

## Detection of infectious hypodermal and hematopoietic necrosis virus (IHHNV) in *Penaeus monodon* by *in-situ* hybridization at transmission electron microscopic level

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### Abstract

*In-situ* hybridization procedure was developed to detect infectious hypodermal and hematopoietic necrosis virus (IHHNV) of infected juvenile *Penaeus monodon* at the ultrastructural level. The tissues were fixed, dehydrated, and embedded in Unicryl<sup>TM</sup> hydrophilic resin. A 692 bp IHHNV-specific DNA probe, labeled with DIG-11-dUTP, was tested on both semi-thin and ultra-thin sections, and the sections were examined under light and transmission-electron microscopes, respectively. The hybridized probe was detected by means of an anti-DIG antibody conjugated to 10 nm gold particles, followed by silver enhancement. This technique allows IHHNV to be tracked in the infected cell, from entry into the cytoplasm, nuclear penetration, through replication in the nucleus, and release from the cell by exocytosis. This report is the first description of *in-situ* hybridization to detect IHHNV at the ultrastructural level. The result showed positive *in situ* hybridization of IHHNV-infected shrimp tissue at TEM in nucleolus and cytoplasm.

**Keywords:** IHHNV, *in-situ* hybridization for TEM, *Penaeus monodon*, virus tracking

### บทคัดย่อ

วิธี *in situ* hybridization ถูกพัฒนาขึ้นมาเพื่อใช้ตรวจหาเชื้อ infectious hypodermal and hematopoietic necrosis virus (IHHNV) ในกุ้งกุลาล่า ค้ำระยะวัยรุ่น ระดับอเล็กตรอนไมโครสโคป จากตัวอย่างกุ้ง ที่ติดเชื้อไวรัส IHHNV เริ่มโดยนำเนื้อเยื่อมา fix ด้วย fixative และตามด้วยขบวนการดึงน้ำ ออกจากเซลล์เนื้อเยื่อ และกำซาบเนื้อเยื่อนั้นด้วยเรซินยูนิคริลเข้าไปในเซลล์ จากนั้นก็เตรียม 692 bp IHHNV DNA probe ซึ่งมี DIG-11-dUTP ติดอยู่ นำ probe นี้ไปตรวจการเชื้อ IHHNV ในตัวอย่างชิ้นเนื้อที่ตัดแบบ semi-thin section แล้วส่องดูด้วยกล้องจุลทรรศน์ และตัดแบบ ultra-thin section แล้วส่องดูด้วยกล้องจุลทรรศน์อเล็กตรอนไมโครสโคป Hybridized probe นี้จะจับกับ แอนติบอดีสำหรับ DIG ที่ติดอยู่กับเม็ดทองที่มีขนาด 10 nm จากนั้น ก็จะเสริมการมองเห็นเม็ดทองให้ชัดเจนด้วยสังกะสี แล้วส่องดูด้วยกล้องจุลทรรศน์อเล็กตรอน เทคนิคนี้จะใช้สำหรับดูขบวนการของไวรัส IHHNV ในการเข้าสู่เซลล์กุ้ง โดยดูการเคลื่อนตัวของไวรัสที่ผ่านเข้า cytoplasm กุ้ง จนกระทั่งเข้าไปในนิวเคลียส แล้วเริ่มมีการจำลองตัวเองและเพิ่มจำนวนไวรัส ในนิวเคลียสกุ้ง แล้วเคลื่อนตัวออกมาใน cytoplasm ของเซลล์กุ้ง วิธีการ *in situ* hybridization ด้วย TEM นี้เพิ่งใช้ในการตรวจหาขบวนการเข้าสู่เซลล์กุ้ง และเพิ่มจำนวนในกุ้งเป็นครั้งแรก ผลพบ positive *in situ* hybridization ของไวรัส IHHNV ในกุ้งที่ติดเชื้อชนิดนี้ทั้งใน nucleolus และใน cytoplasm

