Putative spawner-isolated mortality virus associated with mid-crop mortality syndrome in farmed *Penaeus monodon* from northern Australia

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ABSTRACT: Beginning in 1994, farms in northern Australia experienced a higher than normal mortality rate in 12 to 15 g prawns from growout ponds. The farmers named this problem mid-crop mortality syndrome (MCMS). Intramuscular injection of filtered (450 nm), cell-free extracts of moribund prawns from these ponds killed healthy prawns between 5 to 30 d post-injection. A 20 nm virus was visualized by electron microscopy from a 1.4 g ml⁻¹ band recovered from caesium chloride gradients of extracts from the moribund prawns. DNA was extracted from this band, restriction enzyme digested and ligated into pGEM7zf(+) vector. A digoxigenin-labelled polymerase chain reaction (PCR)-generated, gene probe was subsequently prepared by amplifying an inserted sequence (approximately 2 kb) of one selected clone specific for the virus. Specimens of the moribund prawns stained positively by *in situ* DNA hybridization in endodermal tissues, including the apical ends of hepatopancreatic tubules, the midgut and hindgut caecae, the midgut, and the hindgut folds. In prawns that showed haemocytic enteritis, some haemocytes in the affected midgut showed limited staining. The positively-staining cells showed no cytolysis. In prawns injected with cell-free viral extracts, additional tissues were positive by probe analysis, including strong staining in the male reproductive tract, specifically in the terminal ampoule and the medial vas deferens. Limited staining also occurred in the ovary and in both the stromal matrix and spheroid cells of the lymphoid organ. It was evident that the infection was enteric by natural pathways and systemic by injection. Historical specimens of *Penaeus monodon* experimentally infected with spawner-isolated mortality virus (SMV) were probe-positive in exactly the same pattern as the naturally and experimental MCMS prawns. Altogether, the evidence suggested that the MCMS agent was a parvo-like virus very similar or identical to SMV.

KEY WORDS: *Penaeus monodon* · Mid-crop mortality syndrome · Spawner-isolated mortality virus · Parvovirus

INTRODUCTION

Prior to 1994, the culture of *Penaeus monodon* on farms in northern Australia was characterised by good yields and appeared free of the massive mortalities observed elsewhere in the Indo-Pacific region. However, 4 farms stocked in January 1994 began to experience a higher than normal rate of mortality in 12 to 15 g prawns from their grow-out ponds. The mortalities reached as high as 80 % in some ponds and there

was some evidence that 3 of the farms had been supplied with postlarvae from the same hatchery. The farmers referred to this problem as mid-crop mortality syndrome (MCMS). Due to commercial concerns and the consequent lack of dialogue, the aquaculture industry and government failed to recognize that it was facing a major industry-wide epizootic until early 1995. Thus, coordinated disease investigations did not start until mid 1995, whereby 18 mo of response time were lost. The syndrome produced no histopathognomic lesions and this greatly hampered investigation. Early investigations showed that filtered, cell-free extracts could kill clinically healthy prawns

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between 7 to 20 d post-exposure (Muir, Owens & Anderson unpubl.). Two distinct viral types were observed by electron microscopy in moribund prawns and observations on virogenesis suggested that at least 3 viruses might be involved. This report describes investigations into one of the virus-like particles associated with MCMS.

MATERIALS AND METHODS

Bioassays. *Penaeus monodon*, *Penaeus esculentus*, *Penaeus japonicus*, *Penaeus merguensis* and *Metapenaeus ensis* (3 to 10 g) were caught by cast net in the grow-out ponds of a number of farms and research institutes in northern Australia. Before stocking of shrimp, all tanks and equipment were disinfected for 24 h with a saturated solution of calcium hypochlorite, rinsed twice with tap water, and filled with sea water. The 180 l experimental tanks were equipped with 2 corner filters and 2 air stones and stocked with 10 prawns in each nonreplicated, treatment group. The water temperature was maintained at 21 to 26°C and the salinity at 25 to 37 ppt. The filter wool was changed weekly. Uneaten food, moults, and faecal strings were removed from the tanks daily.

