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Pathological changes in *Fenneropenaeus indicus* experimentally infected with white spot virus and virus morphogenesis

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ABSTRACT

To demonstrate pathological changes due to white spot virus infection in *Fenneropenaeus indicus*, a batch of hatchery bred quarantined animals was experimentally infected with the virus. Organs such as gills, foregut, mid-gut, hindgut, nerve, eye, heart, ovary and integument were examined by light and electron microscopy. Histopathological analyses revealed changes hitherto not reported in *F. indicus* such as lesions to the internal folding of gut resulted in syncytial mass sloughed off into lumen, thickening of hepatopancreatic connective tissue with vacuolization of tubules and necrosis of rectal pads in hindgut. Virus replication was seen in the crystalline tract region of the compound eye and eosinophilic granules infiltrated from its base. In the gill arch, dilation and disintegration of median blood vessel was observed. In the nervous tissues, encapsulation and subsequent atrophy of hypertrophied nuclei of the neurosecretory cells were found. Transmission electron microscopy showed viral replication and morphogenesis in cells of infected tissue. *De novo* formed vesicles covered the capsid forming a bilayered envelop opened at one end inside the virogenic stroma. Circular vesicles containing nuclear material was found fused with the envelop. Subsequent thickening of the envelop resulted in the fully formed virus. In this study, a correlation was observed between the stages of viral multiplication and the corresponding pathological changes in the cells during the WSV infection. Accordingly, gill and foregut tissues were found highly infected during the onset of clinical signs itself, and are proposed to be used as the tissues for routine disease diagnosis.

1. Introduction

White spot virus (WSV) causes significant mortalities to several cultured penaeid species. Since its advent in 1992, WSV has greatly affected the shrimp farming industry (Mohan et al., 1997). This pathogen has tropism for organs of mesodermal and ectodermal origin (Wongteerasupaya et al.,1995, 1996; Chang et al., 1996; Flegel et al., 1997; Rajendran et al., 1999; Wang et al., 1999; Vijayan et al., 2003; Yoganandhan et al., 2003). Evidence of histopathological manifestations in the target tissues is one of the criteria used in the diagnosis of WSV infection (Lightner, 1996; Wang et al., 1997). Studies on histopathology, cytopathology, morphogenesis, pathogenesis, pathogenicity and virulence have been reported for both laboratory raised and wild Asian and American shrimp species.

White spot syndrome virus has a broad host range within decapod crustaceans. At least 18 cultured and/or wild penaeid shrimp (Park et al., 1998), eight caridean species (Pramod-Kiran et al., 2002), seven species of lobster (Rajendran et al., 1999), seven species of crayfish (Edgerton, 2004), 38 crab species (Yoganandhan et al., 2003) six non-decapod crustacean species (Hossain et al., 2001), members of the phyla Chaetognata and Rotifera (Yan et al., 2004), polychaete worms (Supak et al., 2005) and some aquatic insect larva (Ramírez-Douriet et al., 2005) have been found susceptible to the virus. Histopathological observations during WSV infection in brief is available regarding shrimp species such as *Penaes monodon* (Durand et al., 1997; Wang et al., 1999, 2000; Mishra and Shekhar, 2005) *Litopenaeus vannamei* and *Marsupenaeus japonicus* (Lu et al., 1997; Lightner et al., 1998; Escobedo-Bonilla et al., 2007; Perez et al., 2005; Pantoja and Lightner, 2003), species of Crabs (Kanchanaphum et al., 1998; Kou et al., 1998) and lobster (Rajendran et al., 1999; Wang et al., 1998; Jiravanichpaisal et al., 2001).

Fenerropenaeus indicus is one of the major commercial species of shrimp aquaculture in Asia endemic to Indian waters and the most wild caught Indian species. It is a good quality, good flavored shrimp, popular with importers in Japan, Western Europe and the USA, Iran, Bangladesh, Malaysia, Thailand, Indonesia and the Philippines. This species is also susceptible to WSV. Despite its

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importance in commercial catches as well as in culture, histological investigations on the pathological changes in this species due to WSV have been attempted in very limited extend. The only available data is a briefing by Rajendran et al. (1999) and Rajan et al. (2000) who reported nuclear hypertrophy, cell lysis and tissue degeneration as well as the ultra structural of hypertrophied nucleus. This lack of information prompted to undertake the present study on the histopathological changes due to WSV and viral morphogenesis.

2. Materials and methods

2.1. Source of virus

A brood stock of *P. monodon* obtained from a batch of wild spawners brought from Vishakapatnam, Andhra Pradesh in 1997 by Matsyafed, Government of Kerala, for larval production was the source of the virus. The animals had displayed clinical signs of the disease such as white spots on the inner surface of carapace, reddish pleopods and empty intestine. The presence of WSV was confirmed by transmission electron microscopy (TEM) and diagnostic PCR (Lo et al., 1996).

