Correlation of prevalence of the white spot syndrome virus with physicochemical parameters and characteristics of the Pacific white shrimp (*Litopenaeus vannamei*) culture, in Sonora, Mexico.

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*Correlación de la prevalencia del virus del síndrome de la mancha blanca con parámetros fisicoquímicos y características del cultivo de camarón blanco del Pacífico (*Litopenaeus vannamei*) en Sonora, México.*

Abstract

The white spot syndrome virus is the main viral pathogen that causes massive mortality in farmed shrimp and generates large economic losses worldwide. The aim of this study was to estimate the prevalence of WSSV in pooled samples and its relationship with physicochemical parameters and characteristics shrimp culture in Sonora, Mexico. A monitoring was carried out in three shrimp farms from northern, central and southern zones of Sonora, Mexico. Samples of 1,200 juvenile *L. vannamei* were collected during the 2010-2012 growing cycles. Pooled samples were created and analyzed by PCR. Physiochemical parameters and characteristic shrimp culture were recorded. The relationship between physicochemical parameters, shrimp culture management and the prevalence of WSSV was analyzed by multivariate analysis. Based on the results of the PCR and the Epitools calculator, a moderate prevalence of WWSV was registered and through statistical analysis, it was determined that the number of farms in the three zones studied had a significant correlation in the dispersion of the virus, whereas with the physicochemical parameters there was no significant relationship.

Key words: Shrimp farming, pooled samples, PCR, management, epidemiology.

Resumen

El virus del síndrome de la mancha blanca es el principal patógeno que causa mortalidades masivas in granjas de camarón y genera grandes pérdidas económicas a nivel mundial. El objetivo de este estudio fue estimar la prevalencia de WSSV en muestras agrupadas y su relación con parámetros fisicoquímicos y características del cultivo de camarón en Sonora, México. Se realizó un monitoreo en tres granjas de camarón de las zonas norte, centro y sur de Sonora, México. Se colectaron 1,200 camarones juveniles *Litopenaeus vannamei* durante los ciclos de cultivo 2010-2012. Se crearon muestras agrupadas y fueron analizadas por PCR. Los parámetros fisicoquímicos y características del cultivo de camarón fueron registrados. La relación entre los parámetros fisicoquímicos, el manejo del cultivo de camarón y la prevalencia de WSSV fue analizada por un análisis multivariado. Basado en los resultados de PCR y la calculadora Epitools, se registró una prevalencia moderada de WWSV y mediante el análisis estadístico, se determinó que el número de granjas en las tres zonas estudiadas, tuvo una correlación significativa en la dispersión del virus, mientras que con los

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parámetros fisicoquímicos no hubo relación significativa.

Palabras claves: Granja de camarón, muestras agrupadas, PCR, manejo, epidemiología.

Introduction

All over the world, the most cultured shrimp species is Pacific white shrimp *Litopenaeus vannamei* and its total production in 2013 was 331 million tonnes (Xie *et al.,* 2016). Sinaloa, Sonora and Nayarit are the main producers of *L. vannamei* in Mexico and in 2012 the national production was 130 000 tonnes (Hernandez-Llamas *et al.,* 2016). Sonora has contributed significantly to the national production of shrimp and in 2009, reported a production of more than 80 000 tonnes (COSAES 2014). However, the presence of viral and bacterial pathogens has led to a massive mortality of shrimp and, consequently, a decrease in production of approximately 60% in 2012 compared to 2009 (COSAES 2014). In Mexico, WSSV was first reported in June 1999 and since then is the most lethal viral pathogen of the penaeid shrimps, which causes white spot disease (WSD) and can cause a cumulative mortality of up to 100%, causing great economic losses. (Mijangos-Alquisires *et al.,* 2006; Esparza-Leal *et al.,* 2010).

In Sonora state, the most prevalent pathogen in shrimp farms between 2010 and 2012 was the WSSV (COSAES 2014). This virus is a pandemic pathogen disseminated and rapidly dispersed due to its horizontal transmission through cannibalism and predation among the affected organisms and even through water when it occurs in the cultured shrimp *L. vannamei*. (Tendencia *et al.,* 2011; Muniesa *et al.,* 2016). In addition, there are more than 90 hosts and carriers that promote the spread of this virus (Escobedo-Bonilla *et al.,* 2008; Porchas-Cornejo *et al.,* 2017).

