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Early Detection of White Spot Syndrome Virus (WSSV) in Isolated Hemocytes of *Litopenaeus vannamei*

Ariadne Hernández-Pérez[1], Rossanna Rodríguez-Canul[1*], Edgar Torres-Irineo[2,3], Fernando Mendoza-Cano[4], Daniel Eduardo Coronado-Molina[4], Jesús Alejandro Zamora-Briseño[1], Jorge Hernández-López[4]

[1]Laboratorio de Inmunología y Biología Molecular, Centro de Investigación y de Estudios Avanzados del IPN (CINVESTAV-IPN) Unidad Mérida, Mérida, México
[2]Laboratorio de Pesquerías, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional-Unidad Mérida, Mérida, México
[3]UMDI-Sisal, Facultad de Ciencias, Universidad Nacional Autónoma de México, Sierra Papacal, México
[4]Centro de Investigaciones Biológicas del Noroeste, Hermosillo, México
Email: ariadne.hernandez@cinvestav.mx,zambri33@hotmail.com, *rossana.rodriguez@cinvestav.mx, edgar.torres82@gmail.com, Ext.5003.fmendoza@cibnor.mx, dcoronado04@cibnor.mx, jhlopez04@cibnor.mx

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Abstract

To date, White Spot Syndrome (WSS) produced by the White Spot Syndrome Virus (WSSV) causes one of the most severe diseases infecting penaeid shrimps worldwide. Although a vast amount of studies has elucidated pathogenesis in live infection models, there is still little information about the interaction of WSSV infections using *in vitro* models in the whiteleg shrimp *Litopenaeus vannamei* (*L. vannamei*) hemocytes. In this study, a WSSV infection kinetics was performed using total hemocytes isolated from healthy *L. vannamei* organisms and maintained in *in vitro* conditions using isotonic solution for shrimp (ISS). The infected experimental cells received ≈ 30,000 viral copies of WSSV. The viability of the hemocytes (control and infected group) was measured during the kinetics with trypan blue exclusion method and cells were maintained up to 6 hpi (post-infection) with non-significant differences of viability between both groups. WSSV replication was assessed using RT-PCR at the RNA expression level of the early viral gene *Ie*1 and transcripts were detected as early as 30 min pi. Hemocytes from WSSV group showed disrupted integrity, degranulation and irregular shape. This study provides evidence of the capability of WSSV to infect and replicates in *L. vannamei* hemocytes using *in vitro* assays in short times as 30 min.

Keywords

WSSV, *Litopenaeus vannamei*, *Ie*1 Gene, Hemocytes, *In Vitro* Infection, GLS

Analysis

1. Introduction

White Spot Syndrome Virus (WSSV) produces damaging losses to the shrimp aquaculture industry worldwide [1]. The main problem with this pathogen is the cumulative mortalities in a period of 3 to 10 days causing 100% of mortality to the industrial shrimp farming [2]. WSSV is an enveloped circular double-stranded DNA virus with a bacillar shape. The virions are 275 nm in length, and 83 nm in diameter and the size of the nucleocapsid is 216 nm in length and 54 nm in diameter [3]. It has been established that WSSV primarily infects tissue cells of ectodermal and mesodermal origin and its target tissues are hematopoietic, epithelial and connective tissues [4] [5] [6]. Hemocytes from crustaceans are categorized into three main groups, hyaline cells, semigranular cells and granular cell and they are considered the analogue cells of lymphocytes from vertebrates [7]. They derive from mesoderm tissues [8] and are considered the effector cells of the crustacean's immune system because they are actively involved in processes such as pathogen recognition, phagocytosis, melanization, cytotoxicity and cellular communication [9]. The circulatory system in crustaceans is incompletely closed and the hemolymph irrigates all the tissues [10], therefore, hemocytes could play an important role for the dispersion of the WSSV virions through the organism. However, to date, it is still not clear whether WSSV is able to replicate within these cells, especially in the hemocytes of the whiteleg shrimp *L. vannamei*, the most cultivated shrimp worldwide.

In the crayfish *Procambarus clarkii* the expression of *Ie* genes was detected with cycloheximide (CHX) treated primary culture of *P. clarkii* hemocytes, suggesting that WSSV replication occurs in these cells [11]. In contrast, a recent study reported that WSSV replicates in hematopoietic cells of *P. clarkii*, but this process was not entirely successful in hemocytes [12]. Likewise, it was reported that WSSV infects mainly hyaline hemocytes, and granular cells are less susceptible to the virus in *Pacifastacus leniusculus* [5], which lead to the conclusion that hyaline cells were the target cells of WSSV in *P. leniusculus*. To date, these findings have been commonly accepted as part of the pathogenesis of WSSV infection in other crustacean's species.

