Cell Culture Systems from *Penaeus monodon*: Development and Application

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ENVIRONMENTAL BIOTECHNOLOGY

Under the Faculty of Environmental Studies

By

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Certificate

This is to certify that the research work presented in this thesis entitled **"Cell Culture Systems from *Penaeus monodon*: Development and Application"** is based on the original work done by Ms. Seena Jose under our guidance, at the National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Kochi 682016, in partial fulfillment of the requirements for the award of the degree of **Doctor of Philosophy** and that no part of this work has previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title or recognition.

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Declaration

I hereby do declare that the work presented in this thesis entitled **"Cell Culture Systems from *Penaeus monodon*: Development and Application"** is based on original work done by me under the joint guidance of Prof. A. Mohandas (Emeritus Professor) and Prof. I.S. Bright Singh (Coordinator), National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Kochi 682016, and that no part of this work has previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title or recognition.

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CONTENTS

Chapter 1

GENERAL INTRODUCTION

1.1. Shrimp cell culture

1.1.1. Species used

1.1.2. Preparation of animals for aseptic removal of tissues

1.1.2.1. Surface Disinfection

1.1.3. Preparation of tissue for culture - explant method vs enzymatic dissociation vs mechanical dissociation

1.1.3.1. Explant Method

1.1.3.2. Enzymatic Dissociation

1.1.3.3. Mechanical dissociation

1.1.4. Contamination and antibiotics used in shrimp cell culture

1.1.5. Selection/Development of an appropriate culture medium

1.1.6. Osmolality of growth media

1.1.7. pH of growth media

1.1.8. Incubation Temperatures

1.1.9. Sub-culturing and Transfection

1.1.10. Crustacean cell culture for WSSV studies

1.1.11. Crustacean cell culture for cytotoxicity studies

1.2. Possible obstacles and solutions for shrimp cell immortalization

Penaeids shrimp are the most economically important groups of crustaceans distributed throughout Asia, Australia and the Western Hemisphere. Asian countries such as China, India, Indonesia, Vietnam and Thailand account for 55% of the total shrimp catch (FAO, 2009) world over. Globally, penaeid shrimp culture ranks sixth in terms of value amongst all taxonomic groups of aquatic animals cultivated (FAO, 2006). The most important cultured penaeid shrimp species are the giant black tiger shrimp (*Penaeus monodon*), Pacific white shrimp (*P. vannamei*), kuruma shrimp (*P. japonicus*), blue shrimp (*P. stylirostris*) and Chinese white shrimp (*P. chinensis*). World shrimp production is dominated by *P. monodon*, which accounted for more than 50% of the production in 1999 (FAO, 2001). They belong to the largest phylum in the animal kingdom, the Arthropoda. This group of animals is characterized by the presence of paired appendages and a protective cuticle or exoskeleton that covers the whole animal. The subphylum Crustacea is made up of 42,000, predominantly aquatic species that belongs to 10 classes. Within the class Malacostraca, shrimp, together with crayfish, lobsters and crabs, belong to the order Decapoda.

The exterior of penaeid shrimp is distinguished by a cephalothorax with a characteristic hard rostrum, and by a segmented abdomen (Fig 1). Most organs, such as gills, digestive system and heart, are located in the cephalothorax, while the muscles concentrate in the abdomen. Appendages of the cephalothorax vary in appearance and function. In the head region, antennules and antennae perform sensory functions. The mandibles and the two pairs of maxillae form jaw like structures that are involved in food uptake (Solis, 1988). In the thorax region, the maxillipeds are the first three pairs of appendages, modified for food handling, and the remaining five pairs are the walking legs (pereiopods). Five pairs of swimming legs (pleopods) are found on the abdomen (Bell and Lightner, 1988; Baily-Brock and Moss, 1992).

The internal morphology of penaeid shrimp is shown in figure 2. Penaeids and other arthropods have an open circulatory system and, therefore, the blood

and blood cells are called haemolymph and haemocytes respectively. Crustaceans have a muscular heart that is dorsally located in the cephalothorax. The valved haemolymph vessels leave the heart and branch several times before the haemolymph arrives at the sinuses that are scattered throughout the body, where exchange of substances takes place. After passing the gills, the haemolymph returns in the heart by means of three wide non-valved openings (Bauchau, 1981). A large part of the cephalothorax in penaeid shrimp is occupied by the hepatopancreas, the digestive gland. The main functions of the hepatopancreas are the absorption of nutrients, storage of lipids and production of digestive enzymes (Johnson, 1980). One of the haemolymph vessels that leaves the heart ends in the lymphoid organ, where the haemolymph is filtered (van de Braak, 2002[a]). This organ is located ventro-anteriorly to the hepatopancreas (Fig 3). Haemocytes are produced in the haematopoietic tissue. This organ is dispersed in the cephalothorax, but mainly present around the stomach and in the onset of maxillipeds. Figure 4 shows the early stage ovary in *P. monodon.* Ovary lies dorsal to the gut and extends from cephalothorax along the entire length of the tail.

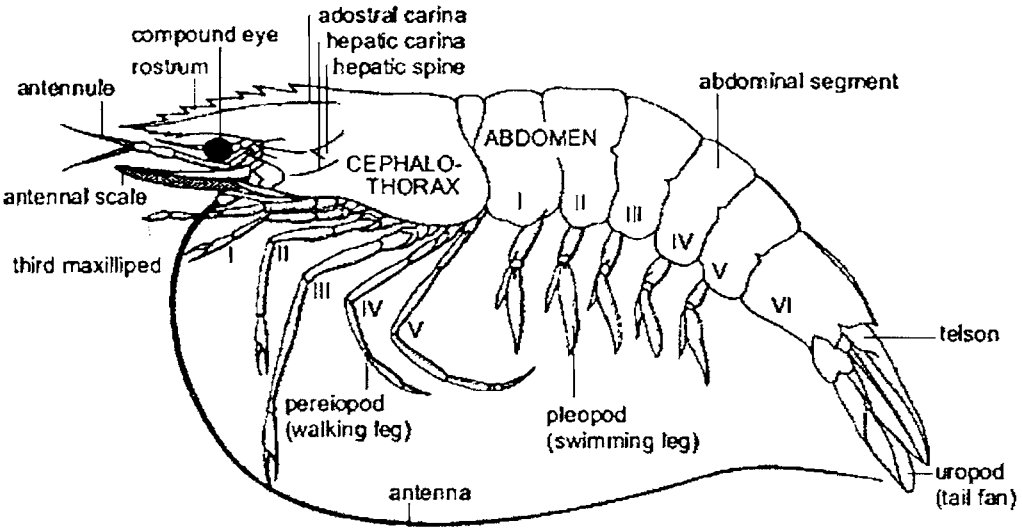


Fig.1. Lateral view of the external morphology of *Penaeus monodon* (Primavera, 1990)

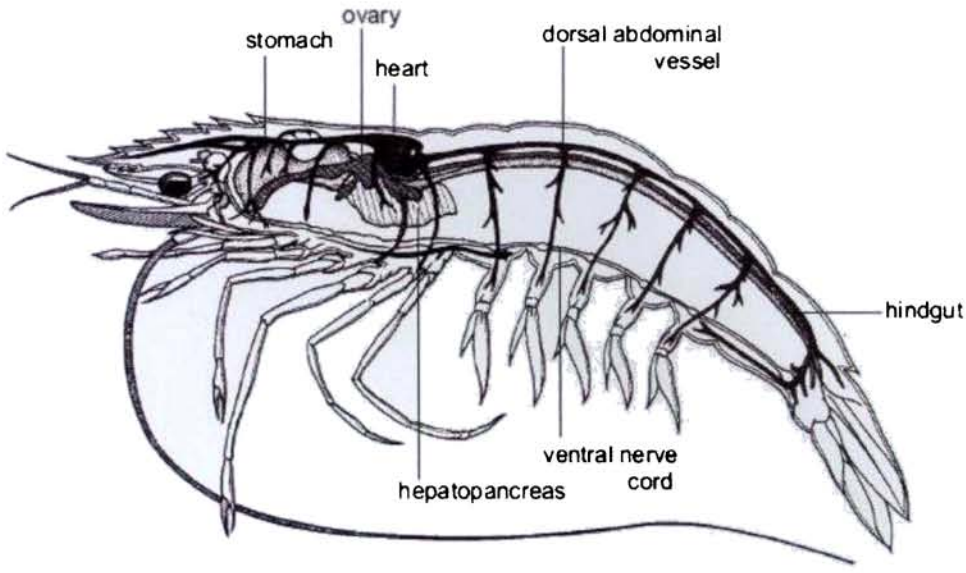


Fig.2. Lateral view of the internal anatomy of female *P. monodon* (Primavera, 1990)

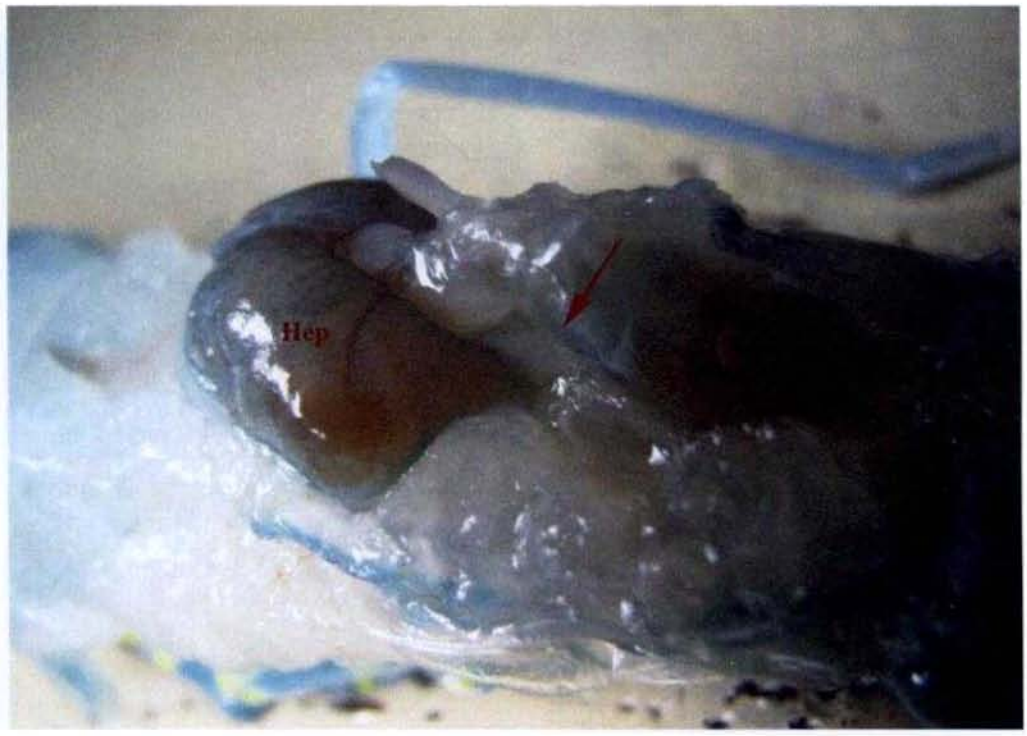


Fig.3. Cephalothoracic region of *P. monodon* showing lymphoid organ (arrow) (Hep-hepatopancreas)

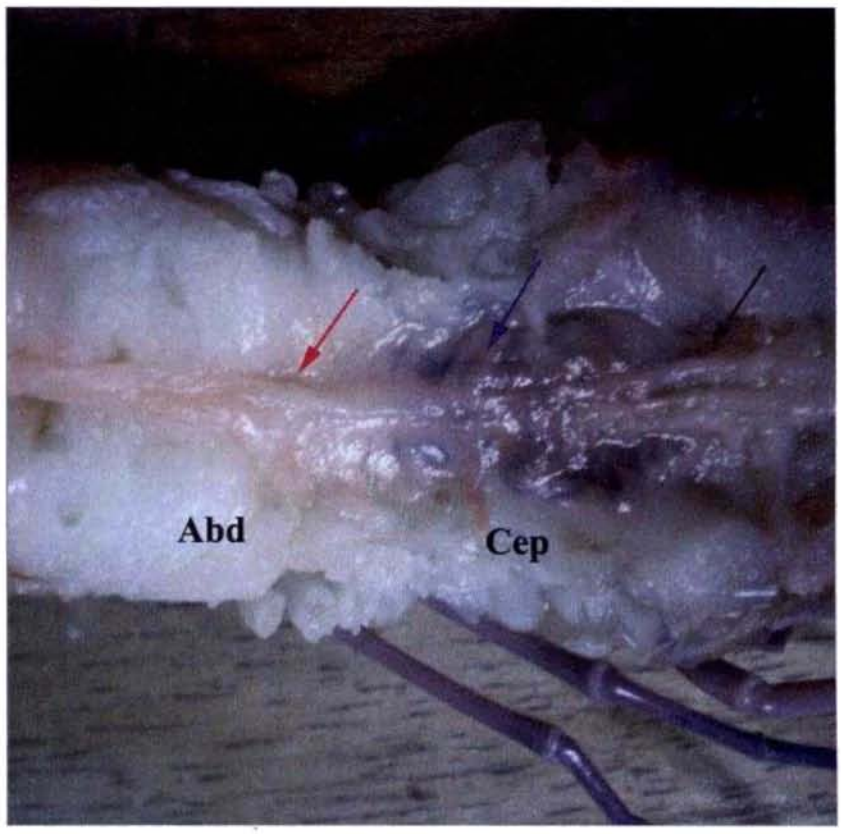


Fig.4. An early stage ovary of *P. monodon* demonstrating anterior lobules (black arrow), lateral lobules (blue arrow) and posterior lobules (red arrow) (Abd-abdominal region; Cep- cephalothoracic region)

1.1. Shrimp cell culture

With the rapid growth of high intensity aquaculture of penaeid shrimp, viral diseases have spread over the shrimp farms worldwide since 1990, causing severe financial losses (Bachere, 2000; Valderrama and Engle, 2004; Chen and Li, 2005). About 20 viruses have been reported in wild and farmed shrimp (Bonami, 2008) including White spot syndrome virus (WSSV), Monodon baculo virus (MBV), Yellow head virus (YHV), Taura syndrome virus (TSV) and Infectious hypodermal and haematopoietic necrosis virus (IHHNV). In order to develop effective strategies for overcoming the plague, detailed studies of shrimp biology and shrimp viruses should be performed. A permanent shrimp cell line

will greatly facilitate the research works in this field. The earliest research on in vitro culture of shrimp cells began in Taiwan where shrimp epizootic broke out first (Chen et al., 1986). Although some successes have been made, no permanent shrimp cell line has yet been made available (Rinkevich, 2005)

1.1.1. Species used

Attempts have been made to develop cell cultures from penaeids such as *P. monodon* (Chen et al., 1986, 1998; Hsu et al., 1995; Chen and Wang, 1999; Fraser and Hall, 1999; Kasornchandra et al., 1999; West et al., 1999; Wang et al., 2000), *P. stylirostris* (Luedeman and Lightner, 1992; Nadala et al., 1993; Lu et al., 1995; Tapay et al., 1995; Shike et al., 2000; Shimizu et al., 2001), *P. japonicus* (Machii et al., 1988; Sano, 1998; Chen and Wang, 1999; Itami et al., 1999; Lang *et al.*, 2002, 2004[a],[b]; Maeda et al., 2003, 2004), *P. chinensis* (Tong and Miao, 1996; Huang et al., 1999; Fan and Wang, 2002; Chun-Lei et al., 2003; Jiang et al., 2005), *P. penicillatus* (Chen et al., 1989; Chen and Wang, 1999), P. *indicus* (Toullec et al., 1996; Kumar et al., 2001), *P. vannamei* (Luedeman and Lightner, 1992; Nadala et al., 1993; Lu et al., 1995; Toullec et al., 1996) and the non penaeids such as *Macrobrachium rosenbergii* (Frerichs, 1996). Besides, initiatives have been made for obtaining cell cultures from *Nephrops norvegicus* (Mulford and Austin, 1998; Mulford et al., 2001)