Many studies have focused on determining the influence of physicochemical parameters in the appearance of WSD epizootics that generate high mortalities in different cultured shrimp species (Zhang *et al.,* 2006; Gunalan *et al.,* 2010; Tendencia and Verreth 2011; Tendencia *et al.,* 2010; Gao *et al.,* 2011; Moser *et al.,* 2012; Lehmann, M., Schleder, D.D., Guertler, C., Perazzolo, L.M., Vinatea 2016; Van Thuong *et al.,* 2016)

On the other hand, only few researches have been carried out to clarify how shrimp farming practices also play an important role in the propagation of WSSV and consequently in the appearance of this disease that causes the massive mortality of cultured shrimp. Some examples of shrimp farming practices are stocking density, proximity between shrimp farms, stocking area and sharing water source between neighborhood shrimp farms (Ruiz-Velazco *et al.,* 2010; Tendencia and Verreth 2011; Tendencia *et al.,* 2011; Hernandez-Llamas *et al.,* 2016; Muniesa *et al.,* 2016).

However, to date there are no effective treatments against the viruses that affect the cultured shrimp and therefore it is advisable to maintain a constant epidemiological surveillance to avoid the propagation of viral pathogens (Sánchez-Paz *et al.,* 2012). Therefore, the objective of this study was to estimate the prevalence of WSSV in pooled samples and its relationship with physicochemical parameters and characteristics of shrimp culture during cycles 2010 to 2012 due to the lack of epidemiological information on how the physicochemical parameters and the shrimp farming practices trigger WSSV outbreaks and cause mass mortalities in *L. vannamei* in Sonora, Mexico.

Materials and methods

Shrimp sampling and data collection

Due to mortality in shrimp farms, the sampling of *L. vannamei* with or without clinical signs (shrimp on the surface, erratic movement, feeding anomalies, reddish color, among others) of WSSV was carried out monthly from May to September during the culture cycles of the shrimp in 2010, 2011 and 2012 in the northern, central and southern zones of Sonora, Mexico (Fig. 1). One shrimp farm per zone was chosen and monitored. The size of the sample was determined according to the standard equation of infinite population and based on data from previous cycles (COSAES 2008), where the WSSV prevalence was of 25% with a 95% confidence interval (*CI*) and 5% of standard error. 400 juvenile white shrimp *L. vannamei* were collected per site of collection per year (n = 80 per month), exceeding the minimum sample size estimated (n = 288). The samples were placed in 96% ethanol bottles and

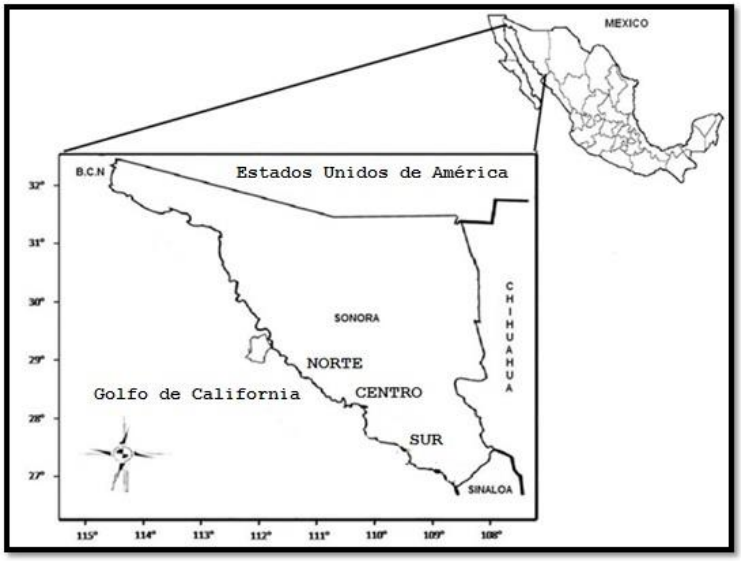


Figure 1. Sonora, Mexico. Locations of the three sampled zones.

transported to the Laboratorio de Analisis de Sanidad Acuicola at the Instituto Tecnologico de Sonora. Muscle tissue (2 mg) of the sixth abdominal segment of each of the 400 shrimps collected per site was dissected. The muscle tissue of ten shrimps was fixed in 1000 µL of 70% ethanol in a 1.5 mL conical microtube. 120 pooled samples per culture cycle were obtained (Table 1). During the shrimp sampling, temperature, salinity and dissolved oxygen were recorded with the YSI 85 portable system (YSI Inc., OH, USA) and pH was recorded with HI98107 pHep (Hanna Instruments, RI, USA). The shrimp farming practice data was provided by the Comite de Sanidad Acuicola del Estado de Sonora (Table 2).

