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Parvo-like virus in the hepatopancreas of freshwater prawns *Macrobrachium rosenbergii* cultivated in Thailand

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ABSTRACT: A survey of cultivated giant freshwater prawns *Macrobrachium rosenbergii* from Thailand revealed the presence of unusual spherical to ovoid inclusions in nuclei of hepatopancreas tubule epithelial cells. These began as small eosinophilic inclusions that became more basophilic as they increased in size. They were present in both R-cells and E-cells but were largest and deeply basophilic only in the E-cells. Confocal laser microscopy revealed that stained nucleic acid fluorescence from the inclusions was lost by treatment with DNase I specific for double- and single-stranded DNA and also lost or reduced by treatment with mungbean nuclease specific for single-stranded nucleic acids. Transmission electron microscopy (TEM) revealed that the inclusions contained tightly packed, unenveloped, viral-like particles of approximately 25 to 30 nm diameter, resembling those produced by shrimp parvoviruses. However, PCR, *in situ* hybridization and immunohistochemical tests for shrimp parvoviruses previously reported from Thailand were all negative. These results suggested that the inclusions contained a parvo-like virus, not previously reported from *M. rosenbergii* in Thailand.

KEY WORDS: *Macrobrachium rosenbergii* · Hepatopancreas · Parvo-like virus · Histopathology · Electron microscopy

INTRODUCTION

Although the giant freshwater prawn *Macrobrachium rosenbergii* is an important aquaculture species in Thailand, cultivation is still dependent on stocked postlarvae derived from captured wild broodstock or from a proportion of harvested shrimp reared, generation to generation, in outdoor, earthen cultivation ponds. In order to overcome production problems caused by disease losses, it is essential to develop domesticated, specific pathogen-free founder stocks maintained under strict quarantine in a permanent central breeding facility. An initial need for development of such stocks is the preparation of a list of excludable pathogens for screening of the founder stocks. Of particular importance for this list would be viral pathogens such as Macrobrachium rosenbergii nodavirus (MrNV) and extra small virus (XSV) that are causally related to white tail disease (Qian et al. 2003, Sahul Hameed et al. 2004, Bonami et al. 2005, Yoganandhan et al. 2006) and result in severe mortality in hatcheries. These viruses may be transmitted vertically from naturally infected broodstock to larvae (Sudhakaran et al. 2007) or via reservoir carriers that may include other shrimp (Sudhakaran et al. 2006a,b) or even aquatic insects (Sudhakaran et al. 2008). The only other virus reported from

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natural infections of *M. rosenbergii* is similar to the one known as hepatopancreatic parvovirus (HPV) in penaeid shrimp (Anderson et al. 1990, Lightner et al. 1994) based on histopathology (Bonami et al. 1995, Lightner 1996). A recent proposal to the International Committee on Taxonomy of Viruses (Tijssen 2008) recommends that these penaeid shrimp parvoviruses be included in a new genus, *Hepanvirus*, in the sub-family *Densovirinae* of the family *Parvoviridae* as Penaeus monodon densovirus (PmDNV), Penaeus merguiensis densovirus (PmergDNV) and Penaeus chinensis densovirus (PchinDNV). These differ in genome size and organization and target tissue from another penaeid shrimp parvovirus also included in the sub-family *Densovirinae*, but in the genus *Brevidensovirus*, as Penaeus stylirostris densovirus (PstDNV) (Tattersall et al. 2005). PstDNV was formerly called infectious hypodermal and hematopoeitic necrosis virus (IHHNV) (Lightner 1996). The newly recommended names (Tattersall et al. 2005, Tijssen 2008) will be used in this manuscript, as will the ICTV method of naming shrimp viruses with non-italicized host species names and abbreviations (Fauquet et al. 2005).

For successful specific pathogen free (SPF) stock development, diagnostic methods would be required for all of the pathogens included in any specific list of pathogens for *M. rosenbergii*. For this species, highly sensitive RT-PCR methods are available for MrNV and XSV (Romestand & Bonami 2003, Sri Widada et al. 2003, 2004, Yoganandhan et al. 2005, Pillai et al. 2006). However, for other viral pathogens, such as the parvo-like virus described above, no molecular methods have been developed.

