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A viral disease associated with mortalities in hatchery-reared postlarvae of the giant freshwater prawn *Macrobrachium rosenbergii*

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ABSTRACT: During mortality outbreaks in hatchery-reared *Macrobrachium rosenbergii* postlarvae (PL) in Guadeloupe Island (French West Indies) during 1997, an associated viral disease was discovered and the agent was subsequently isolated. The clinical signs presented by severely affected PL consisted essentially of an opaque whitish appearance of the abdomen. Histopathological changes in affected PL were characterized predominantly by pale to darkly basophilic, often reticulated, cytoplasmic inclusions in the connective tissue cells of most organs and tissues. The isolated virus was approximately 30 nm in diameter as observed with an electron microscope by negative staining. By its location, structure and size it could be related to different families of the small RNA cytoplasmic viruses such as the Picornaviridae or the Nodaviridae. Its characterization is in progress.

KEY WORDS: Viral disease · Freshwater prawn · Crustacean · *Macrobrachium rosenbergii*

INTRODUCTION

Among the economically important farmed crustacean species, the giant freshwater prawn *Macrobrachium rosenbergii* is most developed in southeast Asian countries. Some Caribbean countries (northern South America and the West Indies) also farm these animals, although the industry is of less importance than the penaeid shrimp farming industry. Nonetheless, the culture of *M. rosenbergii* is important for some Caribbean countries.

Since Vago's (1966) discovery of the first marine crustacean virus, numerous viral diseases and viruses have been reported in crabs, crayfish, prawns, and shrimp. Particularly for penaeid shrimp (Lightner 1996), viral diseases are known to be a limiting factor in farming because they can cause serious production losses during acute epizootics (Adams & Bonami 1991). In marked contrast, except for 2 reports of viruses in *Macrobrachium rosenbergii* (Anderson et al. 1990, Tung et al. 1999), no serious viral diseases have been reported to date in this economically important farmed species. Although a third virus (White Spot Syndome Virus, WSSV) was reported in cultivated *M. rosenbergii* (Lo et al. 1996, Peng et al. 1998) and the disease experimentally induced in this species (Chang et al. 1998, Wang et al. 1998), WSSV should not be considered as a viral disease of *M. rosenbergii* but rather, as stated in Lo et al. (1996) and Peng et al. (1998), as a penaeid shrimp virus capable of developing in a large number of crustacean hosts that include *M. rosenbergii*.

We report here the results of our investigations on mortalities occurring episodically since 1994 in hatchery-reared *Macrobrachium rosenbergii* in Pointe Noire (Guadeloupe) in the French West Indies.

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MATERIAL AND METHODS

Histology. Healthy and diseased postlarvae (PL) *Macrobrachium rosenbergii* were preserved in Davidson's AFA fixative (Lightner 1996, Hasson et al. 1997). Following fixation, PL were embedded in Paraplast X-TRA® tissue embedding medium (Fisher Scientific, Pittsburgh, PA, USA) and 5 µm thick sections were prepared, mounted on glass slides, and stained with hematoxylin & eosin (H&E) for light microscopy examination (Bell & Lightner 1988).

Electron microscopy. Cephalothoraces ('heads') of diseased PL were fixed in 2% glutaraldehyde, post-fixed in 1% OsO_4, and embedded in Epon resin. Semithin sections were stained with toluidine blue. Ultrathin sections were contrasted with uranyl acetate and lead citrate according to Reynolds (1963). Viruses in suspension or in tissue homogenates were studied by negative staining with 2% phosphotungstic acid (PTA), pH 7.0, using carbon-collodium coated grids.

Partial purification of the virus. To better determine details of the viral structure, attempts were made to purify the virus using alternate cycles of low and high speed centrifugation. Infected tissue homogenates in TN buffer, pH 7.4 (Bonami et al. 1990), were first clarified at 3000 × *g* for 20 min, then centrifuged at 100 000 × *g* for 2 h. A few microliters of the re-suspended pellet were placed on carbon-collodium coated grids, negatively stained with 2% PTA, pH 7, and finally observed by transmission electron microscopy (TEM).

RESULTS

History of the disease

The SICA hatchery at Pointe Noire has been in production since 1985 with an optimal production capacity of 20 million PL per year. This hatchery uses a closed recirculating system to produce the larvae in clearwater at densities of between 80 and 110 larvae l^{-1}.

During the 10 yr period from 1985 to 1994, PL production from the Pointe Noire hatchery met planned expectations and it had a history of very reliable performance. Average survival rates in the larval rearing phase of production were close to 80% after 30 d or complete metamorphosis. However, since 1994, abnormal sudden mortalities have been recorded; these were variable (5 to 90% cumulative losses) in intensity, and a function of the reproduction cycles of the broodstock being used. During these cycles, production through the larval stages before metamorphosis seemed to occur normally: growth and nutrition were correct, and survival rates were close to 100%. However, the problems appeared during metamorphosis to the PL stage. Attempts failed to relate the observed mortalities to a possible environmental or toxic agents in the culture system water supply (e.g. anoxia, chlorine residues, paint toxicity, *Artemia* quality, tank osmosis, groundwater pollution, nitrite contamination, etc.). Consequently, investigations were carried out to determine if there was evidence for an infectious etiological agent.