Prawn extracts were prepared from 25 g of frozen cephalothoraxes from both diseased and healthy prawns. The tissue was homogenised in 25 ml of Dulbecco's Modified Eagle's Medium (DMEM, Trace Bioscience PL, cat. no. 50-013-PB) with 1% calf serum, in stomacher bags. The homogenate was made up to 100 ml, frozen and stored overnight at -20°C. After thawing, the homogenate was clarified by centrifugation at 9000 × *g* in an Eppendorf microfuge for 15 min. The extract was filtered through a 450 nm Sartorius Minisart filter using a 2.5 ml Terumo disposable syringe to form the cell-free extract. The filtrate was plated on a blood agar plate and a marine agar plate to ensure sterility. Only once was bacterial contamination observed, so the filtrate was passed through a 220 nm Sartorius Minisart filter and replated. Prawns to be used in the bioassay were intramuscularly inoculated with an extract from other members of their cohort as a test of latent viruses. This group was called the true controls; those receiving only DMEM were called controls, and those intramuscularly inoculated with 20 to 50 µl of moribund prawn extract were the test animals. Any mortalities within 24 h from the time of inoculation were considered to result from toxic protein shock and were replaced. The survival curves were analysed using the Life Tables in SPSS™ software.

Viral isolation. Prawns were collected from farms reporting mortality events and frozen at -20°C. The frozen cephalothoraxes and gut tissues were homo-

genised in a Waring laboratory blender, at a concentration of 10 heads per 200 ml of cold TNE buffer (50 mM Tris-HCl pH 7.4, 10 mM NaCl, 1 mM EDTA) (Bonami et al. 1990) and the homogenates were kept on ice. The homogenate was frozen, thawed and step-clarified by centrifugation at 4°C and 6000, 12 000 and 28 000 × *g* in a Beckman J2-21 centrifuge using a JA17 rotor for 10, 15 and 30 min, respectively. The final supernatant extract was pelleted by ultracentrifugation at 240 000 × *g* for 3 h at 4°C in a Beckman L7-55 ultracentrifuge, and the pellet was resuspended in a small volume of TN buffer (20 mM Tris-HCl, 400 mM NaCl at pH 7.5) (Bonami et al. 1990). This suspension was then centrifuged on a 25 to 45% caesium chloride (CsCl) gradient at 210 000 × *g* for 16 h at 4°C after which bands were localized by transmitted light. The refractive index of the bands was measured on an Atago refractometer and converted to buoyant density.

Electron microscopy. Bands of interest were repelleted as described above. The pellets were drained of supernatant and resuspended in a few drops of TNE. A small drop of these suspensions was placed on Parafilm and a formvar-coated (0.6%) electron microscopic grid placed on top. After 2 to 3 min, the grid was removed and allowed to dry. After rinsing with a drop of TN buffer, it was put onto a drop of uranyl acetate (2%) negative stain for another 2 min, removed and allowed to dry. It was then examined at 80 kV in a Jeol 2000 FX transmission electron microscope (TEM).

DNA probe. DNA was extracted from the CsCl-purified virus using 1% sodium dodecyl sulphate and 100 µg ml⁻¹ proteinase K as described by Sambrook et al. (1989). The ssDNA was then complemented with Klenow polymerase (O'Shade et al. 1986). The resulting dsDNA was then ligated to EcoRI adapters and subsequently digested with BamHI. Finally it was ligated into pGEM7zf(+) which had been doubly digested with EcoRI and BamHI. The vector was used to transform competent *Escherichia coli* strain JM109. Those clones containing large inserts were selected by electrophoresis following EcoRI and BamHI plasmid digestion. Inserts containing prawn DNA were eliminated using dot-blot hybridisation against *Penaeus monodon* DNA. Chosen clones were partially sequenced and compared with GenBank sequences using a Blastn search (Altschul et al. 1990) to determine those most likely to contain viral DNA. The final selected probe was labelled with DIG-11-dUTP (Boehringer Mannheim) using the polymerase chain reaction (PCR) and M13 primers whose complementary sequences were incorporated into the vector. The reaction cocktail included 150 µM dNTP (30 µM DIG-11-dUTP and 120 µM dTTP), 0.5 µM primers, 2.5 mM MgCl₂ and 1 U of *Tth* polymerase (Biotech International Ltd) in a reaction volume of 50 µl. After initial heating of the reaction

mixture at 95°C for 4 min, 35 PCR cycles were carried out with denaturation at 95°C (50 s), annealing at 55°C (50 s) and extension at 72°C (2 min) and with a final extension time of 7 min. Purity of the PCR product was determined by 1% agarose gel electrophoresis of an aliquot. Unincorporated digoxigenin (DIG-dUTP) was separated from the labeled product using ultrafiltration with a Centricon 100 (Amicon) membrane and the concentration of probe was estimated by comparison with a labeled standard (Boehringer Mannheim) following the protocol provided by the manufacturer.