As a preliminary step towards the development of domesticated stocks, cultivated *Macrobrachium rosenbergii* is being surveyed by routine histology for evidence of potential viral pathogens that might have a negative effect on production. The overall objective is to develop a set of molecular tools for their rapid detection. Here we describe the histopathology of a parvovirus-like agent in the hepatopancreas of *M. rosenbergii* from hatcheries in Thailand.

MATERIALS AND METHODS

Shrimp specimens. Samples of 2 postlarvae (PL) batches (collected 27 Feb and 3 March 2007) and 2 female broodstock specimens (14 and 19 June 2007) were obtained from hatcheries in Ratchaburi province Thailand. The living shrimp were transported to the laboratory in Styrofoam boxes containing hatchery water aerated with oxygen.

Histology. The living shrimp were fixed with Davidson's fixative and processed for routine histology using hematoxylin and eosin (H&E) staining as described by Bell & Lightner (1988). The paraffin-embedded tissue sections were also used for *in situ* hybridization tests and immunohistochemical analysis (see below).

Transmission electron microscopy. Hepatopancreatic tissues of approximately 1 mm^3 were fixed for 2 h at 4°C with 4.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. The fixing solution was removed and the tissues were postfixed in 1% (w/v) osmium tetroxide in 0.1 M phosphate buffer at pH 7.4 for 2 h, washed twice with distilled water, and dehydrated in a graded series of ethanol solutions from 50 to 100%, followed by 2 immersions in 100% propylene oxide. Tissues were embedded in Epon-812 resin by successive 1 h infiltrations of 1:1 and 2:1 resin:propylene oxide, and 100% resin. The tissue blocks were then polymerized by incubating at 70°C for 48 h in fresh 100% epoxy resin. Thin sections were stained in 2% (w/v) uranyl acetate and 0.3% (w/v) lead citrate solutions and viewed in a Hitachi H7100 electron microscope at 100 kV.

PCR methods. Shrimp DNA was extracted using a Genomic DNA purification kit (Fermentas), and concentration and quality were measured by spectrophotometric analysis at 260 and 280 nm. For these assays, a commercial detection kit HPV 465/183-IC nested-PCR was used (Ezee Gene kit, Shrimp Biotechnology Business Unit, Thai National Center for Genetic Engineering and Biotechnology) for detection of HPV of *Penaeus monodon* based on the method of Phromjai et al. (2002), which targeted a 441 base target at positions 60 to 500 of GenBank record AF456476, which are equivalent to positions 4189 to 4619 of the complete Thai-HPV sequence (GenBank record NC007218). As indicated in the 'Introduction,' there is a proposal before the ICTV to rename this virus PmDNV in the genus *Hepanvirus*. Also used was an IQ2000 detection kit (Farming Intelligene) for detection of IHHNV that is tentatively listed by ICTV as PstDNV (Tattersall et al. 2005) in the genus *Brevidensovirus*. This kit was produced in 2006, targeted positions 1400 to 1788 of the IHHNV reference sequence (GenBank accession number AF218266), and gave a positive reaction amplicon of 389 bp. All these kits included positive controls and internal controls to verify the integrity of the PCR reactions.

***In situ* hybridization.** Labeled DNA probes for the hepanvirus PmDNV and PstDNV were prepared using a commercial PCR DIG-labeling mix (Roche Molecular Biochemicals) according to the manufacturer's instructions. Primers used for the labeling reactions were obtained from the commercial kits described above and the DNA templates consisted of plasmids containing matching inserted target fragments appropriate for each virus. A ClustalW analysis of the sequences of the 2 probes (www.ebi.ac.uk/Tools/clustalw2/) re-

vealed only 3% sequence identity between the probes, assuring that there would be no cross reaction for target sequences. These labeled probes were used for standard *in situ* hybridization assays as previously described (Sritunyalucksana et al. 2006), with tissue sections derived from hepatopancreatic tissue of *Macrobrachium rosenbergii* showing parvovirus-like inclusions.

