Infectious hypodermal and haematopoietic necrosis virus (IHHNV) in a hybrid penaeid prawn from tropical Australia

L. Owens¹, I. G. Anderson², M. Kenway³, L. Trott³, J. A. H. Benzie³

¹ Graduate School of Tropical Veterinary Science and Agriculture, PO James Cook University of North Queensland, Townsville, Queensland 4811, Australia

² Oonoonba Veterinary Laboratory, Department of Primary Industries, Townsville, Queensland 4811, Australia

³ Australian Institute of Marine Science, PMB No. 3, Townsville Mail Centre, Queensland 4810, Australia

ABSTRACT: Chronic low-grade mortalities became epizootic in a batch of *Penaeus esculentus* hybridised with *Penaeus monodon* prawns in Australia. Histopathological examination revealed extensive tissue changes in the gills, hypodermis, antennal gland, heart, ventral nerve cord, lymphoid organ, haematopoietic tissue, muscle and the connective tissue including the intertubule connective tissue in the hepatopancreas. Infected cells displayed intranuclear Cowdrey type A inclusions with margined chromatin. The inclusions did not stain positively with Feulgen's DNA stain. Electron microscopy showed particles approximately 20 nm in diameter in the cytoplasm near the nucleus but no virions were observed intranuclearly. All the above characters are diagnostic for infectious hypodermal and haematopoietic necrosis virus (IHHNV). Other viruses that were infecting these prawns included lymphoid parvovirus (LPV), *Plebejus* baculovirus (PBV), a haemocytic baculovirus and large icosahedral-shaped virus. This is the first record of IHHNV in a country that has not imported live prawns and suggests that IHHNV occurs naturally in the Australian Indo-West Pacific region.

INTRODUCTION

Due to zoogeographical isolation, aquatic animals in Australia were believed to have a relatively disease-free state. This idea has been transferred from the terrestrial livestock industries, which are relatively disease-free. Furthermore, this belief has been reinforced by years of research on the prawn fisheries, where no large scale disease problems have been encountered. However, the crash in Taiwanese prawn farms (Rosenberry 1988) has led to an awareness of the role of disease in general and viruses in particular in penaeid aquaculture. Since Australia's penaeid aquaculture industries were worth about A\$ 12 million in 1990–91 (Treadwell et al. 1992), there has been some effort to redefine our viral status.

In Australia, hepatopancreatic parvovirus (HPV) was first described in 1985 (Paynter et al. 1985); a *Monodon*-type baculovirus, *Plebejus* baculovirus (PBV), was described in 1987 (Lester et al. 1987); and a new parvovirus-like virus, lymphoid parvovirus (LPV), was discovered

in 1991 (Owens et al. 1991). LPV had many similarities to infectious hypodermal and haematopoietic necrosis virus (IHHNV) but did not readily produce the pathognomonic Cowdrey type A intranuclear inclusions (hereafter abbreviated to Cowdrey A). Both viruses infected tissues of mesodermal and ectodermal origin, with IHHNV being more tissue polytrophic than LPV. IHHNV is one of the most devastating viruses of prawns, particularly in *Penaeus stylirostris*, whilst some losses occur in both *Penaeus vannamei* and *Penaeus monodon* (Lightner 1988). Recently, IHHNV was found in Australia and this paper describes its discovery.

MATERIAL AND METHODS

Single spermatophores from male *Penaeus esculentus* were implanted in freshly moulted female *P. monodon*. The hatch rates varied between 0 and 4 %. The inter-species hybrid larvae were reared in either 80 l conical fibreglass tubs or 1 l Imhoff cones suspended in a 28 °C

constant-temperature water bath. In both systems, larvae were stocked at 100 l^{-1} and reared on a diet of *Chaetoceros gracilis*, *Artemia* and Frippak (registered trade name) microencapsulated particles and Frippak Flake. Larvae in the 1 l cones were terminated at the PL1 (postlarvae 1) stage to establish survival rates, whilst those in the 80 l tubs were reared through until the PL20 stage. At this age, 2000 interspecies hybrids were stocked into a 5 t, U-shaped larval tank and fed commercial prawn pellets according to the manufacturer's recommendations. After they had grown to 4 g, 400 juvenile interspecies hybrids were transferred into a 10 t maturation tank and the diet was supplemented by fresh squid.

Seawater (34 ppt) for the 2 rearing stages was settled for at least 24 h, filtered through sand and cartridge filters down to $1\text{ }\mu\text{m}$ before being treated by ultraviolet light (254 nm) at $9600\text{ }\mu\text{W cm}^{-2}$. The maturation tank was maintained on a recirculation system topped up daily with 10 % new seawater.