The donor tissues from these species used for cell culture development were ovary (Chen et al., 1986, 1989, 1998; Luedeman and Lightner, 1992; Nadala et al., 1993; Tong and Miao, 1996; Mulford and Austin, 1998; Itami et al. 1999; Toullec et al., 1999; West et al., 1999; Chen and Wang, 1999; Shike et al., 2000; Shimizu et al., 2001; Lang et al., 2002; Maeda et al., 2003, 2004); testis (Mulford and Austin, 1998; Toullec, 1999); lymphoid (Chen et al., 1989; Nadala et al., 1993; Tapay et al., 1995; Hsu et al., 1995; Lu et al., 1995, Tong and Miao, 1996; West et al., 1999, Itami et al., 1999;Chen and Wang,1999; Wang et al., 2000; Lang et al., 2002, 2004[a],[b]) heart (Chen et al., 1986; Tong and Miao, 1996; Mulford and Austin, 1998; Chen and Wang, 1999; Lang et al., 2002); hepatopancreas (Chen et al., 1986;

Machii et al., 1988; Ghosh et al., 1995; Mulford and Austin 1998; Toullec 1999; Wang et al., 2000; Lang et al., 2002); gill (Chen et al., 1986; Mulford and Austin, 1998); nerve (Chen et al., 1986; Tong and Miao 1996; Mulford and Austin, 1998; Toullec, 1999; Lang et al., 2002; Chun- Lei et al., 2003) muscle (Chen et al.,1986; Lang et al., 2002); haematopoeitic tissue (Mulford and Austin, 1998; Chen et al., 1998; West et al., 1999; Mulford, 2001); embryonic tissue (Tong and Miao, 1996; Frerichs, 1996; Toullec et al., 1996; Fan and Wang, 2002); haemocytes (Chen and Wang, 1999; Itami et al., 1999; Jiang et al., 2005); eyestalk (Tong and Miao, 1996; Mulford and Austin, 1998; Kumar et al., 2001); epidermis (Toullec et al., 1996; Toullec, 1999) gut (Chen et al., 1986; Mulford and Austin, 1998); and Y organ (Toullec, 1999).

1.1.2. Preparation of animals for aseptic removal of tissues

Aseptic removal of tissues for cell culture development has always been a difficult task due to their aquatic inhabitation and the fact that they carry passive microorganisms in their body fluid with out any pathological signs. To avoid contaminated tissue going in to the tissue culture bottles several steps have been incorporated as part of the protocols over the years.

1.1.2.1. Surface Disinfection

Following have been the different protocols adapted by the earlier workers:

1. Immersed animals in ice cold solutions of 10% bleach X 5 min, 1% povidone iodine for 5min and 70% ethanol for 5min (Shike et al., 2000[a])
2. Immersed the donors in 70% ethanol (Lang et al., 2002 and Maeda et al., 2003)
3. Immersed in 10% sodium hypochloride for 10min and then wiped with 70% ethyl alcohol 5 times at interval of three minutes (Chen et al., 1986)

4. Maintained for 18-96 hours in running sea water sterilized by ultraviolet light and Millipore (0.45) filtration.
5. Maintained in Dakin's fluid for 30-60 seconds prior to dissection and washed three times with balanced salt solution (Machii et al., 1988).
6. Submerged for 5 minutes in chilled 70% ethanol (West et al., 1999).
7. Fertilized eggs were suspended for 1 hour at room temperature in PBS-antibiotic solution (penicillin 400 IU ml^{-1}; streptomycin 400 µg ml^{-1}). Eggs were pelleted and resuspended in a few milliliters of 1:10 buffered iodophore (Buffodine: Evans Vanodine) with added malachite green (0.01mg ml^{-1}) and held for 20 minutes (Fan and Wang, 2002).
8. Disinfected the animals using 5% sodium hypochlorite. Dissected tissues were immersed in a solution containing 3000 $IUml^{-1}$ penicillin and 3000 µg ml^{-1} streptomycin for 5-10minutes (Chen and Wang, 1999).
9. Shrimp anesthetized by placing on ice and were surface sterilized with 70% alcohol followed by 0.02% iodine disinfectant (Wang et al., 2000).
10. Shrimps submerged in iodoform solution (iodoform/water = 1:30, v/v) for about 10-15 min (Chun-Lei et al., 2003).
11. Animals sacrificed by plunging into ice for 3 to 5 minutes and disinfected in ice cold sodium hypochlorite solution (300-350ppm) for 5min, prepared in autoclaved sea water (20g l^{-1}). Subsequently washed with sterile sea water and dipped in cold ethanol (70%) for 2-3minutes, again washed in sea water for 3-4 times (Kumar et al., 2001).
12. Shrimps swabbed with 75% ethanol (Jiang et al., 2005).
13. Maintained in aerated sea water containing 1000 IU ml^{-1} penicillin, 1000 µg ml^{-1} streptomycin for 4-18 hours at room temperature. The embryos and larvae were pretreated with the above antibiotics

combined with fungizone (2.5 μg ml^{-1}) and (Nystatin 100 μg ml^{-1}) for 4 hours, then disrupted by aspiration with a fine tip pipette (Tong and Miao, 1996).

14. Eggs removed from ovigarous *M. rosenbergii* 7-13 days after fertilization and mixed and held for 1h at room temperature in PBS-antibiotic solution (penicillin 100 IU ml^{-1}; streptomycin 100 μg ml^{-1}l; kanamycin 100 μg ml^{-1}; amphotericin B 1 μg ml^{-1}). Treated eggs pelleted by low speed centrifugation and resuspended in a few milliliters of 1:10 buffered iodophore (Buffodine: Evans Vanodine International) with added malachite green (1mg $100ml^{-1}$). Incubated for 20 min (Frerichs, 1996).

15. Immersed shrimp in 0.06% iodine solution dissolved in sea water for 5min and wiped with 70% ethanol (Itami et al., 1999).

16. Shrimp soaked in 100ppm $KMNO_4$ at 4°C for 30 min and then rinsed with sterile sea water (Huang et al., 1999).

17. The animals anaesthetized by immersion in sea water at 4°C for 50-60 min were dipped briefly in 10% (w/v) sodium hypochlorite to inactivate extraneous micro organisms, and rinsed for 10 min in 7% (w/v) iodine tincture followed by rapid immersion in holding medium, which consisted of serum free medium supplemented with penicillin- streptomycin (10^4 IU/ml^{-1}), amphotericin B (10 μg ml^{-1}) and gentamycin (2.5 μg ml^{-1}). Subsequently rinsed several times with 70% ethanol (Mulford and Austin, 1998).

18. After dissection tissues were washed in 2X L-15 containing 1000 μg ml^{-1} streptomycin, 1000units ml^{-1} penicillin and 100 μg ml^{-1} fungizone (Chen et al., 1998; Itami et al., 1999).

19. Sterilized by immersion in freshly prepared 5% (v/v) chlorax containing 5.25% sodium hypochlorite (Chen et al., 1989).

20. Anaesthetized in cold water for 50 min and submerged in freshly prepared 7% iodine disinfectant (Luedeman and Lightner, 1992).

21. Soaked in 10% sodium hypochlorite for 5 minutes. After dissection lymphoid organs pooled in antibiotic incubation medium containing 2XL-15, 100 IU/100µg penicillin/streptomycin per ml, 1% amphotericin B and 0.5% gentamycin with agitation and transferred to fresh medium for further 0.5hr incubation (Tapay et al., 1995)

22. Anaesthetized for 20min and surface sterilized in 0.02% iodine disinfectant for 5min before tissue excision (Hsu et al., 1995).

23. Surface sterilized by 10 min immersion in freshly prepared 1% sodium hypochlorite and rinsed with 70% ethanol (Toullec et al., 1996).

1.1.3. Preparation of tissue for culture - explant method vs enzymatic dissociation vs mechanical dissociation

Once the tissues are aseptically removed they can be stored at 4^0 C for a short period of 30 minutes before seeding in tissue culture bottles. However, the tissues have to be processed further for effective proliferation. Over the years several attempts have been made to evolve an effective preparation of tissue to initiate active proliferation and growth in an appropriate tissue culture medium. Three methods attempted were the explant, enzymatic dissociation and mechanical dissociation. Reports available in literature on implementation of these methods are summarized below:

1.1.3.1. Explant Method

In explant method the tissues are minced into smaller pieces and seeded in growth medium. This method was adapted by several workers (Chen et al., 1986, 1989; Luedeman and Lightner, 1992; Nadala et al., 1993; Lu et al., 1995; Tapay et al., 1995; Toullec et al., 1996; Tong and Miao, 1996; Mulford and Austin, 1998; Chen and Wang, 1999; Itami et al., 1999; Wang et al., 2000; Mulford et al., 2001; Kumar et al., 2001; Lang et al., 2002; Chun-Lei et al., 2003). According to Toullec (1999) the

capability of explants to attach to tissue culture bottle is linked to the strength of haemocytes to adhere to and in fact all tissues contain a small quantity of haemolymph and haemocytes smeared on. Haemocyte-like cells could provide a natural attachment factor. However, hepatopancreas contains only a fewer haemocytes than in the other tissues, but at the same time do exhibit a good capability to attach. This property is provided by the outer membrane of microtubules. But the same outer membrane on the other hand prevents the cell migration delimiting their prospects of becoming a cell line. This suggests that explant technique is not suitable to every tissue; as a general rule, loose tissues are best adapted to this protocol.

1.1.3.2. Enzymatic Dissociation

Enzymes commonly used for the dissociation of shrimp tissues are collagenase, trypsin, pronase and dispase. According to Toullec (1999) trypsin and pronase seem to be too potent for crustacean tissues. Collagenase and dispase are weaker and cause less damage to the cells being more specific to connective tissue. Numerous washes are needed to eliminate the dissociating enzyme. However, enzymatic treatment can weaken the cell membranes and decrease their ability to attach to the substratum. A coating with adhesion factors such as poly- lysine or collagen can compensate this effect.

Maeda et al. (2003) treated tissue pieces with 0.1% collagenase Type V solution at 28°C with shaking at 50 rpm. The suspension was filtered through a metal mesh to remove undigested tissues. Maeda et al. (2004) also used collagenase Type V for 30 minutes at 25°C to disperse the cells from ovarian tissue cut into pieces of 2-3 mm^3. Type IV collagenase was used by Toullec et al. (1996) for the enzymatic dissociation of epidermis, ovary and embryos at a final concentration of 0.25% in culture medium for 30min at 26°C. At the end of this period cells were washed twice with appropriate culture medium containing 10% fetal bovine serum. Cell suspensions were filtered through a 60 µm mesh filter in order to remove tissue fragments.

For the dissociation of midgut gland, Machii et al. (1988) minced the tissue and trypsinised in 0.05% trypsin in Calcium Magnesium free fluid for 20 minutes. Cells were washed twice in a balanced salt solution with 10% FCS or medium Pj-2 with 0.012µg/ml aprotinin. Ghosh et al. (1995) used perfusion technique for the dissociation of hepatopancreas. Prawns were injected with heparin (5000 U) into the periarthroidal space. After 10-15 min carapace was removed and a 20-gauge needle connected to a perfusion reservoir by polyethylene tube was inserted directly into the hepatopancreas and perfused with about 50ml perfusion medium (144mM, KCl; 5mM, KH_2PO4; 1.2mM, $NaHCO_3$; 33mM, EDTA at 7.5 pH) with 0.2g l^{-1} streptomycin sulphate at a flow rate of 12ml/min. The tissue was then carefully removed and transferred to a beaker containing 20ml perfusion medium with EDTA. The hepatopancreas was then meshed with a rubber police man and stirred at 200 rpm with a sterile magnetic stirrer bar with constant bubbling of filtered air.

Chun-Lei et al. (2003) employed trypsin for the dissociation of eyestalk. Eyestalks were removed and rinsed thrice with shrimp saline. Exoskeleton, muscles and some connective tissues were removed in sterile saline, medulla terminalis taken out and placed in Ca^{2+} - and Mg^{2+}-free saline with 0.1% trypsin in the dark for 90 min at 22°C. For the dissociation of haematopoietic tissue Mulford et al. (2001) used pronase (dispase; ex. *Clostridium histolyticum*, Sigma), collagenase (Type 1A; ex. *Clostridium histolyticum*, Sigma) and trypsin (ex. Porcine pancreas, Gibco BRL) prepared to 0.025%-0.2% (w/v) in 3X PBS, calcium and magnesium free with antibiotics at 800 mOsm/kg and at pH 7.4. Small fragments of tissue were added to 4ml volumes of each enzyme with incubation for 15min to 12h at 4°C, and at room temperature.

1.1.3.3. Mechanical dissociation

Mechanical dissociation provides a large number of cells, but seems to reduce the ability of cells to attach and an adhesion factor is sometimes needed to assist their attachment to the substrate. Most fragile cells are often broken by this

drastic treatment. Ruptured cells release proteases into the medium; therefore numerous washes are necessary prior to cell plating to avoid cell digestion. Toullec (1999) and Shike et al. (2000[a]) created cell suspension by sieving the lymphoid organ or ovary through a stainless steel mesh (190nm pore size). Subsequently lymphoid organ cell suspension was passed through a nylon mesh cell strainer (40μm pore size) before plating to remove the debris. West et al. (1999) used a ground glass homogenizer with a clearance of 100 μm for the dissociation of haematopoietic tissue, lymphoid and ovary. Frerichs (1996) and Fan and Wang (2002) gently ground eggs using a mortar and pestle in disinfectant solution, pelleted, resuspended in PBS - antibiotic solution and passed through a stainless steel strainer to separate the cellular component from residual debris. Tong and Miao (1996) disrupted embryo and eggs pretreated with antibiotics by aspiration with a fine tip pipette.

For the dissociation of hepatopancreas, Huang et al. (1999) removed the whole organ from the anterior midgut and put into a sterile beaker in ice bath with 5ml NaCl ($27gl^{-1}$) solution. Hepatopancreas was cut using scalpels and aspirated several times using a dropper. The suspension was passed through a sterile 300 mesh sieve to obtain a single cell suspension. Mulford et al. (2001) pipetted the fragments of haematopoietic tissue several times and sieved through a 40-60 mesh screen tissue grinder for dissociation. Mulford and Austin (1998) also used repeated pipetting for the dissociation of hepatopancreas and ovary. Hsu et al. (1995) minced lymphoid organ and forced through a 23GX11/4 gauge needle for dissociation.

1.1.4. Contamination and antibiotics used in shrimp cell culture

One of the major difficulties experienced in developing cell cultures from shrimp and prawns is the often occurring contamination from various sources. It has to be pointed out that other than contamination from external sources, as the animal body habitually harbors bacteria during various occasions, improper selection of a donor animal may also lead to severe contamination and subsequent losses.

