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Application of gene probes as diagnostic tools for White Spot Baculovirus (WSBV) of penaeid shrimp

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ABSTRACT: Since 1993, similar baculoviruses, which cause high mortalities in penaeid shrimp, have been reported from China, Japan, Korea, Thailand and Taiwan. All these baculovirus-caused diseases are characterized by the presence of white spots in the cuticle. To isolate the agent of the disease referred to as White Spot Syndrome (WSS) and White Spot Baculovirus (WSBV), in this paper, *Penaeus vannamei* and *P. stylirostris* were experimentally inoculated with homogenates of infected *Penaeus monodon* from Thailand. In transmission electron microscopy (TEM), the enveloped WSBV virions have a size of about 350 nm long and 130 nm wide. The nucleocapsids range from 300 to 420 nm in length and 70 to 95 nm in diameter and show a superficially segmented appearance. Also present are unique particles, which have not been observed before, measuring 400 nm in length and 120 nm in width. These particles are larger than most of the usual nucleocapsids and have a cross-hatched superficial appearance. After nucleic acid extraction, *Eco*RI-digested fragments of the WSBV genome were cloned. Four of these fragments were characterized and used as non-radioactive probes labeled with DIG-11-dUTP. By *in situ* hybridization, the probes hybridized with material located in nuclei of all WSBV-infected tissues. Previously reported target tissues, such as connective tissue, epithelial tissue and also hemocytes, clearly showed positive hybridization with the probes. In addition, some light infection was revealed in the muscle and nervous tissue.

KEY WORDS: Non-occluded baculovirus · White Spot Syndrome · Penaeid shrimp · Gene probes · *In situ* hybridization

INTRODUCTION

Since 1993, diseases with cumulative mortalities of more than 80% have been reported in numerous shrimp farms from China, Japan, Korea and Thailand (Inouye et al. 1994, Nakano et al. 1994, Wongteerasupaya et al. 1995), and more recently from Indonesia, Taiwan and India (Chen 1995, Chou et al. 1995, Rosenberry 1995, Wang et al. 1995, Winarno 1995). These new diseases, whose most characteristic sign is the presence of white spots on the cuticle, were attributed to at least 4 viruses reported by different names in the literature: Baculoviral Hypodermal & Haematopoietic Necrosis Virus (HHNBV) by Huang et al. (1994), Rod-shaped Virus of *Penaeus japonicus* (RV-PJ) by Inouye et al. (1994) and Takahashi et al. (1994), Systemic Ectodermal and Mesodermal Baculovirus (SEMBV) by Wongteerasupaya et al., (1995) and White Spot Baculovirus (WSBV) by Wang et al. (1995). The viral agents of these diseases appear to be very similar in size (80 to 120 × 275 to 360 nm), geographic range, and the gross and histological signs produced. For these reasons, the diseases caused by these non-occluded baculoviruses have been regrouped (Lightner 1996) with the various members of the WSBV group under the name of White Spot Syndrome (WSS).

All these viruses were deemed by the authors to be closely related to the family *Baculoviridae* and to the subfamily of non-occluded baculoviruses: the *Nudi-*

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baculovirinae (Francki et al. 1991). But, the recent Sixth Report of the International Committee of Taxonomy of Viruses (I.C.T.V.) (Murphy et al. 1995) has canceled the previous classification and placed these viruses, formerly considered to be members of the subfamily *Nudibaculovirinae*, among the unassigned viruses. For clarity, we have decided to maintain in this paper the old designation of 'non-occluded baculoviruses'.

Natural infections by WSBV have been observed in *Penaeus chinensis* (= *orientalis*), *P. japonicus*, *P. monodon*, *P. indicus*, *P. merguiensis* and *P. penicillatus*. In addition, the viruses produce an experimentally lethal infection in *P. vannamei*, *P. stylirostris* and *P. setiferus* (Lightner 1996).