Processing of specimens for histological analysis. Prawns were fixed in Davidson's fixative by injection and immersion for 24 h after which they were transferred to 70% ethanol for subsequent storage (Bell & Lightner 1988). They were subsequently processed and embedded as described by Culling et al. (1985). Sections of 5 µm were cut and adhered to silane- (Sigma Chemical Co.) treated glass slides and left overnight at 60°C. In addition to these specimens, similarly embedded, stored tissues from *Penaeus monodon* experimentally infected with spawner-isolated mortality virus (SMV; Fraser & Owens 1996) were sectioned and analyzed for comparison.

Hybridisation. The *in situ* hybridisation method used was based on that described by Rolighed & Lindeberg (1996) with some modifications. Briefly, tissue sections were deparaffinised through xylene, washed several times in ethanol and rehydrated in distilled water before final equilibration in TNE buffer. Tissues were then made permeable to probe by digestion with proteinase K 100 µg ml⁻¹ at 37 to 56°C for 10 min. Digestion was stopped by addition of glycine to a final concentration of 0.2% weight/volume (w/v). Tissues were then stabilised in 0.4% paraformaldehyde for 5 min at 4°C. This was followed by 2 washes in double distilled water for 5 min after which tissues were air dried for 5 min. Probe was then applied at a concentration of 1 ng µl⁻¹ in hybridisation buffer (50% formamide, 5% dextran sulphate, 0.5 mg ml⁻¹ sperm DNA, Denhardt's solution, 4× saline sodium citrate (SSC), (0.15 M NaCl and 0.015 M sodium citrate at pH 7.0) followed by placement of a coverslip and simultaneous probe and target DNA denaturation at 95°C for 8 min. Then, slides were immediately quenched on ice for 1 min and hybridised at 37°C overnight. Nonspecifically bound probe was removed by washing in 2× SSC twice for 5 min at room temperature and once in 0.1× SSC at 37°C. Detection of bound probe was carried out according to the protocol described by Durand et al. (1996) with colour development carried out overnight. Tissues were counterstained with 0.5% aqueous Bismarck Brown for 30 s and mounted with Crestia (Scientific Supplies) and Depex (Gurr).

RESULTS

Bioassays

Thirty-two batches of prawns from 6 farms and 2 research institutes were tested from 1995 to 1997. Statistically ($p < 0.01$), all the controls and true controls from 1995 and 1997 were identical with high survival rates (~100%). All the test animals, the controls of 1996 and true controls of 1996 died at a rapid rate with rarely any survivors at the end of a 30 d trial. Late in 1996, there appeared to be 2 types of mortality within 1 source of inoculum: 25% of prawns died within 5 to 10 d; the remainder died between 15 and 30 d. Mortality was bimodal with one mode on Day 8 and the other on Day 15. In bioassays, all the species of prawns tested (*Penaeus monodon*, *P. esculentus*, *P. japonicus* and *P. merguensis*) died at identical rates except for *Metapenaeus ensis*, which survived twice as long as the other species. If the source of the inoculum was from reproductively mature prawns, the mortality was slower than if the inoculum was from juvenile prawns.

Viral isolation and characterisation

A number of light diffracting bands was seen after isopycnic centrifugation through caesium chloride of extracts from moribund prawns. One of the bands that appeared more commonly had a buoyant density of 1.4 g ml⁻¹. Transmission electron microscopy of a negatively stained aliquot revealed particles of approximately 20 to 25 nm in diameter (Fig. 1).

In situ hybridisation

Digoxigenin-labelled probes were used for *in situ* hybridisation studies using histological tissue sections collected from suspected outbreaks of MCMS over several years. Prawns from farms showed blue/black precipitation in endodermal tissues with the most intense staining in the nuclei. Some cells gave staining only in the nucleoli. The cells of the apical end of the hepatopancreatic tubules (Fig. 2), the midgut caecae (Fig. 3), the midgut (Fig. 4), the hindgut caecae, and the hindgut folds all gave positive reactions. In prawns showing haemocytic enteritis, some haemocytes in the affected midguts showed limited staining. The infected cells showed no cytolysis. However, in prawns injected with cell-free extract from moribund MCMS prawns, additional tissues were positive on probe analysis. Of these, the most intensely stained were those tissues of the male

reproductive tract at the terminal ampoule (Fig. 5) and the medial vas deferens. Limited staining occurred in the ovary (Fig. 6) and in both the stromal matrix and spheroid cells of the lymphoid organ (Fig. 7). Occasionally, some cells of the haematopoietic tissue had traces of staining. It appeared that infection was enteric by natural pathways and systemic by injection. Moribund prawns collected from one farm in March 1995, from a different farm through to December 1996, and from bioassay tests using extracts of these farmed prawns were positive by *in situ* hybridisation. This showed that the virus was present in prawn farms in northern Australia for at least 21 mo.

