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MASS MORTALITIES IN HATTCHERY-REARED SEA BASS (*Lates calcarifer*) LARVAE ASSOCIATED WITH THE PRESENCE IN THE BRAIN AND RETINA OF VIRUS-LIKE PARTICLES

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Introduction

Since 1986, an unknown disease causing high mortalities appeared in Ifremer's Pacific Oceanological Center (Tahiti) nursery of sea bass (*Lates calcarifer*). opathology of lesions involving both central nervous system (CNS) and eye retina, and the tuation exists in South-East Asian countries (Indonesia, Philippines, Malaysia and Singapore), and in northern Australia.

This paper describes the histmorphology of a new fish virus, which is believed to be the aetiological agent of high mortalities among sea bass larvae.

Materials and methods.

Animals

Affected fish were sampled from five successive lots of larvae reared at the Center from February 1989 to February 1990. Samples were taken daily from day 8 to day 40 after hatching and were processed for histology or stored at -80°C for virus purification.

Light and electron microscopy

The affected live larvae were fixed *in toto* in Holland Bouin fluid, embedded in paraffin and the 4 and methyl green - pyronin staining, fluorescence examination after acridine orange binding technique.

For electron microscopy, the larvae were fixed in 2.5 % glutaraldehyde in Soerensen buffer and post-fixed in 1 % osmium tetroxide in Palade buffer. Ultrathin sections of the epon embedded specimens were contrasted according to Reynolds (1963) and examined in a Hitachi HU11B electron microscope operating at 75 Kv.

Virus suspensions were negatively stained with 2 % phosphotungstic acid (PTA), pH 7.0.

Virus extraction and purification

Frozen larvae were homogenized in standard PBS buffer using a glass tissue blender. After two clarifications (1,000 g for 10 min. followed by 18,000 g for 30 min.), the final supernatant was filtered on a Celite bed and pelleted at 150,000 g for 2 hrs on a Beckman L7-55 ultracentrifuge. Pellets were resuspended in PBS and 3 times extracted with freon 113 (1,1,2-trichloro 1,2,2-trifluoroethane) before being re-pelleted at 150,000 g for 2 hrs.

The final pellet was resuspended in PBS and layered onto a 20-40 % (w/w) sucrose gradient and centrifuged at 150,000 g for 2.30 hrs. One ml fractions were recovered from the gradient using a Büchler autodensiflow equipped with a UV absorbance monitor (ISCO UA 5) recording the optical density at 254 nm wavelength. Bands were diluted in PBS and pelleted at 200,000 g for 2 hrs in a Beckman SW 40.1 rotor. These pellets and the sucrose gradient pellet too, were layered on a 30-40 % (w/w) CsCl gradient in PBS and isopicnically centrifuged (220,000 g for 15 hrs) in a SW 40.1 rotor. Finally, bands obtained were diluted in PBS and pelleted at 285,000 g for 1.30 hr.

Results

Clinical signs

The main symptoms of the disease are "bleaching" of the 10 - 15 day old larvae, abnormal behaviour, loss of equilibrium and disoriented swimming near the surface. Subsequently, affected fish sink to

the bottom and die.

Histopathology

Histological examination of the larvae revealed abnormal neurons in different parts of the brain (mainly optic tectum, cerebellum, tegmentum, vagal lobes, medulla oblongata and spinal cord). This abnormality consisted of enlarged basophilic cells with very basophilic round-shaped inclusion bodies and cytoplasmic vacuolation (Fig. 1). The vacuoles were ofter very extensive and the destruction of the affected cells complete. The resulting loss of neural substance gave a spongiform appearance to the tissue ("spongiosis"). The retina itself displayed the spongiotic aspect and the cells containing inclusion-bodies (Fig. 2). The lesions were limited to the nueronal layers. In all cases, the inflammatory response was very discrete. The cerebellum, the optic tectum and the retina appeared to be the primary sites of the lesions.

These histopathological changes were visible in 10 to 20-22 day old larvae. The extent of the lesions varied considerably from fish to fish, although the "spongiosis" appeared to be more extensive as the larvae were affected younger. 35 day old larvae showed the same lesions, but only in the cerebellum.

All the other organs examined by light microscopy, namely gills, heart, digestive system, liver, exocrine pancreas, kidney, spleen, skin and skeletal muscles showed no conspicuous lesions (Fig. 3).

