Short communication

Attempts at producing a hybridised *Penaeus monodon* cell line by cellular fusion

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**A R T I C L E   I N F O**

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**A B S T R A C T**

The lack of a standardised system for the isolation, identification and purification of prawn viruses, is a major obstacle to the control of viruses in penaeid aquaculture. To date, spontaneous and induced transformation of somatic penaeid cells has failed. Hybrid cells with the aim of supporting the growth of penaeid viruses were created using polyethylene glycol (PEG)-mediated fusion with two immortal cell lines, Epithelioma papulosum cyprinid (EPC) and *Spodoptera frugiperda* pupal ovarian cells (SB), fused with *Penaeus monodon* haemocytes. The immortal cell lines were biochemically blocked with actinomycin D and puromycin before fusion occurred. A total of 78 hybrid clones were created. The methods used to confirm the presence of *P. monodon* genes and proteins in the hybrid cells did not detect crustacean components, nor was any viral amplification detected by real-time PCR after hybrid cells were inoculated with two *P. monodon* paroviruses, *Penaeus merguiensis* densivirus and infectious hypodermal and haematopoietic necrosis virus. These results suggest although the creation of the hybrid cells appeared successful, the cell lines lacked key crustacean cell components required for their use as an *in vitro* system for virus replication.

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1. Introduction

The lack of a standardised system for the isolation, identification and purification of prawn viruses is a major obstacle to the control of viruses in penaeid aquaculture. In addition to the known viral pathogens, previously unknown or newly emerging viruses are posing further threats to this industry. In an attempt to try and keep up with both established and emerging penaeid viruses, the use of cell cultures is vital, not only to aid identification as a sensitive diagnostic tool, but also for the analysis of interactions between viruses and their host cells, in an effort to identify some of the mechanisms involved in the steps of viral infection [1].

Several investigators have demonstrated that prawn primary cell cultures can support in vitro growth of monodon baculovirus [2], yellowhead virus [3–7], whitespot syndrome virus (WSSV) [4,8–10] penaeid rod-shaped baculovirus (PRBV — synonym of WSSV) [11] and systemic ectodermal and mesodermal baculovirus (synonym of WSSV) [12]. However, primary cell cultures are tedious to develop, contain a heterogeneous mix of cells, have a limited life span and are prone to contamination. Immortal cell lines have several advantages over primary cell cultures as a research and diagnostic tool such as their ability to be continuously cultured in vitro, eliminating the need to return to live animals as a source of cells; the cell lines are often homogeneous with respect to geno-type and phenotype and therefore exhibit stable properties; and can be mass cultured within a short period, which may be essential in the face of a serious and extensive disease outbreak [13]. Currently no verified crustacean cell line exists despite numerous attempts.

Considering spontaneous and induced transformation of somatic penaeid cells has failed to date, creation of hybrid cells capable of supporting the growth of penaeid viruses was attempted by fusing cells from an immortal cell line with prawn cells. In the development of modern biology, the creation of cell hybrids by artificially-induced cell fusion has been a vital tool for the creation of monoclonal antibody-producing hybridoma lines in which fusion is induced between an antibody producing B lymphocyte and an immortalised myeloma cell [14–17].

The aim of this study was to first create *Penaeus monodon* cell hybrids, and then assess their viability and devise methods to confirm the presence of crustacean cellular components. Cells were also tested for their ability to support two penaeid paroviruses, using real-time PCR for assessment of viral replication.
2. Materials and methods

2.1. Source of animals

P. monodon were collected from wild stocks off the coast of northern Queensland, Australia, or from a commercial farm located at Innisfail, Queensland, Australia. Following collection, P. monodon were maintained at 28 ± 3°C at James Cook University in 1 t Reln® recirculating tanks with biological filtration.

2.2. Cell lines

Two immortal cell partners, Epithelioha papulosum cyprinid (EPC) and Spodoptera frugiperda pupal ovarian cells (Sf9) were investigated in cell fusion experiments. Cells were maintained in 25 cm² flasks containing 10 ml of Leibovitz 15 (L-15) (Gibco, Brisbane, Australia) or tissue culture medium 100 (TC-100) (Gibco, Brisbane, Australia) for the maintenance of the EPC and Sf9 cells respectively, and supplemented with 10% foetal bovine serum (FBS) (Trace Biosciences, Castle Hill, Australia). The cells were observed daily with an Olympus IMT-2 inverted phase-contrast microscope, and once a monolayer formed, cells were trypsinised, split and reseeded.