DNA extraction and PCR

The pooled samples (20 mg of muscle tissue) were centrifuged at 10,000 rpm / 5 min in Spectrafuge ™ 16M (Labnet, NJ, USA) and the ethanol was decanted. Then, the pooled samples were homogenized with 500 µL of DNAzol buffer (Invitrogen, CA, USA) following the manufacturer's instructions for DNA extraction. The extracted DNA was diluted in 200 µL of nuclease-free water. The PCR reactions for presence of WSSV in each of the 360 pool samples were carried out following the IQ2000^{TM} WSSV Detection and Prevention System kit protocol (GeneReach Biotechnology Corp, Taichung, Taiwan) proposed by the World Organization for Animal Health (OIE), which is highly sensitive (20 viral particles per reaction) and specific. 2 µL of DNA were used for each reaction and incubation was performed in a Veriti® Thermal Cycler (Life Technologies, CA, USA). The amplified DNA products were stained with GelRed (Biotium, CA, USA) and visualized on a 1.5% agarose gel electrophoresis.

Statistical analysis

According to the molecular validation carried out by the OIE, a diagnostic sensitivity of 96.33% (95% *CI* 93.53 - 98.16%) and a diagnostic specificity of 100% (95% *CI* 98.78 - 100%) were calculated for the PCR using IQ2000^{TM} WSSV. With previous data, prevalence of WSSV in pooled samples was estimated using the AusVet Epitools online calculator, assuming a known sensitivity and specificity with a 95% *CI* (Sergeant 2009). The prevalence of WSSV in pooled samples was estimated according to the prevalence levels: high (> 50%), moderate (10-50%) and low (1 -10%) (Mendoza-Cano *et al.*, 2016). The STATGRAPHICS XV.II software was used to determine the variation of the physicochemical parameters by one-way ANOVA and the relationship between factors and WSSV prevalence were determined by multivariate analysis. A *post-hoc* Fisher LSD test with 95% CI for comparisons of means was realized in cases in which the results

Table 1. Sampling of *L. vannamei* and WSSV prevalence during culture cycles 2010-2012.

Zone	2010					2011					2012				
	NSA	NPA	NPP	%	95% *CI*	NCA	NPA	NPP	%	95% *CI*	NCA	NPA	NPP	%	95% *CI*
N	400	40	0	0	----	400	40	14	4.4	2.1-6.7	400	40	34	19.3	11.4-27.2
C	400	40	16	5.2	2.6-7.8	400	40	0	0	----	400	40	0	0	----
S	400	40	38	34.8	1.7-67.9	400	40	37	27.6	12.1-43.0	400	40	26	10.7	6.4-14.8
Total	1200	120	54	6.1	4.4-7.7	1200	120	51	5.7	4.1-7.2	1200	120	60	6.7	5.3-8.2

N: Northern, C: Central, S: Southern, %: Prevalence, NSA: Number of shrimps analyzed, NPA: Number de pools analyzed, NPP: Number of positive pools, CI: Confidence Interval

Table 2. Summary of physicochemical parameters and characteristics of shrimp culture recorded during culture cycles 2010 – 2012.

Year	Zone	SF	SA	SD	S			pH			T			DO		
					Min-Max	Mean	*s*	Min-Max	Mean	*s*	Min-Max	Mean	*s*	Min-Max	Mean	*s*
2010	N	40	11578	23	37.1-39.2	39.2	1.9	8.3-8.5	8.4	0.1	25.9-31.9	30.0	2.4	4.1-5.8	4.8	0.7
2010	C	20	1337	23	41.5-44.2	42.6	1.3	8.1-8.4	8.3	0.1	25.4-32.6	30.0	2.8	4.5-5.1	4.9	0.3
2010	S	84	9798	23	39.6-44.1	41.6	2.0	8.3-8.5	8.4	0.1	27.3-32.3	30.4	1.9	4.7-5.4	5.0	0.3
2011	N	41	12919	17	38.7-42.4	40.0	1.4	8.1-8.4	8.3	0.2	25.9-33.8	30.6	2.9	4.7-5.8	5.1	0.5
2011	C	20	1747	17	40.4-44.7	42.4	1.7	8.1-8.5	8.3	0.2	24.6-33.0	30.3	3.4	4.5-5.4	4.9	0.3
2011	S	88	10796	17	42.5-45.3	44.0	1.1	8.2-8.4	8.3	0.1	29.9-32.0	31.1	0.8	4.9-5.7	5.1	0.4
2012	N	35	13473	17	38.0-39.9	39.3	0.8	8.2-8.5	8.3	0.2	26.2-32.0	30.2	2.4	4.7-5.3	4.8	0.3
2012	C	22	1919	17	41.1-44.1	42.6	1.1	8.3-8.4	8.3	0.1	28.1-31.8	30.2	1.4	4.7-5.2	4.9	0.2
2012	S	79	9389	17	40.7-45.0	42.6	1.7	8.3-8.4	8.3	0.1	27.7-32.0	30.5	1.6	4.8-5.5	5.0	0.3