**คำสำคัญ:** IHHNV, *in-situ* hybridization for TEM, *Penaeus monodon*, ขบวนการเคลื่อนตัวของไวรัสในเซลล์กุ้ง

### 1. Introduction

In shrimp farming, most failures are due to viral pathogens. The two most prominent viruses causing mass mortality in Thailand have been yellow-head disease (YHD) and white-spot syndrome (WSD). In addition, several other diseases, while not fatal to shrimp, retard growth and impair their health; these include infections by hepatopancreatic parvovirus (HPV), monodon baculovirus (MBV) and infectious hypodermal and hematopoietic necrosis virus (IHHNV) (Flegel & Fegan, 1995; Flegel 1997; Lightner & Redman,

1998; Flegel, Thamavit, Pasharawipas, & Alday-Sanz, 1999; Chayaburakul, Nash, Pratanpipat, Sriurairatana, & Withyachumnarnkul, 2004; Chayaburakul, Lightner, Sriurairatana, Tang-Nelson, & Withyachumnarnkul, 2005).

The disease caused by IHHNV, a non-enveloped 22 nm DNA virus, was first recognized in 1981 at an experimental shrimp culture facility on Oahu, Hawaii. Pacific blue shrimp, *Litopenaeus stylirostris*, were severely affected by a highly acute and lethal disease (Lightner, Redman, & Bell, 1983a, b; Bell and Lightner, 1984). Natural infections have

been observed in *L. stylirostris* and *L. vannamei*, which are American Pacific species, and in *P. monodon* and *P. semisulcatus*, which are Indo-Pacific species (Lightner, 1999). The virus has been experimentally shown to infect other penaeid species, and can probably infect all penaeids (Lightner, 1999).

IHHNV infection has different outcomes among different penaeid shrimp species. In *L. stylirostris*, it results in severe mass mortality, in *L. vannamei* it causes runt-deformity syndrome (RDS), while in *P. monodon*, the disease has a low impact on shrimp growth and reproduction (Withyachumnarnkul, Chayaburakul, Loa-Aroon, Plodpai, Sritunyalucksana & Nash, 2006).

The detection of IHHNV in shrimp by polymerase chain reaction (PCR) methods has become more complicated, since a fraction of positive cases may not be infections, but rather IHHNV DNA fragment insertions into the shrimp genome (Saksmerprome, Jitrakorn, Chayaburakul, Laiphrom, Boonsua, & Flegel, 2011).

## 2. Objectives

The purpose of this study is to be tracked in the IHHNV infected *P. monodon* cell, from entry into the cytoplasm, nuclear penetration, through replication in the nucleus, and release from the cell by exocytosis.

## 3. Materials and methods

**Shrimp species.** IHHNV-infected juvenile *Penaeus monodon* from the East coast of Thailand were brought to our laboratory.

**Fixation, embedding, and sectioning.** Three different fixatives were tested to determine which preserved ultrastructure without interfering with *in-situ* hybridization with a DIG-labeled IHHNV probe (515 bp). The fixatives were 6% glutaraldehyde. All fixatives were prepared with 0.15 M Millonig's phosphate buffer (pH 7.0), supplemented with 1% sodium chloride and 0.5% sucrose (Lightner, 1996). Ice-cold fixative, approximately 1/10 of the total volume of the shrimp, was first injected into the hepatopancreas prior to it being dissected and cut into small pieces (~1 mm<sup>3</sup>) in ice-cold phosphate buffer. Tissue pieces from each shrimp were transferred to 1 ml of the same fixative and fixed for 6 h under refrigeration at ~4°C. Gill lymphoid organ samples from 5 shrimp per species were fixed in each fixative.

**After fixation,** tissues were rinsed twice with ice-cold phosphate buffer and divided into 2 portions, one of which was post-fixed with 1% osmium tetroxide (in phosphate buffer) for 1 h at room temperature (RT = 25°C) and the other not. From this point, all samples were processed in the same way. Specimens were dehydrated at RT in a graded series of ethanol (15 min each in 30, 50, 70, 80, 95%, and twice in absolute ethanol) and infiltrated at 4 °C with increasing concentrations of the hydrophilic resin, Unicryl™ (British Bio Cell International Ltd, Golden Gate, Cardiff, UK), as follows: 24 h in resin: absolute ethanol (1:2), 24 h in resin: absolute ethanol (2:1) followed by 24 h in pure resin. Resin-infiltrated specimens were transferred into Beem capsules containing fresh resin and polymerized at -10°C for approximately 72 h by exposure to UV light provided by 2x15 W Phillips UV lamps, 360 nm wavelength, set about 15 cm below the Beem capsules.