The clinical signs of the WSS include a sudden reduction in food consumption, lethargy, a loose cuticle (and often a reddish to pink discoloration) and the presence of white spots of 0.5 to 2.0 mm in diameter in the cuticle. Histologically, the characteristic sign of the disease is nuclear hypertrophy due to the development and accumulation of intranuclear virions. The target tissues are connective and epithelial tissues, particularly epithelial cells of the gills and stomach (Inouye et al. 1994, Chen 1995, Wongteerasupaya et al. 1995). Therefore, Wongteerasupaya et al. (1995) concluded that these were infections limited to tissues of ectodermal and mesodermal embryonic origin.

Until recently, diagnostic procedures for viral diseases of penaeid shrimp were largely dependent upon history, clinical signs, and histological examination of moribund animals (Lightner & Redman 1992, Lightner 1993, 1996). The practical application of these procedures is sometimes difficult and sensitivity is often limited.

Since 1993, biotechnological diagnoses using DNA probes, such as those currently used in human and veterinary medicine, have been developed for some viral diseases of penaeid shrimp (Mari et al. 1993a). These DNA probes are highly specific and have a high degree of sensitivity. They are currently used in our laboratories as diagnostic and research tools for different virus diseases such as Infectious Hypodermal and Haematopoietic Necrosis (IHHN) (Mari et al. 1993a), *P. vannamei* (PvSNPV) = (*Baculovirus penaei*-type) BP-type virus (Bruce et al. 1994, Bonami et al. 1995), Nuclear Polyhedrosis Virus of *P. monodon* (PmSNPV) = (Monodon Baculovirus) MBV (Mari et al. 1993b, Poulos et al. 1994), Hepatopancreatic Parvovirus (HPV) (Mari et al. 1995), and Taura Syndrome Virus (TSV) (Mari, Bonami & Lightner unpubl. data).

Here, we report on the partial cloning of WSBV-DNA and the development of gene probes to be used as a diagnostic tool by *in situ* hybridization.

MATERIALS AND METHODS

Infected animals. For virus purification, we used *Penaeus vannamei* and *P. stylirostris* experimentally infected at the University of Arizona, Aquaculture Pathology Center (USA). The inoculum for the infection was provided by DiagXotics (Wilton, CT, USA) and originated from infected *P. monodon* from Thailand. The inoculum was prepared from a homogenate of infected shrimp tissues (head and tail) in TN buffer (0.02 M Tris-HCl, 0.4 M NaCl, pH 7.4). After centrifugation at 2500 rpm (1000 × *g*) for 10 min, the supernatant fluid was diluted 1:20 in 2% NaCl and filtered (0.2 µm filter). Experimental infection was induced by 0.2 ml of the filtered suspension injected into each shrimp in the dorsal lateral area of the fourth abdominal segment, injecting between the tergal plates forward into the third abdominal segment.

Virus purification assays. Hemolymph of moribund and dead shrimps was withdrawn on the third and fourth days after experimental injection using 10% sodium citrate as an anticoagulant. The hemolymph was frozen for 5 min and thawed for 10 min. This procedure was repeated twice to disrupt infected cells. After a dilution (1:2 in TN buffer), the hemolymph was clarified by low speed centrifugation at 500 × *g* for 10 min, followed by centrifugation at 3000 × *g* (SS34 Sorvall rotor) for 15 min. The supernatant fluid was then centrifuged at 71 900 × *g* (AH629 Sorvall rotor) for 1 h. The pellet was resuspended in 1 ml TN buffer.

Electron microscopy. Purified virus fractions were negatively stained on carbon-collodium-coated grids using ultrafiltered (0.2 µm) 2% sodium phosphotungstate (PTA at pH = 7.0) and observed with a Hitachi HU 12 C Transmission Electron Microscope (TEM).