The culture of hemocytes could help in the evaluation of the mechanisms associated with the host-parasite infections [13]-[18]. However, due to the variability in methodologies and reagents used, this strategy is not entirely feasible yet [18]. This could be solved by implementing the use of non-expensive reagents for the detection of early stages of WSSV infection in target cells.

The aims of this study were i) to confirm if the WSSV is capable of replicating in the hemocytes of *L. vannamei* and ii) to evaluate the use of an isotonic solution for shrimp (ISS) to maintain the viability of *L. vannamei* hemocytes under

these conditions.

2. Materials and Methods

2.1. Hemolymph Donors

Healthy juvenile shrimps (*L. vannamei*) (12.72 ± 0.83 g of weight and 11.98 ± 1.29 cm in length), were purchased from a local farm in Sonora, Mexico, and acclimatized in the laboratory for ten days with constant aeration at 35 practical salinity units (PSU). Tanks were cleaned daily, and water was changed at 20% of its total volume. Shrimp were fed *ad libitum*, twice a day with commercial food (35% protein). Before the experiments, they were screened to be free of WSSV, IHHNV, TSV, YHV, and IMNV using IQ2000 kits, following the manufacturer's instructions with qPCR or RT-PCR, depending on the case.

2.2. WSSV Inoculum Preparation and Viral Load Quantification

2.2.1. Inoculum Preparation

The inoculum was prepared from gills, pleopods, and muscle of *L. vannamei* infected with WSSV. Tissues were disrupted with a tissue homogenizer with 4 ml of saline solution (150 mM NaCl) and centrifuged at 3000 × *g* for 20 min at 4 ˚C. The supernatant was filtered through a 0.22 μM pore size membrane and centrifuged in a micro-filter Millipore Sigma Amicon® of 100 kDa cut-off [19]. The material retained in the filter was collected and kept frozen at −80˚C, until its use.

2.2.2. Inoculum's Viral Load Quantification

The inoculum's viral load was quantified by qPCR. DNA was extracted using the Geneclean® kit (MP Biomedicals), and the copy number was quantified with a commercial qPCR kit (IQ REAL™ WSSV Quantitative System) following the manufacturer's instructions. Calibration curve included 10^1, 10^2, 10^3, 10^4 and 10^5 WSSV copies/μL, and was recorded using a Corbett thermocycler. Data were processed with the Rotor-Gene Software Version 6.1.

2.2.3. Inoculum's Infectivity Evaluation Viral Load Quantification

The pathogenicity of the WSSV inoculum was corroborated in five shrimps *L. vannamei* injected with 50 μL of the inoculums between the third and fourth abdominal segment. After 24 hpi, hemolymph was collected from the ventral sinus of each shrimp with a 1 ml syringe, containing the ISS in 1:3 ratio [20]. This solution is composed of 450 mM NaCl, KCl 10 mM, and 10 mM HEPES, with pH adjusted to 7.5 ± 0.2 and osmolarity to 903 ± 13 mOsmol kg^{-1} to mimic hemolymph characteristics. Additionally, to avoid coagulation and cellular activation, EDTA 10 mM was added to the ISS. The hemocytes were separated from the hemolymph after centrifugation with 1000 × *g* for 5 min and 4˚C. The supernatant was discarded, and the cell pellet was re-suspended gently with 50 μL of ISS without EDTA in order to avoid interference with subsequent molecular analyses. Then, DNA from hemocytes was extracted with the Geneclean® kit

(MP Biomedicals). The presence of WSSV was detected by PCR using the IQ SYBR® green super mix kit (Biorad) and the primers *Ie*1-F (5 '-GAC-TCT-ACA-AAT-CTC-TTT-GCC-A-3') and *Ie*1-R (5'-CTA-CCT-TTG-CAC-CAA-TTG-CTA-G-3') [21], which produce an amplicon of 502 bp, according to the following protocol: 60 min 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.