Common contaminating agents are bacteria, yeast, fungus, protozoans and thraustochytrids. Thraustochytrids are marine and freshwater heterotrophic protests, that feed as saprophores as parasites or as bacterivores (Porter, 1990; Raghukumar, 1992). Their evolutionary relationships and taxonomy are still poorly understood (Porter, 1990; Cavalier-Smith et al., 1994) and they were characterized as neither protozoa nor fungi, but as heterotrophic heterokontchromists (Cavalier-Smith et al., 1994). Incidence of thraustochytrid contamination was reported in cell cultures from mollusk (Ellis et al., 1985; Ellis and Bishop, 1989), sponges (Ilan et al., 1996; Blisko, 1998), corals (Frank et al., 1994), oysters (Awaji, 1997) and tunicates (Rinkevich and Rabinovitz, 1993, 1994, 1997). They appear in a variety of forms as rapidly dividing cells, round cells with filopods forming a stellate pattern around the cells, cells connected by net like ectoplasmic processes or, as spherical-to-ellipsoid cells (Rinkevich, 1999). Rinkevich (1999) after the detailed examination of the literature especially the studies that described highly proliferating cultures reported suspicious thraustochytrid contamination in shrimp cell culture works published by Itami et al. (1989), Hsu et al. (1995), Toullec et al. (1996). There are several ways to identify thraustochytrids in vitro; unfortunately none of them is conclusive. By electron microscopy sagenogenetosome a specialized structure unique to thraustochytrids (Porter, 1990) can be diagnosed. This structure is difficult to find in some thraustochytrids as there may be only one in a cell of up to 100µm. Under epifluorescence microscopy, the use of acriflavine hydrochloride, which stains the sulfated polysaccharide cell walls of these organisms is highly recommended (Raghukumar and Schaumann, 1993). Other features are cytoplasmic extensions without any organelles and formation of biflagellated zoospores in some genera of thraustochytrids. A confirmative method that distinguishes thraustochytrids from animal cells is by their typical 18S mRNA signatures (Cavalier-Smith et al., 1994).

This issue of contamination by microscopic organisms was addressed by several researchers and the various antibiotic preparations used by them during different occasions are summarized in Table 1.

Table 1. Antibiotics used in shrimp cell culture

Sl.No	Reference	Penicillin	Streptomycin	Amphotericin B	Gentamycin	Fungizone
1	Toullec et al., 1996	0.16 g l^{-1}	0.1g l^{-1}	-	-	-
2	Lu et al., 1995	100 IUml^{-1}	100 µg ml^{-1}	5µg ml^{-1}	10 µg ml^{-1}	-
3	Tapay et al., 1995	100 IU ml^{-1}	100 µg ml^{-1}	-	-	-
4	Ghosh et al.,. 1995	72mg l^{-1}	100mg l^{-1}	-	75mgl^{-1}	-
5	Chen et al., 1989	100 IU ml^{-1}	100 µg ml^{-1}	-		-
6	Mulford and Austin, 1998	10^4 IU ml^{-1}	10^4 µg ml^{-1}	10 µg ml^{-1}	-	-
7	Mulford et al., 2001	100 IU ml^{-1}	0.1mg ml^{-1}	2.5 µg ml^{-1}	-	-
8	Itami et al., 1999	1000 IU ml^{-1}	1000 µg ml^{-1}	-	-	25 µg ml^{-1}
9	Frerichs, 1996	-	-	1 µg ml^{-1}	500 µg ml^{-1}	
10	Tong and Miao, 1996	100 UI ml^{-1}	100 µg ml^{-1}	-	-	-
11	Jiang et al., 2005	100 UI ml^{-1}]	100 µg ml^{-1}	-	-	-
12	Chun-Lei et al., 2003	200U ml^{-1}	100U ml^{-1}	-	80U ml^{-1}*	-
13	Fan and Wang, 2002	100 UI ml^{-1}	100 µg ml^{-1}	-	-	-
14	Maeda et al., 2003, 2004	1000U ml^{-1}	1000 µg ml^{-1}	-	-	-
15	Lang et al., 2002	100,000 IU l^{-1}†	100,000 IU l^{-1}	-	-	-
16	Shike et al., 2000[a]	100 UI ml^{-1}	100 µg ml^{-1}	2.5 µg ml^{-1}	-	-
17	Shimizu et al., 2001	100 UI ml^{-1}†	100 µg ml^{-1}	2.5 µg ml^{-1}	-	-
18	Luedeman and Lightner,. 1992	10^4 unit ml^{-1}	10^4 µg ml^{-1}	10 µg ml^{-1}	-	-

*gentamycin sulphate; †Penicillin G

1.1.5. Selection/Development of an appropriate culture medium

Absence of an appropriate growth medium especially for shrimp have been hampered the progress in cell line development to a certain extent. What has been done so far is to modify and use the available media which otherwise have been designed for mammalian cell culture systems.

The media generally used are 0.2X L-15 (Shimizu et al., 2001), 1X L-15 (Chun-Lei et al., 2003), 2X L-15 (Chen et al., 1986, 1989, 1998; Nadala et al., 1993; Lu et al., 1995; Tapay et al., 1995; Tong and Miao, 1996; Toullec et al., 1996; Mulford and Austin, 1998; Chen and Wang, 1999; Wang et al., 2000; Kumar et al., 2001; Shike et al., 2000[a]; Maeda et al., 2003& 2004; Jiang et al., 2005) M199 (Ghosh et al., 1995; Toullec et al., 1996; Itami et al., 1999; Shimizu

et al., 2001; Lang et al., 2002), Pj-2 (Machii et al., 1988), MPS (Tong and Miao, 1996; Fan and Wang, 2002), NCTC 135 (Wang et al., 2000), Grace's Insect Medium (Luedeman and Lightner, 1992; Nadala et al., 1993;Toullec et al., 1996; Wang et al., 2000), MM Insect medium (Nadala et al., 1993), and TC 100 medium (Nadala et al., 1993).

Considering the inadequacy of these media several attempts have been made to supplement them with growth factors in isolation as well as in multiples. Shike et al. (2000[a]) supplemented the medium with 20% FCS. Meanwhile, Lang et al. (2002) used 20% FBS along with NaCl-11g l^{-1}, KCl- 0.4g l^{-1}, $MgSO_4$. $7H_2O$- 3g l^{-1}, $MgCl_2$. $6H_2O$- 3.3g l^{-1}, $CaCl_2$. $2H_2O$ - 0.9g l^{-1}, Na_2HPO_4. $12H_2O$- 0.1g l^{-1}, HEPES- 2.38g, L- Glutamine- 0.15g, Lactalbumin Hydrolysate- 0.1g, $NaHCO_3$- 2.2g. The medium used by Maeda et al. (2003, 2004) consisted of 10% FBS, glucose 1g l^{-1}, proline- 0.1g l^{-1}, TC Yeastolate- 1g l^{-1} and lactalbumin hydrolysate- 1g l^{-1} .Chen et al. (1986) added 18%FCS, 30% muscle extract, 0.006gml^{-1} NaCl and 10% lobster haemolymph to the growth medium. The additives provided by Machii et al. (1988) consisted of 300mg/l glucose, 100mg l^{-1} lactalbumin hydrolysate and 20% FCS. At the same time Fan and Wang (2002) incorporated 15-20% heat inactivated FBS, 0.55g l^{-1} sodium pyruvate, 0.75g l^{-1} $NaHCO_3$, 2.0g l^{-1} chitosan, 100 µl/flask of nerve nodule extracts, and 12g NaCl to give an osmolality of 2.4%. Chen and Wang (1999) added 20% FCS along with ovary extract, muscle extract and lobster haemolymph. 20% FBS alone was supplemented by Wang et al. (2000) and Frerichs (1996). Kumar et al. (2001) used 27% prawn muscle extract, 10% prawn haemolymph and 10% FBS. Jiang et al. (2005) additionally added 20% FBS, 2g l^{-1}glucose, 2.4% NaCl, GIT medium, AKN salt solution and inorganic acid of MPS. Itami et al. (1999) used 11g NaCl, 0.4g KCl, 3g MgSO4. H_2O, 3g $MgCl_2.6H_2O$, 0.9g $CaCl_2.2H_2O$, 0.05g $NaH_2PO_4.2H_2O$, 0.15g l- glutamine and 1g lactalbumin hydrolysate. Mulford et al. (2001) added 10% heat inactivated FBS, 5% Nephrops serum, 5% Nephrops muscle extract, 0.06g l^{-1} of L-proline, 1 gl^{-1} glucose to the media. FBS and L-proline were

added by Mulford and Austin (1998). Chen et al. (1998) added FCS, muscle extract of grass prawn and lobster haemolymph and Chen et al. (1989) added 5% FCS, 10% shrimp muscle extract, 15% lobster haemolymph. Luedeman and Lightner (1992) added 10% hybridoma quality fetal bovine serum along with proline (2mg ml^{-1}), sodium bicarbonate (40mg/ml), magnesium chloride (2M), and sodium chloride (5M). Nadala et al. (1993) added 20% FBS, 8% shrimp extract, 20 ng/ml EGF and Ghosh et al., (1995) added 3.75mM HEPES, 2.1mM sodium bicarbonate, 0.2g l- glutamine L^{-1} Tapay et al. (1995) used a combination of 20% fetal bovine serum, 8% shrimp extract, 20ng ml^{-1} epidermal growth factor of murine submaxillary origin, 10 units ml^{-1} human recombinant interleukin 2 and salt solution. Lu et al. (1995) added 20% FBS, 4% shrimp extract, 30ng ml^{-1} epidermal growth factor. Toullec et al. (1996) added proline 0.06g l^{-1}, 10mM HEPES in M199 and L-glutamine in Grace at a final concentration of 0.3g l^{-1}. Ten percent heat inactivated fetal bovine was added in all media.

1.1.6. Osmolality of growth media

Osmolality requirements for successful cell culture development are well known. As mentioned above since the growth media used for mammalian and avian cell cultures were used for shrimp/prawn cell culture there was the requirement of modifying the osmolality by addition of extra salt. In most of the cases NaCl was used for adjusting osmolality. Meanwhile a few researchers used a mixture of salts to bring up the salt content preferably to that of haemolymph (Mulford and Austin 1998; Chen et al., 1989; Mulford et al., 2001). Osmolality ranged from 520 to 820 mOsmol for saline water species while an osmolality of 450mOsmol was preferred for fresh water species such as *M. rosenbergii*.

1.1.7. pH of growth media

The pH values used in growth media have been those of haemolymph of the animals where from the tissues for the cell culture development have been

used. This ranges between 7.0 and 7.5 (Toullec, 1999). Specific reports of the pH value used for cell culture development are given in Fan and Wang (2002), (7-7.2); Chen and Wang (1999), (6.8-7.2); Wang et al. (2000), (7.4); Chun-Lei et al. (2003), (7.5); Kumar et al. (2001), (6.8-7.2); Jiang et al. (2005), (7.0-7.2); Tong and Miao (1996), (7.0-7.2); Itami et al. (1999), (7.6); Mulford et al. (2001), (7.4); Mulford and Austin (1998), (7.4), and Toullec et al. (1996), (7.0).

1.1.8. Incubation Temperatures

In aquatic Asia pacific region shrimps/prawns are referred to have a water temperature of 25°C to 32°C as the optima. Naturally, the cell cultures derived from such animals also prefer to have this range of temperature. Therefore, attempts have been made by almost everyone to incubate the cultures at a particular temperature within this range. However, there were instances of maintaining a temperature of 15 to 16°C (Ghosh et al., 1995; Mulford and Austin, 1998; Mulford et al., 2001). In one instance Chen et al. (1998) used a temperature range of 21-32°C, the optimum being 28±1.

Majority of the workers have been using a closed system with media containing bicarbonate. Meanwhile workers like Lang et al. (2002) have attempted to grow the culture in 5% CO_2 atmosphere. In another instance Luedeman and Lightner (1992) employed an atmospheric gas phase with open system under which a cell monolayer with 80% confluence was formed within a period of 2 days from ovarian tissue.

1.1.9. Sub-culturing and Transfection

Ultimate objective of every shrimp/prawn cell culture development programme was establishment of corresponding cell lines. However, this objective has not been achieved so far. In most of the cases passaging has not been attempted and the efforts were to maintain the culture for a long duration by change of media. Meanwhile, Chen et al. (1986) attained 3 passages in ovarian culture and Chen et al. (1989) attained 2 passages in lymphoid culture. Chen and

Wang (1999), attained three passages in ovarian and lymphoid cultures, Kumar et al. (2001) attained 4 passages in eyestalk culture, Freirichs (1996) and Fan and Wang (2002) attained 10 passages in embryonic culture, and Mulford et al. (2001) and Mulford and Austin (1998) attained 1 passage in haematopoietic tissue and ovarian culture respectively. Hsu et al. (1995) claimed to have attained more than 90 passages for the culture of lymphoid organ which was later reported as thrausochytrid contamination by Rinkevich (1999). At the same time Tapay et al. (1995) reported to have attained 44 passages for lymphoid cultures. Even though not able to be sub cultured, various researchers could maintain cell cultures for different duration. Accordingly, Lang et al. (2002) could maintain the culture for more than a month, Maeda et al. (2003) for 45 days, Chen et al. (1986) for 2 months, and West et al. (1999) for 5 months. Chen and Wang (1999) maintained the heart tissue cell culture for 4 days and lymphoid and ovary for 20 days. Wang et al. (2000) maintained the culture for >1 week, Chun-Lei et al. (2003) for 8-15 days, Kumar et al. (2001) for 3 months, Tong and Miao (1996) for 3 months, Itami et al. (1999) for 54 days, Mulford et al. (2001) for >21 days, Mulford and Austin (1998) for greater than 3 months, and Luedeman and Lightner (1992) for 10 days. Nadala et al. (1993) maintained lymphoid for greater than 3 weeks and nerve for 3 months. Toullec et al. (1996) maintained embryonic and ovarian cultures for several months. Haemocyte cultures were maintained by Jiang et al. (2005) for 20 days, Itami et al. (1999) for 10 days, and Chen and Wang (1999) for 4 days.

Transfection of lymphoid cultures with SV40 large T antigen containing vectors were reported (Tapay et al., 1995; Hu et al., 2008). Tapay et al. (1995) employed pSV3-neo, a shuttle vector to attain 44 passages and Hu et al. (2008) employed a pantropic retroviral vector containing envelope glycoprotein of vesicular stomatitis virus (VSV-G) to attain 21 passages. Firefly luciferase and *Escherichia coli* ß galactosidase reporter gene expressions was recorded in *P. stylirostris* lymphoid and ovarian cell cultures mediated by transfection with

retroviral vectors pseudotyped with VSV-G (Shike et al., 2000[a]). The VSV-G binds to the phospholipids moieties in the target cell membrane and no specific protein receptor is required for vector entry into the cell. Therefore VSV-G pseudotyped retroviral vector has an extremely broad host cell range (Que et al., 1999; Mizuarai et al., 2001; Sarmasik et al., 2001; Dreja and Piechaczyk, 2004) and can integrate stably into the genome of dividing cells, allowing for a stable and heritable expression of heterologous gene.