Tissues of prawns showing other viral or microbiological infections were tested for specificity with the gene probe. *Monodon baculovirus*, hepatopancreatic parvovirus, Australian infectious hypodermal and haematopoietic necrosis virus (AIHHNV) (Owens et al. 1992), and a *Mycoplasma* from prawns (Ghadersohi & Owens 1998) failed to stain with the gene probe. However, all of these agents were probe-positive with their own respective gene probes, except AIHHNV, which was negative when tested with a probe derived from an isolate of IHHNV from *Penaeus vannamei* (DiagXotics Inc.). *P. monodon* experimentally infected with SMV (Fraser & Owens 1996) was probe-positive in exactly the same pattern as prawns experimentally infected with extracts from MCMS prawns.

Fig. 1. A negatively stained, transmission electron micrograph of presumptive virions recovered from a 1.4 g ml^{-1} band in a caesium chloride gradient of moribund MCMS prawn extract. Presumptive virions in the center of the photograph are spherical whilst some have straight sides and angular profiles characteristic of the genus *Parvovirus* (Murphy et al. 1995) (scale bar = 100 nm)

DISCUSSION

There is considerable evidence that one of the viruses associated with MCMS is a parvo-like virus. Parvoviruses have a buoyant density of 1.39 to 1.43 g

Fig. 2. *Penaeus monodon*. Photomicrograph of cells at the apical end of hepatopancreatic tubules of experimentally infected prawn showing scattered nuclei staining positive with the DIG-labeled gene probe (scale bar = 27 μm)

Fig. 3. *Penaeus monodon*. Photomicrograph of the anterior midgut caecum from farmed MCMS prawn showing positive staining with the DIG-labeled gene probe. Note that in addition to the heaviest staining associated with nuclei that many nucleoli are also stained (scale bar = 36 μ m)

Fig. 4. *Penaeus monodon*. Photomicrographs of midgut cells from MCMS prawn tested with the DIG-labeled gene probe. (a) Farmed prawns showing probe-positive cells on the left (I = infected) and probe-negative cells (N = normal) on the right (scale bar = 53 μ m). (b) Experimentally infected prawns showing one lobe heavily stained, some lobes with scattered staining and cells beneath the basement membrane unstained. (Scale bar = 53 μ m)

Fig. 5. *Penaeus monodon*. Photomicrograph of the terminal ampoule of the male reproductive tract from experimentally infected MCMS prawn tested with the DIG-labeled gene probe. (a) Low magnification of the tissue showing an intense positive intranuclear reaction (scale bar = 36 μ m). (b) High magnification showing unstained, uninfected nuclei and intensely stained, infected nuclei in noncytolytic cells (scale bar = 14 μ m)

Fig. 6. Photomicrograph showing focal, positive gene probe staining in the ovary of experimental MCMS prawn (scale bar = 36 μ m)

Fig. 7. Photomicrograph showing limited gene probe staining (arrowheads) of both stromal cells and spheroids (S) in the lymphoid organ of experimental MCMS prawn (N = normal; scale bar = 14 μ m)