2.3. Prawn haemocytes

A total of 300 µl of haemolymph from each P. monodon was extracted using a 25-gauge needle inserted into the dorsal thoracic chamber. A 50% dilution with Alsever's anticoagulant (27 mM Na3C6H5O7, 336 mM NaCl, 226 mM glucose, 9 mM EDTA) was used to avoid clotting. After brief centrifugation (500 × g for 5 min), the haemocytes were resuspended in L-15 medium with 10% FBS supplemented with antibiotics (penicillin 200 IU/ml, streptomycin 250 µg/ml, kanamycin 80 µg/ml, polymyxin B 5 µg/ml, and fungizone® 2.5 µg/ml). Haemocytes from one animal were used for each fusion.

2.4. Fusions of immortal cell lines and prawn haemocytes

EPC and Sf9 cells at 4 × 10⁶ cells per flask were suspended in 20 ml culture medium without FBS. The cells were treated with actinomycin D (0.25 µg/ml) and puromycin (2 × 10⁻⁴ M) [17]. Culture flasks were incubated at 28°C for 3 h and rocked gently every 30 min to mix the cell suspension. To determine the amount of viable cells, equal volumes of a cell suspension and 0.2% trypan blue solution were mixed, loaded into the counting chamber of a haemocytometer, and examined using an Olympus IMT-2 inverted phase-contrast microscope. The viable cells, which were not stained blue, were counted and converted into viable cells/ml. The three flasks of biochemically-blocked cells were combined to provide a total of 1.20 × 10⁶ viable cells. The cells were washed twice with culture medium without FBS and pelleted in a 10 ml tube. Freshly harvested prawn haemocytes were added to the pelleted biochemically-blocked cells in a 1:1 ratio. The tube was made up to 10 ml with culture medium and then centrifuged. The supernatant was carefully removed and pre-warmed PEG-4000 (Koch-Light) (50%) was added dropwise over a 45 s with constant swirling in a 27°C waterbath. The cells were left for 60 s and then 10 ml of culture medium was added dropwise to dilute out the PEG solution. The cells were gently inverted to mix the PEG and left undisturbed for a further 5 min. The cells were pelleted, then resuspended in 10 ml of culture medium with 20% FBS. To obtain single cell colonies, the cells suspension was seeded out over a 96-well plate at 200 µl per well. Columns 2 through to 7 were used, with columns 1 and 8 containing culture medium only to help protect the cell-containing wells from drying out.

The plate was sealed inside a plastic container and incubated at 28°C. Cells were examined on days 1 and 6 post-fusion and 100 µl of medium exchanged. On day 14 post-fusion, cells of viable appearance were transferred to 24-well plates. Once confluent in the 24-well plates (generally from 7 d post-transfer), the cells were transferred to 25 cm² flasks and supported in culture medium supplemented with 10% FBS. When confluent in the flasks, cells were trypsinised and stored in liquid nitrogen, and later tested for crustacean components.

2.5. Tests for crustacean components in surviving cells

To verify that crustacean DNA was incorporated into the fused cells, two PCRIs were conducted using arthropod specific 18S rRNA primers 143F/143R [18] and primers designed on the P. monodon haemocyanin gene (GenBank accession no. AF431737) [19]. Once a confluent monolayer of cells had formed, cells were trypsinised and pelleted using slow centrifugation (500 × g for 5 min). DNA was extracted from the cells using a High Pure PCR Template Preparation Kit (Roche Sciences, Brisbane, Australia).

For PCR analysis, 1 µl of the DNA template (50–150 ng) was added to 49 µl of PCR reaction mixture consisting of MBI ferments PCR buffer (750 mM Tris–HCl pH 8.8, 200 mM (NH4)2SO4, 0.1% Tween20) 200 µM dNTPs, 1 µM of each primer, 1.5 mM MgCl2 and 1 U Tag polymerase (MBI Fermentas, Hanover, USA). The arthropod 18S rRNA primer sequences and methodology are described elsewhere [18]. For the haemocyanin PCR, primer sequences were H-299, 5′-TCA-AGA-CCG-CTG-GTA-ATC-ATA-3′ and H-594, 5′-CCG-GGA-ATT-GTG-TCT-3′ with an expected amplicon of 295 bp. The optimised PCR profile consisted of an initial 3 min denaturing at 95°C followed by 40 cycles of 95°C for 1 min, 59°C for 1 min, and 72°C for 1 min and a final 5 min extension at 72°C.

To analyse the PCR products, 10 µl of the PCR reaction mixture was used in electrophoresis on 1.5% agarose containing 1 mg/ml ethidium bromide and visualised under ultraviolet transillumination. Amplicons selected for DNA sequencing were purified from the agarose gel using QIAquick Gel Extraction Kit (Qiagen, Melbourne, Australia) and sent to Macrogen Corporation (Seoul, South Korea). Analysed sequences were then compared to available databases using the Basic Local Alignment Search Tool (BLAST) to determine approximate phylogenetic affiliations.