Nucleic acid extraction. The purified virus suspension was treated with proteinase K (final concentration: 50 µg ml^{-1}, 2 h, at 37°C), followed by sarkosyl (final concentration. 0.5%, 2 h, at 65°C). The DNA was then extracted twice with buffer-equilibrated phenol, once with phenol/chloroform/isoamyl alcohol (25:24:1) and twice with chloroform/isoamyl alcohol (24:1). The nucleic acid was precipitated in cold absolute ethanol containing 0.3 M sodium acetate overnight at –20°C (Sambroock et al. 1989). After a washing with 70% alcohol and a centrifugation, the final pellet was resuspended in 0.1 × TE (Tris-ethylenediaminetetraacetic acid) (Sambroock et al. 1989)

Restriction enzymes. The following restriction enzymes *Aat* I, *Bam*H I, *Bgl* II, *Cla* I, *Eco*R I, *Eco*R V, *Hind* II, *Hind* III, *Kpn* I, *Pst* I, *Sac* I, *Sal* I, *Sma* I, *Sph* I, *Ssp* I and *Xba* I (Boehringer, Mannheim) were used according to the manufacturer's instructions.

Agarose gel electrophoresis. One percent agarose gels in 0.5 × TBE (Tris-Borate EDTA) buffer containing 0.5 µg ml^{-1} ethidium bromide were used (Sambroock et al. 1989). Marker II, III and X (Boehringer, Mannheim) and RF ΦX174-HaeIII DNA digest were used as molecular weight (MW) markers. After separation by gel electrophoresis (about 1 h migration at 120 V in a submarine electrophoresis tank), the DNA bands of interest were excised from the gel and recovered using the Geneclean II kit (BIO 101, Inc.).

Cloning and screening. The *Eco*R I DNA restriction fragments were directly ligated with T4 DNA ligase (Appligene) in the dephosphorylated *Eco*R I site of *Puc*18 vector. Transformation was done according to standard methods (Sambroock et al. 1989) using competent *Escherichia coli* DH 5α cells. Alkaline lysis minipreparations, restriction enzymes and gel electrophoresis were used to analyze each recombinant plasmid. In addition, for the plasmids of interest, mapping of the inserts was done.

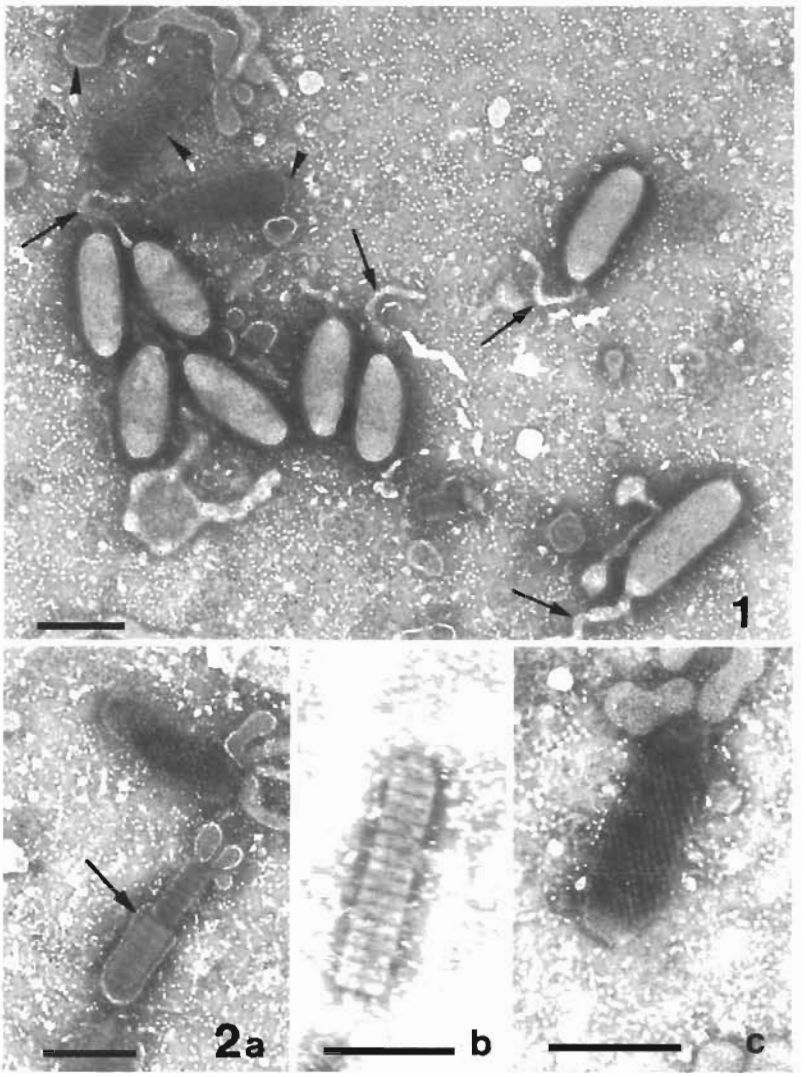
DNA labeling. The cloned DNA fragments were labeled by incorporation (random primed method) of DIG-11-dUTP using the Genius I Kit TM (Boehringer, Mannheim) according to the protocol suggested by the manufacturer. Southern transfers (Sambroock et al. 1989) and dot-blots (according to the method suggested in the application manual of the manufacturer) were performed on positively charged nylon membranes (Boehringer, Mannheim).