Moribund prawns were collected just after the major epizootic (Day 100; see Fig. 1) from holding tanks and fixed in Davidson's fixative. Histological processing and staining followed the methods outlined by Culling et al. (1985). For electron microscopy, diced gill tissue was fixed in 2.5 % glutaraldehyde/2 % paraformaldehyde in cacodylate buffer or 10 % formalin and postfixed in 1 % osmium tetroxide. The tissue was dehydrated and mounted in Spurr's (Tm) resin. Sections were cut on an LKB Ultratome at $500\text{ }\text{\AA}$ (50 nm), stained with uranyl acetate/70 % methanol and lead citrate and viewed at 80 kV on a Joel 2000FX transmission electron microscope.

RESULTS

Gross signs

Survival of the interspecies hybrids to the PL1 stage was greater than 80 % with no obvious abnormalities detected. Although densities were not measured during the subsequent postlarval stages until PL20, survival was estimated to be greater than 50 %.

When the prawns reached approximately 3 to 4 g, the major mortality occurred (Fig. 1) and consequently the survivors were moved to the maturation tank. There, chronic low-grade mortalities persisted over a 1 mo period. Approximately 5 prawns were dying every day when the prawns were 5 to 10 g. The prawns became weak, then stopped moving, and died. There was no noticeable change in coloration of the prawns, with the interspecies hybrids still reflecting a mixture

Fig. 1. Mortality curve of *Penaeus esculentus* × *Penaeus monodon* hybrids during juvenile growth. For comparison, data on the mortalities of *Penaeus stylirostris* described by Lightner et al. (1983) are shown.

of their parental colours and patterns. Fouling with epicommsals was not noticeable. However, the gills had many small melanised foci.

The mortality curve of the interspecies hybrids was compared to the mortality curve of *Penaeus stylirostris* as described by Lightner et al. (1983) (Fig. 1). The *P. stylirostris* epizootic occurred in much younger prawns than the interspecific hybrids, so the curves were transposed horizontally until the 50 % mortality point was common. When the shape of the 2 curves was tested with the Kolomogorov-Smirnov goodness-of-fit test, they were identical ($K-S = 0.03$, $p > 0.98$).

Histopathology

Single, rounded, intranuclear, eosinophilic occlusions were present in the hepatopancreatocytes of some of the prawns. The lesions are typical of PBV infections (Lester et al. 1987). The intensity of infection was low and was considered not to contribute to the mortalities.

All the major mesodermal and ectodermal tissues showed prominent Cowdrey type A intranuclear inclusions. The cells in the hypodermal tissues of the gills (Fig. 2) and oral appendages, the brachioistegial membrane, the general hypodermis (Fig. 3), antennal gland (Fig. 4), heart (Fig. 5), the gonads (Fig. 6), the lymphoid organ (Fig. 7), the connective tissue (Fig. 8), the haematopoietic tissue (Fig. 9), rarely the skeletal muscle (Fig. 10), and nerve cord (Fig. 11) displayed inclusions. The tissues/organs most heavily infected

Figs. 2 to 5. Infected *Penaeus esculentus* × *Penaeus monodon* hybrid. Fig. 2. Gills showing intranuclear Cowdrey A's (arrows) and an LPV inclusion (curved arrow). Note the hypertrophied nature of the infected nuclei, which are 2 to 3 times larger than uninfected nuclei. × 549; scale bar = 18.2 μm. Fig. 3. Hypodermis with Cowdrey A's (arrows). Note the large, amorphous nature of the Cowdrey A's. Compare with those in Fig. 4. × 686; scale bar = 14.6 μm. Fig. 4. Antennal gland with subepithelial layers displaying Cowdrey A's (arrows) and an LPV inclusion (curved arrow). Note the small rounded nature of the Cowdrey A. × 686; scale bar = 14.6 μm. Fig. 5. Heart with Cowdrey A's (arrows). × 686; scale bar = 14.6 μm.

Figs. 6 to 9. Infected *Penaeus esculentus* × *Penaeus monodon* hybrid. Fig. 6. Male vas deferens displaying Cowdrey A's (arrows), × 439; scale bar = 22.8 µm. Fig. 7. Lymphoid organ showing many Cowdrey A's (arrows) and a few LPV inclusions (curved arrows). Note the lack of normal, uninfected nuclei. × 686; scale bar = 14.6 µm. Fig. 8. Connective tissue around a blood vessel showing numerous Cowdrey A's (arrows). One in the centre has a very rounded eosinophilic inclusion (curved arrow) similar in size to an LPV basophilic inclusion. × 521; scale bar = 19.2 µm. Fig. 9. Haematopoietic tissue showing Cowdrey A's (arrows), × 549; scale bar = 18.2 µm.