1.1.10. Crustacean cell culture for WSSV studies

WSSV, the most serious pathogen ever recorded in shrimp (Lo et al., 1996; Chen et al., 1997) causes total devastation of shrimp culture industry within 3 to 7 days of infection (Mamoyama et al., 1994; Hao et al., 1999). WSSV has a remarkably broad host range among crustaceans. Almost every species of penaeid shrimp is susceptible to WSSV. Moreover, the virus can effect other marine, brackish water, and fresh water crustaceans including cray fishes, crabs, spiny lobsters and even hermit crabs (Lo et al., 1996; Flegel 1997, 2006). WSSV was originally classified as an unassigned member of the Baculoviridae family, but has been recently re-classified as a new virus family, the Nimaviridae (genus *Whispovirus*). Complete WSSV virions are ellipsoid to bacilliform-enveloped particles, with a distinctive tail like appendage to one end.

The WSSV genome is a large circular dsDNA of approximately 300kbp. Three WSSV isolates from China (WSV-CN, accession no.AF332093), Thailand (WSV-TH, accession no. AF369029), and Taiwan (WSV-TW, accession no. AF440570), have been completely sequenced, and their genome sizes are 305, 297, 307kbp, respectively. The ICTV whispovirus study group committee recently chose the China isolate, WSV-CN as the type strain (Leu et al., 2008).

Various researchers have proved the WSSV susceptibility of lymphoid organ cell culture (Tapay et al., 1997; Kasornchandra et al., 1999; Itami et al.,

1999; Wang et al., 2000), ovarian culture (Maeda et al., 2004), haemocyte culture (Jiang et al., 2005) and haematopoeitic stem cells of the crayfish, *Pacifastacus leniusculus* (Jiravanichpaisal et al., 2006). Wang et al. (2000) studied the ultrastructure and morphogenesis of WSSV in lymphoid culture and proposed an alternative pathway for viral assembly. Instead of packing nucleoprotein into a partially enveloped empty capsid (Leu et al., 2008), the alternative pathway proposes that the electron dense nucleocapsid is assembled first and then enveloped by viral membrane. RT-PCR expression of VP 28 envelope protein of WSSV was demonstrated in crayfish haematopoietic stem cells from as early as 36 hour post infection by Jiravanichpaisal et al. (2006).

1.1.11. Crustacean cell culture for cytotoxicity studies

Cytotoxicity is considered an important index for evaluating safety of antimicrobials and management chemicals prior to their administration in aquaculture. For its accomplishment cell lines can be used which out-rightly eliminates animal experimentation as part of the bioassays as being followed now. Such an approach can forecast their in vivo effects as well as assisting their optimization for field level application. This is specifically because under field conditions realizing their negative impacts happens to be quite cumbersome (MacGowan et al., 2001). Hauton and Smith (2004) employed *Homarus gammarus* granulocyte culture to investigate the cytotoxicity of aquaculture immunostimulants and 2, 4-dinitrophenol using neutral red uptake assay while Sung et al. (2003) studied the effects and toxicity of phthalate esters on the isolated haemocytes of *Macrobrachium rosenbergii* utilizing haemocytic adhesion, pseudopodia formation, superoxide anion production and phenoloxidase activity.

1.2. Possible obstacles and solutions for shrimp cell immortalization

Primary cells have a limited proliferative capacity in culture due to cellular senescence. The cell cycle consists of three major interphases

beginning with an initial gap phase (G1 phase), a DNA synthesis stage (S phase), a second gap phase (G2 phase) and eventual mitosis, where division of the cell occurs (Freshney, 2000). A cell may pause in the G1 phase before entering the S phase and enter a state of dormancy (G0 phase). Entry into the S phase is tightly regulated by signals from the environment, with cycle duration dependent upon cell type, as well as species variation. For all somatic cells, after a number of progressions through the cell cycle, cellular senescence will occur and the cell will eventually die.

This finite life span is regulated by a group of dominantly acting senescence genes, the products of which negatively regulate cell cycle progression (Sasaki et al., 1994). Disrupting those genes which are involved in limiting the proliferative potential of cells should effect escape from the senescence pathway and push cells towards immortalization and transformation (Crane, 1999). Many viruses have evolved genes which encode for proteins which effectively suppress or delay apoptosis long enough for the production of sufficient quantities of virions (Teodoro and Branton, 1997). Adenovirus, Simian virus 40 (SV40), polyomavirus, and human papillomaviruses (HPV) are some of the viruses that have evolved such strategies (Teodoro and Branton, 1997; O'Brien, 1998). Each of these viruses encodes proteins that interact with key regulators of the cell cycle to stimulate unscheduled DNA synthesis. Cell cycle regulatory genes such as p53 and the retinoblastoma (Rb) gene are the most commonly affected (Sager, 1992). These genes act at the G1 checkpoint ensuring completion of DNA replication and the integrity of the genome prior to cells entering the synthesis phase. In normal cells, p53 and the Rb gene are usually inactive, and cells proceed around the normal cell cycle. However, in response to cellular stress or DNA damage, these genes become activated by phosphorylation and will often result in either growth arrest at the G1/S interface or apoptosis (Sager, 1992; Levine, 1997). However, for continual cellular proliferation, suppression of both p53 and Rb genes may be necessary.

A variety of cell physiological stimuli can provoke a cell to enter senescence. Following extensive passage in culture (Hayflick and Moorehead, 1961) and exposure to oxidative damage (Chen et al., 1995; von Zglinicki et al., 1995;) or activation of an oncogene (Serrano et al., 1997; Zhu, et al., 1998; Zindy et al., 1998; de Stanchina et al., 1998), primary cultures of mammalian cells enter into irreversible growth arrest. Replicative senescence that takes place following extensive passage in culture may be the cellular manifestation of progressive telomere shortening. Telomeres are the structures at the ends of mammalian chromosomes that 'cap' the chromosomes and provide a protective function, preventing end-to-end chromosomal fusions (Greider and Blackburn, 1996). With each round of cell division and DNA replication, the telomeric DNA sequence is under-replicated, leading to the progressive shortening of the telomere (Olovnikov, 1973). Eventually the shortened telomere may no longer be able to protect the end of the chromosome, and the unprotected chromosomal DNA end may release a senescence inducing signal to the cell. In humans, telomeres are made of the simple double-stranded hexameric DNA repeat (TTAGGG)n reiterated for over 2-30kbp, and by a G-rich single stranded 3' overhang of 50-200 nucleotides in length (Blackburn, 1994). Telomere of Pacific white leg shrimp, *P. vannamei* is having pentanucleotide repeating units, (TTACC)n (Alcivar-Warren, 2006) where as that of *P. japonicus* is (TTAGG)n repeats (Lang et al., 2004[a]). In many cell lines, telomere maintenance is provided by the action of the ribonucleoprotein enzyme complex, telomerase (Colgin and Reddel, 1999). Telomerase is expressed in germ cells and has moderate activity in stem cells, but is absent from normal somatic cells. Deletions and/or mutations within senescence genes and transfection can allow cells to escape from the negative control of the cell cycle and re-express telomerase (Bodnar et al., 1998; Colgin and Reddel, 1999). The ectopic expression of the catalytic subunit of the human telomerase gene (hTERT) restores telomere length in certain cell types and allows early passage cultures of cells to circumvent senescence and become immortalized (Bodnar et al., 1998;

Vaziri and Benchimol, 1998). To date only one study has been performed on in the in vitro telomerase activity in shrimp cells. Using telomeric repeat amplification protocol assays Lang et al. (2004 [a]) reported active telomerase activity in cultured lymphoid organ cells for up to 30 days.

Oxidative damage is another cellular stress that can induce senescence-like growth arrest. The culturing of cells in conditions of mild hyperoxia shortens replicative lifespan and induces accelerated telomere shortening (von Zglinicki et al., 1995). Conversely, cells cultured in low oxygen tension have an extended replicative lifespan and delayed senescence (Chen et al., 1995; Saito et al., 1995). Reactive oxygen species (ROS) have been implicated more directly in the process of senescence. Dilute hydrogen peroxide can induce cells to enter into a senescence-like growth arrest or, at higher concentrations, to undergo apoptosis (Chen and Ames, 1994).

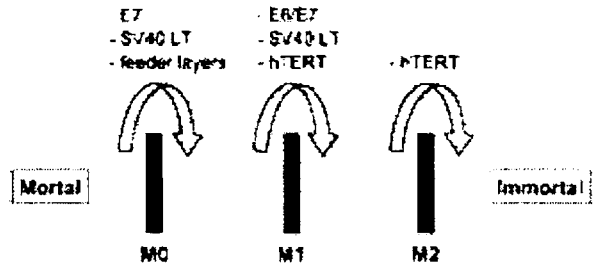


Fig.5. Multiple obstacles blocking the path to cell immortality (Lee et al., 2004)

At least three immortality stages, M0, M1 and M2 (Fig 5) have been described in literature that can limit the life span of primary cells. While M1 and M2 are caused by the shortening of telomeres, M0 appears to represent a delayed response to inadequate culture conditions. Feeder layers have successfully been used to overcome M0, while M1 and M2 are most efficiently bypassed by the expression of exogenous hTERT. Viral oncogenes, such as the SV40large T antigen and the E6/E7 proteins of the human papilloma virus (HPV16), can overcome M1 and provide cells with an extended life span (Fig 5), but this strategy invariably gives rise to cells that display cancer-associated changes.

However, cells expressing these oncogenes which can bypass cell cycle checkpoint pathways leading to extended life span are not yet immortal as telomeres continue to shorten with divisions (Shay and Wright, 1996). Terminal telomere shortening eventually leads to M2 (for mortality stage 2), an anti-proliferative state characterized by massive cell death. Immortal clones can sometimes emerge from M2 (at a frequency of 10^{-7}) that have gained the ability to maintain the size of their telomeres (Counter et al., 1992; Shay et al., 1993; Counter et al., 1994). Lee et al. (2004) proposes that in conjunction with feeder layers, exogenous hTERT can bypass all three obstacles (M0, M1, M2) without causing significant changes in phenotypic properties. Meanwhile, cotransfection of multiple genes was proposed for cell immortalization. For example with human diploid fibroblasts the only successful expression reported thus far used a combination of hTERT, Ras and SV40 large T (Hahn, 1999; Elenbass, 2001).

For many cell types, the main obstacle to immortality is their inability to proliferate in vitro. This failure to proliferate can be an intrinsic property of the cells, as in the case of post-mitotic terminally differentiated cells, which have lost all proliferative capacity upon differentiation (e.g. neurons). Alternatively, this failure can result from our inability to emulate in vivo conditions that support growth. Indeed, there are several examples of cells with a capability for in vivo proliferation that are unable to divide in the artificial environment of the laboratory (e.g. hepatocytes). Catalytic subunit of the telomerase gene alone should not be expected to overcome these obstacles, as the enzyme does not appear to inhibit differentiation, alter phenotypic properties, or decrease growth requirements (Lee et al., 2004). Selection of appropriate tissue with mitotic potential such as haematopoietic tissue, ovarian tissue and embryonic tissue for in vitro culture is crucial in the case shrimp culture. Lymphoid cells readily form monolayer in culture. Owens and Smith (1999) explain this as cell migration from the explant rather than by cell division. Observations made by van de Braak et al. (2002^{b}) confirmed limited mitotic activity in lymphoid organ.

While a number of invertebrate cells have demonstrated spontaneous transformation in vitro, other cell types have required manipulation to assist transformation. The lack of spontaneous transformation in crustacean cells may be due to the lack of oncoviruses that infect these animals (Crane and Williams, 2002). The neoplastic transformation of vertebrate cells was first achieved by transfection with active oncogenes (Ratner et al., 1985), yet this technique has had limited application in crustacean and aquatic invertebrate cells (Claydon and Owens, 2008). The current techniques for the introduction of foreign genes into crustacean cells are quite underdeveloped, and transfection of plasmid DNA into shrimp cells has proven to be difficult (Shike et al, 2000). Much research of crustacean and aquatic invertebrate cell cultures has involved the adjustment of the culture media via nutrition supplementation in an effort to enhance continual mitosis (Claydon and Owens, 2008). Limitation in the transfection of shrimp cells with viral vectors used in mammalian cell culture is the lack of specific protein receptor for these viruses in the shrimp cell. The application of vectors pseudotyped with vesicular stomatitis virus envelope glycoprotein (VSV-G) is a possible solution for this problem (Shike et al, 2000[a]; Hu et al., 2008). The VSV-G protein binds to phospholipids moieties of the cell membrane, thus circumventing the need for a specific protein receptor on the target cell surface (Mastromarino et al., 1987). Viral genes can integrate stably into the genome of dividing cells allowing a stable and hereditable expression.

Gene delivery to cells, especially non-dividing ones is limited to a large extent by multiple extracellular and intracellular barriers, the major one being the nuclear envelope. Once in the cytoplasm, plasmids must make their way into the nucleus in order to be expressed. Numerous studies have demonstrated that transfections works best in dividing populations of cells in which the nuclear envelope dissembles during mitosis, thus largely eliminating the barrier. It is well appreciated that non-dividing or growth arrested cells cannot be easily transfected by almost any method (Dean et al., 2005). However, since shrimp

cells do not actively undergo cell division, the mechanisms of nuclear transport of plasmids in non-dividing cells are of critical importance. Plasmids can be transported into the nuclei of non-dividing cells via the nuclear pore complex (NPC) (Dowty et al., 1995) but do so in a sequence-specific manner (Dean, 1997).The sequences that support DNA nuclear import were termed DNA nuclear targeting sequences or DTS (Dean et al., 2005).The 72 bp enhancer region from SV 40 genome sequence was found to support the nuclear import of cytoplasmically localized plasmids (Dean et al., 1997; 1999). Other DTS sequences reported were smooth muscle gamma actin promoter (SMGA) and the flk-1 promoter (Vacik et al., 1999; Dean, 2002). Protein factors involved in DNA nuclear import include the nuclear localization signals (NLS) within the amino acid sequences of transcription factors. SV 40 enhancer contains binding sites for number of transcription factors such as AP1, AP2, Oct-1, TEF-1 etc. (Wildeman, 1988). Complexing DNA with NLS containing peptides and proteins increase the nuclear import of plasmids. These various molecular approaches together with the selection of appropriate tissue and growth media might lead to shrimp cell immortalization. The present study was undertaken in the light of these developments and understandings with the following objectives:

1. Development of primary cultures from *Penaeus monodon* with special reference to lymphoid organ
2. Development of primary haemocyte culture from *Penaeus monodon* and its application in white spot syndrome virus (WSSV) titration and viral and immune related gene expression
3. Primary haemocyte culture of *Penaeus monodon* as model for cytotoxicity and genotoxicity studies
4. Transfection of *Penaeus monodon* primary cell cultures, primary oocytes and sperm cells in vitro

Chapter 2

DEVELOPMENT OF PRIMARY CULTURES FROM *PENAEUS MONODON* WITH SPECIAL REFERENCE TO LYMPHOID ORGAN

2.1. Introduction

Penaeid shrimp culture has taken up the central stage in aquaculture production sector attaining globally the sixth position in terms of value among all taxonomic groups of animals cultivated (FAO, 2006). It has to be pointed out that this achievement is amidst the multitudes of infectious diseases, which stand out as the major impediments in the aquaculture production. *Penaeus monodon*, one of the most widely cultured penaeid shrimps, is under threat of several diseases, among which white spot disease caused by white spot syndrome virus (WSSV) spells out the greatest havoc. Eventhough extensive investigations have been made on this virus, lack of a valid cell line has been hampering the progress of research in elucidating pathogenesis and morphogenesis as well as the development of prophylactic and therapeutic measures.