The inclusions present in affected tissues were Feulgen negative and stained red with pyronin methyl green. This coloration disappeared after enzymatic digestion with ribonuclease. Observation after acridine orange staining showed a red fluorescence of the inclusions revealing the presence of RNA.

Electron microscopy

Electron microscopy showed the presence of round-shaped osmiophilic inclusion-bodies in the cytoplasm of degenerating cells from the brain and retina (Fig. 4). The inclusion bodies appeared to be limited by a unit-membrane and filled with small (25 nm) regular (icosahedral) particles. These particles sometimes showed a pseudo-crystalline arrangement and are identified as viral units. The particles were often free in the cytoplasm of affected cells.

Virus isolation and purification

In the sucrose gradient, two bands were evident containing particles (full and empty particles) and some tubular-shaped structures with a diameter identical to the viral particles; but the majority of the material was found in the pellet, associated with the cell debri.

After the CsCl gradient, purified full and empty virus particles showed a typical icosahedral shape with a diameter of 25 nm. Small projections were often visible at the edges of isolated particles (Fig. 5).

Discussion

The existence of virus affecting the CNS and the retina of hatchery reared sea bass is demonstrated in the present study. This virus seems to be intrinsically neurotropic. In fact, there is no evident tissue lesions in other organs. Because if the viral distribution, the association of the clinical and histological signs and the absence of other specific tissue lesions, a viral aetiology of the mass mortalities among sea bass fry is suspected. There is very little information on the existence of neurotropic fish viruses (Wolf, 1984; Dorson *et al.*, 1984). In addition, the present virus affecting the CNS and the retina of sea bass has a very small size, and is one of the smallest fish viruses recorded. Recently, the existence of small viruses affecting the CNS of different marine fish was demonstrated (Bellance Gallet de Saint-Aurin, 1988; Glazebrook, 1990; Yoshikoshi Inoue, 1990). In all these cases, the symptomatology and the virus morphology are very similar.

The virus found in the present work is tentatively identified as a member of the family Picornaviridae based on its ability to replicate in the cytoplasm, the presence of a unit-membrane, its size (25 nm) and RNA content (large Feulgen re-

gative and RNA positive inclusions formed entirely by virions). Further investigations on viral properties are necessary to classify the virus more precisely.

This virus seems to be very virulent and causes high losses. Extensive research in virology and immunology is needed to find a long term solution to this problem.

Summary

Since 1986 high mortality of hatchery-reared sea bass (*Lates calcarifer*) larvae appeared in Ifremer's Pacific Oceanological Center (Tahiti). Light and electron microscopy show vacuolation and cells with cytoplasmic inclusion bodies in the brain, spinal cord and retina. Numerous non-enveloped particles identified as viral units, icosahedral in morphology and measuring about 25 nm in diameter were found in the cytoplasm of the degenerating cells. The brain and the retina degeneration seem to be the cause of the high mortalities of hatchery-reared sea bass larvae.

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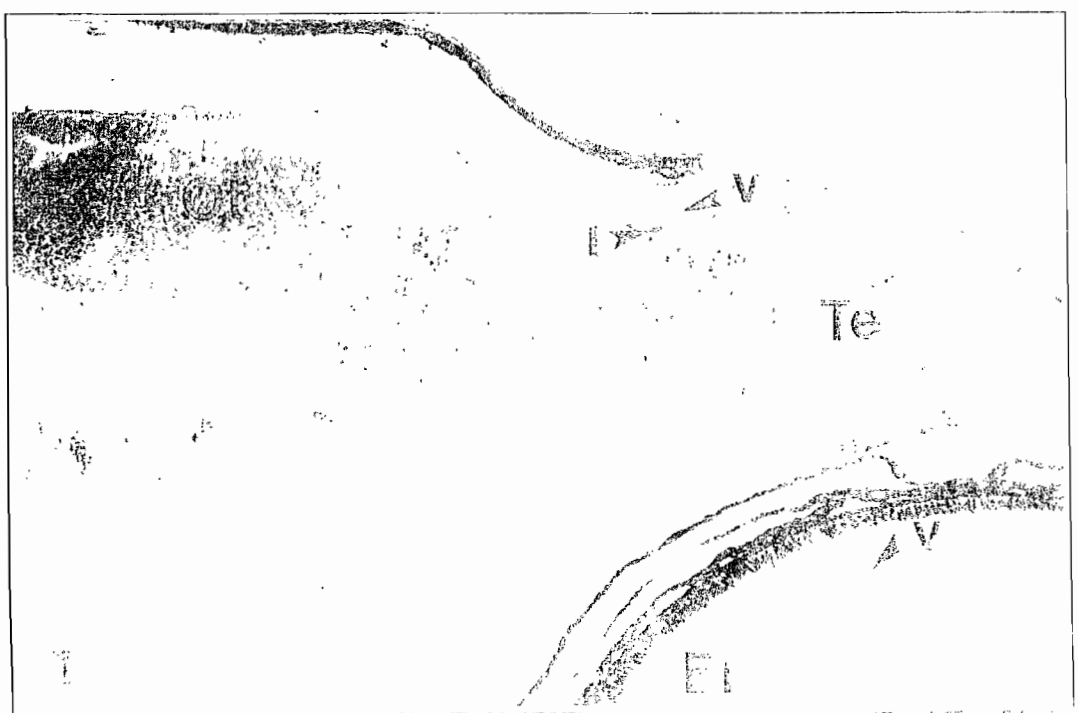