N: Northern, C: Central, S: Southern, SF: Shrimp farms, SA: Stocking area (Hectares), SD: Stocking density (shrimps/m^2), S: Salinity (mg/L), T: Temperature (°C), DO: Dissolved oxygen (mg/L), Min-Max: Minimum and maximum value, *s*: Standard deviation.

of ANOVA were significant.

Results

According to the results obtained from the PCR, it was determined that 45.8% (165/360) of the pooled samples were diagnosed as positive to WSSV in the state of Sonora. A moderate prevalence of WSSV was reported in pooled samples of the 2010 and 2011 culture cycles in the southern zone, 34.8% (95% *CI* 1.7-67.9%) and 27.6% (95% *CI* 12.1-43.0%) respectively. A moderate prevalence of WSSV was also observed in the 2012 cycle with 19.3% (95% *CI* 11.4-27.2%) in pooled samples from the shrimp farm in the northern zone; whereas a low or no prevalence of WSSV was determined in the central zone (Table 1).

Regarding to physicochemical parameters, salinity varied from 37.1 to 45.3 practical salinity units (mg/L), pH from 8.1 to 8.5, temperature from 24.6 ° C to 37.8 ° C and dissolved oxygen from 4.1 to 5.8 mg / L (Table 2). Salinity was the only parameter that varied significantly between the zones and the sampling time, while the other physicochemical parameters did not show significant variation (Table 3). No significant correlation was observed between physicochemical parameters and the prevalence of WSSV (Table 4).

According to the data of shrimp farming practices obtained from COSAES, the aquaculture method is semi-intensive. The northern zone has almost the same stocking area as the southern zone, but has fewer shrimp farms; therefore, the shrimp farms in the northern zone are larger than those in the southern zone. The central zone has less stocking area with smaller shrimp farms (Table 2). Was observed a significant correlation of number of shrimp farms per zone with WSSV prevalence in Sonora, Mexico, while a non-significant correlation was reported between the stocking area and the stocking density in the prevalence of WSSV (Table 4).

Table 3. Variation of physicochemical parameters

Parameter	*Source of variation*	*Sum of squares*	*Degrees of freedom*	*Mean sum squares*	*F-statistic*	*P- value*
Temperature	Inter groups	4.94308	8	0.617886	0.12	0.9983
	Intra groups	192.162	36	5.33784		
	Total	197.105	44			
Salinity	Inter groups	109.778	8	13.7222	6.08	0.0001*
	Intra groups	81.2	36	2.25556		
	Total	190.978	44			
pH	Inter groups	0.052	8	0.0065	0.51	0.8385
	Intra groups	0.456	36	0.0126667		
	Total	0.508	44			
Dissolved oxygen	Inter groups	0.532151	8	0.0665189	0.50	0.8462
	Intra groups	4.76528	36	0.132369		
	Total	5.29743	44			

***** High statistical significance (*P-value ≤ 0.01*)

Table 4. Statistic correlation between with physicochemical parameters and characteristics of shrimp culture and WSSV prevalence.

	Shrimp farms	Stocking area	Stocking density	Salinity	pH	Temperature	Dissolved oxygen
Pearson coefficient	0.7808	0.4543	0.1161	0.1968	0.1949	-0.2340	0.4220
Degree of freedom	9	9	9	9	9	9	9
P-value	0.0130*	0.2193	0.7661	0.6118	0.4904	0.544	0.2579

***** High statistical significance (*P-value ≤ 0.01*)

Discussion

When a pooled sample is positive, it is very difficult to determine if it is for one or more infected organisms, therefore, it is impossible to determine the prevalence with statistical precision (Mendoza-Cano *et al.,* 2014). A previous study, also in Sonora, reported a prevalence of WSSV of 0% in wild organisms collected in marine waters surrounding farms in the southern zone of the state (Macías-Rodríguez *et al.,* 2014). On the other hand, the state authorities reported a moderate to high prevalence for 2010 (> 70%), 2011 (> 40%) and 2012 (> 50%) culture cycles in pooled samples of cultivated *L. vannamei* (COSAES 2014). However, the studies described above, based the estimation of prevalence in individual samples of wild organisms and only some in pooled samples of *L. vannamei* cultivated in farms of Sonora and they used the standard equation for prevalence estimation, which statistically limits the analysis and its certainty in the estimation of prevalence of WSSV (Button *et al.,* 2013; Mendoza-Cano *et al.,* 2014). Therefore, the use of pooled samples, validated PCR and statistical methods or programs that accurately calculate the prevalence of diseases that affect shrimp culture would help to improve epidemiological surveillance strategies and reduce the costs of analysis by the use of expensive reagents for the development of molecular diagnostics (Singer *et al.,* 2006; Shipitsyna *et al.,* 2007; Zhou *et al.,* 2014; Edouard *et al.,* 2015).