First reported cell culture from *Penaeus monodon* was by Chen et al. (1986), following which Chen and Wang (1999), West et al. (1999), Fraser and Hall (1999) and Wang et al. (2000) made attempts to establish cell lines from the same species without much success. Cell cultures from different species of penaeids were reported from *P. stylirostris* (Leudeman and Lightner, 1992; Tapay et al., 1995; Shike et al., 2000[a] and Shimizu et al., 2001*)*, *P. japonicus* (Chen and Wang, 1999; Lang et al., 2002, 2004[a,b]; Maeda et al., 2003), *P. chinensis* (Tong and Miao, 1996; Fan and Wang, 2002; Chun-Lei et al., 2003; Jiang et al., 2005), *P. penicillatus* (Chen et al., 1989; Chen and Wang, 1999), *P. indicus* (Toullec et al., 1996; Kumar et al., 2001), *P. vannamei* (Leudeman and Lightner, 1992; Nadala et al., 1993; Toullec et al., 1996) and non - penaeids such as *M. rosenbergii* (Frerichs, 1996). Besides, initiatives were also made for obtaining cell cultures from *Nephrops norvegicus* (Mulford et al., 2001; Mulford and Austin, 1998). Among them Tapay et al. (1995), Freirichs (1996), Fan and Wang (2002), and Hu et al. (2008) could attain the progress of cell cultures up to or more than 10 passages. In spite of these relentless efforts world wide no

immortal cell line could be developed so far. Nevertheless, infection of WSSV in primary cultures of lymphoid organ was observed by Lu et al. (1995) and Wang et al. (2000), while Maeda et al. (2004) and Jiang et al. (2005) employed cultures of ovary and haemocytes, respectively, for the same.

In this context a study was undertaken to develop primary cell cultures from ovary, heart, muscle and lymphoid tissues of *P. monodon,* which would later be taken forward for establishment. In this process the effect of growth and attachment factors and BrdU incorporation in the primary cell cultures generated from lymphoid organ was investigated. Subsequently, the primary cell cultures generated from the lymphoid tissue was subjected for WSSV susceptibility and expression of WSSV genes and shrimp immune related genes could be elucidated.

2.2. Material and methods

2.2.1. Experimental animals

WSSV and Monodon Baculo virus (MBV) negative *P. monodon* larvae obtained from a local hatchery were stocked and reared in a Recirculating Aquaculture System for shrimp integrated with nitrifying bioreactors (Kumar et al., 2009) in sea water having 15g l^{-1} salinity Water quality was maintained by the addition of a probiotic Detrodigest ™ (NCAAH, India) to manage detritus and Enterotrophotic ™ (NCAAH, India) to control *Vibrio.* The larvae were fed with commercially available pelleted feed (Higashimaru, India). They were confirmed WSSV negative by nested PCR (WSSV detection kit, Genei, India) when they grew to 8-12g, and used for all experiments. For isolation of ovarian tissue female animals weighing 80-120g obtained from local hatcheries were used.

2.2.2. Surface sterilization of the animals

The animals were sacrificed by immersing in crushed ice and disinfected by maintaining in 800 mg l^{-1} sodium hypochlorite solution prepared in ice cold sea water (salinity 15 g l^{-1}) for 10 minutes. Subsequently, they were washed 5

times in sterile ice cold sea water, dipped in 70% alcohol and rinsed in ice cold sea water.

2.2.3. Development of primary cell cultures of heart, ovarian and muscle tissues

Heart tissue, dorsally located in the cephalothorax was excised from the surrounding tissues and held in Hank's Balanced Salt Solution (HBSS) with osmolality adjusted to 720±10 mOsm. Muscle tissue was cut from the abdominal region and the ovarian tissue from the anterior dorsal region was (from anterior dorsal region, Chapter 1, Fig 4) also excised and held in HBSS. The tissues were washed thrice with HBSS to remove the contaminating haemocytes and cut into pieces of $1mm^3$ size. Tissue pieces were further disaggregated by pipetting and seeded into $25cm^2$ flasks (Greiner Bio One, India) with 2 ml growth medium. The medium used was Grace's Insect supplemented with magnesium chloride hexahydrate (2g l^{-1}), 10% sodium bicarbonate solution (3.5ml l^{-1}), L-proline (20mg l^{-1}), sucrose (200mg l^{-1}), trehalose (200mg l^{-1}), fetal bovine serum (10%), fish muscle extract (4%), shrimp muscle extract (4%), shrimp haemolymph (8%), 0.06µg ml^{-1} chloramphenicol, 100µg ml^{-1} streptomycin and 100 IU ml^{-1} penicillin. Cultures were incubated at 25°C and observed daily under inverted phase contrast microscope (Leica, Switzerland).

Fish muscle extract was prepared from *Clarias gariepinus* muscle tissue. Muscle tissue(10%, w/v) was macerated in PBS, centrifuged at 10,000g for 20 minutes, supernatant incubated at 56°C for 30 minutes, centrifuged to remove the coagulated proteins, passed through poly vinylidene fluoride membrane (PVDF, 0.22µ) (Millipore, India) and maintained at - 20°C. For preparing shrimp muscle extract (10%, w/v) muscle tissue was isolated from *P. monodon*, after removing the cephalothorax region. The same protocol for fish muscle extract was followed. Haemolymph was collected from healthy P. *monodon.* Haemolymph was withdrawn asceptically using capillary tubes containing 100µl anticoagulant (Tris HCl 0.01M, Sucrose 0.25M, Tri Sodium Citrate 0.1M) from rostral sinus. Haemolymph was

diluted with PBS (1:1), centrifuged at 10,000g for 20 minutes, supernatant incubated at 56°C for 30 minutes, centrifuged to remove the coagulated proteins, passed through poly vinylidene fluoride membrane (PVDF, 0.22μ) and maintained at -20°C.

2.2.4. Development of primary culture of lymphoid organ

Lymphoid organ consists of two distinct lobes located ventro-anterior to the hepatopancreas (Chapter 1, Fig 3). Shrimps were dissected to remove the lymphoid organ and the tissue pieces were immediately immersed in HBSS. Before seeding tissue pieces were washed thrice, cut into $1mm^3$ pieces, pipetted vigorously and seeded into 25 cm^2 flask. Media used were Grace's Insect (composition given above) and Leibovitz's L-15 (Sigma) at double strength supplemented with 2% glucose, 20% FBS, 0.06μg ml^{-1} chloramphenicol, 100μg ml^{-1} streptomycin and 100 IU ml^{-1} penicillin. For further experiments L15 based medium was used. Performance of the primary culture in L - 15 basal medium and basal medium supplemented with MEM Vitamins (1X) and tryptose phosphate broth (2.95 g l^{-1}) were compared. This was accomplished by MTT assay of 96 well plate (Greiner Bio One, India) cultures of lymphoid organ at 3rd and 5th days of culture. Triplicates were kept for each medium.

2.2.5. Effect of attachment factors on lymphoid cell culture

Attachment factors tested were laminin (20 μg ml^{-1}, Sigma, USA), fibronectin (20 μg ml^{-1}, Sigma, USA) and poly-L-Lysine (200μg ml^{-1}, Sigma, USA). Stock solutions were prepared in PBS and 34μl per well of each solution was added to 96 well plate. The plate was incubated at 37°C for 2 hours and excess was removed. To the coated wells 200μl of the growth medium containing explants of uniform size were added and incubated at 25°C. MTT assay was done after 48 hours. Triplicates were kept for the attachment factors and control wells were devoid of the same.

2.2.6. Effect of growth factors on lymphoid primary cell culture

Growth factors tested were fibroblast growth factor-basic (bFGF, from bovine pituitary glands (Sigma, USA)), epidermal growth factor (EGF, from mouse submaxillary glands (Sigma, USA)), insulin like growth factor-1 (IGF-1, human, recombinant expressed in *E. coli* ((Sigma, USA)), insulin like growth factor-2 (IGF-2, human, recombinant expressed in *E. coli* ((Sigma, USA)) and transforming growth factor-ß1 (TGF- ß1, from porcine platelets ((Sigma, USA)). Primary stock solutions of bFGF and EGF was prepared in media while IGF-1 was prepared in water, IGF-2 in 10mM acetic acid containing 10µg ml^{-1} bovine serum albumin (BSA) and TGF- ß1 in 4mM HCl containing 1 µg ml^{-1} BSA. Primary stocks were diluted to required concentrations with growth media. 96 well plate cultures of lymphoid organ was prepared and growth factors were added to get a final concentration of 10 ng ml^{-1}, 20 ng ml^{-1} and 40 ng ml^{-1} for bFGF, 1 ng ml^{-1}, 10 ng ml^{-1}, and 30 ng ml^{-1} for EGF and 10 ng ml^{-1}, 25 ng ml^{-1} and 50 ng ml^{-1} for IGF-1, IGF-2 and TGF- ß1. MTT assay was done after 48 hours. Triplicates were kept for each concentration of growth factors, and control wells without the addition of growth factors were kept.

2.2.7. MTT assay

The assay is a colorimetric method based on the determination of cell viability utilizing the reaction of a tetrazolium salt (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide, MTT) with the mitochondria of metabolically active cells. The reduction of the tetrazolium salt by nicotinamide adenine dinucleotide dehydrogenase (NADH) and nicotinamide adenine dinucleotide phosphate dehydrogenase (NADPH) within the cells produces insoluble purple formazan crystals, which are later solubilized yielding a purple-colored solution (Mosmann, 1983).

After replacing the medium, 50µl of MTT (Sigma) solution (5mg ml^{-1} in PBS; 720mOsm) was added to each well and incubated for 5 hours in dark.

Control consisted of medium alone with MTT added. After incubation, the medium was removed and MTT-formazan crystals were dissolved in 200µl dimethylsulfoxide. Absorbance was recorded immediately at 570nm in a micro plate reader (TECAN Infinite Tm, Austria).

2.2.8. 5-bromo-2'-deoxyuridine (BrdU) assay of lymphoid cell culture

A sample of 20µl of 10mM BrdU solution was added to each well of 96 well plate lymphoid organ cultures with 200µl medium. Wells without the addition of BrDU were kept as control. After 24 hour incubation, medium was removed, washed with PBS, fixed with 4% paraformaldehyde for 15 minutes and washed again with PBS. 2M HCl was added to each well, incubated for 20 min, neutralised with 0.1M sodium borate (pH 8.5) for 2min and washed with PBS. Cells were permeabilised with PBS containing 0.2% triton X-100 and 3% BSA for 5 minutes. After blocking with 3% BSA in PBS for 1 hour, 1:1000 dilution (in 3% BSA) of the mouse monoclonal anti BrdU antibody (Sigma, USA) was added and incubated for 1 hour. Cells were washed thrice with PBS for 5 minutes each and incubated for 1 hour with rabbit anti mouse FITC conjugate, 1: 40 dilution (Sigma, USA). Wells were washed with PBS, stained with DAPI (0.2µg ml^{-1}) and observed under inverted fluorescent microscope (Leica, Switzerland). DAPI and FITC were viewed under filters with excitation wavelength 360-370nm and 470-490nm respectively. Test wells were compared with the wells without BrdU (negative control). The images were processed and merged using the "Leica Application Suite" software (Leica Microsystems, Switzerland).

2.2.9. Preparation of WSSV lysate from gill tissue and infection of lymphoid cell culture

Gill tissue (500 mg) from WSSV infected shrimps weighing 8-12g under laboratory conditions was macerated with mortar and pestle kept in ice bath in 10ml cell culture medium with glass fiber wool. The extract was centrifuged at 10,000g for 10minutes at 4°C and the supernatant passed through 0.22µm polyvinylidene fluoride (PVDF) membrane (Millipore). The extract obtained was

diluted 30 times and added to lymphoid organ cultures and observed for cytopathic effect (CPE).

2.2.10. Immunofluorescence assay for detection of WSSV in lymphoid cell culture

For the immunofluorescence detection of WSSV, lymphoid cultures were prepared in Leighton tubes with cover slips (10 x 22mm; Micro-Aid) and incubated at 25°C. After 48 hours WSSV inoculum was added. After 12 hours cover slips were removed from Leighton tubes, washed twice in PBS (720mOsm) and immersed in 10% paraformaldehyde, and maintained in 70% ethanol at -20°C until use. These fixed cover slip cultures were used for the immunofluorescence detection of WSSV. The cover slips were attached to a glass slide, free sites were blocked using 3% BSA in PBS and incubated in a humidified chamber for 1 hour. The slides were washed in PBS/Tween 20 (0.01%) and added WSSV monoclonal (C 38) antibody (Anil et al., 2002) on the slide, incubated for 1 hour and washed three times in wash buffer. It was again incubated for an hour after addition of rabbit anti mouse FITC conjugate, 1: 40 dilution (Sigma), and subjected to washing. After incubation with general nuclear stain DAPI (10 µl, 0.2µg ml^{-1}) for three minutes, the slides were rinsed with distilled water, air dried, mounted (Vectashield, USA) and observed under fluorescent microscope (Olympus, Germany). DAPI and FITC were viewed under filters with excitation wavelength 360-370nm, and 470-490nm, respectively. The slides were compared with uninfected lymphoid organ cultures (negative control). The images were processed and merged using the "Image pro-express" software (Media Cybernetics Inc, MD, USA).

2.2.11. Expression of WSSV genes and shrimp immune related genes in WSSV infected lymphoid cell culture

2.2.11.1. RNA isolation from WSSV infected haemocyte culture

Lymphoid explant cultures prepared in 24 well plate in modified 2X L15 medium was used for RNA extraction. After 48 hours of incubation the medium was removed and 500 µl WSSV inoculum was added. RNA isolation was done

after 8^{th} and 24^{th} h from 10 wells each. Wells added with heat inactivated (56°C for 1 hour) WSSV were kept as controls. RNA was isolated from control cells after 24 hours. For RNA isolation the medium was removed, wells washed with ice cold PBS, explants detached and removed by repeated pipetting and TRI reagent (Sigma) added to each well. Complete lysis of cells was allowed by repeated pipetting and the reagent was collected in 1.5 ml MCTs. The samples were stored for 5 minutes at room temperature to ensure complete dissociation of nucleoprotein complexes. 0.2ml chloroform was added to 1ml TRI reagent, shaken vigorously for 15 seconds, and allowed to stand for 15 minutes. The resulting mixture was centrifuged at 12,000g for 15 minutes at 4°C. Colourless upper aqueous phase was separated carefully from the three layers formed and transferred to a fresh tube. 0.5ml isopropanol was added and stored for 10 minutes at RT and centrifuged at 12,000g for 10 min. RNA was found precipitated on the sides and bottom of the tube after centrifugation at 12,000g for 10 min at 4°C. Supernatant was discarded and the pellet was washed twice with 75% ethanol. The RNA pellet was air died and dissolved in 20µl of DEPC treated sterile water by repeated pipetting at 55°C. These RNA samples were subjected to DNase treatment with RNase-free DNase 1 (New England Biolabs). 0.2 units of the enzyme was added per µg RNA and incubated at 37°C for 10 minutes. The enzyme was inactivated at 75°C for 10 minutes. Concentration and quality of RNA were measured by taking the absorbance at 260/280nm in a UV-Visible spectrophotometer.