Fig. 1: Light micrograph of a saggital section of a 15 day old larva showing vacuolation (V) and basophilic inclusions (I) in the brain and the eye (Te: telencephale; Ot: optic tectum; Er: eye retina). Giemsa stain

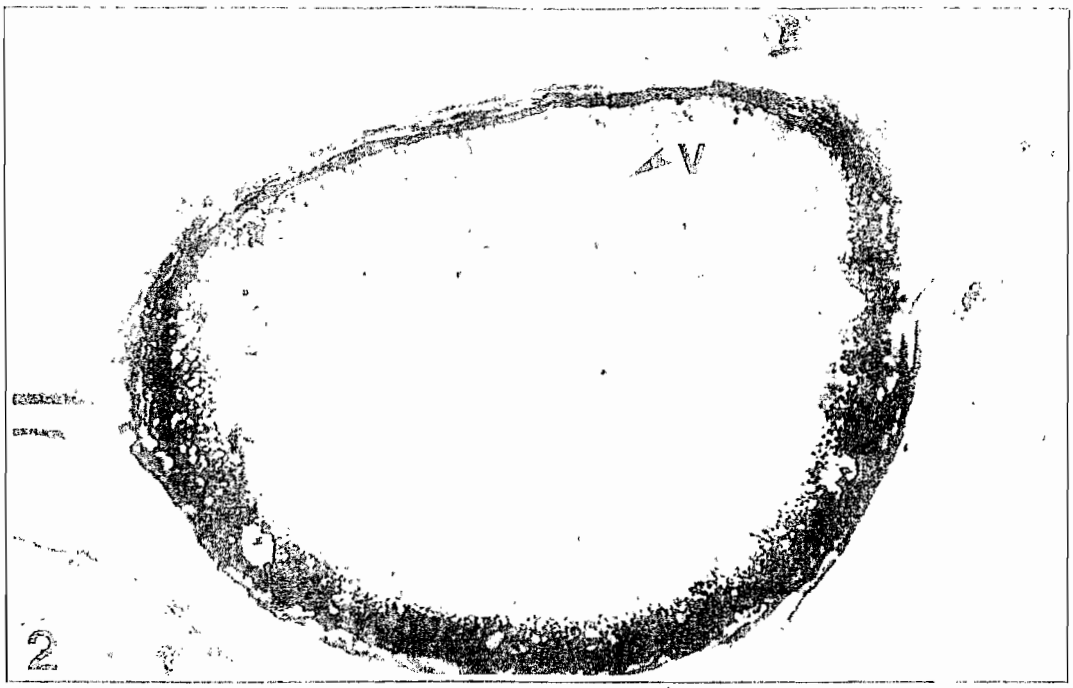

Fig. 2. Light micrograph of a sagittal section of a 12 day old larva: extensive vacuolation (V) in the eye retina. Toluidine blue.