***In situ* hybridization.** Shrimp (*Penaeus setiferus*) were infected experimentally with the WSBV inoculum from infected *P. monodon* at the University of Arizona, Aquaculture Pathology Center (USA). Infected animals were fixed in Davidson's fixative according to Bell & Lightner (1988) and paraffin-embedded. Sections (4 µm thick) were mounted onto positively charged microscope slides (Superfrost/Plus, Fisher Scientific) and were used for *in situ* hybridization using Digoxygenin labeled probes according to the technical manual of the manufacturer (Boehringer, Mannheim). Sections of healthy shrimp and PvSNPV-(BP-type baculovirus) infected animals were also used to test the probe specificity.

RESULTS

Isolated particles

The pellet of the final centrifugation was resuspended in TN buffer and examined by TEM after negative staining. It contained enveloped virions, free nucleocapsids and free viral envelope fragments (Fig 1) Compared to the numerous nucleocapsids and virions, few cell-derived contaminants were present; this allowed us to extract the DNA directly from this preparation.



Figs 1 & 2 Fig. 1. Negatively stained White Spot Baculovirus (WSBV) particles Enveloped virions with the typical appendage at one extremity (arrows) and nucleocapsids (arrowhead) The preparation is lightly contaminated by fragments of envelope and hemocyanin molecules in the background 2% PTA TEM Scale bar = 250 nm Fig. 2. Degradation steps of WSBV particles (a) Disruption of the envelope (arrow) showing the inner component, the nucleocapsid (b) The non-enveloped nucleocapsid exhibits a superficial segmented appearance. (c) Swollen and ovoid nucleocapsid with a cross-hatched appearance 2% PTA. TEM. Scale bars = 250 nm

Ultrastructure

Intact enveloped particles appeared slightly ovoid and were about 350 by 130 nm with size variations ranging from 300 to 420 and 110 to 140 nm, respectively. Several of them possessed an appendage at one extremity which looked like an envelope extension and measured 270 to 310 nm in length by about 30 nm in diameter.

Nucleocapsids were observed in different states: some without envelopes (free nucleocapsids), and others surrounded with envelope fragments (Fig. 2a). Free rod-shaped nucleocapsids were 300 to 420 by 70 to 95 nm and had a superficially segmented appearance, with segments displaying an angle of 90° to the long axis of the particle (Fig 2b). The segments, which were about 23 nm thick, were separated from one another by an electron dense 6 nm band.

Different particles with a size of 400 nm (300 to 460 nm) by 120 nm (100 to 150 nm) were also observed. These appeared larger than the nucleocapsids and the virions described above. These particles were ovoid and exhibited a cross-hatched surface with the cross-hatching at a variable angle of 35° to 45° to the long axis of the particle. Free nucleocapsids also had a segmented appearance which here appears poorly defined. This leads us to consider that they might be swollen or 'relaxed' nucleocapsids (Fig. 2c). In addition, a few short, truncated, and enveloped nucleocapsids that possesed an appendage at the flat end were also noted in our preparation.