Semi-thin sections (1 µm thickness) were placed on a drop of double-distilled water on a standard-microscope glass slide and heat-dried at 60°C for 1 to 2 min, stained with 1% toluidine blue in 1% sodium borate at 60°C for 1 min, and then observed with a light microscope for the presence of diagnostic IHHNV intranuclear inclusions (Lightner, 1996). Consecutive semi-thin sections were placed on drops of high performance liquid chromatography (HPLC) quality water on a superfrost/Plus, positively charged, microscope slide (Fisher Scientific, Pittsburgh, PA), heat dried and stored at room temperature until needed (2 to 3 d). Four such slides were prepared from each block. Two to 3 consecutive ultra-thin sections (gold interference color) from the same blocks were placed on each of 5 carbon/Formvar-coated 100 mesh nickel grids and stored, unstained, at RT until needed (not more than one week)

**Preparation of CK 515 probe.** A 515 bp fragment from the HPV DNA genome was amplified by PCR using purified IHHNV genome as a template (supplied by the Department of Veterinary Science and Microbiology, University of Arizona, Tucson, AZ, USA) and specific primers designed by Primer 4 program (Scientific & Educational Software, PA). The amplified product was electrophoresed in 1% low-melting-point agarose gel, the band was excised, and the DNA purified using agarose (Roche Molecular Biochemicals),

according to the manufacturer's protocol. DNA labeling (designated CK 515): the purified 515 bp product was randomly labeled with digoxigenin (DIG)-11-dUTP using a Genius I kit (Roche Molecular Biochemicals) according to the manufacturer's protocol. Labeled DNA was visualized with alkaline phosphatase-conjugated anti-digoxigenin antibody and the substrate nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indoyl phosphate (BCIP), supplied with the Genius I Kit.

***In-situ* hybridization for light microscopy.** The procedures used were based on Carlos and Lightner (2001). Hybridization of the CK 515 probe to IHHNV nucleic acids in the resin-embedded gill was first assessed by light microscopy. Positive and negative controls for this phase of the study were paraffin sections of IHHNV-infected *Penaeus monodon* and IHHNV non-infected *P. monodon*, respectively. Paraffin sections were subjected to standard procedures reported elsewhere (Lightner, 1996), except that the probe detection step was modified for use with plastic sections, as described below.

*In-situ* hybridizations on semi-thin plastic sections were performed in duplicate, with 1 group of slides used as a negative control (i.e., no probe added to the hybridization solution). Sections were re-hydrated at RT by immersion for 5 min in HPLC water followed by 5 min TNE (50 mM Tris-HCl, 10 mM NaCl, 1 mM EDTA, pH 7.4). To each section, 100  $\mu\text{g ml}^{-1}$  Proteinase K (Sigma Chemical, St. Louis, MO) in 1 TNE were added for 15 min at 37 °C.

Proteinase K was inactivated by a 5 min rinse in ice-cold 0.4% formaldehyde and sections were soaked 5 min in 2x standard saline citrate (SSC; 1x = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0). 0.5 ml of hybridization solution (50% formamide, 0.02% Ficoll 400, 0.02% polyvinylpyrrolidone (PVP) 360, 0.02% bovine serum albumin [BSA], 5% dextran sulfate, 0.5  $\text{mg ml}^{-1}$  denatured salmon DNA, 4xSSC) were poured onto the sections and the slides were incubated in a humid chamber at 37°C for 30 min, quenched on ice, and diluted to 17.5  $\text{ng } \mu\text{l}^{-1}$  in ice-cold hybridization solution. A probe (250  $\mu\text{l}$ ) was placed onto each of the slides, which had been incubated overnight at 37°C in a humid chamber. Negative control slides were incubated in hybridization solution only.

