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Detection of White Spot Syndrome Virus in Brazil using Negative Staining, Immunoelectron Microscopy and Immunocytochemistry Techniques

Detección del Virus del Síndrome de Mancha Blanca en el Brasil Utilizando Inmunomicroscopía e Inmunomarcación con Partículas de Oro Coloidal

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SUMMARY: In this study thirty shrimp samples from commercial marine shrimp *(L. vannamei)* farms of southern region of Brazil were obtained. Hepatopancreas and shell scrapings fragments collected in these animals were processed by transmission electron microscopy using negative staining (rapid preparation), immunoelectron microscopy and immunocytochemistry (immunolabelling with colloidal gold particles) techniques. On the transmission electron microscopy a great number of white spot virus particles, ovoid or bacilliform-to-ellipsoid, measured 230-290 nm in length and 80-160 nm in diameter with intra-nuclear projections were visualized by the negative staining technique in 27 (90%) out of 30 samples examined. Using immunoelectron microscopy technique, the anti-VP 664 serum agllutinated a large number of particles formed by antigen-antibody interaction. In the immunocytochemistry technique, the antigen-antibody reaction was styrongly marked by the particles of colloidal gold over the virus. Notably, this is the first report, to our knowledge, describing use of these microscopy techniques to study Brazilian *L. vannamei* marine shrimp samples; moreover, this methodology also appears to be a viable complementary tool for diagnosing the presence of the white spot virus within shrimp tissues. Importantly, these are the first photoelectron micrographs of the WSSV in Brazil.

KEYWORDS: White spot syndrome virus; Negative staining; Immunoelectron microscopy; Immunocytochemistry; Marine shrimp; *Litopenaeus vannamei.*

INTRODUCTION

The first experimental cultivation and production of penaeid shrimp was reported in the early 1970s, when French researchers in Tahiti developed techniques for the breeding and intensive farming of several exotic species, including *Penaeus japonicus, P. monodon* and later, *P. vannamei* and *P. stylirostris*, the latter later renamed *Litopenaeus vannamei* and *L. stylirostris*, respectively. In the late 1970s and early 1980s, *L. vannamei* and *L. stylirostris* were removed from their natural breeding area (west coast of Latin America between Mexico and Peru) to the United States, Belize, Nicaragua, Colombia, Venezuela, Philippines, China and Brazil (Rosenberry, 2002; Briggs *et al.*, 2005).

In 1983, the white shrimp, popularly known as *L. vannamei*, was imported to Brazil for cultivation because it is a specie with high reproductive capacity, good adaptation to captivity, rapid growth, efficient feed conversion and high survival rates. However, it was only in 1995 that the commercial production of this exotic species began. Currently, the white shrimp accounts for 95% of Brazilian marine shrimp production (Briggs *et al*.), having reached 76,000 tons in 2004 (FAO, 2006). However, shrimp farming in the Americas and worldwide has been severely impacted by various types of pathogenic viruses, notably by the White Spot Virus (Paez-Osuna, 2001; Lightner, 2005a, b; Sanchez-Martinez *et al*., 2007).

The White Spot Disease (WSD), or White Spot Syndrome, is caused by the virus from which its name derives (WSSV). It has this name because of the

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characteristic of this disease that is the formation of white spots in the exoskeleton, in the rostrum and in the epidermis. This virus belongs to the genus Whispovirus and the family Nimaviridae (Mayo, 2002), but was initially classified as a member of the Baculoviridae family (Wang *et al.*, 1995).

The disease emerged in Mainland, China between 1992 and 1993, and was quickly dispersed throughout the Asian continent by infected seeds and animal stock. The first outbreaks were reported in 1993 on a *Macrobrachium japonicus* farm in Japan (Nakano *et al.*, 1994). Shrimp exhibiting the characteristic signs and histopathology of white spot disease were also reported in Korea, India, the Philippines and the USA, and this same virus also struck shrimp farms in southeast Europe (1997) and the Middle East (1999) (Lightner,2005 b). During 1999, the disease had a serious impact on the shrimp industry in Central and South America and resulted in large losses, especially in Ecuador, which was, until then, one of the major world producers. Between 2000 and 2001, the WSSV reached Spain and Australia; however, cases of successful containment and eradication have been reported, and for both events, the import and utilization of infected frozen shrimp as animal feed was implicated as the route of introduction (Lightner, 2005b; Sánchez-Martínez *et al*., 2007). In November 2004, commercial farms of *L. vannamei* produced in the town of Laguna in southern Brazil were infected with WSSV. Mortality rates reached 90%, causing losses of more than 3 million USD. This first outbreak in Brazil was reported by OIE on January 20, 2005 and, more recently, a new outbreak of the virus has been reported on farms in Ceará, northeast Brazil (CEI, 2005; Seiffert *et al*., 2005; Cavalli *et al.*, 2008).