Nucleic acid

After extraction, viral DNA was electrophoresed on 1% Agarose gel (Fig. 3). Only 1 band was observed, migrating at the same distance as the higher MW marker (23.1 kbp), suggesting a high MW for the viral DNA (at least equal to or higher than 23.1 kbp)

Cloning and screening

The nucleic acid was digested with *Eco*R I and electrophoresed in a 1% agarose gel (Fig 4). Numerous bands (at least 25) were revealed. Their estimated size, compared to DNA MW markers, ranged from 15 to 0.6 kbp. To construct probes, 10 small restriction fragments were chosen ranging in size from 0.6 to 2 kbp. They were ligated separately in the *Eco*R I site of *Puc*18 and used to transform competent cells.

From a total of 250 screened clones, we selected those containing a recombinant plasmid displaying 2 *Eco*R I sites and with an insert size similar to the genomic *Eco*R I restriction fragment used for the cloning.

Mapping of the inserts

To characterize each selected insert and to reveal possible homologies, restriction maps were constructed (Fig. 5). Table 1 lists the insert sizes and the number of restriction sites located in each.

Probe construction

Four of the cloned inserts were chosen to be labeled with DIG-11-dUTP and used as probes. The size of these inserts was 1.2, 1.4, 1.5, and 1.6 kbp, respectively. The probes were called E1.2, E1.4, E1.5, and E1.6.

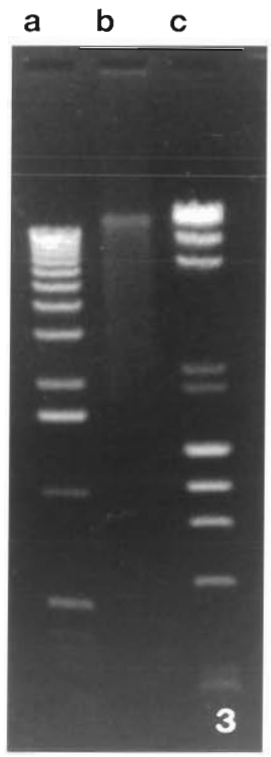


Fig. 3. Agarose (1%) gel electrophoresis of WSBV-DNA (lane b); lane a: Marker X (Boehringer, Mannheim); lane c: Marker II (Boehringer, Mannheim) + RF ΦX *174-DNA-Hae* III digest

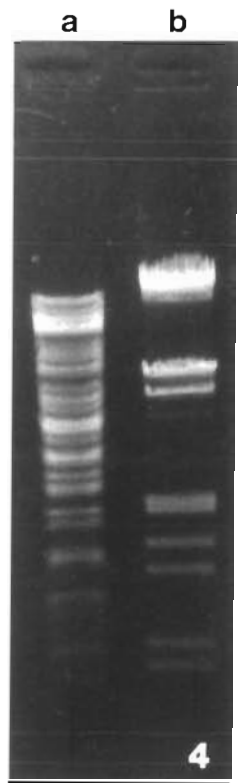


Fig. 4. Agarose (1%) gel electrophoresis of *Eco*RI digested WSBV-DNA (lane a); lane b: Marker III (Boehringer, Mannheim)