Immunohistochemistry. We placed paraffin-embedded tissue sections of 7 µm thickness on albumin-coated glass microscope slides and deparaffinized them in xylene 3 times for 5 min before re-hydration with a graded solvent series (N-butyl alcohol followed by ethanol 95%, 90%, 80%, and 70%) for 5 min each. After washing with distilled water for 5 min, the sections were re-fixed with 10% formalin for 10 min, and then washed with PBS, pH 7.2, 3 times for 15 min each. After blocking with P_1^+ (10% calf serum in PBS, pH 7.2) for 30 min, they were incubated with MAb specific to PstDNV (Bui 2007) or PmDNV (Rukpratanporn et al. 2005) for 5 h at 37°C or overnight at 4°C. The MAb was removed by washing the sections in PBS, pH 7.2, 3 times for 10 min each. Then 2 drops of GAM-HRP diluted 1:1000 was added to cover the sections and they were incubated at 37°C for 3 hr. After washing in PBS, pH 7.2, 3 times for 10 min each, they were incubated in substrate-chromogen mixture working solution (0.03% DAB and 0.006% H_2O_2) for 5 min. The slides were rinsed with distilled water and counterstained with hematoxylin and eosin-Y (H&E) or with eosin-Y only. Positive immunoreactions were visualized as brown coloration at the site of viral infection against the purple-blue of hepatopancreatic nuclei and pink cytoplasm with H&E or against pink cytoplasm with eosin-Y. Positive controls for these tests were *Penaeus monodon* tissues infected with PmDNV and *P. vannamei* tissues infected with PstDNV.

***In situ* enzyme assays.** Sections (5 µm) from paraffin-embedded tissues of *Macrobrachium rosenbergii* showing intranuclear inclusions were de-waxed with xylene and rehydrated through an ethanol series to distilled water before being treated with 100 µl of Proteinase K solution (20 µg ml^{-1} in 1× TNE buffer) for 30 min at 37°C in a humid chamber. They were then washed 3 times with TNE buffer and immersed in distilled water for 5 min before excess solution was blotted off. The sections were then incubated for 24 h with 100 µl of enzyme reaction mixtures that comprised 50 U of either DNase I (Fermentas) or mung bean nuclease (Biolabs). Controls consisted of sections incubated with buffer solution only. The sections were washed 3 times for 5 min each with 0.05% Tween® 20 in PBS, stained for 1 h with the nucleic acid stain To-Pro-3® iodide (Invitrogen) diluted 1:1000 in PBS and washed 3 times for 5 min each with 0.05% Tween® 20 in PBS. Finally, a drop of antifade reagent (ProLong® Gold: Molecular Probes) was added before covering with a coverglass and viewing with a confocal laser microscope.

RESULTS AND DISCUSSION

Histopathology

The hatchery larvae and broodstock of *Macrobrachium rosenbergii* showed unusual spherical to ovoid inclusions in tubule epithelial cells of the hepatopancreas. These ranged from small, eosinophilic inclusions of graded sizes to larger, more basophilic inclusions (Fig. 1), suggesting graded development. They were present in both R-cells and E-cells but were largest and deeply basophilic only in the E-cells. In early stages, the affected E-cell nuclei were not greatly enlarged when compared to normal, uninffected nuclei. In all cases, chromatin was displaced, as were the nucleoli, although the latter were difficult to distinguish from the almost equally large chromatin fragments in the nuclei. The larger, deeply basophilic inclusions in E-cells were contained in enlarged nuclei and were surrounded by a region of unstained nucleoplasm, giving an appearance very similar to inclusions seen with PmDNV in Thailand (Flegel 2006).

Confocal microscopy using the nucleic acid specific fluorescent stain To-Pro-3 gave positive results with the intranuclear inclusions and fluorescence was diminished or lost, together with that of the chromatin, by treatment with DNase I, which is specific for both single- and double-stranded DNA (Fig. 2c,d). In addition, fluorescence from the inclusions (but not chromatin) was diminished by mung bean nuclease, which is specific for single-stranded nucleic acids (Fig. 2e,f). Despite this loss in fluorescence, the size and shape of the inclusions by phase contrast microscopy remained unchanged. These results indicated that the inclusions contained single-stranded DNA that was digested by the enzymes used while the associated protein remained intact. The intranuclear inclusions could also be seen in squash mounts of hepatopancreatic tissue from postlarvae (PL) of *Macrobrachium rosenbergii* stained with malachite green (Fig. 3). In some cases, the squash-mount inclusions appeared to contain smaller bubble-like sub-inclusions.