During the last two decades, a combination of poor management practices and intensive penaeid shrimp farming has led to the emergence of several viral diseases. The WSD is reported as the most devastating among these viral diseases and can cause 100% mortality of the farmed shrimp (Lightner 2005 a, b; Sanchez-Martinez *et al*.). This virus infects many tissues, multiplies in the nucleus of the target cell, leads to cellular disintegration at the final phase of infection and causes the destruction of the infected tissues (Flegel, 1997; Leu *et al*., 2009).

The WSSV has a cylindrical shape, is slightly elliptical, measures between 121±9 nm x 26±276 nm (Wongteerasupaya *et al.*, 1995) and is a double-stranded DNA virus with a genome of approximately 300 bp. The virion is composed of a trilaminar nucleocapsid enveloped by a membrane that often shows a tail-like extension (Wu & Yang, 2006). The White Spot Syndrome Virus 1 is the only representative of the genus Whispovirus described, showing no apparent immunologic diversity, although some genetic polymorphism has been observed in several isolates (ICTVdB, 2007).

One of the characteristics of this virus is the ability to infect numerous hosts, including a great variety of crustaceans (shrimps, lobsters, copepods), insects and larvae; in penaeid shrimp specifically, the virus has been detected at all stages of growth (Chang *et al.*, 1998). Transmission can be horizontal through water or by the practice of cannibalism of infected shrimps, or vertically, from females to eggs, resulting in infected larvae (Lo *et al.*, 1997).

In accordance with recommendations by the WOAH/OIE (2003a, b), *in situ* hybridization and nested polymerase chain reaction are used for the detection and diagnosis of WSD. The presence of the whole virus is not required for these techniques, because viable DNA segments can be amplified by specific primers. Other techniques, such as histology and electron microscopy, are used for the observation of lesions and visualization of the virus and, more recently, methods based on antibody-based pathogen detection have been used (Lightner 2005a).

Specifically, the use of transmission electron microscopy (TEM) allows for the study of structural and morphological details of the virus and the subcellular constituents with which these organisms may or may not be associated. The use of TEM along with immunoelectron microscopy (IEM) and immunocytochemistry is particularly beneficial when the number of virus particles in a sample is very low, since it allows for the identification of the virus not only by a specific antigen-antibody reaction but also via morphology (Katz & Kohn, 1984; Hayat & Miller, 1990). Furthermore, it allows for the detection and identification of antigens from virus-induced structures and their localization in infected cells, serotyping of viral strains (Kjeldsberg, 1986) and the determination of antigenic variants of an isolate (Patterson & Oxford, 1986). These techniques can be used individually, in series or in parallel, in order to confer increased sensitivity or specificity of virus detection.

The objective of this study was to demonstrate the viability of negative staining, immunoelectron microscopy and immunocytochemistry (immunolabeling with colloidal gold particles) as a complementary diagnosis of white spot disease in field samples.

MATERIAL AND METHOD

Samples. Thirty shrimp samples from commercial marine shrimp (*L. vannamei*) farms of southern Brazil (North coast of Santa Catarina), in growout phase, were obtained between 2006 and 2007. All animals showed white spots on the exoskeleton, in the rostrum and in the epidermis. The shrimp were desensitized by refrigeration and frozen. Fragments of the hepatopancreas and shell scrapings were suspended in phosphate buffer (0.1 M, pH 7.0).

Negative Staining Technique. Drops of the obtained suspensions were placed on metal grids, previously covered with colloidal film and stabilized with carbon. Subsequently, the grids were drained with filter paper and negatively contrasted with 2% ammonium molybdate, pH 5.0 (Brenner & Horne, 1959; Hayat & Miller, Madeley, 1997).