Post-hybridization washes employed decreasing concentrations of SSC buffer at 37°C (2 x 5 min each in 2 x SSC, 1 x SSC, 0.5 x SSC and 0.1 x SSC). Slides were soaked for 5 min at RT in Buffer I

(0.1 M Tris-HCl, 0.15 M NaCl, pH 7.5) and blocked for 15 min at 37°C with 0.5 ml of 10  $\text{ng ml}^{-1}$  Blocking reagent (Roche Molecular Biochemicals) in Buffer I. Hybridization probe was detected with sheep anti-digoxigenin antibody conjugated to 10 nm gold particles (British BioCell International Ltd; OD [optical density]<sub>520</sub> = 3.0) diluted to 3.49  $\mu\text{g ml}^{-1}$  in blocking buffer. Diluted conjugate (10  $\mu\text{l}$ ) was placed onto the sections, which were covered with a glass cover slip and incubated in a humid chamber at 37°C for 30 min. Unreacted gold conjugate was removed by rinsing slides 4 times for 5 min each in Buffer I at RT, followed by 4 separate 5-min rinses in HPLC water. Silver enhancement was performed with a silver enhancing kit (British BioCell International, Ltd). Silver enhancing solution (20  $\mu\text{l}$ ) was placed onto sections, which were covered with a cover-slip and incubated at 28°C for 1.5 h in the dark. The reaction was stopped by immersion in HPLC water for 15 min. Slides were heat-dried in 1% sodium borate at 60°C for 0.5 min, mounted with Permount (Fisher Scientific) and examined using a bright field light microscope for black silver precipitate, which indicated probe hybridization.

***In-situ* hybridization for electron microscopy** After having confirmed the ability of probe CK 515 to detect IHHNV in semi-thin plastic sections, ultra-thin sections were hybridized with the same probe. Except for a few modifications, the hybridization protocol for ultra-thin sections used a similar methodology. All reactions were performed by floating the grids, section side down, on 20  $\mu\text{l}$  drops of reagent. All buffers, solutions and incubation temperatures were the same, unless stated otherwise. Excess solution on the grids was blotted away between steps and all incubations were conducted inside a humid chamber.

Briefly, grids were re-hydrated, incubated for 5 min in TNE, and treated with 100  $\mu\text{g ml}^{-1}$  Proteinase K for 5 min. Proteinase K was inactivated as above and grids incubated for 5 min in 2 x SSC and for 30 min in hybridization solution. The grids were incubated overnight (approximately 16 h) on drops of denatured CK 515 probe (17.5  $\text{ng ml}^{-1}$ ) in hybridization solution (test group), or in hybridization solution alone (negative controls). Post-hybridization washes were performed on decreasing concentrations of SSC buffer, as above, after which the grids were soaked for 5 min in Buffer I and for 15 min in blocking buffer.

The DIG-labeled probe was detected by floating the grids on 10  $\mu\text{l}$  drops of anti-DIG gold

conjugate for 30 min. Grids were rinsed 4 times for 5 min each in Buffer I, followed by 4 x 5 min rinses in HPLC water prior to silver enhancement in 20  $\mu$ l drops of silver enhancing solution for 5 min at 18 °C, in the dark. The reaction was terminated by floating the grids on HPLC water for 15 min followed by air-drying. Finally, sections were stained with lead citrate and uranyl acetate and viewed using a Phillips CM 12 electron microscope operated at 80 kV.

#### 4. Results

##### *In-situ* hybridization for light microscopy

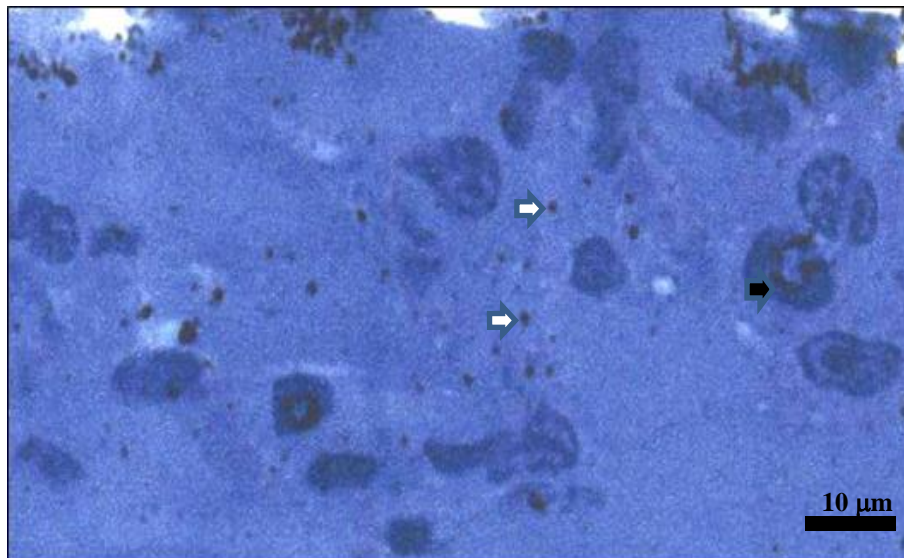
With *in-situ* hybridization at the light-microscope level of semi-thin sections, a black precipitate indicating the site of hybridization was observed on the cytoplasm and the peripheral zones of nuclei of infected cells. Significant non-specific deposition of silver was detected within the cytoplasm of infected gill cells in semi-thin sections (Figure 1).