Examination of the affected nuclei by transmission electron microscopy (TEM) revealed that the intranuclear inclusions contained small viral-like particles of approximately 25 to 30 nm in diameter (Fig. 4), similar to those reported for other parvoviruses in marine shrimp (Lightner 1993, Flegel 2006). Some of the inclusions showed vacant spaces that corresponded with

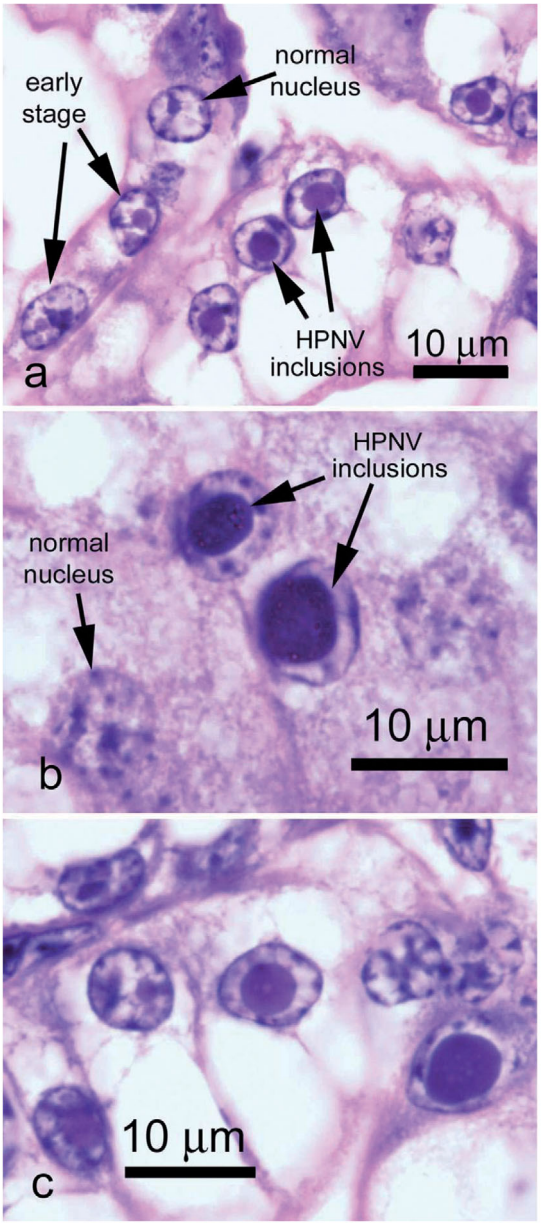

Fig. 1. *Macrobrachium rosenbergii.* Photomicrographs of tissue sections stained with H&E showing hepatopancreatic parvovirus-like intranuclear inclusions in hepatopancreas tubule epithelial cells of postlarvae. (a) Tissue section in the region of R-cells and B-cells showing only small, eosinophilic, circular inclusions (HPNV inclusions). (b) Tissue section in the region of E-cells showing large, basophilic oval inclusions in E-cells. (c) Tissue section showing transitional forms of the 3 stages in inclusion formation

the bubble-like sub-inclusions seen in the hepatopancreatic squash mounts described above.

Standard PCR methods for detection of PmDNV (Phromjai et al. 2002) and PstDNV from *Penaeus monodon* (Lightner 1996) gave negative results with our *Macrobrachium rosenbergii* samples but positive results with positive control material (not shown). *In situ* hybridization tests using PCR-labeled DNA probes prepared from both of these viruses also gave negative results with *M. rosenbergii* samples, although they gave positive results with the respective positive controls using infected tissues of *P. monodon* and *P. vannamei*, respectively (not shown). These negative test results suggested that the genome of the virus in *M. rosenbergii* differed sufficiently from those of PmDNV and PstDNV not to give a hybridization signal.