Immunoelectron Microscopy Technique (IEM). The copper grids were prepared as described above, sensitized for 15 minutes with primary anti-WSSV polyclonal antibody against protein VP664 (Abcam®, diluted at 1:200) and were washed with PBS buffer. Upon incubation with the WSSV viral suspension for 10 minutes, grids were washed successively with distilled water and negatively contrasted with ammonium molybdate under the same conditions (Katz & Kohn; Hayat & Miller; Wu & Yang).

Immunocitochemistry Technique (Immunolabeling with colloidal gold particles – ISCG). Previously prepared copper grids were incubated for 30 minutes in drops of viral suspension, sensitized with the same antibody diluted at 1:80 and washed with PBS buffer. Grids were incubated sequentially with secondary antibody (protein A conjugated with colloidal gold particles of 10 nm in diameter (Electron Microscopy Sciences ®) diluted 1:20 in 0.5% PBS for 30 minutes and negatively contrasted with ammonium molybdate (Knutton, 1995).

The samples were examined by transmission electron microscopy using a Philips EM 208 microscope. As a technical control, for every 10 samples incubated, one was treated as described previously, but the antibody was replaced by distilled water.

RESULTS

Negative Staining Technique. Analyses performed by TEM revealed a large number of particles with ovoid or bacilliform-to-ellipsoid morphology, measuring about 230-290 nm long and 80-160 nm in diameter, showing intra-nuclear projections (Fig. 1) in 27 (90%) of 30 samples examined (Table I).

In some samples, the capsids were empty due to nucleocapsid release, and the viral envelopes were separated from their virions. In other samples, only the nucleocapsids were observed, revealing grooves located along the major axis (Fig. 2). Another samples showed ring-like particles connected to the virion (Fig. 3).

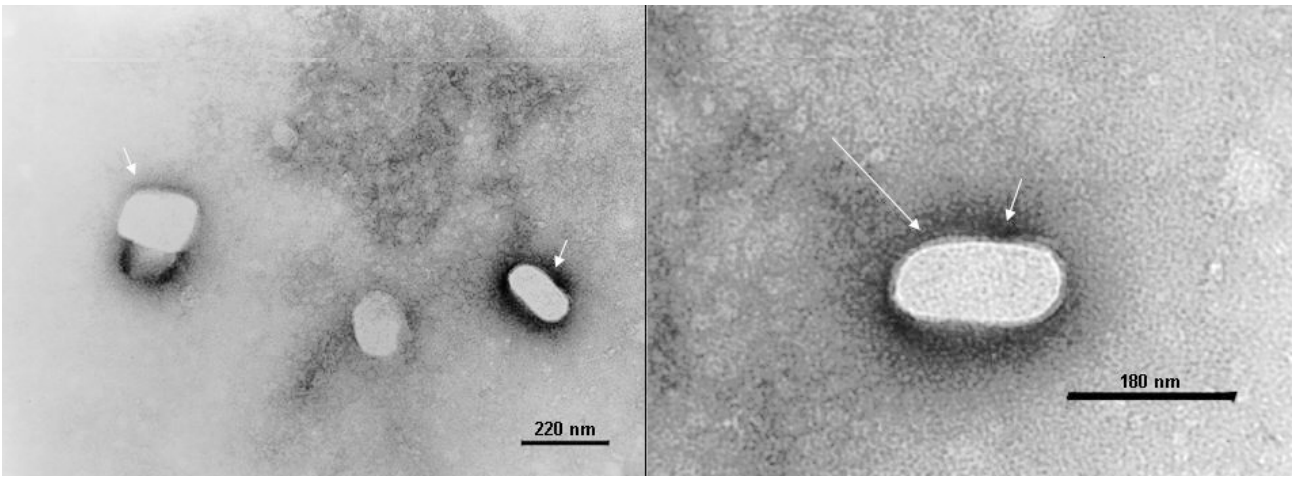

Fig. 1. Photoelectronmicrograph of the WSSV viral suspension from *L. vannamei* hepatopancreas. Oval-shaped and bacilliform particles (large arrow) and outer envelope (lower arrow)

Immunoelectron Microscopy Technique. Results from IEM showed a positive reaction in 90% of the samples analyzed (Table 1). The anti-VP664 serum agglutinated a large number of particles (i.e., antibody-virus aggregate) (Fig. 4a).

Immunocytochemistry Technique. In the immunocytochemistry technique, the antigen-antibody reaction was increased by colloidal gold particles that labeled Whispovirus effectively (Figs. 4b, c, d), thus confirming the results previously obtained by negative staining and immunoelectron microscopy techniques (Table 1).