Clinical signs of the disease

The first gross sign of disease in a rearing tank was the presence of a few whitish PL 2 to 3 d after the first PL emerged. The whitish PL were visible only against a dark background (i.e. on a dark tank bottom). Floating exuviae (molts) in the tanks were abnormal and resembled 'mica flakes'. Some of the whitish PL were cannibalized by healthier PL in the same tank. During the following few days, the affected PL became more 'milky' and opaque in appearance, and the prevalence of opaque, milky PL often increased dramatically to as high as 90%. The abdomens (tails) were particularly milky and opaque. The discoloration seemed to start at the tail extremity (telson) and gradually progress towards the head. Eventually all muscles in the abdomen and cephalothorax were affected. At the time the PL became more milky and opaque, the first deaths were recorded. Mortality reached the maximum about 5 d after the appearance of the first gross signs. Very few PL presenting these signs survived beyond 15 d. After release into grow-out ponds, survivors seemed to grow normally.

Histopathology

The tissues most affected in moribund prawns were striated muscles of the abdomen and cephalothorax and intratubular connective tissue of the hepatopancreas. No bacterial, microsporidial, fungal or sporozoan pathogens pathogens were seen. However, there were foci of affected cells which contained conspicuous, cytoplasmic viral inclusions. These cytoplasmic inclusions were generally discrete, pale to darkly basophilic (with H&E) and from <1 to ~40 µm in diameter (Fig. 1). Viral inclusions were also apparent, but much less conspicuous, in connective tissue of the subcutis and rarely present in the gills.

The striated muscle presented multifocal areas of hyaline necrosis of muscle fibers. Moderate edema was often also present in necrotic muscles and produced abnormally large open spaces among affected muscle cells. The sarcoplasm of necrotic muscle cells stained more eosinophilic and lost the banded (in longitudinal sections) or granular (in cross sections) appearance apparent in normal fibers. Focal to multifocal areas of