In a previous report on a parvovirus (called hepatopancreatic parvo-like virus or HPV-type virus) from *Macrobrachium rosenbergii* in Malaysia (Anderson et al. 1990, Lightner et al. 1994), the intranuclear inclusions resembled the larger basophilic inclusions in our Fig. 1b and a commercial probe derived from PchinDNV of *Penaeus chinensis* from Korea failed to hybridize with those inclusions. These results differ from a recent report from Taiwan (Hsieh et al. 2006), where intranuclear inclusions in their Fig. 1a somewhat resemble those in our Thai material (our Fig. 1a), while those in their Fig. 1b are distinctly different. In addition, our sections (Fig. 1a) do not show a distinct clear zone between the intra-nuclear inclusion and the marginated chromatin. Nor does the Taiwanese material show a gradation of inclusions from eosinophilic to basophilic, as in our Thai material. Curiously, the Taiwanese samples gave positive PCR and *in situ* hybridization results with tests designed for PstDNV from *P. monodon* (Hsieh et al. 2006). We do not know the reason for the differences between our results and those of Hsieh et al. (2006), but it is curious that positive results for PstDNV in the Taiwanese study were obtained in tubule epithelial cells of the hepatopancreas of *M. rosenbergii*. This seems unlikely given that PstDNV in *P. monodon* and other susceptible penaeid shrimp species is always associated with tissues of ectodermal and mesodermal origin and never with those of endodermal origin (i.e. hepatopancreatic tubule epithelial cells and midgut columnar epithelial cells) (Lightner 1996). Unfortunately, the published figure for the *in situ* hybridization results (Hsieh et al. 2006) is of low resolution and is not accompanied by an adjacent section stained with H&E so that the nuclei showing apparent positive hybridization reactions cannot be identified for comparison with the histological sections shown in their Fig. 1. Further, the Taiwanese report does not include TEM or enzyme digestion results. Thus, comparison with our work must await further clarification.

In addition to *in situ* hybridization tests, we carried out immunohistochemical tests using antibodies prepared specifically against heterologously expressed proteins of PmDNV (Rukpratanporn et al. 2005) and PstDNV (Bui 2007). The results of these tests were also negative, although positive with respective control