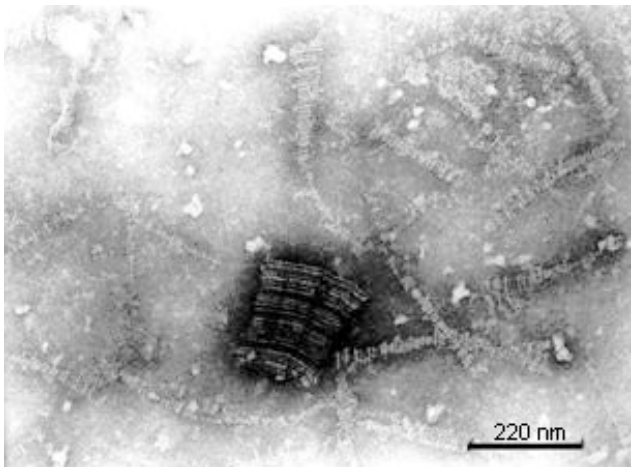

Fig. 2. Photoelectronmicrograph of the WSSV viral suspension from *L. vannamei* shells showing WSSV nucleocapsids with grooves. Negative staining technique.

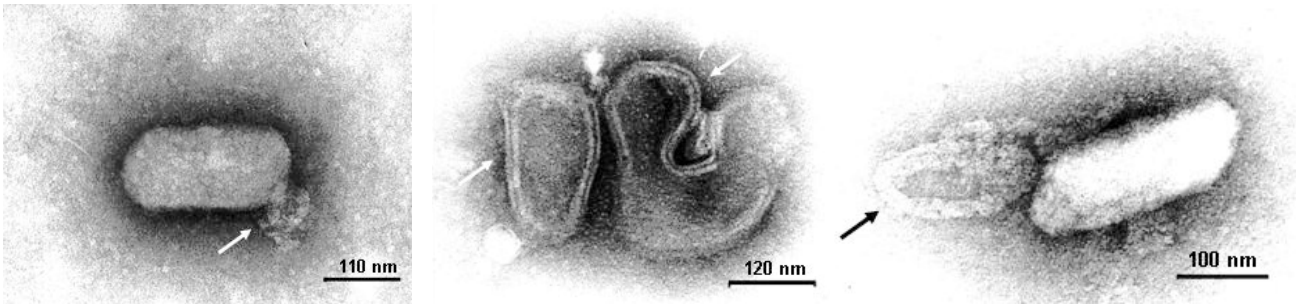

Fig. 3. Photoelectronmicrograph of the WSSV viral suspension showing ring-like particles connected to the virion (arrows). Negative staining technique.

Sample	NS	IEM	ISCG	Sample	NS	IEM	ISCG
1	+	+	+	16	+	+	+
2	+	+	+	17	+	+	+
3	+	+	+	18	-	-	-
4	+	+	+	19	+	+	+
5	+	+	+	20	+	+	+
6	+	+	+	21	+	+	+
7	+	+	+	22	+	+	+
8	+	+	+	23	+	+	+
9	+	+	+	24	+	+	+
10	+	+	+	25	+	+	+
11	+	+	+	26	+	+	+
12	+	+	+	27	-	-	-
13	+	+	+	28	-	-	-
14	+	+	+	29	+	+	+
15	+	+	+	30	+	+	+

Table I. Comparation of results obtained by applying the negative staining (NS), immunoelectron microscopy (IEM) and immunocytochemistry (ISCG) techniques for WSSV. Samples of marine shrimp, Santa Catarina State, Brazil, 2007-08.