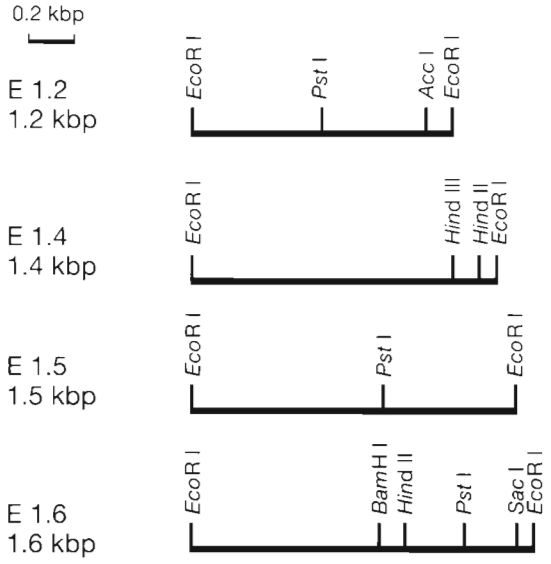


Fig. 5. Restriction maps of the cloned inserts

Table 1. Number of restriction sites of WSSV probes

Enzymes	Probes			
	E1.2	E1.4	E1.5	E1.6
Aat I	0	0	1	0
*Bam*H I	0	0	0	1
Bgl II	0	0	0	0
Cla I	0	0	0	0
*Eco*R V	1	1	0	0
Hind II	0	1	2	2
Hind III	0	1	0	1
Kpn I	0	0	1	0
Pst I	1	0	1	1
Sac I	0	0	0	1
Sal I	0	0	0	0
Sma I	0	0	0	0
Sph I	0	0	1	0
Ssp I	1	0	0	0
Xba I	0	0	0	0

Some tests were done by dot-blot hybridization against log dilutions of extracted WSBV DNA. We noted strong hybridization of these 4 probes with the viral DNA genome.

In situ hybridization

The 4 probes were tested separately by *in situ* hybridization in paraffin sections of WSBV-infected and healthy shrimps and PvSNPV-(BP-type) infected shrimps (another type of baculovirus). No reaction was found in healthy shrimp tissue or in PvSNPV-infected shrimp. By contrast, a strong positive reaction was obtained in WSBV-infected shrimp tissue. These results indicate the specificity of our constructed probes. When used separately, and at the same concentration, the 4 probes detected all the same areas in the histological sections with identical intensity.

The hypertrophied nucleus of an infected cell was strongly labeled. The nuclei exhibiting the most intense reaction for WSBV were located in epithelial cells of the gills (Fig. 6a) and the stomach, in the epidermis (Fig. 6b) and in all connective tissues. The gills showed an intense labeling in both the cuticular epithelium and hemocytes. In the digestive tract, labeling was limited to cuticular epithelial cells of the stomach. Many fixed and circulating hemocytes showed a positive reaction, and these were observed throughout the hemocoel as well as in the hemal sinuses of the hepatopancreas and amongst the striated skeletal muscles. A weak positive reaction was observed in nuclei of nervous tissue (Fig. 7a) and muscle tissue (Fig. 7b) but nuclear hypertrophy was lacking.

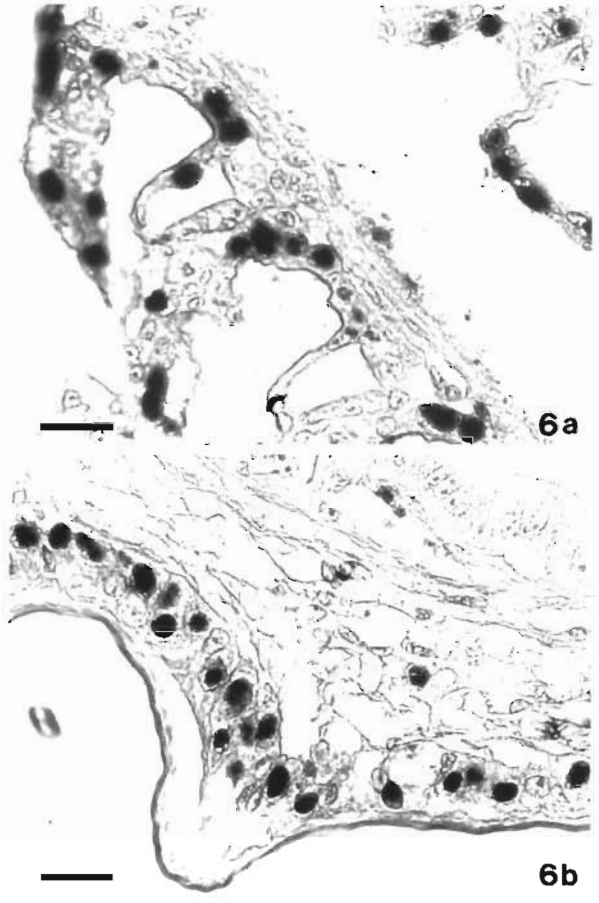


Fig. 6. *In situ* hybridization of WSBV-infected *Penaeus setiferus* using an equimolar mixture of the 4 prepared probes. The darkly stained nuclei demonstrate a strong positive reaction. (a) Gills with positive epithelial cells. (b) Cephalothoracic epidermis in which almost all nuclei are positive. Light microscopy. Scale bars = 20 µm