A monoclonal antibody (MAb) developed by Ainsworth [20], was used to assess the presence of crustacean proteins in hybrid cell populations. After assessing contamination and responsiveness of the antibodies, antibody 1B2-S was selected for immunostaining of the hybrid cell lines which was categorised as a type I antibody with general haemocytic specificity, staining approximately 78% of P. monodon haemocytes [20].

Polyclonal antibodies (PAb) were also used to assess crustacean proteins in hybrid cell populations. In contrast to monoclonal antibodies, which are derived from a single cell line, a PAb contains a mixture of immunoglobulin molecules secreted against many antigens. The polyclonal antibodies used in the present study were initially developed in chickens against gill-associated virus (GAV) of P. monodon [21]. This particular PAb was found to lack reactivity against GAV and was instead concluded to have a presumptive prawn cell origin which therefore may contain an array of non-specific proteins that react with P. monodon cells, making it an ideal candidate to use against the developed hybrid cells.

For immunohistochemistry (IHC) cells were spun onto positive charged microscope slides and fixed in acetone for 20 min. Endogenous peroxidase activity was blocked by adding 0.3%...
hydrogen peroxide in methanol to each slide for 30 min. After washing with phosphate buffered saline with 1% Tween20 (PBS-T), slides were incubated with either the MAB or PAb (1:100) for 1 h at 37 °C in a humid enclosed chamber. Slides were then exposed to three 5 min washes with PBS-T, and incubated with the secondary antibody (anti-mouse horseradish peroxidase (HRP) conjugate, 1:120; Jackson Immuno Research, West Grove, USA) in a humid chamber for 1 h at RT. After washing the slides in three 5 min washes with PBS-T, slides were incubated with fresh diaminobenzidine substrate for 10 min, washed briefly in water, and counter-stained with haematoxylin for 2 min. Following a brief water wash, slides were then exposed to Scott’s tap water substitute for 30 s before final water wash. Slides were then dehydrated through grades of ethanol, cleared with xylene, mounted and examined using an Olympus BH-2 microscope.

For quantitative analysis of crustacean proteins, an indirect ELISA was also developed using anti-chicken polyclonal antibodies [21]. Hybrid cells were trypsinised from 25 cm² flasks and centrifuged at 500 x g for 5 min. The cell count was determined by haemocytometer. Approximately 1 x 10⁵ cells were lysed by adding 125 µl of Celllytic MT reagent (Sigma, Brisbane, Australia). After 15 min of incubation with lysis reagent, cells were centrifuged at 5000 x g for 10 min, and the protein containing supernatant was removed for use as an antigen in the ELISA. Cell supernatant was diluted (1:1) in bicarbonate-carbonate coating buffer (TropBio, Townsville, Australia); 50 µl was loaded into each well of a 96-well U-bottom plate (Sarstedt, Adelaide, Australia), and incubated overnight in a humid environment. Following coating, 150 µl of post-coating buffer (TropBio Townsville, Australia) was added and the plate incubated for 1 h before washing three times in wash buffer (Trop Bio, Townsville, Australia). Fifty µl of diluted (1:80) PAb were added to each well, incubated for 1 h in a humid environment and washed three times with wash buffer. The secondary conjugated antibody was diluted (1:120), and 50 µl were added to each well. Following 1 h incubation, the plate was washed six times with wash buffer. Finally, 100 µl of ABTS peroxidase substrate solution were added to each well, and the plate was again incubated for 1 h. Colour formation was determined with dual absorbance readings at 415 nm and 490 nm, using a Labsystems Multiskan EX ELISA plate reader (Pathtech, Preston, Australia). All incubation steps were performed at RT.

A second ELISA was performed to detect the crustacean protein tropomyosin by sending samples to a commercial company, Elisa Systems (Windsoor, Brisbane, Australia). Hybrid cells were also exposed to two prawn viruses and quantification levels monitored. The viruses were the Australian Penaeus merguiensis densovirus (PmergDVN) and the Australian strain of infectious hypodermal and haematopoietic necrosis virus (IHHNV), both of which are known to infect P. monodon. The more lethal viruses such as white spot syndrome virus (WSSV) and/or yellow head virus (YHV) might have been more suitable for viral inoculation of cell cultures, but both viruses are exotic to Australia and therefore unavailable.