ml⁻¹ (Murphy et al. 1995) whilst our sample came from a band at 1.4 g ml⁻¹. The size of the presumptive icosahedral virions visualised by TEM was 20 to 25 nm, which is consistent with the size range of parvoviruses (18 to 26 nm) (Murphy et al. 1995). Many parvoviruses cause enteritis in their hosts (e.g. human parvovirus, canine parvovirus and bovine parvovirus) and the gene probe here stained the enteric tissues of the prawn in natural infections. Parvoviruses can only replicate in actively dividing cells as they require cellular replication enzymes for virogenesis. Much of the staining in the prawns was associated with the zone of proliferation of the hepatopancreas (i.e. the outer edge of the organ). Likewise, midgut caecal cells (also with a very high mitotic index) gave strong probe-associated staining. Staining was mostly intranuclear and rarely cytoplasmic. Murphy et al. (1995) state that the progeny of infectious parvovirions accumulate in the cell nucleus. Cells showing a positive *in situ* hybridization reaction only in the nucleolus may have been at a non-proliferating stage of infection. Parvoviruses have an intimate association with the nucleolus and it is often changes in the nucleoli, including the separation of the fibrillar and granular portions, which are the first signs of infection, as in the genus *Densovirus* (family *Parvoviridae*) (e.g. Edgerton et al. 1997). In addition, the noncytolytic nature of the infections studied here is also characteristic of the genus *Dependovirus* (family *Parvoviridae*).

The relationships amongst the Australian parvo-like viruses of prawns needs clarification. Lymphoidal parvo-like virus (Owens et al. 1991) and Australian IHNV (Owens et al. 1992) have been tentatively

grouped together by Lightner (1996) due to their simultaneous occurrence in prawns dying of IHNV. Hepatopancreatic parvovirus (HPV) as characterized by Lightner (1996) has never been found in the hepatopancreas of Australian juvenile *Penaeus monodon* either by the presence of pathognomic intranuclear inclusions or by extensive HPV-gene probe analysis. Animals that were gene-probe positive for SMV, were gene-probe negative for HPV (Kahn 1998). Hepatopancreatic strains of HPV do exist in Australian *P. esculentus* (Paynter et al. 1985), *P. merguensis* (Darmono 1987) and *P. japonicus* (Spann et al. 1997a), although their interrelationships are unknown. The most parsimonious conclusion for *P. monodon* is that SMV and the parvo-like virus described here for MCMS are either very closely related or identical. Apart from our positive probe results with the original SMV-infected prawns, this is further supported by the fact that the MCMS-associated virus appears to be enteric and to infect the midgut, the only place SMV virions were visualised using TEM (Fraser & Owens 1996). In our opinion, the gene probe analysis data available at this time suggests that the various parvo-like viruses found in Australian penaeids are different. Further characterisation of these agents is required before definitive statements can be made as to whether the differences are at the strain or species level.

The aetiological agent(s) of MCMS still remains to be elucidated. The necessity of using mixed prawn homogenates which are then injected into farmed prawns possibly already harbouring a suite of viruses makes bioassays a crude investigative tool at best. The fact that the 1996 true controls died at rates equivalent

to the test animals in 1995, 1996 and 1997 demonstrates this point. Which members of the viral cocktail were the most important is unknown and it may be that it is the mix itself that causes the mortality. Ether-treated prawn extract killed at a slower rate than untreated extract (15 or more additional days to mortality), suggesting that the parvovirus was capable of causing mortality but that its virulence was enhanced by the presence of other coinfecting viruses such as an enveloped, filiform virus (gill-associated virus, Spann et al. 1997b).

In our opinion, the epidemiological behaviour of this parvo-like virus lends some support to the viral accommodation hypothesis of Flegel & Pasharawipas (1998). Their hypothesis proposes that prawns are capable of actively accommodating new viral infections by specifically suppressing viral-induced apoptosis, and that this capability can lead to a significant decline in the severity of epizootics from a new virus within 1.5 to 2 yr of its first appearance in a population. However, according to their hypothesis, a consequence of the process for the shrimp industry is the occurrence of occasional batches of tolerant carrier prawns that are still vulnerable to massive mortality triggered by environmental or other stress which overrides the apoptosis suppression mechanism. SMV produced epizootics with acute mortality in spawners in 1993 and 1994 (Fraser & Owens 1996). Early descriptions of these mortalities uncannily described what might be interpreted as apoptosis of haemocytes in infected prawns (O'Neill 1994). During 1994, MCMS epizootics occurred on farms with prawns that were probe-positive for this virus. By 1995, when researchers became involved, the virus/prawn relationship had apparently changed, such that probe studies showed the presence of the viral genome in the surprising absence of cytolysis (i.e. absence of apoptosis?). Furthermore, farmers in 1996/1997 described environmental factors (e.g. low oxygen stress, rapid salinity change) as being the reasons for mortalities in ponds where we found strongly probe-positive shrimp. A similar scenario occurred in Thailand with yellow-head virus disease (Pasharawipas et al. 1997) and led to the formulation of the viral accommodation hypothesis (Flegel & Pasharawipas 1998).

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