##### *In-situ* hybridization for electron microscopy

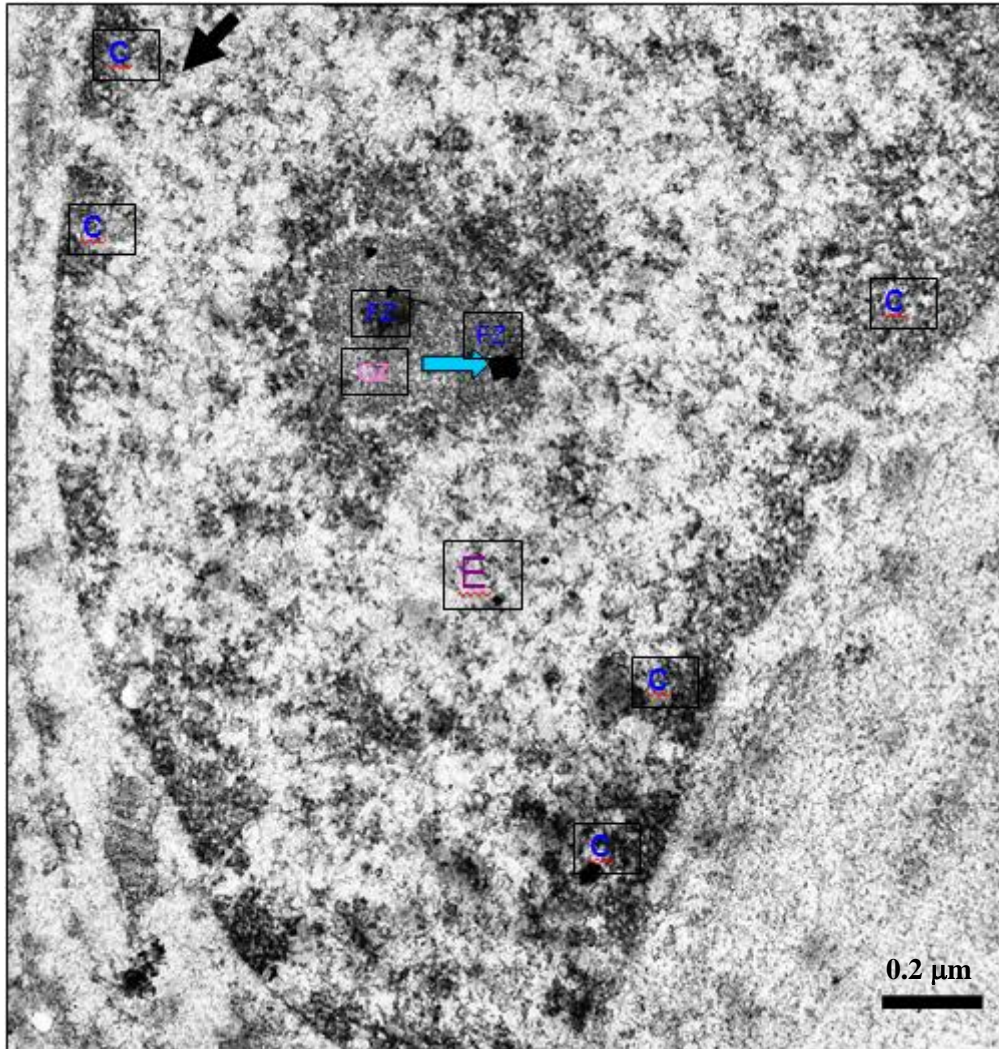
Positive IHHNV blocks were examined for *in-situ* hybridization at the transmission electron-microscopic level using the specific probe conjugated with 10 nm gold particle instead of DIG, as in conventional *in-situ* hybridization by light microscope. The results of *in-situ* hybridization were checked by electron microscopy for agreement with those obtained by light microscopy on semi-thin sections. The cellular structure of intranuclear IHHNV-infected cells displayed the strongest reaction to probe CK515.