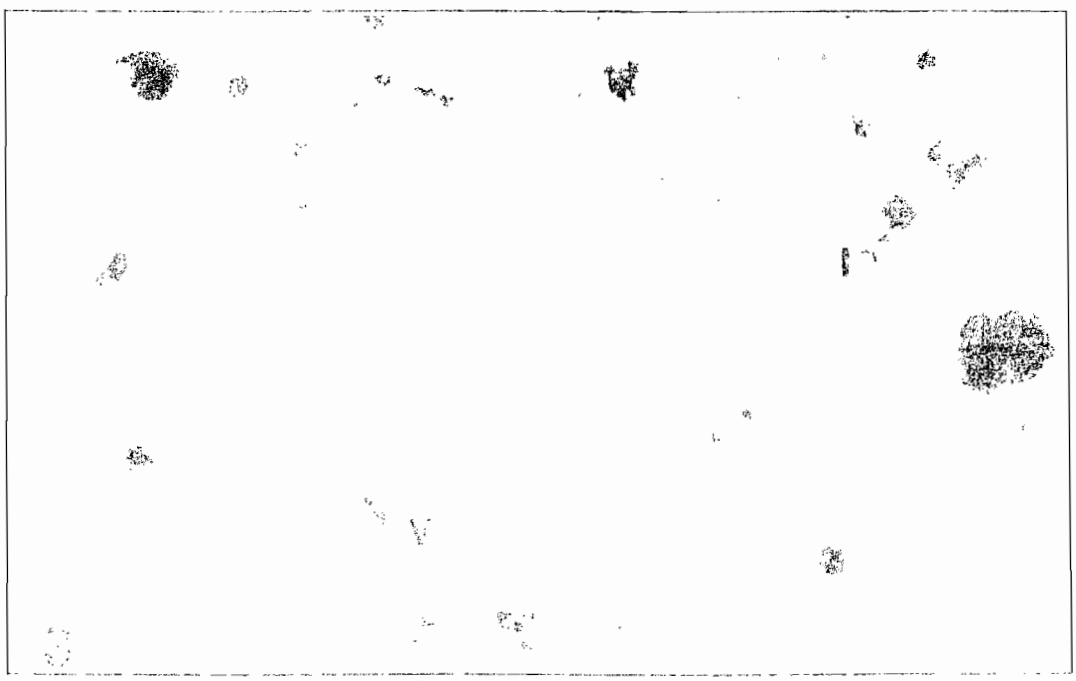

Fig. 3. Light micrograph of a sagittal section of a 15 day old larva showing vacuolation (V) and abnormal cells containing inclusion bodies (I). Giemsa stain