2.2. Viral inoculum

WSV infected gills (500 mg) from *P. monodon* were macerated in an ice-bathed mortar. A volume of 10 ml of cold PBS (NaCl 8 g, KCl 0.2 g, Na_2HPO_4 1.15 g, KH_2PO_4 0.2 g, double distilled water 1000 ml) was used to make a tissue suspension. The homogenate was centrifuged at 8200*g* in a refrigerated centrifuge (REMI C.24, India) at 4 °C, and the supernatant was filter-sterilized using a 0.22 μm nitrocellulose membrane (Sartorius India (P) Ltd.). An aliquot (10 μl) was streaked on ZoBell's 2216E agar plates and incubated at 28 ± 1 °C for 72 h to determine the bacterial load of the inoculam.

2.3. Experimental animals

A batch of *F. indicus* from a single broodstock was reared in a hatchery. Juvenile shrimp ($n = 50$, mean body weight (MBW) = 3 ± 1) were used for virus amplification and subsequently to perform the experiments. Prior to the experimental infections, shrimps were subjected to a formaline stress test for one hour in seawater (20 g/l) containing 100 ppm formaline with adequate aeration. They were subsequently observed for three days for the manifestation of diseases and mortality. Animals were confirmed to be WSV negative by PCR analysis.

2.4. Experimental infection

An aliquot of 0.01 ml filtrate was injected at the dorsal side of the abdomen of *F. indicus* between telson spine and 6th abdominal segment using 1 ml tuberculin syringe. Animals ($n = 25$) were intramuscularly inoculated (with the WSV inoculum). Groups of Five animals were placed in a fiber-glass tank (40 × 25 × 10 cm) with sea water having 20 g/l salinity at ambient temperature (28 ± 1 °C) with continuous aeration. Shrimps were fed *ad libitum* with a commercial feed ('Grower' from Higashimaru, India) containing 40% protein. Fresh filtered seawater (about 7 l) with the same salinity and temperature was daily replaced. Animals were monitored for clinical signs of infection such as feeding cessation, lethargy and mortality. Moribund shrimps were fixed for histopathology and electron microscopy as described below. A group of uninfected animals ($n = 10$; two from each set) was used as a control and processed in the same way as the experimental shrimps.

2.5. Histopathology

Moribund as well as control animals having MBW 3 ± 1 g were fixed by injecting 1–3 ml (depending on size) Davidson's fluid on the dorsal side of 6th segment. Immediately after the injection, cuticle was split sagittally and the whole animal immersed in 15 ml Davidson's fluid per animal in screw capped tubes for 24 h. Subsequently, the animals were dissected, and gills, heart, nerve cord, stomach, foregut, mid-gut, hindgut, hepatopancreas, eye, integument and ovary were transferred to 70% ethyl alcohol, processed for histopathology and double stained with haematoxylin and eosin (Bell and Lightner, 1988), and examined under light microscope (Nikon Type 104, Japan).

2.6. Electron microscopy

For electron microscopy gill, foregut, heart, hepatopancreatic connective tissue, hindgut and nerve tissues from WSV infected *F. indicus* as well as those of healthy control animals were removed, minced into 1 mm sized pieces and fixed in 2.5% glutaraldehyde in PBS (1 M, pH 7.4) for 24 h at 4 °C and post fixed in 2% osmium tetroxide in PBS (1 M, pH 7.4) for 2 h at 4 °C. After dehydration through an ascending series of acetone, the tissue pieces were embedded in epoxy resin (Electron Microscopy Sciences, USA). Ultra thin (0.5 μl) sectioned, stained with uranyl acetate and lead citrate, and examined under transmission electron microscope (Philips, CM-10). Based on the ultrastructure of the infected nuclei viral morphogenesis was investigated.

3. Results and discussion

3.1. Histopathology

PCR negative healthy animals which survived formaline stress test were used for the experiment. Presence of WSV in the source tissue as well as the inoculum was confirmed by TEM (Fig. 1a) and diagnostic PCR (Fig. 1b). The viral inoculum showed zero CFU/ml in ZoBell's 2216E agar plate confirming the absence of bacteria in the preparation. Injected animals showed erratic swimming and cessation of feeding after 30–48 h, and with out waiting for mortality they were fixed and processed as described above.

3.2. Digestive system

3.2.1. Foregut

The ventral median channel, ventro lateral folds, and the dorsal grooves, dorsal median folds and intra lateral cardiac plate of the

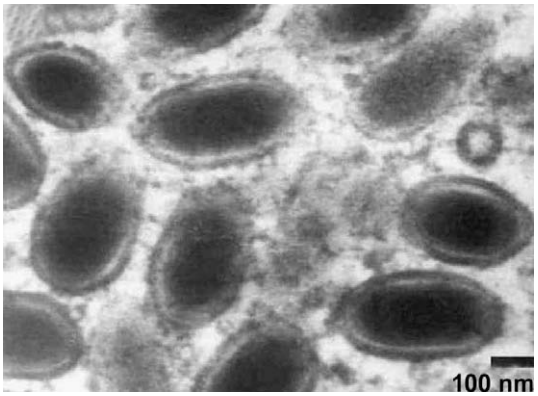

Fig. 1a. TEM of hypertrophied nucleus of gill tissue of *F. indicus* stained with uranyl acetate and lead citrate. Characteristic rod-shaped virions of WSV are seen.