According to the results, gold particle presented at the central fibrillar zone (FZ; nucleolar organizer chromosome) of the nucleolus of the cells and scattered in the karyoplasms at peripheral clumps of heterochromatin of the nucleus of the cells (Figure 2). However, the cytoplasmic probe signal was rather scarce.

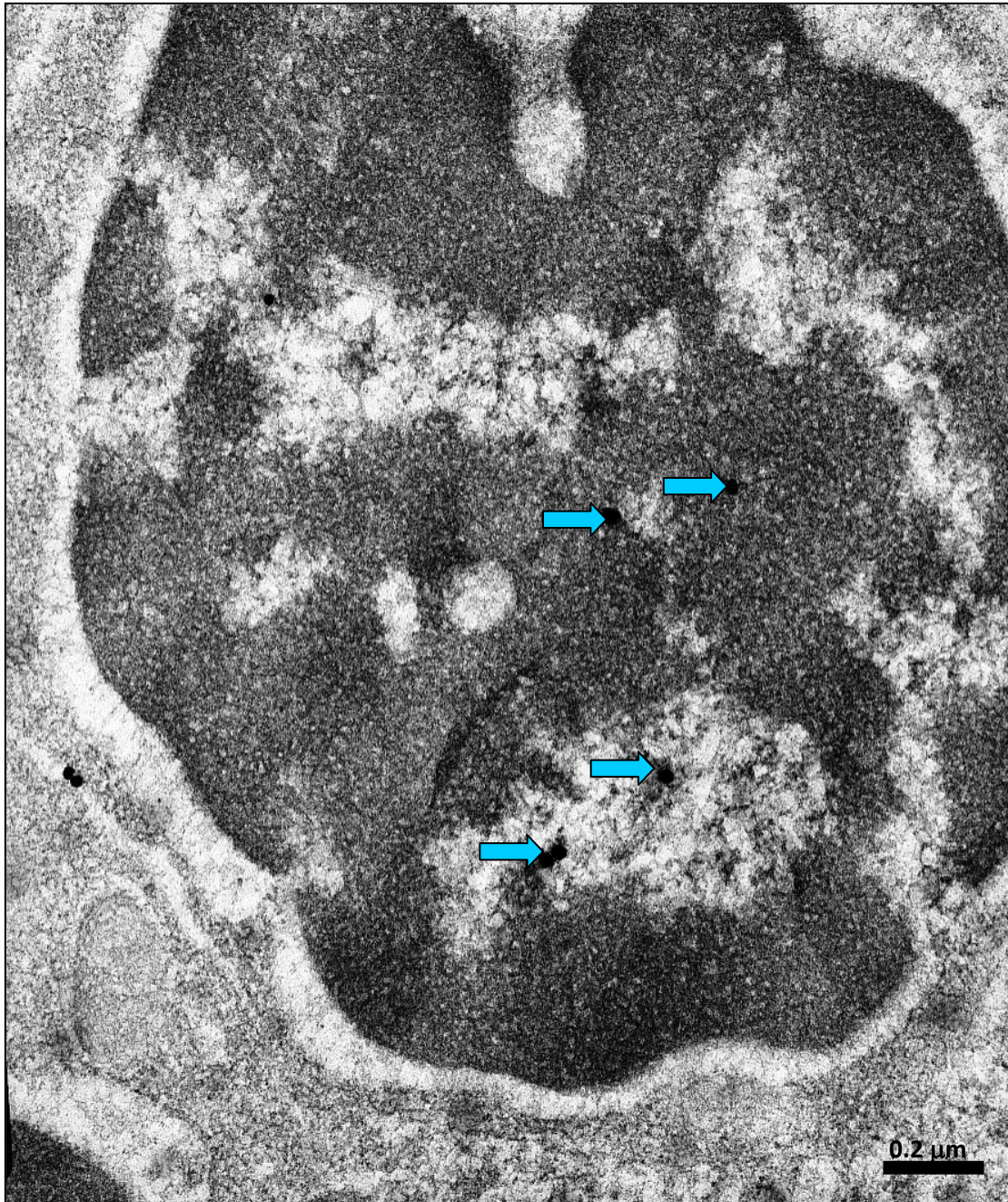
The result also found the early apoptotic body that was triggered by IHHNV to the shrimp cells, and showed positive *in-situ* hybridization at the electron-microscopic level (Figure 3).