2.2.11.2. RT-PCR of WSSV genes and shrimp immune related genes

1µg RNA was subjected to cDNA synthesis with 20µl reaction mix containing M-MuLV reverse transcriptase (80U), RNase inhibitor (8U), Oligo $(dT)_{12}$ primer(40pmoles), dNTP mix (1mM), RTase buffer (1X) and $MgCl_2$ (2mM) at 42°C for 1 hour. All reagents were purchased from New England Biolabs. Expression of 8 WSSV genes and 8 immune related genes were amplified. Shrimp ß actin gene was also amplified as a reference. PCR using 2µl of cDNA was

subsequently performed with specific primer sets as given in Table 1. 25 µl PCR reactions were done with 0.5U of Taq DNA polymerase, 200µM dNTP mix, 10pmoles of each forward and reverse primer and 1X PCR buffer. The hot start PCR programme used for WSSV genes was 94°C for 2min, followed by 35 cycles of 94°C for 30s, annealing for 30s, 68°C for 30s followed by final extension at 68°C for 10 min. Annealing temperatures were 50°C for endonuclease, 53°C for latency 1 and ribonucleotide reductase (rr1), 54°C for DNA polymerase (dnapol), immediate early gene1 (ie1), Vp 28 and thymidine kinase and thymidilate kinase (tk-tmk) and 55°C for protein kinase 1(pk1) and ß actin. For immune genes also hot start PCR was done with 94°C for 2min, followed by cycles of 94°C for 2 min, annealing for 1min, 72°C for 1min followed by final extension at 72°C for 10 min. Annealing temperature and number of cycles employed for PCR of Astakine and peroxinectin were 56°C and 30 cycles, for prophenoloxidase (Pro PO) and transglutaminase 56°C and 35 cycles, for crustin and penaeidine 55°C and 35cycles, for alpha 2 macroglobulin 65°C and 30cycles and for haemocyanin 56°C and 35cycles.Ten µl of each PCR product was analysed by 1% agarose gel electrophoresis, stained with ethidium bromide and visualized under ultraviolet light.

2.2.12. Statistical analysis

The results in the figures are average values of 3-6 replicates ± standard deviation. The effects of treatments were statistically analyzed by single factor and two factor analysis of variance (ANOVA) wherever applicable. Differences were considered significant at $P < 0.05$.

2.3. Results

2.3.1. Primary cell cultures from heart, ovary and muscle tissues

Primary cell cultures from heart, ovary and muscle tissues were developed. In this process migration of cells from the explants and cell proliferation could be noticed with in four hours of incubation. There were remarkable differences in the morphology of cells generated from these tissues. The primary cell culture developed from the heart tissue was rounded with little pseudopodia (Fig 1).

These cells could be maintained for three weeks with intermittent change of medium with out sub-culturing. Subsequently the cells were found to get detached and get disintegrated.

The primary cell cultures developed from the ovarian tissue was fibroblastic along with the primary oocytes having larger nuclei (Fig 2). Some of the primary oocytes were found with dividing nuclei but with out cytoplasmic division (Fig 3). Primary cell cultures developed from the early stage and immature ovaries were found better in attachment and proliferation of cells than that of mature ovaries. Ovarian primary cell culture was maintained for 2 weeks with intermittent change of medium.

The primary cell cultures generated from muscle tissue were thread like and most of the cells were in suspension. The cells developed could be maintained for 3-4 days (Fig 4).

2.3.2. Primary cell culture from lymphoid organ

Performance of the primary cell cultures in double strength L-15 medium was found to be better than that in Grace's Insect medium. The explants were found getting attached to the culture vessel within 2 hours of seeding. Once the explants were attached, round cells in large numbers were seen to migrate from the tissue and remained (Fig 5) the same in morphology. Apart from, fibroblastic (Fig 6) and epithelioid cells (Fig 7) were also found developing as part of the primary cell culture (Fig 8). Cells in the mitotic phase were frequently seen among the epithelioid cells (Fig 9) while mitotic events were rare in round cells and fibroblastic cells. Epithelioid cells which were released to the medium were transferred to another flask where it got attached to and formed a monolayer (Fig 10). Attempts to subculture the fibroblastic cells did not yield satisfactory results as only a few cells were able to be reattached on passage. From the studies conducted it was noticed that the firoblastic cells formed monolayer quickly while epitheloid cells were not. Their occurrence was sporadic and inconsistent.

Performance of lymphoid organ cell cultures in the basal L-15 and modified 2XL-15 media was compared utilizing MTT assay. No significant ($P>0.05$) difference was found on the third day of culture between the two media (Fig 11). However, on the fifth day, significant difference in the MTT readings was found ($P<0.05$) between the two, with higher MTT values for modified L-15 medium. In the modified medium metabolic activity of the cells was significantly high on the 5th day ($P<0.05$) which was in the declining order during the same period (3rd to 5th day) in the basal medium. Accordingly, the modified medium (L – 15) containing Leibovitz's L-15 (Sigma) at double strength supplemented with 2% glucose, MEM Vitamins (1X), tryptose phosphate broth (2.95 g l^{-1}), 20% FBS, 0.06µg ml^{-1} chloramphenicol, 100µg ml^{-1} streptomycin and 100 IU ml^{-1} penicillin was finalized to be used for all subsequent studies.

2.3.3. Effect of attachment factors on lymphoid primary cell culture

Efficacy of attachment factors such as laminin, fibronectin and polylysine were evaluated by growing lymphoid cells on microwell plates coated with the same in terms of MTT readings. Accordingly in laminin (20µg ml^{-1}) coated wells, negative absorbance was recorded after 48 hours of incubation (Fig 12). Absorbance was not significantly ($P>0.05$) different from the control with respect to fibronectin (20 µg ml^{-1}) coated wells while absorbance for poly-L-lysine (200 µg ml^{-1}) coated wells were significantly much higher than that of the control. In terms of attachment fibronectin and poly lysine coated wells were found better than laminin coated wells.

2.3.4. Effect of growth factors on lymphoid primary cell culture

Among the growth factors tested bFGF (Fig 13), EGF (Fig 14), IGF-2 and TGFß did not have any significant effect ($P>0.05$) in the growth and multiplication of the cells measured in terms of MTT assay compared to that of the control. IGF-1 (Fig 15) at 10ng ml^{-1} was found suitable for supporting better growth than in the control *($P<0.05$)*.

2.3.5. 5- bromo-2'-deoxyuridine (BrdU) assay of lymphoid primary cell culture

Green positive fluorescence of FITC was observed in a very few cells (Fig 16) while most of the cells were negative with only blue signals from DAPI.

2.3.6. WSSV infection of lymphoid cell culture and immunofluorescence detection of WSSV

Cytopathic effect was visible within 12 hours of inoculation with WSSV and it was more prominent at 24 hours. Infected cells were found shrunken with refractile granules accumulated (Fig 17). Finally, the cells were found detached from the culture plate and lysed. Green positive signals with FITC conjugated monoclonal antibodies against WSSV were observed (Fig 18) from the nuclei of infected cells and no such positive signals could be obtained from the control. Blue fluorescence of DAPI was observed in the cell nuclei in all preparations.

2.3.7. Expression of WSSV genes and shrimp immune related genes in WSSV infected lymphoid cell culture

2.3.7.1. Expression of WSSV genes in virus infected lymphoid cell culture

Expressions of an immediate early gene (ie1), 5 early genes (pk1, tk-tmk, rr1, dnapol, endonuclease), a late gene (Vp 28) and a latency gene (latency 1) were investigated at 8^{th} and 24^{th} h post infection (Fig 19). All the genes, except the latency 1 gene, were expressed at 8^{th} and 24^{th} hours; the latter only at the 8^{th} hour. However, there noticed down regulation of pk1 and DNA polymerase at the 24^{th} hour post infection. None of the viral genes was found expressed in the control cell cultures exposed to heat inactivated WSSV.

2.3.7.2. Expression of immune related genes in WSSV infected lymphoid cell culture

Immune related genes investigated included prophenoloxidase, astakine, peroxinectin, alpha 2-macroglobulin, haemocyanine, transglutaminase, crustin and penaeidin (Fig 20). All the genes could be amplified at 8^{th} h post infection except the transglutaminase. At 24^{th} hour only alpha 2 macroglobulin and penaeidin were alone found expressed, among which that of the former was

weak. In the control, exposed to heat inactivated WSSV, faint expression of peroxinectin, alpha 2-macroglobulin and crustin, and strong expression of penaeidin were detected.

2.4. Discussion

This was an attempt to generate primary cell cultures from various tissues of heart, ovary, muscle and lymphoid and to take forward the strengthening of the cultivation technique to get it established or/and use it for the isolation of WSSV. On comparing the results with those of previous workers it has been found that primary cell culture of heart tissue has been attempeted by various workers with varying level of success (Chen et al., 1986; Tong and Miao, 1996; Mulford and Austin, 1998; Owens and Smith, 1999). Similar to the present study Chen et al. (1986) and Lang et al. (2001) observed adherence of the cells but without multiplication while Tong and Miao, (1996) observed mitotic figures in heart primary cell culture in the medium for penaeid shrimp (MPS). While round cells were observed in the present study, Mulford and Austin (1998) had noticed fibroblastic cells. Primary culture of muscle tissue was reported by Wang et al. (2001) in *Macrobrachium nipponense* where the authors passaged the culture for 3 times. In the present study muscle cells developed could be maintained for only 3-4 days.

Cell culture from the ovarian tissue of the penaeid shrimp was reported more frequently than that of heart tissue (Leudeman and Lightner, 1992; Nadala et al., 1993; Tong and Miao, 1996; Toullec et al., 1996; Chen et al., 1998; Mulford and Austin, 1998; West et al., 1999; Chen and Wang, 1999; Fraser and Hall, 1999; Shike et al., 2000[a]; Shimizu et al., 2001; Lang et al., 2002; Maeda et al 2003). Both fibroblastic and epithelioid cells were observed (Leudeman and Lightner, 1992; Chen et al., 1998; Mulford and Austin, 1998; Fraser and Hall, 1999) similar to the present study while Maeda et al. (2003) reported epithelioid cells only. Grace's Insect Medium was used by Leudeman and Lightner (1992)

and Nadala et al. (1993) while others used L 15 (Chen et al., 1998; Mulford and Austin, 1998; Maeda et al 2003) and M 199 (Toullec et al., 1996; Lang et al., 2002). Superior performance of early stage immature ovaries noted in the present study was in agreement with the observations of Toullec et al. (1996), Mulford and Austin (1998) and Fraser and Hall (1999). Mitosis was reported in ovarian cell cultures (Lang et al., 2002; Maeda et al 2003). Lang et al. (2002), similar to the present study, noticed different developmental stages of oocytes in culture.

Although explant cell cultures were obtained from various tissues and organs of penaeid shrimps, the cells from lymphoid organ formed monolayer more rapidly and remained stable for longer periods of time (Nadala et al., 1993). This is the reason for many researchers for choosing lymphoid organ as their preferred tissue (Chen et al., 1989; Nadala et al., 1993; Tapay et al., 1995; Lu et al., 1995; Tong and Miao, 1996; Tapay et al., 1997; West et al., 1999; Chen and Wang, 1999; Itami et al., 1999; Kasornchandra et al., 1999; Owens and Smith, 1999; Wang et al., 2000; Shike et al., 2000[a]; Lang et al., 2002). The lymphoid organ or oka organ was first described by Oka (1969) followed by Martin et al. (1987) and Hose et al. (1992). Although the latter authors attributed a haematopoietic function for lymphoid organ, van de Braak et al. (2002[b]) did not support this function due to limited number of mitotic figures observed, and proposed lymphoid organ as a filter for all foreign materials encounterd in the haemolymph.

L-15 medium has been described as the most suitable one for penaeid shrimp cell culture and has been employed by various workers (Chen et al., 1989, Nadala et al., 1993; Kasornchandra et al., 1999; Owens and Smith, 1999). Kasornchandra et al. (1999), Itami et al. (1999) and Wang et al. (2000) reported fibroblastic and epithelioid morphology of the cells in the lymphoid cell culture, meanwhile, Wang et al. (2000) described round cells as well. Round cells migrating out of the explant, observed in the present study, appear to be haemocytes present in the lymphoid organ, the filtering organ of haemolymph. van de Braak et al. (2002[b]) reported the presence haemocytes of granular and

semi granular types within the central haemal lumen of lymphoid organ. Difficulty experienced in subculturing the cells by mechanical or enzymatic means has been reported by several researchers (Chen et al., 1989; Owens and Smith, 1999; Itami et al., 1999), while Kasornchandra et al. (1999) reported up to three passages. Even though researchers such as Lang et al. (2002) reported cell division in lymphoid organ culture none was able to passage the culture beyond a few rounds. Owens and Smith (1999) attributed the ready formation of monolayer in lymphoid cell cultures to cell migration from the explant rather than by cell division. Hsu et al. (1995) claimed it upto 95 passages but Rinkevich (1999) reported, after careful examination of the figures published by Hsu et al. (1995), this as thraustochytrid contamination, which went on unaware of the authors. That might be the reason why the works of Hsu et al. (1995) could not be later projected as the attainment of establishment of cell line from shrimp. Subsequently, more than 21 passages of the cultures transfected with simian virus 40 large T antigen employing a pantropic retroviral vector has been reported by Hu et al. (2008). In spite of these developments none of cell cultures has been made available to the scientific community till date.

Laminin and fibronectin are extracellular matrix proteins and poly-L-lysine is a positively charged aminoacid, which enhance attachment and proliferation of cells. Of the three, poly-L-lysine (200 $\mu g\ ml^{-1}$) was found to enhance attachment and proliferation of cells from the explants. However, Frerichs (1996) observed no increase in adhesiveness of embryonic cells of *Macrobrachium rosenbergii* in poly lysine coated plates. In the same pattern Cook et al. (1989) and Braasch et al. (1999) did not observe any positive results for laminin and fibronectin coating for crab, *Cardisoma carnifex* and lobster, *Panulirus marginatus* neurons and *P. vannamei* haemocytes, respectively. However, in our study, lack of attachment on laminin and fairly better attachment on fibronectin coated plates could be observed for cells from lymphoid organ. Fan and Wang (2002) noticed enhanced growth and proliferation in embryonic cells of *P. chinensis* after the

administration of IGF-2 and bFGF, while Fraser and Hall (1999) reported no stimulation of growth after the application of EGF and bFGF. In the present study, IGF-1 enhanced cell proliferation at a concentration of 10ng ml^{-1}.

Attempt of BrdU assay has not been reported in the lymphoid cell culture of penaeid shrimp while 1-2% Brdu positive cells was reported in *P. vannamei* haemocyte culture (Braasch et al., 1999) and 35% in *P. japonicus* (Maeda et al., 2003) ovarian primary cell cultures. Very few positive fibroblastic cells have been noted in the present study, which supported the findings of van de Braak et al. (2002[b]) who observed limited number of mitotic figures in the lymphoid organ.

Wang et al., 2000 have proved the susceptibility of WSSV in lymphoid primary cell culture while Maeda et al. (2004), Jiang et al. (2005) and Jiravanichpaisal et al. (2006) studied the WSSV infectivity of ovarian cell culture of *P. japonicus*, haemocyte culture of *P. chinensis* and haematopoeitic stem cells of the crayfish, *Pacifastacus leniusculus* respectively. Shrinkage, rounding and detachment of infected cells were the common cytopathic effects reported in the event of WSSV infection (Wang et al., 2000; Maeda et al., 2004; Jiravanichpaisal et al., 2006). Additionally, in the present study, accumulation of retractile granules was also observed. Infected haemocytes exhibited clumping (Jiang et al., 2005). Wang et al. (2000), Maeda et al. (2004) and Jiravanichpaisal et al. (2006) employed electron microscopy and in situ hybridization (Jiravanichpaisal et al., 2006) to demonstrate the WSSV infectivity of the cells while Jiang et al. (2005), similar to that of in the present study, employed immunodetection utilizing monoclonal antibodies against WSSV.