2.3. *In Vitro* Maintenance of Hemocytes and WSSV Infection

2.3.1. Isolation and *in Vitro* Maintenance of the Hemocytes

Hemolymph from 20 healthy shrimps *L. vannamei* (50 - 150 µL per shrimp) in intermolt-stage was extracted from the ventral sinus and mixed with ISS-EDTA in 1:3 ratio and allotted equally in experimental and control groups in 10 mL tubes. The hemocytes of the two 10 mL tubes (control and experimental) were separated from the hemolymph after centrifugation at 1000 × *g* for 5 min and 4˚C. The supernatant was discarded, and the cell pellet was re-suspended gently with 500 µL of ISS without EDTA, maintaining the samples at 4˚C. Both groups were divided into five aliquots of 100 µLand placed in 96-well sterile microplates (inertGrade™ BrandTech® Scientific), especially suited for cell cultures when adhesion is not desired.

2.3.2. WSSV Infection in Isolated Hemocytes

The experimental and control plates containing the hemocytes in ISS were challenged with 50 µL of the WSSV inoculums and 50 µL of ISS respectively. Plates were incubated at 29˚C and 35 RPM during 6 h. Observations were performed at 30 min, 1, 2, 4 and 6 h post-infection (h pi) and included: i) assessment of cell viability and ii) fixation of the samples to analyze the transcripts of WSSV.

2.3.3. Assessment of Cell Viability

Once finished the observation times, hemocytes from the corresponding well were gently re-suspended with a micropipette to homogenize cells that could be attached to the bottom. Then, viability was evaluated through trypan blue stain exclusion method (Invitrogen 0.4%), mixing 10 µL of each sample with 10 µL of the stain, and live and dead cells were counted twice/sample in an automated cell counter (Countess™ Invitrogen). The number of viable cells was divided by the number of total cell per 100 and expressed as a percentage of viability= [(live cells)/(total cells)] × 100.

2.3.4. Hemocytes Samples Fixation and RNA Isolation

The remaining cells from each well were fixed with TRIzol® Reagent (Invitrogen) for RNA isolation following the manufacturer's recommendations, and recuperation of total well content was ensured after revision of the plates with an optical microscope. Total RNA concentration was measured with a Nano Drop 2000 spectrophotometer (Thermo-Scientific) at an A260/280nm ratio. RNA concentration was adjusted to 100 ng/µL.

For all RNA samples, PCR reactions were performed to confirm they were

DNA-free (data not shown), evaluating the *Ie*1 gene using the IQ SYBR® green supermix kit (Biorad) and the primers *Ie*1-F and *Ie*1-R [21] with the same amplification protocol described above.

2.4. WSSV Transcripts Detection Using RT-PCR

For the absolute quantification of WSSV by RT-PCR, the 502 bp amplicon of the *Ie*1 gene was amplified using DNA from the inoculum as a template and cloned into the CloneJET PCR Cloning Kit (Thermo Scientific™), according to the manufacturer's instructions. The recombinant plasmid was sequenced, quantified and used as a template to perform serial dilutions with known copy numbers ranging from 2×10^6 to 20 copies·μL^{-1}. cDNA was synthesized from experimental and control RNA samples using the iScript™ (Biorad) system, following manufacturer's recommendations. Reactions of RT-PCR were performed to detect the *Ie*1 gene using the IQ SYBR® green supermix kit (Biorad) and the primers *Ie*1-F and *Ie*1-R [21] with the same amplification protocol described above. Amplification specificity was assessed by melting curve analysis.

2.5. Detection of Uninfected and WSSV Infected Hemocytes Using SEM

The effect of WSSV was assessed at the end of the experiment (6 h pi). Hemocytes were analyzed by scanning electron microscopy (SEM). A sub-sample of hemocytes were fixed with a fixation solution (3% glutaraldehyde and 0.1 M sodium cacodylate), washed two times with saline phosphate buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4), washed one more time with distilled water and dehydrated for intervals of 15 min in ethanol series (30%, 50%, 70%, 90%, and 100%) [22]. The samples were dropped on a 13 mm Thermanox Plastic® coverslips (Nunc) pretreated with 0.2% gelatin and finally dried using a critical point drier (Quorum®, model K850) and coated with gold-palladium using an ion sputter coater (Quorum, Q150R ES). The hemocytes were observed under the scanning electron microscope (FESEM Jeol 7600F) with 25 kV at 2500X.