**Figure 1** Semi-thin section of *in-situ* hybridization for TEM: positive for IHHNV in the nucleus (black arrows) and cytoplasm (white arrow).



**Figure 2** TEM micrograph showing the interphase nucleus with chromatin DNA lying at the periphery of the nucleus, as well as the nucleolus and RNA. DNA is localized in the central fibrillar zone (FZ; nucleolar organizer chromosome) of the nucleolus, surrounded by a coarser granular zone (GZ), rich in RNA. The DNA-containing chromatin (heterochromatin; dense coiled chromosome) appears as peripheral clumps of material (Ch). Some also occurs near the nucleolus. The less dense region of the nucleoplasm represents the uncoiled regions (euchromatin; Eu), where messenger RNA is being actively synthesized. Positive ISH TEM in FZ (arrows) may be the location of IHNV replication.



**Figure 3** TEM micrograph showing the early apoptotic nucleus (condensation of chromatin) appears as a dense nucleus (coiled chromatin). DNA fragments in the apoptotic nuclei are likely detected by DNA probe for IHHNV (arrows). Apoptosis is caused by IHHNV.

## 5. Discussions

The differences in hybridization intensity between the semi-thin sections and the ultra-thin sections may be due to the ultra-thin sections not heating sufficiently to denature the double-stranded DNA into single-stranded DNA, so that some IHHNV DNA in the ultra-thin sections, which were already double-stranded DNA could not bind with the single-stranded DNA probe, even though IHHNV is a single-stranded DNA virus.

As would be expected, the strongest hybridization signal was observed in intranuclear-infected IHHNV cells, the site of replication center of parvovirus and marginated chromatin. The results showed IHHNV in the peripheral zone of the nucleus, and also the central fibrillar zone of the nucleolus, where the DNA in normal cells is located (Porter, and Bonneville, 1973). It is possible that IHHNV, a single-stranded DNA virus, duplicates itself in the central fibrillar zone of the nucleolus, and matures in the peripheral zone of the heterochromatin or marginate chromatin.

These findings suggest that IHHNV replicates in the nucleolus and maturation occurs in the nucleoplasm. The finding of signal hybridization in some nuclei with chromatin margination also suggests that marginate chromatin in the nucleoplasm may be the site of precursors to virogenic stromata, because, in the sample in this experiment, the inclusion body (believed to be the site of accumulation of mature virions in other viruses) could not be found. The IHHNV-positive results in the peripheral zone of the heterochromatin and nucleolus suggest that the IHHNV virus might duplicate itself within the nucleus, like other DNA viruses.

Normally, virus particles are located in the nucleolus; however, high TEM magnifications are unable to confirm or visualize the particles of a specific virus in the nucleolus. However, *in-situ* hybridization at electron-microscopic level has enabled image enhancement for the investigation of viruses, especially in the central fibrillar zone of the nucleolus and the marginated chromatin in the nucleoplasm.

Moreover, *in-situ* hybridization at the electron-microscopic level enabled the finding of a positive result in the apoptotic body, which normally could not be found by conventional TEM and *in-situ* hybridization by light microscope alone. When these two techniques were combined, the signal could be enhanced, enabling the investigation of smaller

particles not possible using conventional TEM and *in-situ* hybridization by light microscope alone. Another benefit of this technique is that it can distinguish viruses of similar size and shape as IHHNV from others, such as Leamsigha virus (Pratoomthai, Sakaew, Sriurairatana, Wongprasert, and Withyachumnarnkul, 2008).

Shrimp may use apoptosis to limit viral Sriurairatana, Tang-Nelson, and Withyachumnarnkul, 2005), triggered by a given level of viral infection; this would help increase shrimps' tolerance to IHHNV. However, if large amounts of IHHNV infect shrimp simultaneously, the shrimp may generate excessive apoptosis, and die from the apoptosis.

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