Figs. 10 to 12. Infected *Penaeus esculentus* $\times$ *Penaeus monodon* hybrid. Fig. 10. Striated muscle with a single Cowdrey A (arrow). $\times 686$; scale bar = 14.6 μm . Fig. 11. Ventral nerve cord with a Cowdrey A (arrow) next to an LPV inclusion (curved arrow), both slightly out of focus as they were in different planes. $\times 686$; scale bar = 14.6 μm . Fig. 12. Infected intertubule connective tissue with both Cowdrey A's (arrows) and LPV (curved arrow). $\times 686$; scale bar = 14.6 μm .

were the lymphoid organ and the connective tissue throughout the prawn. Every cell of the lymphoid organ was infected, including the tubule cells. In some prawns, the connective tissue in-between the hepatopancreatic tubules was also infected (Fig. 12). This elicited a haemocytic infiltration which started to separate adjoining tubules. Also present in this area were inclusions of LPV. The hearts of some prawns had focal collections of haemocytes and in some prawns melanised nodules were contained within haemocyte aggregations in the connective tissue. None of the gut tissues displayed the inclusion bodies.

All the above tissues also displayed much less commonly the round, basophilic intranuclear inclusions of LPV. IHNV is a much more aggressive virus, with approximately 30 cells infected with IHNV to every one infected with LPV. Whilst the Cowdrey A inclusions were Feulgen negative, the LPV basophilic inclusion stained positively with Feulgen.

The first sign of an infected cell is margination of the chromatin which leads to the nucleus becoming clear. The cell becomes slightly to markedly hypertrophic. Then the nucleolus, which retains its rounded shape, becomes eosinophilic. The nucleolus is, at this stage, very similar in size to both the LPV inclusion and the IHNV basophilic inclusion. Many infected cells displayed this rounded, eosinophilic inclusion in a large, clear hypertrophied nucleus (Figs. 4 & 8). The intranuclear inclusion then grows non-symmetrically in size to give the typical Cowdrey A.

Electron microscopy

With transmission electron microscopy (TEM), 3 possible virus types were visualised: small IHNV-like virions; large (900 × 136 nm), cytoplasmic, haemocytic baculovirus virions, and large (100 to 180 nm), cytoplasmic, un-enveloped, icosahedral virions (cf. an iridiovirus). The last 2 virion types will be described in a separate paper.

Nuclei of cells presumptively displaying Cowdrey A's had a homogeneous mass that almost filled the whole nucleus (Fig. 13). There was no ring of margined chromatin but rather a wide separation of the 2 layers of the nuclear membrane (the nuclear envelope). The intranuclear matrix could not be resolved into virions even when magnified up to 50 000 times. However, in the cytoplasm of cells with presumptive Cowdrey A inclusions, there was a ring of non-enveloped particles about 18 to 20 nm in diameter around the outer nuclear membrane in particular (Fig. 14), and around other vacuoles and swollen mitochondria. The nuclear pores were also filled with these

particles and in one cell there appeared to be an out-pouring of these particles from one of the nuclear pores (Fig. 14). It appears that assembly of the virions is taking place in the cytoplasm at the membrane boundaries. Some of the mitochondria of infected cells were greatly swollen, to almost one-third the size of the nucleus (Fig. 13). The picture is remarkably similar to Figs. 3 & 4 of Lightner (1988) without the paracrystalline arrays, and to that of the densovirus from *Carcinus mediterraneus* (in Mari & Bonami 1988: their Fig. 2).

As well as the Cowdrey A's, there were the perfectly rounded, electron-dense, intranuclear inclusion bodies of LPV (Fig. 15). Again the inner matrix could not be resolved into individual virions but it seemed to be finely granular. Similar to cells displaying Cowdrey A's, the chromatin was not margined but rather appeared to be incorporated into the inclusion body (Fig. 16). A range of cells from newly infected to a full LPV inclusion body were found. Initially, there were cells with very dense and thickened chromatin, surrounded by a nuclear membrane in the process of splitting (Fig. 17). In one cell, a pocket in the chromatin contained virion-like particles (Fig. 17). Secondly, only the centre of the nucleus was not electron-dense and the nuclear membrane was split along its whole length, except for the nuclear pores where the mem-

Fig. 13. Infected *Penaeus esculentus* × *Penaeus monodon* hybrid. A Cowdrey A intranuclear inclusion of IHNV in the gills. The nuclear membranes (n) are split except at the nuclear pores (p) and there is a layer of particles around the nuclear membranes. The mitochondria (m) are greatly swollen. TEM, ×21 000; scale bar = 476 nm

Fig. 14. Infected *Penaeus esculentus* $\times$ *Penaeus monodon* hybrid. An enlargement of IHNV infected gill cell with an inclusion (I). Note the many particles 18 nm in diameter around the nuclear membrane and associated with the nuclear pores (p). Virions are apparently flowing out of the nuclear pore (arrow). Note the swollen mitochondria (m) close by. TEM, $\times 41\,000$; scale bar = 165 nm. Inset shows non-enveloped virions at the arrow magnified 2.8 times. TEM, $\times 115\,000$; scale bar = 462 nm.

