sonora and Sinaloa, the prevalences of IHHNV were up to 19.5% between 2008 and 2009 [9]. Furthermore in the Gulf of Mexico, IHHNV has been reportedin Tamaulipas in *Farfantepenaeus aztecus* (Ives, 1891) and *Litopenaeus setiferus* (Linnaeus, 1767) with prevalences of 4.4% between 2005 and 2006 [11] (**Figure 1**). Although results from this study are similar to those reported in other regions of Mexico, the presence of IHHNV and WSSV has not been reported before this work in the Yucatan Peninsula [19]. Likewise, this is the first report of IHHNV in *P. notialis* or *P. brasiliensis*. In this sense, it is important to address that in Mexico there is only one report of WSSV in wild organisms from the Mexican Pacific Coast [9], and to our knowledge, WSSV is absent in the Gulf of Mexico. Although WSSV was not detected in this study, the surveillance of this pathogen is relevant, because WSSV has been reported in more than 100 species of crustaceans, including numerous wild species [20]. Thus, early detection of a given disease is a key strategy for biosecurity and monitoring [21], because viral infections occur in all stages of the life cycle of the shrimp (eggs, larvae,

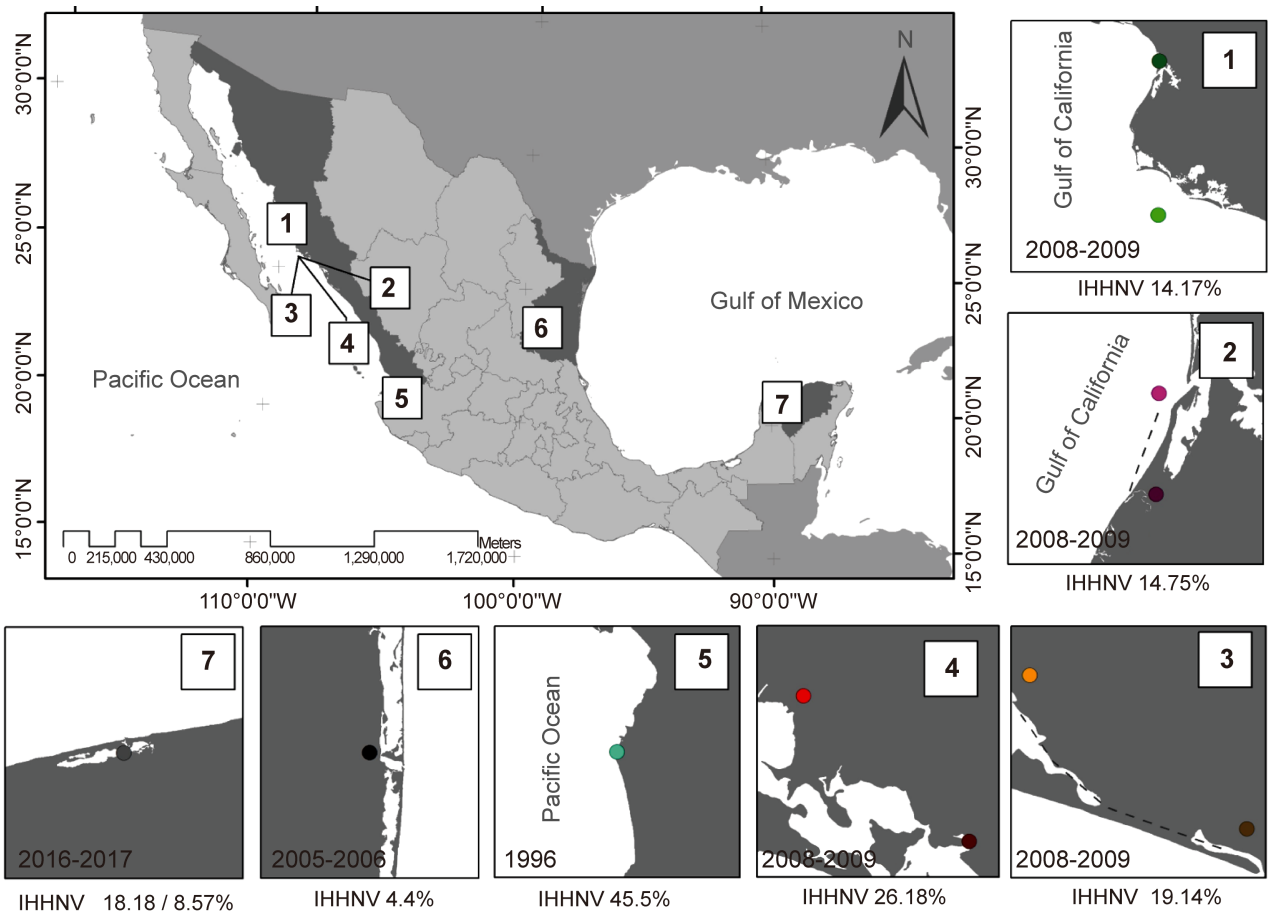

Figure 1. Historic IHHNV prevalence reported in different sites in Mexico: Sites 1 - 5: Wild shrimps collected from the states of Sonora, Sinaloa and Nayarit in the Pacific Ocean. Site 6: Wild shrimps collected from the State of Tamaulipas. Site 7: Progreso, Yucatan in the Gulf of Mexico. For WSSV, prevalences have been reported in sites 1 & 2. And absent in the Gulf of Mexico. Map was constructed using ArcGIS software version 9.3. Dotted line means fishing transect.

post-larvae, juvenile, and adults) [21]. In natural conditions is difficult to evidence the effect of a given disease in wild populations [1].

The presence of IHHNV in southern Mexico, support the hypothesis that IHHNV has become an enzootic species in Mexico [22], despite the fact that no major shrimp farms exist in the area. An extension of this study is on its way in a wider area and a wide range of stages to monitor the prevalence of IHHNV and WSSV.

5. Conclusion

This study represents the first effort to evaluate the small local shrimp fishery in the coast of the state of Yucatan by mtDNA COI barcode. The presence of IHHNV in two new species provides insight evidence that the virus is enzootic in Mexico. However, more areas need to be surveyed. The absence of WSSV in southern Mexico is of local ecological and economic importance. Long-term monitoring is needed to evaluate the dynamic of prevalences in wild shrimp populations and fisheries.

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