2.6. Statistical Analyses

For each experiment, three replicates were performed. The data obtained from the three replicates, including cell viability and viral charge, were averaged. This study consisted of repeated measures over time, which implies that measures were dependent. Consequently, a generalized least squares (GLS) model was used to evaluate the prognosis of the viral load against time post infection from both groups. The general model was expressed as $y = X\beta + \varepsilon$, where y is the (n) values of the viral load observed at each time. X is a matrix of dimension n × 2, the first column consists of only ones (for the intercept) and the second variable is the time effect. β contains the intercept α, and the coefficient β_1. ε represents the residual error. The residual error is normally distributed with

mean 0 and variance-covariance structure contained in a matrix Ω, $\varepsilon \sim N(0, \sigma^2 \Omega)$. For the covariance structure of the repeated measures (individuals), four possible models were examined; 1) compound symmetry, 2) completely unstructured, 3) autoregressive with equal variances, and 4) autoregressive with unequal variances [23]. Finally, the model with the lowest Akaike Information Criterion (AIC) value was selected.

The generalized estimating equations (GEE) were used to evaluate the effects of cell viability and time effects. For this, cell viability was assumed to follow a binomial distribution. GEE allow for the use of a correlation matrix structure which takes into account the lack of independence from the same cluster (each group of wells). The conditional mean $E(Y_{it}|X_{it}) = \mu_{it}$ is related to independent variables through a link function $g(\mu_{it}) = X_{it}\beta$. Cell viability corresponds to the well *i* in time *t*. Variance structure of Y_{it} is given by var $(Y_{it}|X_{it}) = \mu_{it}(1 - \mu_{it})$ for proportional data [23]. An auto-regressive correlation structure was assumed because it was also assumed the association between points to be time dependent.

The GLS was performed using the *nlme* package [24] and the GEE using the *gee* package, both implemented in R, Core Team.

3. Results

3.1. Screening of Viral Pathogens in *L. vannamei*

All shrimps tested negative for WSSV, TSV, IHHNV, YHV, and IMNV (data not shown), meaning all shrimps were free of viral presence.

3.2. Viral Inoculum Preparation and Inoculum's Infectivity

The WSSV viral load in the inoculum was of 600 copies/μl. The five shrimps *L. vannamei* injected with 50 μL of the inoculum (≈30,000 viral copies) began to show clinical signs of the disease after 24 hpi; *i.e.* a sudden reduction of food consumption, lethargy, loose cuticle and reddish coloration. The presence of viral *Ie*1 transcripts in their hemocytes confirmed the infection by WSSV (data not shown).

3.3. Hemocytes Viability

Cell viability in the infected group was of 89.60% (±1.58) at 30 min pi, 84.20 (±2.15) at 1 h pi, 83.30 (±7.89) at 2 h pi, 61.70 (±14.22) at 4 h pi, and 34.60 (±11.06) at 6 h pi. In the control group (uninfected) it was of 89.08% (±0.82) at 30 min pi, 86.86% (±1.79) at 1 h pi, 84.59% (±3.89) at 2 h pi, 62.57% (±10.78) at 4 h pi, and 45.00 (±10.58) at 6 h pi.

In vitro viability of the control and the infected groups showed no significant differences (**Table 1** top part). The viability showed a decreasing tendency as time progressed, showing the lowest proportion of viable cells at 6 h pi. Notice that coefficients of 1 h pi with respect to 0.5 h pi, and 2 h pi with respect to 1 h pi, were no statistically significative different, while coefficients for 4 h pi with

respect to 2 h pi and 6 h pi with respect to 4 h pi were statistically different (Table 1 top part). However, the viability rate in control group was never less than 40% (Figure 1(a)).

3.4. Viral Load of Hemocytes Maintained in ISS

The number of viral copies/cell was estimated in both groups by using a quanti-

Table 1. Coefficients from the generalized estimating equations applied to the proportion of viable cells (top part). Coefficients from the generalized least squares applied to the number of viral replicates per cell viable (bottom part).

	Estimate	Std. Error	z-value	*p*-value
Intercept	2.09074	0.04815	43.4199	<0.0001
Treatment-Infection	0.06998	0.07404	0.9452	0.3446
1 hpi	−0.18978	0.11525	−1.6468	0.0996
2 hpi	−0.38205	0.19453	−1.9639	0.0495
4 hpi	−1.58702	0.24888	−6.3765	<0.0001
6 hpi	−2.29141	0.15687	−14.6075	<0.0001
Infection:1 hpi	−0.29609	0.12888	−2.2974	0.02
Infection:2 hpi	−0.02654	0.23806	−0.1115	0.9112
Infection:4 hpi	−0.05576	0.30306	−0.184	0.8540
Infection:6 hpi	−0.49324	0.21768	−2.2658	0.02
	Estimate	**Std. Error**	**t-value**	***p*-value**
Intercept	0.000017	0.000003	6.47	<0.0001
1 hpi	0.000036	0.000012	2.87	<0.01
2 hpi	0.000001	0.000003	0.38	0.7088
4 hpi	0.000098	0.000024	4.13	<0.001
6 hpi	0.000501	0.000329	1.52	0.1353