Similar methods were used to isolate and purify PmergDVN and IHHNV from prawn tissue. For PmergDVN, 25 hepatopancreas were removed from PCR-positive P. merguiensis [22]. For IHHNV, 100 pleopods were removed from 10 PCR-positive P. monodon [23]. Hepatopancreasi or pleopods were homogenised in 250 ml TN buffer (0.02 M Tris—HCl, 0.4 M NaCl, pH 7.4) and centrifuged at 4500 x g for 15 min to pellet tissue debris. The supernatant was re-centrifuged at 13 000 x g for 15 min, the liquid supernatant was collected and vacuum filtered through 0.45 µm filter membrane. Ultracentrifugation was then performed according to the method described in [24]. The final viral pellet was resuspended in 3 ml of TN buffer and stored at –80 °C until required. To verify the presence of viral DNA, a DNA extraction was performed on 200 µl of purified virus using the previously described primers and PCR methodology for PmergDVN [22] and IHHNV [23].

Duplicate 75 cm² flasks of hybrid cells, plus control cell lines of EPC and SF9 were grown to 60–80% confluency. Following removal of the culture media, each flask was inoculated with either 100 µl of purified virus or 100 µl of TN buffer. The flasks were gently agitated for 20 min to promote viral absorption [25]. Then, 15 ml of fresh culture medium (pH7.4) with 5% FBS was added, without removing inoculum. The cells were incubated at 28 °C and monitored once a day for 14 d for cytopathic effect (CPE). One ml of cell culture medium was collected at days 0, 4, 7, 10 and 14 d post-exposure and replaced with equal volume of fresh medium. Two hundred µl of these samples were used to perform a DNA extraction.

Extracted DNA was used in quantitative real time PCR to determine if viral replication was evident. Real time PCR was performed in accordance to the developed protocols for PmergDVN [22] and Australian IHHNV [26]. To accommodate small quantification differences, if the number of viral copies present at the end of the experiment (day 14) was approximately one log different to the number on day 0 (i.e. >10 fold difference), viral copies were considered to increase/decrease. A stable value was reported otherwise. Serial dilutions from previously constructed PmergDVN and IHHNV plasmids were used to determine a standard linear relationship for quantification.

3. Results

3.1. Hybrid cell lines

Homogenous cultures were produced by single cell cloning of the viable hybrid cells from three fusions; F11, F12 and F13 (Table 1).

3.2. Cell fusion confirmatory tests for crustacean components

Crustacean components in the fused cell populations were not demonstrated using any of the selected confirmatory methods. For the arthropod 18S rRNA gene, all hybrid cell lines produced PCR-positive amplicons of varying intensity at the expected size of 848 bp (Fig. 1). Sequencing amplicons from the cell hybrids showed no evidence of crustacean 18S rDNA, but contained 18S rDNA from the EPC or SF9 hybrid partner despite the presumed primer specificity. On the other hand, amplicons from P. monodon DNA matched (99%) the P. monodon 18S rDNA sequence.

All hybrid cells were PCR-negative for the P. monodon haemocyanin gene. A PCR-positive amplicon was obtained using DNA extracted from P. monodon haemocytes with subsequent sequencing matching the P. monodon haemocyanin gene (GenBank accession no. AF431737). The designed PCR primers were found to be highly specific for P. monodon haemocyanin detection and did not cause amplification in other crustacean species such as P. merguiensis, P. vannamei, P. stylirostris; Cherax quadricarinatus, Portunus pelagicus, Scylla serrata or Macrobrachium rosenbergii (data not shown).

Using IHC, no positive staining was observed when P. monodon haemocytes and fused cell populations were tested with MAb 1.9B2-5. Positive staining of P. monodon haemocytes, F12, and F13

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cell Fusion</th>
<th>Culture media</th>
<th>Number of clones</th>
</tr>
</thead>
<tbody>
<tr>
<td>F11</td>
<td>SF9 cells × P. monodon haemocytes</td>
<td>TC-100 + 10% FBS</td>
<td>52</td>
</tr>
<tr>
<td>F12</td>
<td>EPC × P. monodon haemocytes</td>
<td>L-15 + 10% FBS</td>
<td>6</td>
</tr>
<tr>
<td>F13</td>
<td>EPC × P. monodon haemocytes</td>
<td>L-15 + 10% FBS</td>
<td>20</td>
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cells was achieved with the PAb (Fig. 2). However, EPC cells, which were included as a negative control, also produced positive staining, suggesting this antibody is not specific for *P. monodon* proteins. This was further confirmed through use of a capture ELISA that found EPC cells produced a higher absorbance reading than any of the hybrid cell populations (Fig. 3) suggesting non-specificity of the PAb. The tropomyosin ELISA was also negative for the crustacean protein, suggesting a lack of tropomyosin expression in the hybrid cells.