Figs. 15 to 18. Infected *Penaeus esculentus* × *Penaeus monodon* hybrid. **Fig. 15.** Three IHNV infected cells (top) and one LPV intranuclear inclusion (bottom). TEM, ×5000; scale bar = 2 µm. **Fig. 16.** Late-stage LPV inclusion with split nuclear membranes and chromatin incorporated into the inclusion body. TEM, ×10 000; scale bar = 1 µm. **Fig. 17.** Early-stage presumptive LPV-infected nucleus with partial splitting of the nuclear membranes, hyperchromasia and a possible pocket of virions in the chromatin (arrow). TEM, ×17 000; scale bar = 588 nm. **Fig. 18.** Middle stage of development of an LPV inclusion with the central portion of the nucleus not fully incorporated into the inclusion. TEM, ×17 000; scale bar = 588 nm

branes are still joined (Fig. 18). Thirdly, the whole inclusion was electron-dense and it had small, irregularly positioned less dense parts to it (Fig. 16). No cells were seen that simultaneously displayed both types of pathology. Cells were either producing the LPV-type inclusion or the IHNV-type.

Immunodiagnosics

Samples of gills identified histologically as having IHNV-like lesions were sent to D. Lightner, University of Arizona (USA), to be tested with a monoclonal antibody to IHNV in an ELISA. The tissue gave

values of 38 to 71 % intensity of the known IHHNV positive control (Lightner pers. comm.).

DISCUSSION

The widespread, systemic occurrence of the Cowdrey A inclusion bodies, i.e. chromatin-haloed, Feulgen-negative, eosinophilic intranuclear inclusion bodies, is pathognomonic for IHHNV (Lightner et al. 1983, Lightner 1988) as no other prawn virus produces them in the same numbers. The diagnosis in the interspecific hybrid prawns was confirmed by TEM similarities between this case and IHHNV and by the results of the ELISA. Furthermore, the shapes of the mortality curves for IHHNV in *Penaeus stylirostris* and the interspecies hybrid were identical, but separated by age and size. Therefore, IHHNV or a virus that is indistinguishable from IHHNV within the current limits of diagnostic abilities is present in Australia.

As Australia has had a stringent quarantine policy, it is believed that no live prawns have been imported into the country, and the current case is a record of an endemic infection of IHHNV. This is supported by the presence of low numbers of Cowdrey type A inclusions in wild-caught *Penaeus merguensis* in northern Australia (Owens & Hall-Mendelin 1990). Furthermore, culture facilities in SE Asia using only wild broodstock have been infected with IHHNV (Lightner et al. 1990). This gives support to the notion that the West Pacific may be the original geographical source for IHHNV.

Owens & Hall-Mendelin (1990) noted that only prawns that did not have a long (geological time frame) exposure to IHHNV commonly produced Cowdrey A's whilst those that had been under long term (geological time frame) selection pressure did not. It took a new genetic mix, the cross between *Penaeus esculentus* and *P. monodon*, for IHHNV to become evident in Australia, even though research in this field has been ongoing since 1984. It is believed that had IHHNV occurred previously to this case, it would have been diagnosed.

Owens et al. (1991) recorded many similarities between IHHNV and LPV and concluded they were very closely related. There are 2 mutually exclusive hypotheses regarding these 2 viruses. Firstly, they could be 2 separate parvoviruses (Bonami et al. 1990, Owens et al. 1991). Alternatively, they could be the same parvovirus with different expression modes in different hosts or cells. The infection of prawns with both IHHNV and LPV should support the first hypothesis. However, cells of the interspecific hybrid prawns could be either expressing paternal, maternal or recombinant genes which may allow different cells in the same organ to express differently. All the surviving inter-

specific hybrids were males, suggesting the female mix of genes was lethal, and the basis of the interspecific hybrids' resistance to the viral infections was compromised. Therefore, the data from this case do not support either hypothesis unequivocally.

The presence of IHHNV in Australia gives further evidence that in terms of disease, tropical marine Australia is part of the SE Asian faunal province. Over recent years, more and more disease organisms thought to be absent from Australia have been discovered here, e.g. the sequential discovery of the prawn viruses themselves (loc. cited), lymphocystis virus (Pearce et al. 1990), luminescent *Vibrio harveyi* disease of prawns (Muir 1990), bopyrid parasites (Owens 1990) and the pathogenic strain of the freshwater bacteria *Edwardsiella tarda* (in Eaves et al. 1990). It seems apparent that isolation of Australia's terrestrial and freshwater faunas is not matched in its marine fauna or consequent diseases.

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