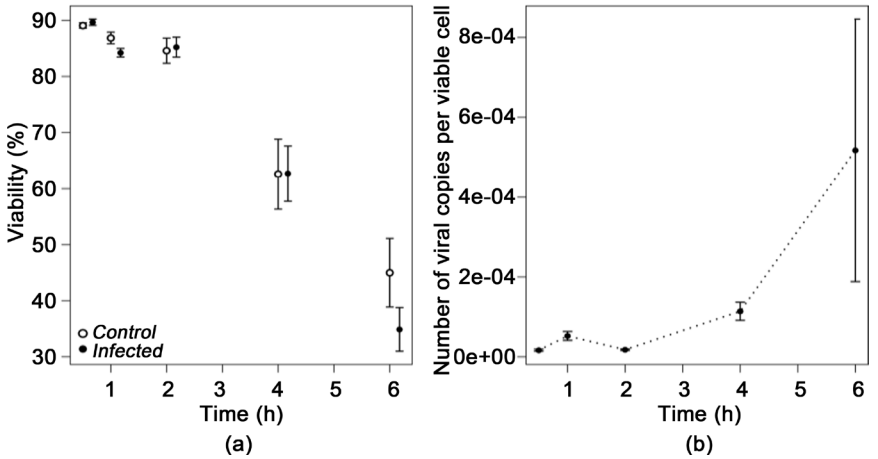

Figure 1. Hemocytes in ISS infected with WSSV. (a) Mean value of cell viability for control and infected groups; (b) Mean value of the number of viral replicates per viable cell in the infected group. Bars are standard errors.

fication curve by RT-PCR. The total viral load was 82.88 (±39.89) viral copies at 30 min pi, 257.62 (±191.80) viral copies at 1 h pi, 78.20 (±20.30) viral copies at 2 hpi, 343.82 (±164.77) viral copies at 4 h pi, and 233.85 (±229.71) viral copies at 6 h pi. The total number of viral copies was divided into the number of live cells in Figure 1(b). Only in the infected group, the *Ie*1 gene was up-regulated during all the experiment.

The viral load's observations showed positive coefficients during the complete kinetics, being the highest at 6 h pi (Table 1 bottom part). The standard error at 6 hpi was the highest (Table 1 bottom part). From these observations, we could confirm that in this group the virus was replicating along the WSSV infection kinetics, while hemocytes in the ISS negative control group were free of WSSV contamination.

3.5. Cytopathic Effect of WSSV on Hemocytes

Granular cells from the uninfected group showed membrane integrity, spherical shape, the presence of granules and a size of 6.8 µm in diameter (Figure 2(a) and (Figure 2(c)). In contrast, cells isolated from WSSV infected hemocytes