For viral inoculation of the hybrid cells, the presence of viral DNA was confirmed using conventional PCR. Real-time PCR analysis found virus levels to either decrease or remain stable over the 14 d period, suggesting that no viral replication occurred, but that some residual virus remained in the inoculum.

### 4. Discussion

Most hybrid cell lines have been created by somatic cell fusion, requiring a fusion partner which is unable to survive in selection medium if it does not fuse with a primary cell partner. Littlefield [27] developed a cell selection procedure that showed by blocking the main biosynthetic pathway of guanosine by the folic acid antagonist aminopterin, an alternative ‘salvage’ pathway exists in which the nucleotide metabolites hypoxanthine or guanine are converted to guanosine monophosphate. This alternative pathway utilises the enzyme hypoxanthyl guanosyl phosphoribosyl transferase (HGRPT). Blocked cells that lack this enzyme, such as mouse myeloma cells used for the creation of hybridomas, cannot survive in media containing hypoxanthine, aminopterin and thymidine (HAT medium), as both DNA synthesis pathways are blocked. However, fused cells in which the missing enzyme is provided by the unblocked somatic cell partner, will survive and can be selected for.

Wyss [28] established several insect hybrid cell lines using a similar process of blocking the ‘salvage pathway’ of the enzyme adenosyl phosphoribosyl transferase (APRT) with thymidine, adenine and methotrexate (TAM) containing medium. However, similar to previous research [17,29] our research found limited success in producing hybrid cells using these methods (data not

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**Fig. 1.** Arthropod 18S rRNA PCR on cell hybrids, control cell lines and *P. monodon* haemocytes. Lane M: GeneRuler (1 kb); Lane 1: F11 cell hybrid; Lane 2: F12 cell hybrid; Lane 3: F13 cell hybrid; Lane 4: EPC cells; Lane 5: SF9 cells; Lane 6: *P. monodon* haemocytes.

**Fig. 2.** Immunostaining with a polyclonal antibody from *P. monodon*. (A) *P. monodon* haemocytes (positive control), (B) F13 cell hybrids, (C) EPC cells, and (D) EPC cells not exposed to PAb (negative control).

**Fig. 3.** Mean ELISA absorbance readings for EPC cells and EPC hybrid cell lines.
shown). Instead, this study fused *P. monodon* haemocytes with EPC and S9 cells that were pretreated with actinomycin D, an inhibitor of ribosomal RNA synthesis, and puromycin, a protein inhibitor [17]. Fused cells were able to proliferate because non-fused cells died due to the loss of active ribosomes and protein synthesis, whereas cells fused with primary cells were likely to survive due to the supply of intact ribosome’s and a protein synthesis ability. This fusion method was found to be successful in producing healthy viable hybrid cells that were able to be cloned post-fusion and could be examined for gene content.

However, the demonstrated absence of crustacean genes and proteins in the hybrid cells, along with the observed inability of the cells to support crustacean viral replication, suggest that these hybrid cell populations lack key crustacean cell components required for their use as a crustacean in vitro system. The make-up of the chromosomes, genes and proteins of the hybridised cells remain unknown. Although cell-to-cell fusion is universally involved in processes such as fertilisation and other biological events, the mechanisms involved in fusion vary among cell types and are not fully understood [30]. The deficiency of crustacean genes and proteins observed in the hybrid cells may be due to the absence of crustacean chromosomes. Many studies of cross-species cell hybrids have found that the chromosomes of one species are preferentially lost [31] or gradually lost over time [32]. Cell membranes can also blend or merge without fusion of the cellular contents [33]. Future research is therefore required to continue to confirm the true identity of the created hybrid cells. Karyotypic analysis of the parental and hybrid cells could be compared to confirm the makeup of the hybrid cells. If future hybrids were created, new fusion technologies are now on the horizon. Typically, PEG chemical fusions rely on random cell pairing often resulting in low overall fusion efficiencies and require lengthy subculturing to isolate desired hybrids. Fusion efficiencies have somewhat improved over time and methods using microfluidic devices show promise [34].

For many animals, cultured cell lines are considered indispensable tools for studying the molecular and cellular mechanism of pathogenesis [14]. In addition to this, the ability to grow virus in culture provides a potentially limitless source and thus facilitates its characterisation. These current attempts of cell line fusions were not successful at producing a crustacean hybrid cell line that was able to support viral growth. However, this methodology could be worthwhile for other research groups to pursue in the future as the goal of a continuous crustacean cell line that supports viral replication is still vital for breakthroughs in combating viral diseases.

References


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