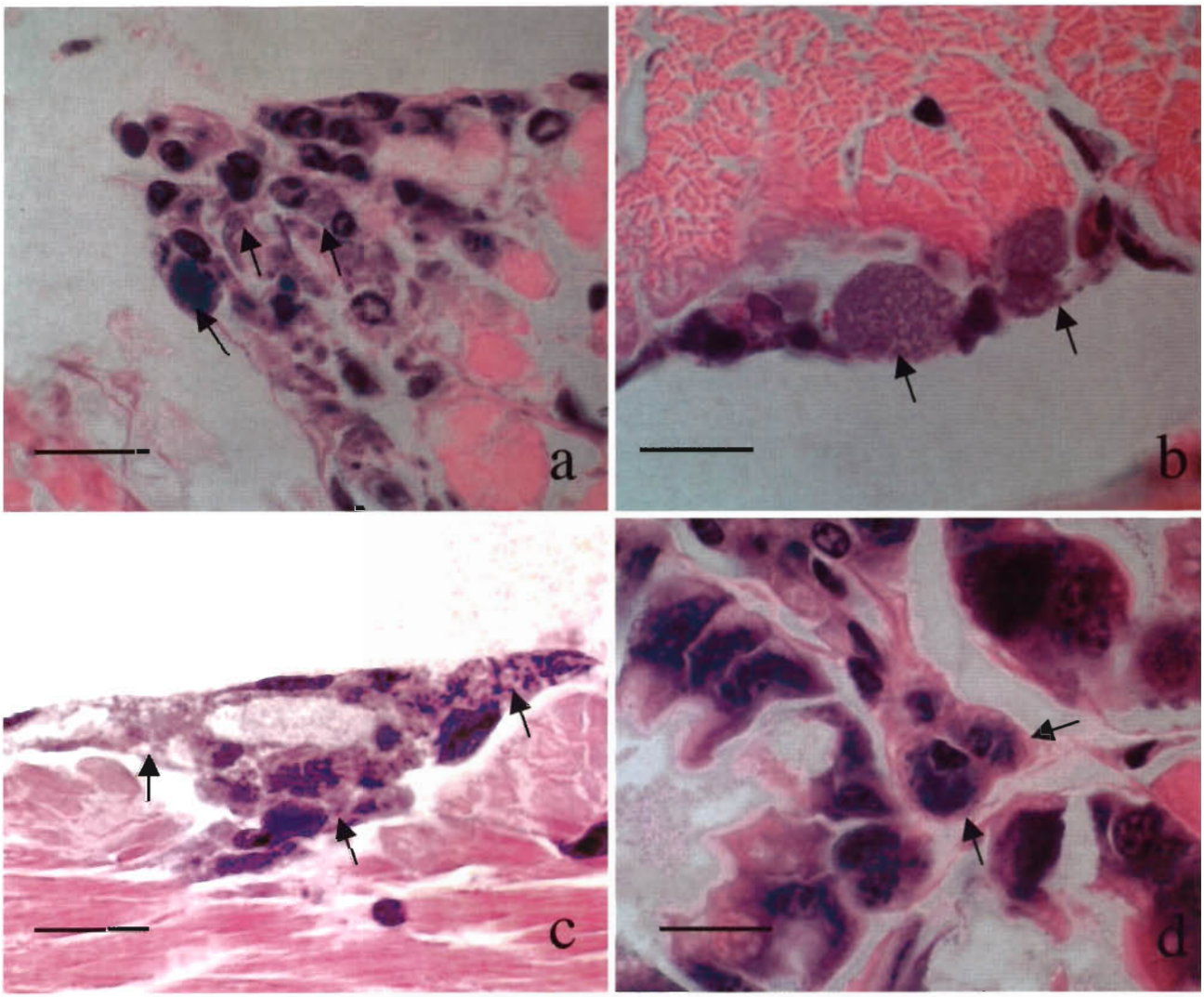

Fig. 1. *Macrobrachium rosenbergii*. Histological sections of diseased PL that illustrate viral inclusion bodies. Pale to darkly basophilic, intracytoplasmic inclusion bodies of <1 to ~40 µm are apparent in a number of cells (some are indicated by arrows) in connective tissue and fixed phagocytes in striated muscle (a,b,c). (d) A prominent cytoplasmic viral inclusion displacing the nucleus of an intratubular connective tissue cell in the hepatopancreas. All H&E. Bars = 26 µm

hemocytic infiltration and fibrosis were apparent within and between affected muscle fibers. Present in some muscle fibers, especially at ligament connections to the cuticle and in the connective tissues between muscle fibers, were foci of cells with conspicuous, cytoplasmic viral inclusions (Fig. 1a,b,c). Viral inclusions were also noted in the cytoplasm of infiltrating fibrocytes in some necrotic muscle foci.

Intratubular connective tissue cells and fixed phagocytes were often found to contain large conspicuous cytoplasmic viral inclusions. Most were pale to darkly basophilic, generally irregular bodies. A few were large and displaced the host cell nucleus (Fig. 1d). No viral inclusions were observed in epithelial cells of hepatopancreatic tubules or in midgut mucosal epithelial cells.

Ultrastructure of infected cells

By TEM, infected cells appeared necrotic, exhibiting a disorganized cytoplasm. Within the cytoplasm, 2 types of large electron-dense inclusions were observed (Fig. 2). Within one type, virus-like particles of about 25 to 28 nm diameter were interspersed within an electron-dense matrix. These were considered to be viroplasmic or virogenic areas. In proximity to these virogenic areas were other better defined masses which contained dense viral particles, often associated in small groups. These 2 areas, by their structure and size, seem to correspond to the basophilic cytoplasmic inclusions found in histological sections of diseased PL by light microscopy.

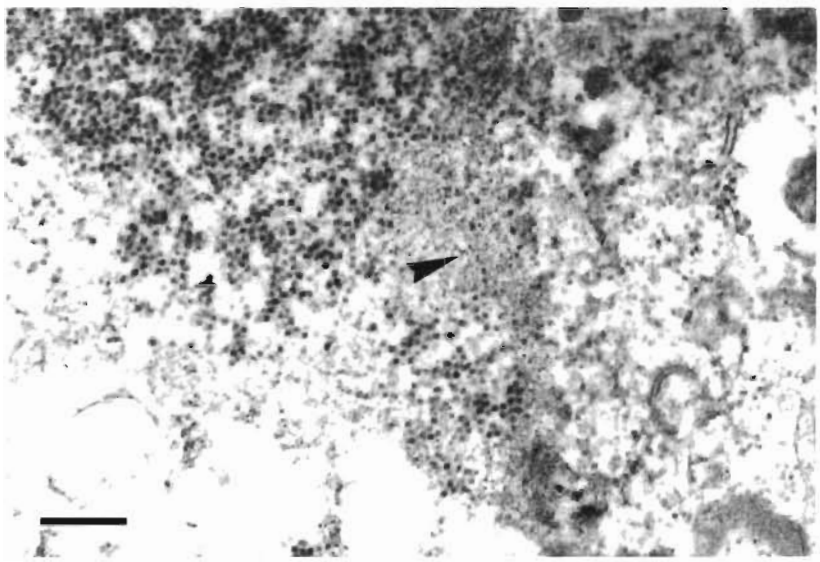

Fig. 2. *Macrobrachium rosenbergii*. Infected connective cell. Note the presence of a virogenic area (arrowhead). TEM. Bar = 200 nm

Virus isolation

For virus isolation we collected diseased PL exhibiting the characteristic signs of disease from the SICA hatchery during one peak of mortality. These PL were immediately frozen and mailed to the Montpellier laboratory on dry ice.