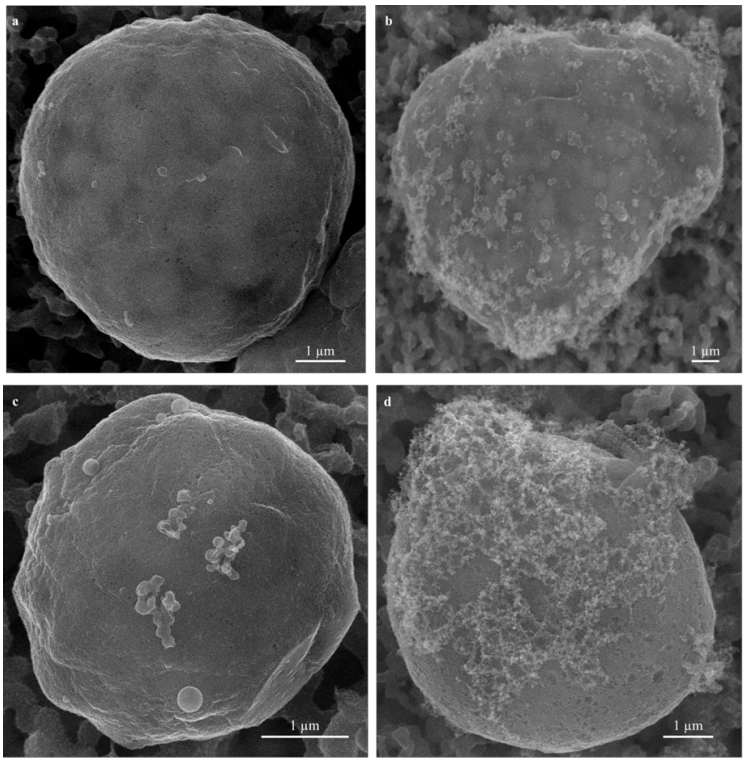

Figure 2. Electron micrographs of gold-palladium coated granular hemocyte from both WSSV infected and control ISS conditions. (a) Uninfected hemocyte showing a spherical shape and a size of 6.8 µm in diameter; (b) WSSV infected hemocyte showing disrupted integrity and irregular shape and a size of 11.6 in length and 14.8 in width; (c) Uninfected hemocyte showing a spherical shape and a size of 4 µm in diameter; (d) WSSV infected hemocyte showing disrupted integrity and irregular shape and dense content surrounding the cell, and a size of 7 µm in diameter. Scale bars = 1 µm. The hemocytes were observed under a scanning electron microscope (FESEM Jeol 7600F) with 25 kV at 2500X.

showed disrupted integrity, degranulation, irregular shape and a size of 11.6 in length and 14.8 in width ((**Figure 2(b)**, (**Figure 2(d)**).

4. Discussion

In this decade, substantial effort has been done in order to establish a culture of crustacean's hemocytes, and significant achievements were obtained for *in vitro* manipulation of viral infections in different crustacean's species [11] [16] [18]. In this study, we proposed the use of ISS [20] to assess *L. vannamei* hemocytes viability and to detect viral replication during early stages of WSSV infection in this species.

Shrimps experimentally infected showed main signs of WSSV [25], therefore, the differential filtration during the inoculum preparation allowed the successful recovery of infective virions. This purification method process was also useful to prevent the hemocytes activation by remains of debris by separating them from the WSSV virions [26]. WSSV infection was also corroborated by amplification of the *Ie*1 gene in isolated hemocytes, an early-immediate transcription gene that plays a fundamental role in promoting viral replication and proliferation [21].

The hemocytes of *L. vannamei* were maintained up to 6 hpi in ISS during the *in vitro* infection. Results of viability, showed a similar decreasing pattern in both, the infected and the control groups at the end of the assays. Based on the statistical analyses, the ideal time of preservation of hemocytes goes between 0.5 and 2 hours. The hemocytes are highly reactive cells, and they start a process of controlled exocytosis when they are in contact with any strange pathogen like bacterial and fungi wall compounds, β-glucans or lipopolysaccharides, therefore, hemocyte population decreases rapidly [18]. *In vivo*, this action is compensated by the releasing of new hemocytes from the hematopoietic tissues into the hemolymph [27], whereas in *in vitro* conditions, hemocytes can react when they are attached to strange surfaces and their viability decreases over time with no compensation of new hemocytes. This had been marked as one of the main limitations of the implementation of suitable cell culture conditions [18]. The above mentioned reactivity could explain why the viability in this study was decreasing in both experimental and control groups, as well as some other factors such as that the media-solution in which the cells were suspended began to be insufficient to maintain the cells and the induction of apoptosis in the infected group.

In the infected hemocytes group, the *Ie*1 transcript was detected by RT-PCR as early as 30 min pi as well as during the kinetics. Although the viral load didn't show a linear proportion, our analysis showed that there is an increase in viral RNA (infection) each time with respect to the previous.

This *Ie*1 WSSV gene has been detected after 2 hpi, 4 hpi [28] and 6 hpi [29], but infection times of less than one hour have not been reported so far. In *L. vannamei* injected with WSSV was found that the early host transcriptome profile of hemocytes changed slightly in an early infection (5 hpi), while large tran-

scriptional differences were identified at 48 hpi (late infection) of WSSV [30], showing the importance of studying early stages of the WSSV infection to comprehend the pathogeny of the disease.

The scanning electron microscopy analysis was used in this study to attest that the cytopathic effect was caused by WSSV and not by the manipulation of the cells. However, the cytopathic index was not performed because of the lack of enough samples.

The confirmation of infection in hemocytes by WSSV in times as short as 30 min generates interesting expectations to potential times for *in vitro* manipulation.

5. Conclusion

The detection of WSSV analyzed with the *Ie*1 gene, confirmed viral replication in hemocytes of *L. vannamei* as early as 30 min pi.

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Authorship and Conflicts of Interest Statement

All authors agree with the content of the manuscript and there are no conflicts of interests between them.

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