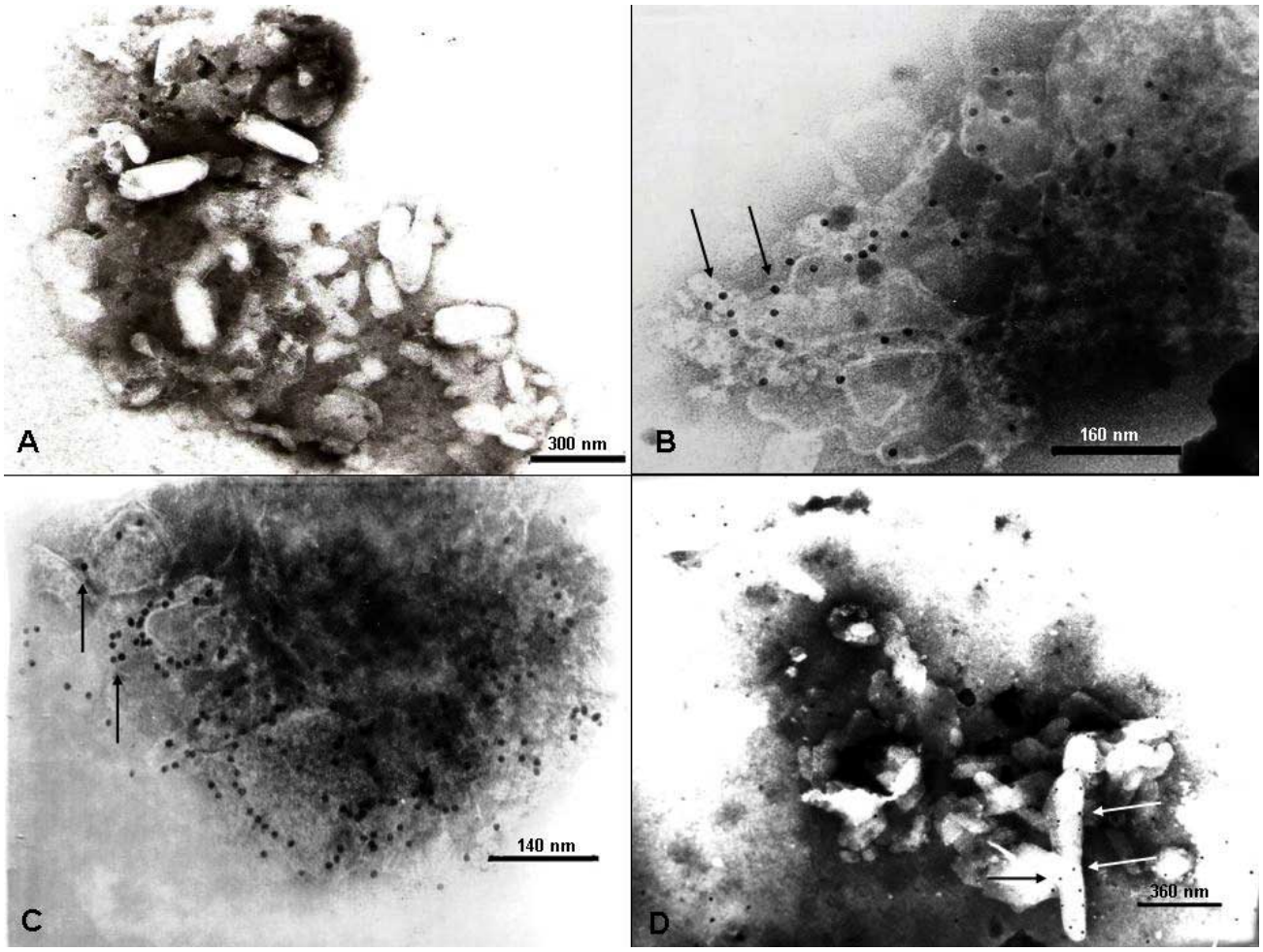

Fig. 4. Immunoelectron microscopy and immunocytochemistry techniques. A - WSSV particles aggregated to the immune complex. B, C, D - a strong antigen-antibody interaction was evidenced by the gold particles on the virion (arrows).

DISCUSSION

In the present paper thirty shrimp samples from commercial marine shrimp (*L. vannamei*) farms of southern Brazil were obtained.

The examined animals presented formation of white spots in the exoskeleton, in the rostrum and in the epidermis.

Similarly, these symptons were described by other authors in other shrimps species (Nakano *et al*.; Lo *et al*.; Seiffert *et al*.; Cavalli *et al*.; Muller *et al*., 2010).

Analysis performed by TEM revealed a large number of particles with ovoid or bacilliform-to-ellipsoid morphology, showing intranuclear projections and ring-like particles connected to the virion in 27 (90%) out of 30 samples examined.

These morphological aspects also were been observed by other authors in ultrastructural study for the WSSV (Chou *et al*., 1995; Durand *et al*., 1997; Wang *et al*., 2000; Rajendran *et al*., 2004; Van Hulten *et al*., 2001; Afsharnasab, 2006; Escobedo *et al*., 2008; Zhu *et al*., 2009).