Direct observations by TEM of negatively stained tissue homogenates from diseased prawns exhibited numerous non-enveloped virus-like particles of about 30 nm in diameter.

After one cycle of low-high speed centrifugation, the final pellet showed a very large number of these particles (Fig. 3). Some appeared empty and others full. In some virions, capsomers could be distinguished on the surface.

DISCUSSION

Although Koch's postulates were not completed by experimental transmission of the disease, the large amount of particles isolated from these diseased prawns, the presence of quite similarly sized viral particles within cytoplasmic inclusions in diseased PL, and the epidemic character of disease development strongly supported viral etiology. Viral etiology was also supported by the fact that similar problems to those observed at the Pointe Noire hatchery were also reported from 4 other hatcheries (which used different larval rearing methods) in the French West Indies. After several years of satisfactory production, 3 of the 4 hatcheries reported identical problems of mortality preceded by 'white-tailed' PL in the tanks. Even the newest hatchery at Morne Vert, Martinique, Virgin Islands, has very recently reported identical symptoms and mortality after 3 yr of successful production.

Apart from WSBV (white spot baculovirus) causing mortalities in *Macrobrachium rosenbergii* (Peng et al. 1998), which is probably a transmitted penaeid virus, the viral agent here described and the newly reported MMV (*Macrobrachium* mussel virus) (Tung et al. 1999) appear as the first 2 species-specific viruses of *M. rosenbergii* causing disease outbreaks and mortalities in this species. Based on size, shape, cytoplasmic location, and formation of discrete cytoplasmic inclusions in connective tissue cells, the virus described herein could be related to small cytoplasmic RNA viruses, such as those in the Picornaviridae or Nodaviridae families (Murphy et al. 1995). However, it differs in size and location from a previously reported intranuclear virus-like particle in *M. rosenbergii* (Anderson et al. 1990), which is more likely to be related to the parvoviruses. The pathodiagnostic lesions, affected tissues, clinical signs, and size of the viral particles (about 30 nm in negative staining and 22.9 ± 3.6 nm measured in section) are consistent with those described for MMV

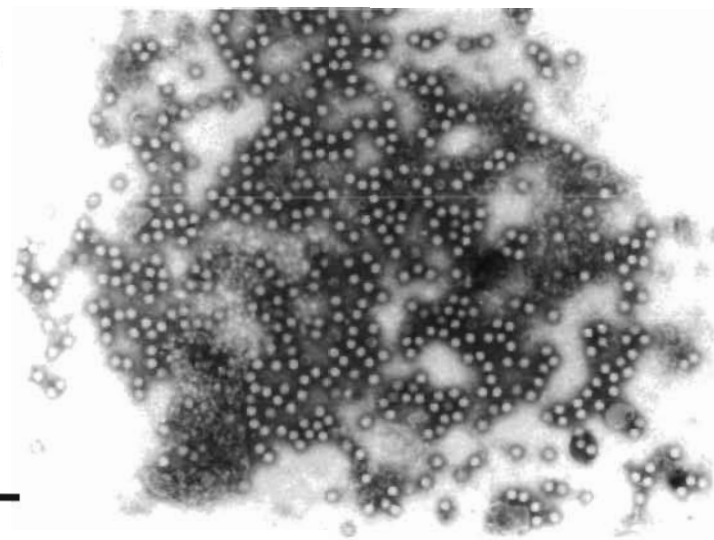

Fig. 3. Negatively stained viral particles prepared directly from diseased PL homogenates. Bar = 200 nm

(Tung et al. 1999). A virus similar to that in *M. rosenbergii*, but infecting different target tissues, was recently reported in *Penaeus vannamei*. This virus, which shares similar characteristics (i.e. size, shape, lack of an envelope, and cytoplasmic replication) has been named TSV (Taura Syndrome Virus), and characterized as a picornavirus (Bonami et al. 1997).

Macrobrachium rosenbergii PL presenting similar gross signs of white tails and similar histopathological changes were found in pathological cases collected in Puerto Rico, and submitted to the Tucson Aquaculture Pathology Laboratory (University of Arizona) in the period 1990 to 1992. These findings suggest that the disease may have been present in the Caribbean region for several years before it was recognized in Guadeloupe. It is possible that commercial exchanges among different hatcheries and farms located in the West Indies contributed to the spread of this disease and such exchanges should be considered an important potential production risk for *M. rosenbergii* in the Caribbean region and elsewhere.

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