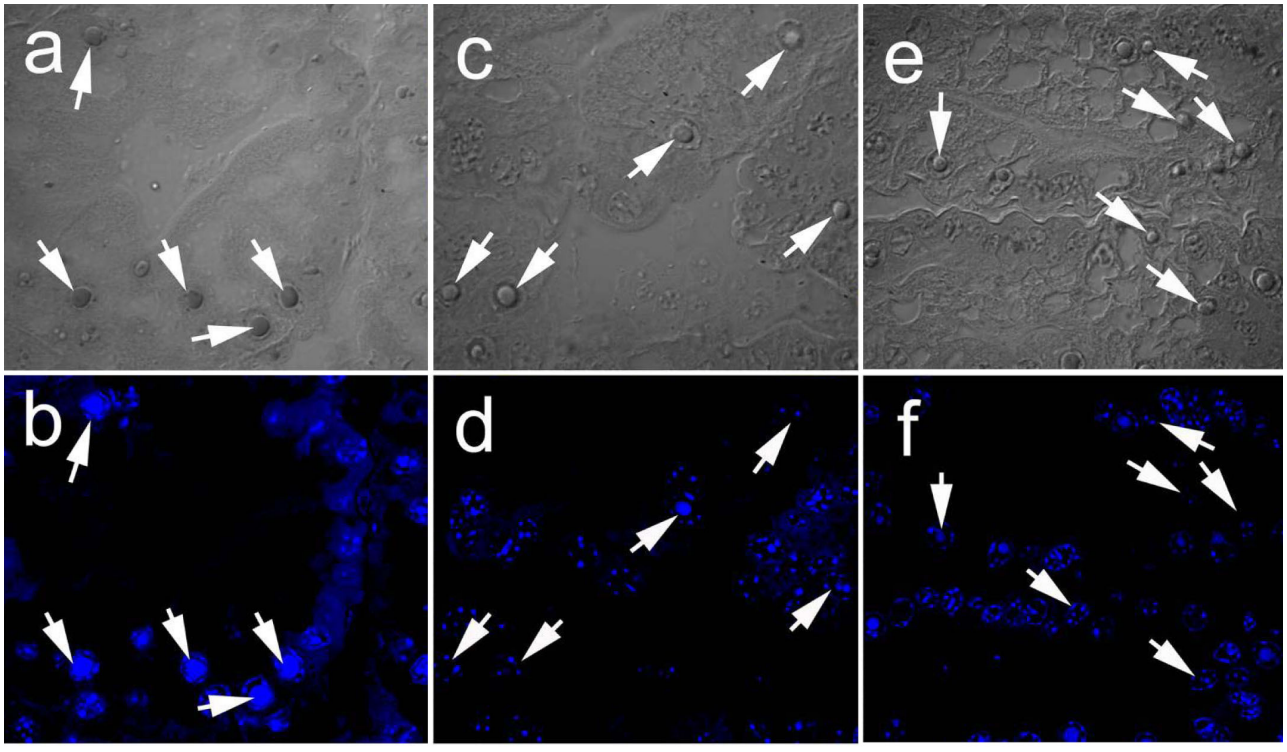

Fig. 2. *Macrobrachium rosenbergii.* Confocal photomicrographs of hepatopancreatic tissue sections of an adult specimen after enzyme treatment. (a) Differential interference contrast (DIC) micrograph of control specimen treated with buffer and showing intranuclear inclusions (arrows). (b) Same as (a) but by fluorescence imagery showing bright blue fluorescence of chromatin and the inclusions (arrows at the same positions as in a). (c) DIC micrograph of tissue treated with DNase I and showing some lightening of the intranuclear inclusions (arrows) when compared to those in (a). (d) Same field as in (c) but by fluorescence imagery showing loss or lightening of fluorescence for both chromatin and the intranuclear inclusions (arrows at the same positions as in c). (e) DIC micrograph of tissue treated with mungbean nuclease and showing some lightening of the intranuclear inclusions (arrows) when compared to those in (a). (f) Same field as in (c) but by fluorescence imagery showing loss or lightening of fluorescence for inclusions (arrows at the same positions as in e)

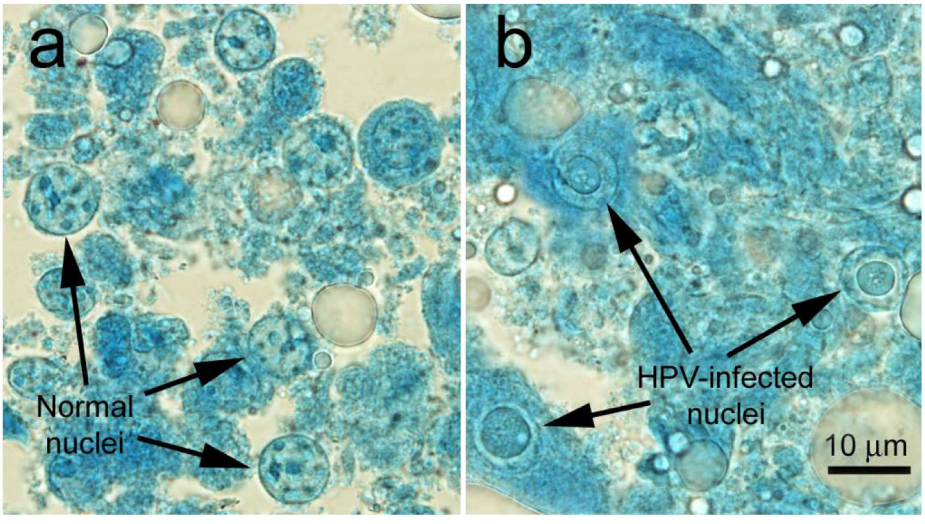

Fig. 3. *Macrobrachium rosenbergii.* Photomicrographs of a squash-mount of hepatopancreatic tissue of postlarvae (PL) stained with malachite green. (a) Tissue from a normal, uninfected PL. (b) Tissue from an infected PL showing hepatopancreatic parvovirus-like (HPV) intranuclear inclusions. Scale bar applies to both photomicrographs