Genes of the most double stranded DNA viruses are expressed in the host in a cascade fashion. Immediate early and early genes are expressed before viral DNA replication, while expression of late genes occurs after replication of the viral genome (Honess and Roizman, 1974; Friesen and Miller, 1986; Blissard

and Rohrmann, 1990; Blissard, 1996). All the tested genes including immediate early gene 1, protein kinase, thymidine kinase and thymidylate kinase, ribonucleotide reductase 1, endonuclease, DNA polymerase, Vp 28 and latency 1 genes were expressed in WSSV infected lymphoid cell culture. This demonstrated the suitability of lymphoid cell culture in WSSV morphogenesis related investigations.

Haemocytes in shrimp play a crucial role in cellular defense mechanisms and are responsible for releasing humoral defense molecules. All the immune related genes studied except transglutaminase were expressed in lymphoid cell culture. This could be attributed to the haemocytes present in the lymphoid organ which migrated during incubation (van de Braak et al., 2002[a]). Among the immune genes investigated, haemocyanin is expressed in hepatopancreas and gets processed under acidic conditions to produce an antibacterial peptide astacidine1 as reported in cray fish (Lee et al., 2003) and shrimp (Destoumieux et al., 2001), specifically during bacterial infection. However, in our study this was found weakly expressed in the WSSV infected *P. monodon* lymphoid cell culture.

Table 1. Primer sequences of WSSV genes, shrimp immune related genes and ß actin

Sl. No.	Gene	Primer sequence (5'-3')	Size (bp) of PCR product	Reference
WSSV genes				
1.	Immediate early gene (ie1)	F-GACTCTACAAATCTCTTTGCCA R-CTACCTTTGCACCAATTGCTAG	502	
2	Protein kinase (pk1)	F-TGGAGGGTGGGGACCAACGGACAAAAC R-CAAATTGACAGTAGAGAATTTTGCAC	512	
3	Thymidine kinase and thymidylate kinase (tk-tmk)	F-GAGCAGCCATACGGGTAAAC R-GCGAGCGTCTACCTTAATCC	412	
4	Ribonucleotide reductase (rr1)	F-ATCTGCTAGTCCCTGCACAC R-AAAGAGGTGGTGAAGGCACG	408	
5	DNA polymerase (dnapol)	F-TGGGAAGAAAGATGCGAGAG R-CCCTCCGAACAACATCTCAG	586	
6	Endonuclease	F-TGACGAGGAGGATTGTAAAG R-TTATGGTTCTGTATTTGAGG	408	Liu et al., 2005
7	Vp 28	F-CTGCTGTGATTGCTGTATTT R-CAGTGCCAGAGTAGGTGAC	555	
8	Latency 1	F-CTTGTGGGAAAAGGGTCCTC R-TCGTCAAGGCTTACGTGTCC	647	
Shrimp immune related genes				
1	Prophenol oxidase (pro PO)	F -TGGCACTGGCACTTGATCTA R -GCGAAAGAACACAGGGTCTCT	590	Jiravanich paisal et al., 2007
2	Astakine	F -GTCGCGCATTTAACAAGGAG R -CCCTGTGGATTGAGCTCACT	455	
3	Peroxinectin	F -CGAAGCTTCTTGCAACTACCA R -GCAGGCTGATTAAACTGGCTT	547	
4	Alpha 2 macroglubilin	F -ATGGCCAATCCCGAGAGGTACCTACTG R -TGTTGCTGCAGAAGTTTGTTATCCTCAT	345	Lin et al., 2007[a]
5	Haemocyanin	F-GTCGACGAACTTCACTGGGA R-GTTCAGTGTCATCAACGGCA	598	
6	Transglutaminase	F-TGGGYCTTCGGGCAGTT R-CGAAGGGCACGTCGTAC	627	Jiravanich paisal et al., 2007
7	Crustin	F-GCACAGCCGAGAGAAACACTATCAAGAT R-GGCCTATCCCTCAGAACCCAGCACG	430	
8	Penaeidin-3	F-AGGATATCATCCAGTTCCTG R-ACCTACATCCTTTCCACAAG	240	
9	ß Actin	F-CTTGTGGTTGACAATGGCTCCG R-TGGTGAAGGAGTAGCCACGCTC	520	Zhang et al., 2007

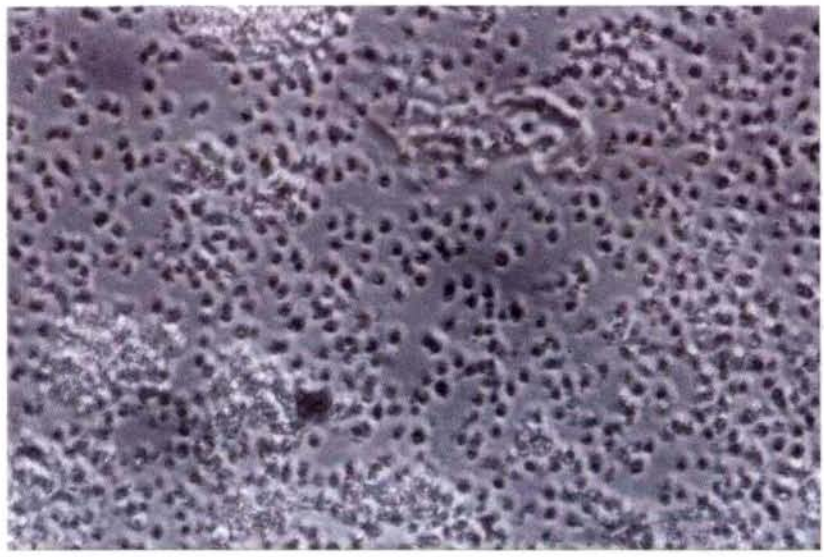


Fig.1. Primary cell culture from heart tissue of *P. monodon* (20X)

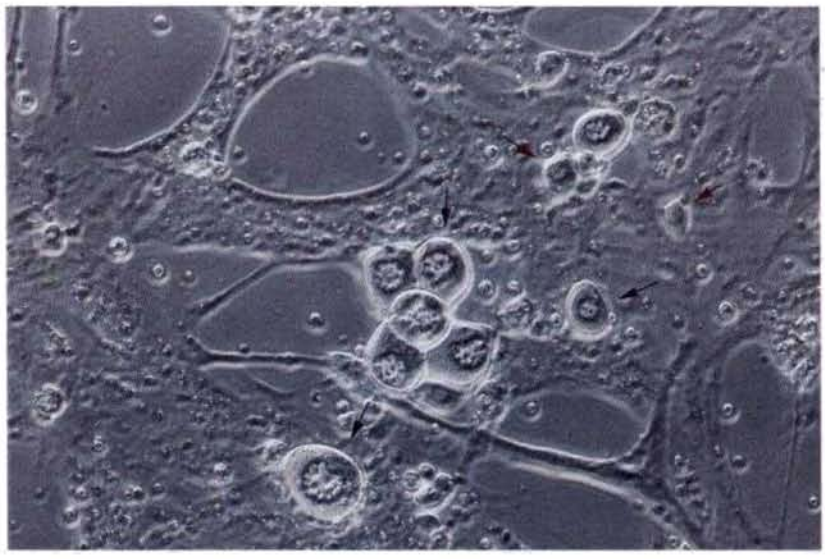


Fig.2. Primary culture from ovarian tissue of *P. monodon* showing primary oocytes (20X) among fibroblastic cells (black arrows). Oocytes in different stages of development can also be seen (red arrows).

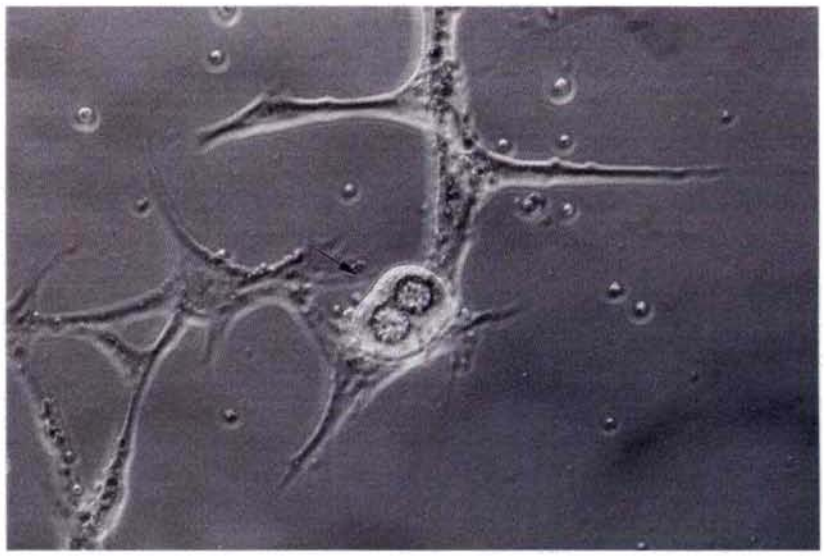


Fig.3. Nuclear division of primary oocytes in ovarian cell culture (20X)

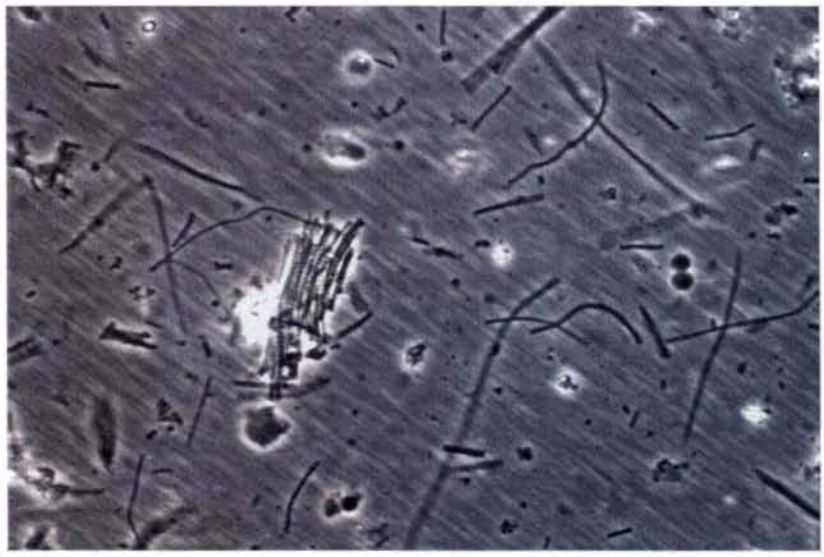


Fig. 4. Primary cell culture from muscle tissue of *P. monodon* in culture. Elongated thread like cells seen (40X)

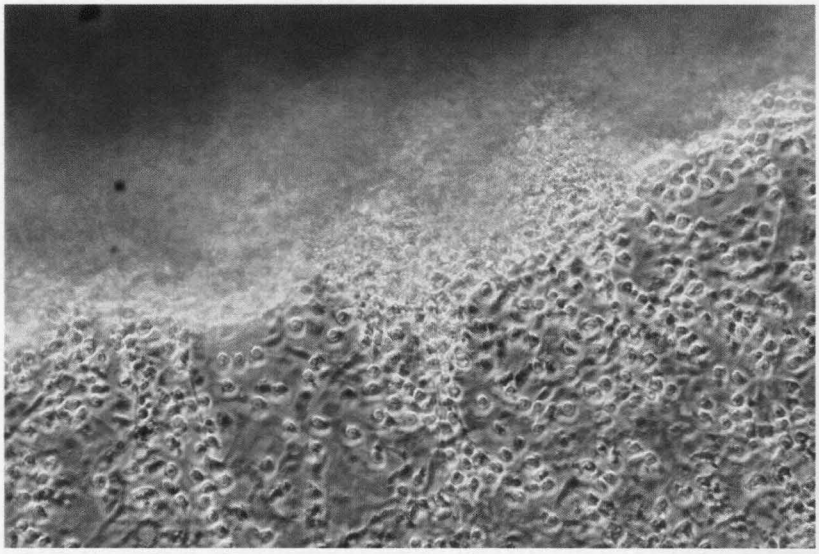


Fig.5. Round cells migrating from explant (20X) of lymphoid tissue

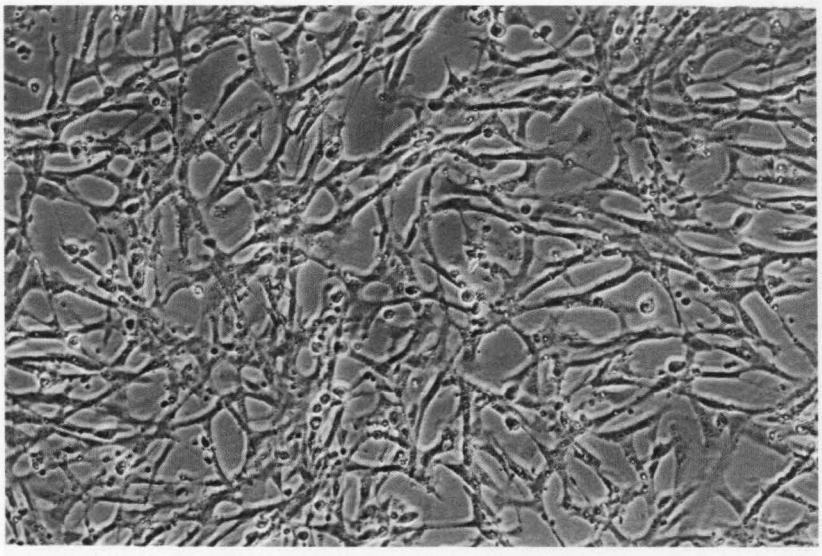


Fig.6. Monolayer of primary cell culture (fibroblastic) from lymphoid tissue (20X)

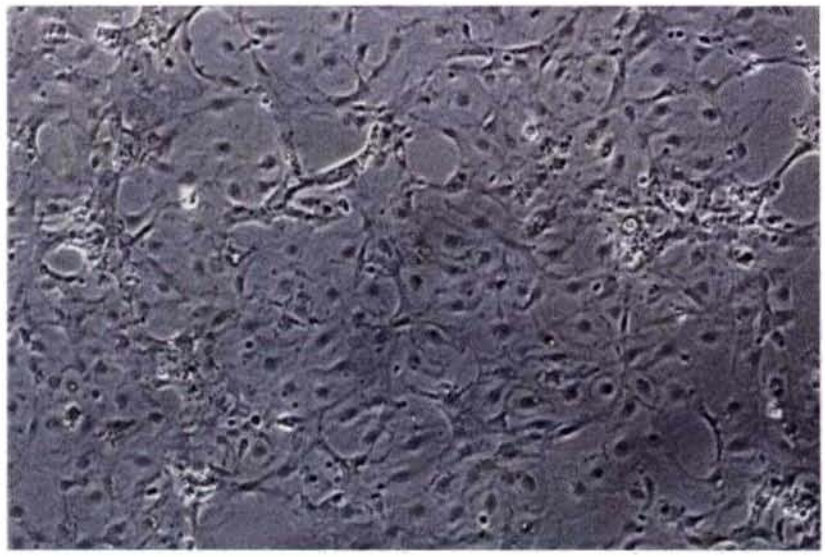


Fig.7. Monolayer of primary cell culture (epitheloid) from lymphoid tissue (20X)