The physicochemical parameters of water and shrimp farming practices can cause stress and increase the probability of infection by pathogens in *L. vannamei*. In addition, physicochemical stressors that fluctuate above or below optimal limits cause a reduction in the immune response in cultured penaeid shrimp, as well as an acceleration in the replication and propagation of the WSSV (Ponce-Palafox *et al.,* 1997; Gunalan *et al.,* 2010).

Salinity is an environmental factor that influences the survival, growth and immune response of *L. vannamei* (Lu-Qing, *et al.,* 2005) and although this shrimp species has euryhaline capacity, it has been determined that the range of 25 to 40 mg/L is the best for a good growth of cultured shrimp (Ponce-

Palafox *et al.,* 1997). Although a statistically significant relationship was not observed between salinity and the prevalence of WSSV, it has been described that abrupt changes in this parameter increase host susceptibility and promote the transition from WSSV to the lytic state in a short time (Peinado-Guevara and López-Meyer 2006; Gao *et al.,* 2011; Vaseeharan *et al.,* 2013; Van Thuong *et al.,* 2016).

The pH influences all the physiological processes of the shrimp, since it is an environmental factor present in all the metabolic reactions and also directly influences the phenomena that occur in the bodies of water; and it has been determined that marine shrimp develop better in a pH range between 7.0 and 9.0 (Zhang *et al.,* 2017). The registered pH values were kept within the optimum limit for the development of the shrimp culture. There was no significant variation in the comparison analysis and the correlation of the pH with WSSV prevalence was not significant. However, in vitro experiments have reported that the optimal proliferation of WSSV occurs in a range of pH 8.0 to 9.0 (Gao *et al.,* 2011).

Temperature is an environmental factor that influences feeding processes, immune response and growth of shrimp culture and it has been reported that the optimum temperature range is 28 to 30 ° C (Ponce-Palafox *et al.,* 1997; Tendencia and Verreth 2010). The comparison of the temperature values recorded during the present investigation did not result in significant variation and a non-significant statistical correlation of this factor was reported with WSSV prevalence. But previous studies have reported that a temperature higher than 29 ° C and lower than 32 ° C accelerates the proliferation of WSSV (Gao *et al.,* 2011; Moser *et al.,* 2012).

Dissolved oxygen is vital in all stages of life of shrimp and plays an important role in the culture of all species of crustaceans, so it is suggested that it remains above 5 mg/L since its saturation levels are directly related to water quality (Cheng *et al.,* 2003; Lehmann *et al.,* 2016). The oxygen values reported here are slightly below or above the recommended, but no significant variation was observed according to the statistical comparison and there was no significant statistical correlation with WSSV prevalence. In short, it is important to maintain adequate levels of oxygen saturation, since the low level of this parameter, known as hypoxia, can affect the processes of the immune response and consequently cause mass mortalities of cultured shrimp (Cheng *et al.,* 2003; Lehmann *et al.,* 2016).

When the groups of shrimp farms are very close to each other, they share the water source, which allows the mobilization of carriers and viral particles between neighboring farms, so that the process of viral dispersion can be accelerated (Tendencia *et al.,* 2011). This would explain that the moderate prevalence of WSSV in southern Sonora might be due to the large number of grouped farms that share the same water reserves. On the contrary, in the northern zone, a moderate prevalence was observed in the 2012 cycle. This could be due to the fact that the farms are less but larger, which makes the management and control of the physicochemical parameters and shrimp health monitoring more complicated (Muniesa *et al.,* 2016), which allowed the prevalence of the WSSV to increase compared to the 2010 and 2011 cycles.

In conclusion, WSSV prevalence was determined from pooled samples collected in shrimp farms from three zones of Sonora, Mexico and wasn´t related with physicochemical parameters, meanwhile, the shrimp culture characteristic influence the virus dispersion.

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Conflicts of interest

The authors declare they have no conflicts of interest with regard to the work presented in this report

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