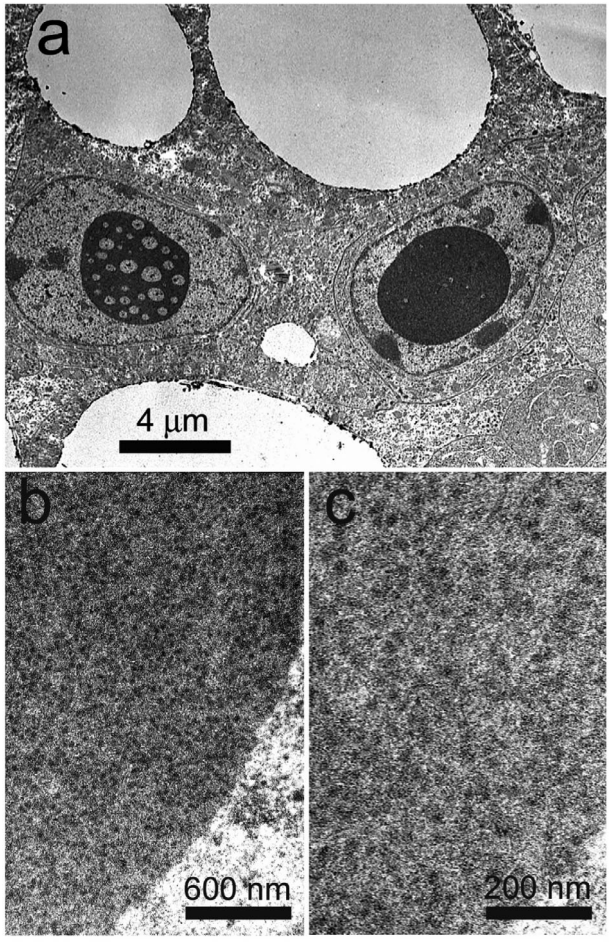

Fig. 4. *Macrobrachium rosenbergii*. Transmission electron micrographs of hepatopancreatic parvovirus-like intranuclear inclusions in hepatopancreatic tubule epithelial cells of a post-larval specimen. (a) Low magnification electron micrograph, (b) medium magnification of an intranuclear inclusion and (c) high magnification of an intranuclear inclusion

samples of *Penaeus monodon* infected with PmDNV and *P. vannamei* infected with PstDNV (not shown), further supporting the distinct nature of the parvo-like virus from *Macrobrachium rosenbergii* when compared to hepanviruses and PstDNV reported from some other penaeid shrimp species.

Based on the target tissue, on the TEM results and on histopathology, the virus described appears to most closely resemble hepanviruses characterized from penaeid shrimp. It is possible that the virus described herein is the same or closely related to that previously described in *Macrobrachium rosenbergii* from Malaysia (Anderson et al. 1990, Lightner et al. 1994). Results from a molecular probe designed from PchinDNV were also negative. On the other hand, the published photomicrographs of the intranuclear inclusions from the Malaysian material resemble only the large, intensely basophilic inclusions seen in E-cells of our Thai material. The smaller, more eosinophilic inclusions seen in the Thai material were not described in the publications from Malaysia (Anderson et al. 1990, Lightner et al. 1994), although this difference may have resulted from the life stages or the stage of infection of the shrimp examined. In addition, the TEM results for the Malaysian material did not precisely match ours in that some of the virions from Malaysia were enclosed by a 'thick electron-dense structure' (Anderson et al. 1990, p. 447) not seen in our material. Further, no enzymes were used to confirm the nucleic acid content of the Malaysian inclusions. Thus, no firm conclusions can be made regarding the relationship of the Thai virus to the genus *Hepanvirus* or the genus *Brevidensovirus* or to the viruses reported from Malaysia and Taiwan until the genomes of all of the isolates have been at least partially characterized. On the other hand, we could confirm that the nucleic acid in the Thai material was single-stranded DNA. According to the latest edition of Virus Taxonomy (Fauquet et al. 2005), the only ssDNA viruses reported from invertebrates are in the family *Parvoviridae*. In the absence of genome sequence information, those involved in specific pathogen free stock development with *M. rosenbergii* will have to depend on histological analysis when screening and monitoring specimens for this new virus.

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