In other samples only the nucleocapsids were observed, revealing grooves located along the major axis. These aspects also has been related by other researchers (Durand *et al*., 1997; Afsharnasab, 2006).

In the immunoelectron microscopy technique the anti VP 664 serum agglutinated a large number of particles formed by antigen antibody interaction.

This technique was successfully applied to agglutinate various types of viruses, such as paramyxovirus, retrovirus and coronavirus (Catroxo *et al.*, 2010).

Despite the fact that these are non-purified field samples, satisfactory distribution of numerous gold particles can be observed throughout the entire nucleocapsid as opposed to one specific region when we apply the immunocytochemistry technique, confirming the results fo the negative staining and immunoelectron microscopy techniques.

In other experiments, WSSV particles were enhanced by this technique (Wu & Yang; Liu *et al.*, 2006; Tsai *et al*., 2006; Chang *et al.*, 2008).

The VP664 protein is a giant polypeptide chain that functions as a structural protein and, due to its size, is considered to be the largest protein isolated thus far from shrimp; this protein provides the basis for the production of polyclonal antisera. Encoded by an open reading frame (ORF) of 18,234 nucleotides, it generates a long polypeptide of 6077 amino acids with an as yet unknown function (Leu *et al.*, 2005).

In this study, we used the anti-VP664 serum to confirm a positive result from *L. vannamei* in shell scrapings and hepatopancreas samples. Because the shrimp organs were flaccid and easily perishable, they were immediately frozen at -20ºC and processed in twenty days. Notably, the NS, IM and ISCG techniques were performed using frozen field samples, wherein the number of viral particles is smaller than that obtained from cell culture, a procedure used to enrich the material for subsequent use by TEM. It is evident that the process of freezing the samples immediately after collection, did not interfere in the result indicating that the virus particle remained integrated.

The results in Table I, show a perfect concordance between the samples, showing a perfect affinity between these techniques.

All techniques used herein were sensitive, thus demonstrating their value at a diagnostic level.

Electron microscopy is becoming increasingly important in the diagnosis of viral agents (Hayat & Miller) and constitutes an essential tool in the detection of emerging diseases by providing a rapid diagnosis of viral infections through processing of clinical samples (Gelderblom & Mãnnel, 2003; Leu *et al*.). The association of TEM with serodiagnostic methods using mono- or polyclonal antisera enriches this methodology and stands out as an accurate and fast diagnostic tool.

Notably, this is the first report, to our knowledge, describing use of these microscopy techniques to study Brazilian *L. vannamei* marine shrimp samples; moreover, this methodology also appears to be a viable complementary tool for diagnosing the presence of the white spot virus within shrimp tissues.

Importantly, these are the first photoelectron micrographs of the WSSV in Brazil.

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RESUMEN: Se obtuvieron para el estudio 30 muestras de camarones marinos comerciales *(L. vannamei)* de las granjas de la región sur de Brasil. Fueron procesados fragmentos de hepatopáncreas y raspados internos del cefalotórax recogidos en estos animales por microscopía electrónica de transmisión con tinción negativa (preparación rápida), inmunomicroscopía y técnicas de inmunocitoquímica (inmunomarcación con partículas de oro coloidal). En la microscopía electrónica de transmisión de un gran número de partículas de virus de la mancha blanca, ovoide o elipsoidal a baciliformes, medían 230-290 nm de longitud y 80-160 nm de diámetro. En 27 (90%) de las 30 muestras examinadas intra-nuclear proyecciones se visualizaron mediante la técnica de tinción negativa. Utilizando una técnica de inmunomicroscopía electrónica, el anti-suero VP 664 reunió a un gran número de partículas formadas por la interacción antígeno-anticuerpo. En la técnica de inmunocitoquímica, la reacción antígeno-anticuerpo fue fuertemente reforzada por las partículas de oro coloidal en los virus. En particular, en Brasil este es el primer informe, a nuestro entender, que describe el uso de estas técnicas de microscopía en muestras de camarón marino *L. vanamei.* Además, esta metodología también parece ser una herramienta complementaria viable para diagnosticar la presencia del virus de la mancha blanca en tejidos de camarón. Es importante destacar que estas son las primeras fotos en microscopia electrónica del WSSV obtenidas en Brasil.

PALABRAS CLAVE: Vírus de la mancha blanca, tinción negativa, inmunomicroscopía electrónica; inmunocitoquímica; camarón marino, *Litopenaeus vannamei*.

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