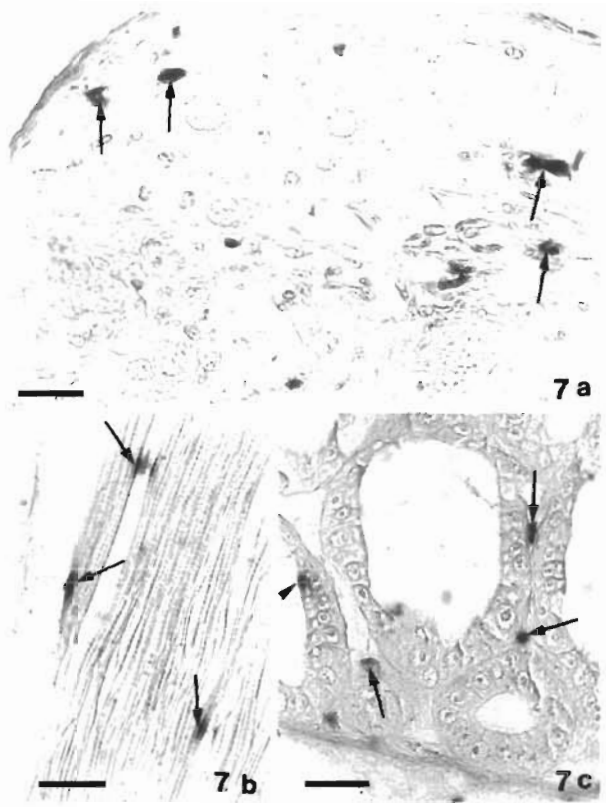


Fig. 7. *In situ* hybridization of WSBV-infected *Penaeus setiferus* using a mixture of the 4 probes. (a) Nervous tissue (here a segmental ganglion) demonstrates a weak positive reaction (arrows). (b) The striated muscle of the cephalothorax exhibits some signs of the infection (arrows). (c) Some nuclei of the spongy connective tissue of the hepatopancreas show a positive reaction (arrows). Note the weak positive reaction on the apical side (in the microvillous border and the cytoplasm) of 1 digestive cell (arrowhead). Light microscopy. Scale bars = 20 µm

Some necrosis of the epithelial tissues, caused by the virus infection, was noted. In 1 infected specimen, there was a necrotic area of the stomach epithelium and labeling was particularly strong due to the accumulation of infected hemocytes in the connective tissue.

No reaction was observed in the epithelial cells of the hepatopancreatic tubules or the midgut mucosa. In 1 specimen, superficial labeling was observed in the apical part of some epithelial cells of the hepatopancreas, but the nuclei were clearly unaffected (Fig 7c).

DISCUSSION

After purification, negatively stained viral particles of WSBV originating from infected *Penaeus monodon* were ovoid and possessed an envelope extension. The unenveloped nucleocapsids exhibited a superficially segmented appearance. In addition, some nucleocapsids showed a larger size and a different superficial structure which has not been reported before. These nucleocapsids seemed to be distended, particularly because of the variation in size and the angle of the cross-hatching. Thus, we attribute this phenomenon to an osmotic effect which occurred during the purification or the negative staining steps. These particles could also correspond to degradation steps (Fig. 2). For the moment, we have no explanation regarding the presence of short truncated nucleocapsids (enveloped or not), but attempts to isolate these particles are in progress.