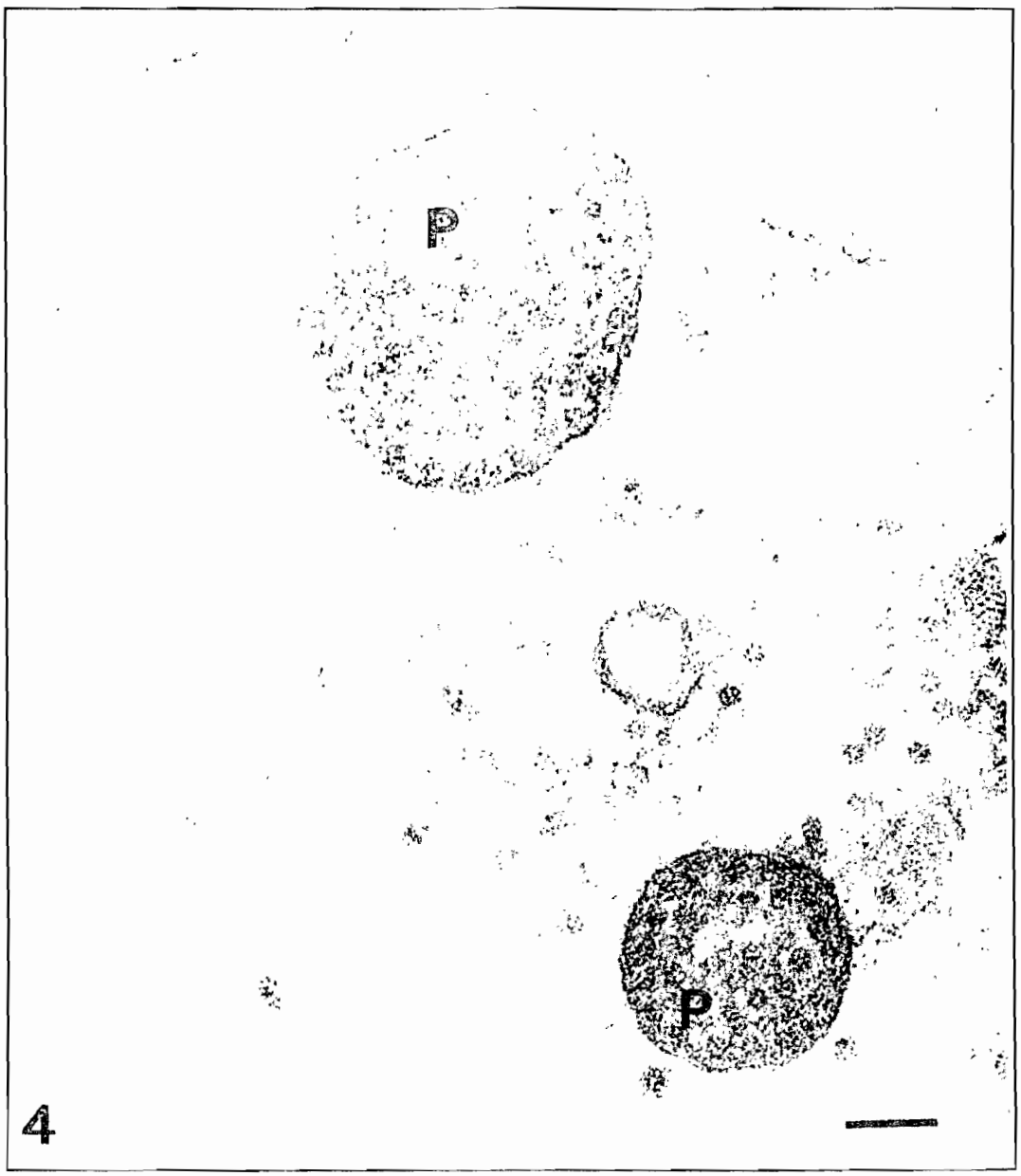

Fig. 4. Electron micrograph showing the presence of inculsion bodies within the cytoplasm of a nervous cell: paracrystalline arrays of virions (P). (Bar = 0.1 μ).

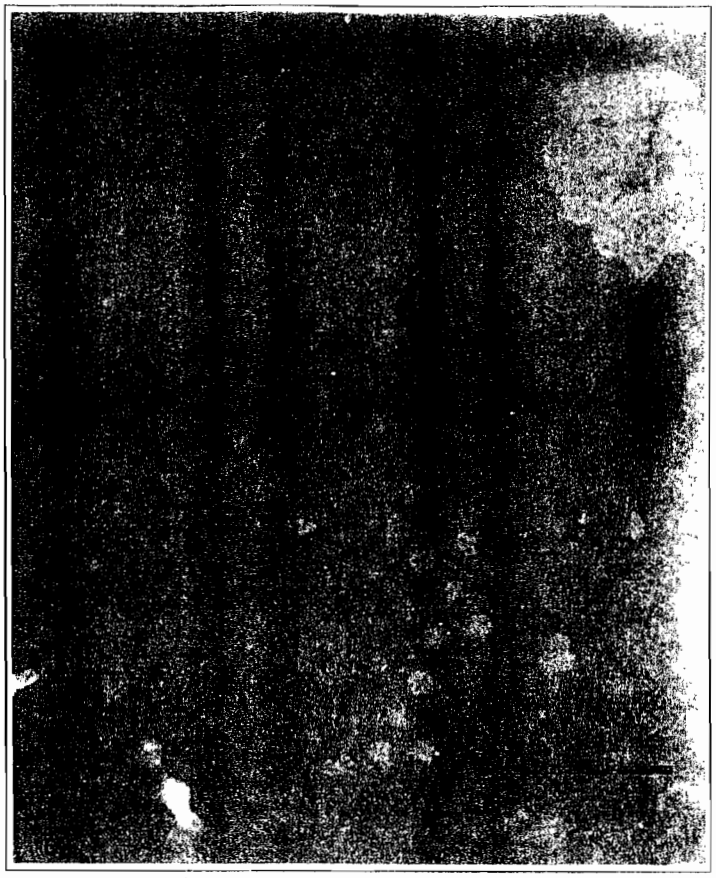

Fig. 5. Electron micrograph of negatively stained (PTA) purified virions. (Bar = 0.1 μ).