Negatively stained virions and nucleocapsids appeared to be larger than the published measurements of other WSS viruses that were obtained from ultra-thin sectioned infected tissue (Huang et al. 1994, Inouye et al. 1994, Nakano et al. 1994, Takahashi et al. 1994, Chou et al. 1995). This size difference can be attributed to different measurement techniques being used (in negatively stained preparations or in ultra-thin sections). The viruses observed in negatively stained preparations and described as SEMB virus (Wongteerasupaya et al. 1995), WSS virus (Wang et al. 1995) and RV-PJ (Momoyama et al. 1995) are morphologically very similar to WSBV. They also have in common the segmented superficial appearance of the nucleocapsids and an envelope extension of the virions. Considering the structure of the particle in negatively stained suspensions, WSBV appears to be closely related to the baculovirus of the crab *Carcinus maenas* (Bazin et al. 1974, Bonami 1980) and to the baculovirus called B2 in *C. mediterraneus* (Mari 1987). These 3 baculoviruses have in common an ovoid virion with an envelope extension (Bazin et al. 1974, Mari 1987), a superficial segmentation of the nucleocapsid (Mari 1987, Bonami 1980), and a development in connective tissue cells and hemocytes. Baculovirus B described by Johnson (1977) in *Callinectes sapidus* also develops in connective tissues and hemocytes, but no data are available on the particle structure as observed by negative staining

Using the constructed probes, *in situ* hybridization in WSBV-infected shrimp sections clearly localized the viruses. Strong labeling was limited to infected nuclei and was stronger when there was hypertrophy of the nucleus. Infection was detected in the connective tissues, hemolymph, gills, epidermis and the stomach. Tsing (1987) determined that hemocytes fixed to the tissues are phagocytes that are morphologically similar to those that circulate in the hemocoel. The differentiation of these 2 hemocyte types with the light microscope is very difficult. However, in several infected specimens, strong labeling of hemocyte aggregations in association with epithelial necrosis of the stomach confirmed the infection of circulating hemocytes. We

suppose that fixed hemocytes were also infected, but we could not conclusively demonstrate this possibility. The target tissues for WSBV detected by the probes are the same as those reported by Chen (1995), Inouye et al. (1994) and Wongteerasupaya et al. (1995). Other tissues, such as the muscle and nerve, showed weak labeling This labeling indicated a light infection of WSBV, although these tissues had not been reported before by histology to be target tissues for WSBV. With *in situ* hybridization, we detected light infections in others tissues of mesodermal and ectodermal embryonic origin, confirming the results of Wongteerasupaya et al. (1995).

Epithelial cells of the midgut and hepatopancreas were not infected. However, in 1 shrimp, labeling of the apical part of columnar epithelial cells of hepatopancreatic tubules was observed. The presence of viral nucleic acid there could have resulted from the absorption of virions from released infected cells, from fragments of infected, ingested animals (cannibalism), or from necrosis of the epithelium of the stomach where disrupted cells could release virions into the lumen of the midgut and hepatopancreatic tubules during ecdysis.

When the level of infection is very low, histological diagnosis is particularly difficult. In contrast, diagnosis with these gene probes is highly specific and sensitive and it could be used as an early diagnostic tool for WSBV infection using hemolymph samples from live shrimp.

This virus consists of a singly enveloped rod-shaped nucleocapsid. Its nucleic acid is a single molecule of double-stranded DNA with a high MW. Replication appears to be exclusively in the nucleus, and occlusion bodies are not produced. Therefore, WSBV is closely related to the *Baculoviridae* family and to the *Nudibaculovirinae* subfamily according Francki et al. (1991).

WSBV and the baculoviruses already reported to occur in WSS have common target tissues and histological characteristics. These similarities suggest that they may be the same type of virus. On the other hand, comparison of the *Eco*R I electrophoretic patterns of WSBV (Fig. 4) and SEMBV (Wongteerasupaya et al. 1995) showed differences that indicated a possible virus diversity. However, this difference might also be attributed to incomplete digestion of the WSBV genome. For the moment we do not have enough data on these virus genomes to conclude whether they are the same or related viruses. The complete cloning of the WSBV genome and the use of gene probes could be useful in determining the possible differences between these WSS viruses. To date, the use of these constructed gene probes gives the pathologist a new specific and sensitive tool for the diagnosis of this disease, particularly for low level infections.

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