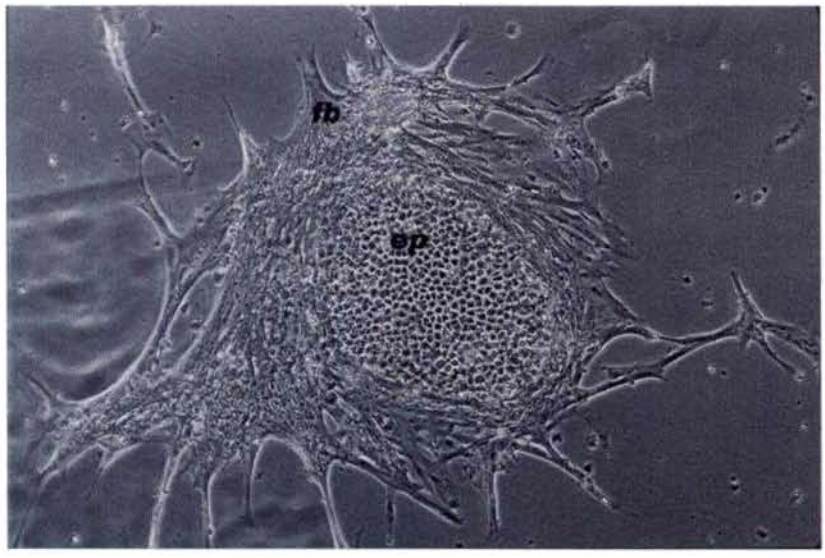


Fig.8. Primary cell culture from lymphoid tissue (Epithelioid among fibroblastic cells) (10X) (fb- fibroblastic; ep-epithelioid)

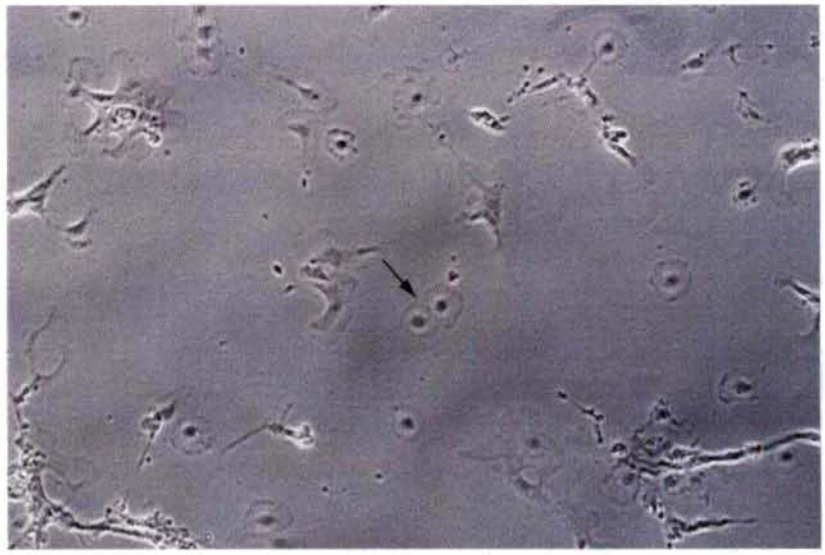


Fig.9. Epithelioid cells in division generated from primary lymphoid cell culture (20X)

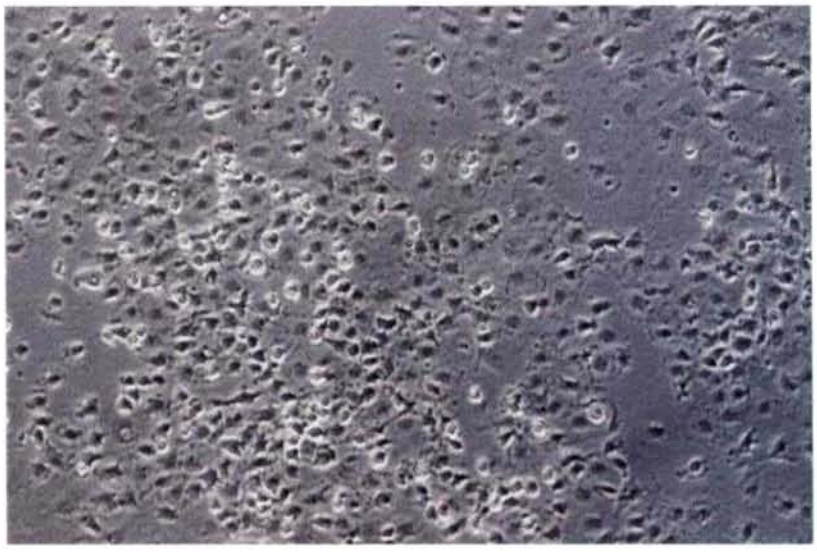


Fig.10. First passage of epithelioid cells from a primary cell culture generated from lymphoid tissue (20X)

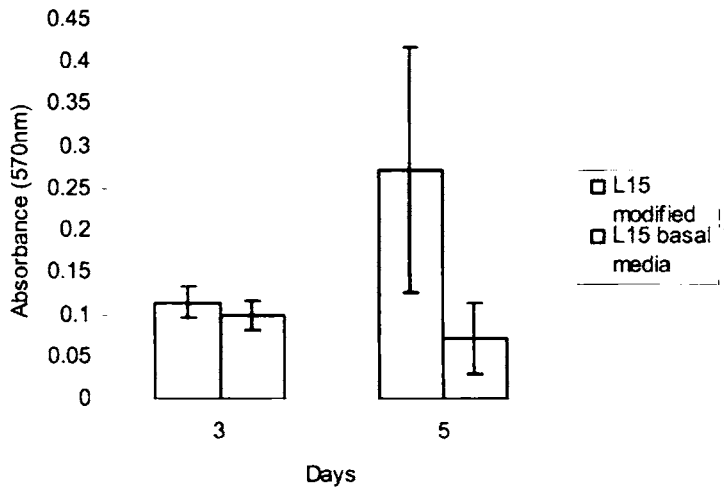


Fig.11. Comparison of 2X L-15 basal medium and modified L – 15 medium for sustaining lymphoid primary cell culture in terms of MTT readings (n=4)

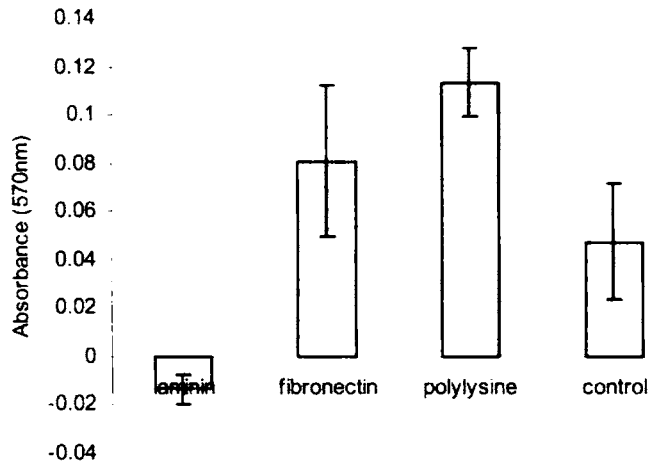


Fig.12. Effect of attachment factors on the growth of primary cell culture from lymphoid tissue in terms of MTT readings (n=3)

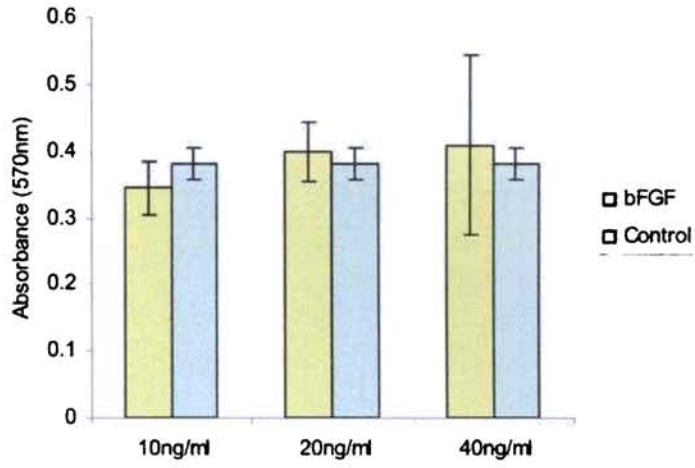


Fig.13. Effect of basic fibroblastic growth factor (bFGF) in the growth of primary cell culture from lymphoid tissue in terms of MTT readings (n=3)

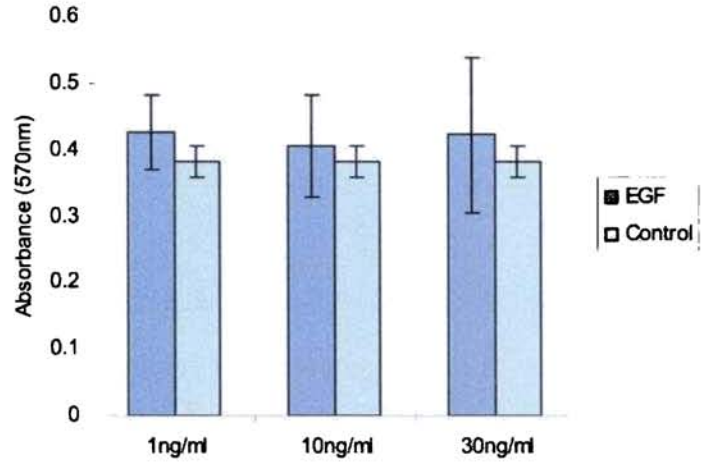


Fig.14. Effect of epidermal growth factor (EGF) in the growth of primary cell culture from lymphoid tissue in terms of MTT readings (n=3)

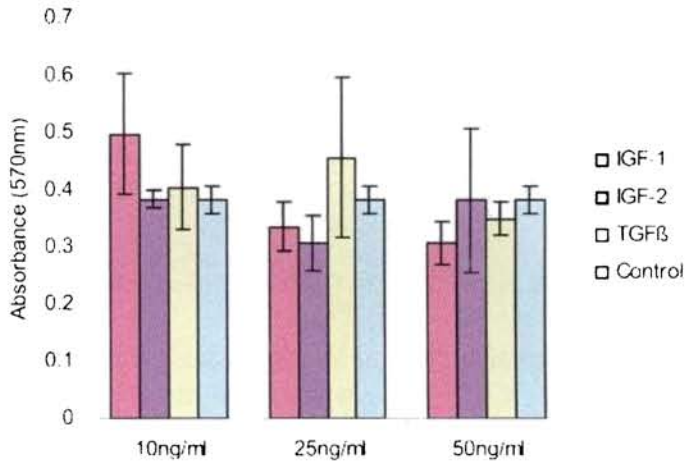


Fig.15. Effect of insulin like growth factor 1 (IGF1), insulin like growth factor 2 (IGF 2) and transforming growth factor ß1 (TGF ß) in the growth of primary cell culture from lymphoid tissue in terms of MTT readings (n=3)

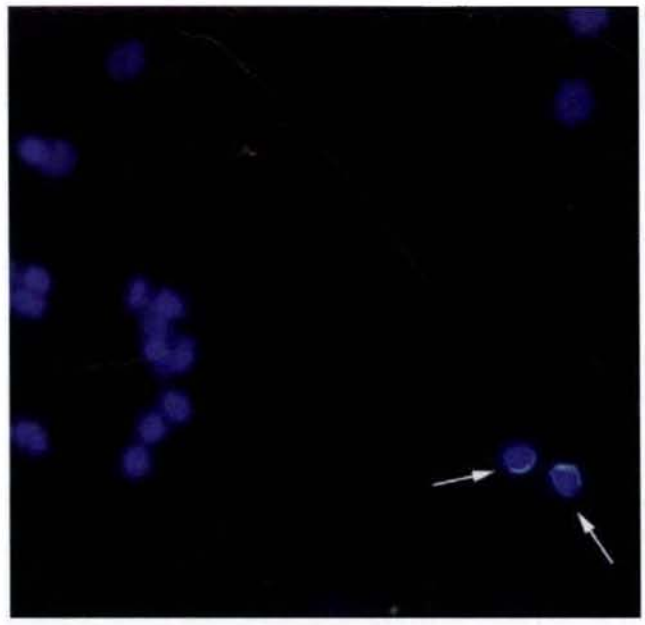
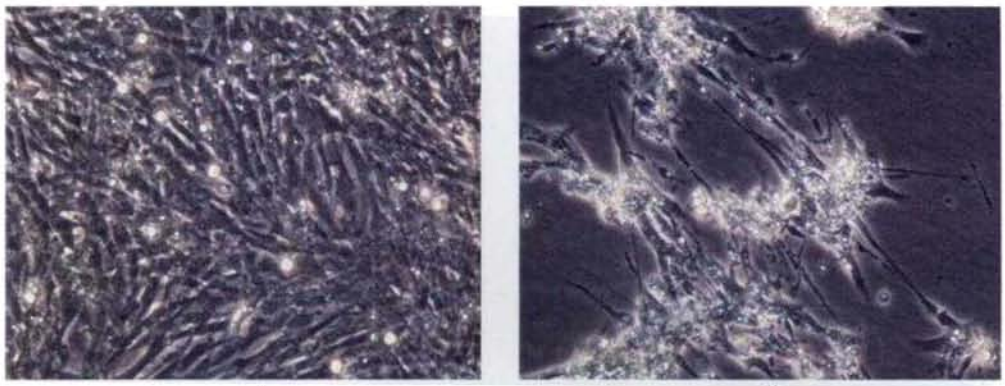
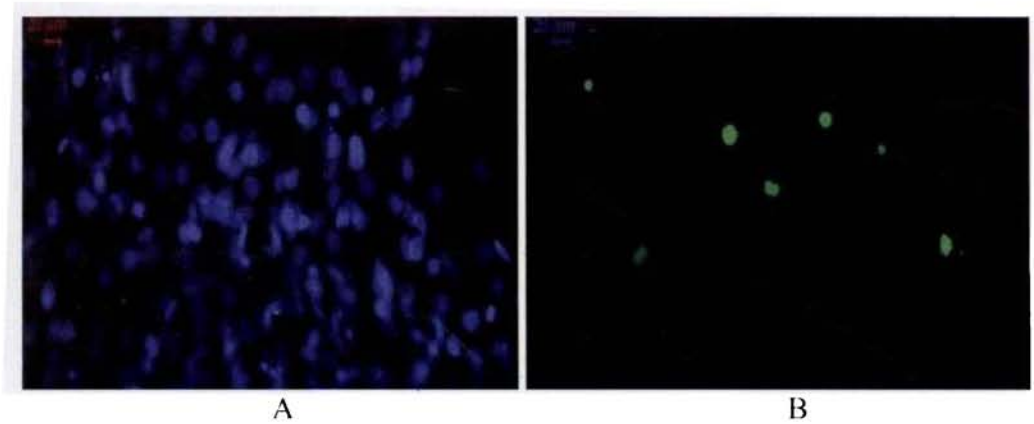


Fig.16. BrdU assay on the primary cell culture from lymphoid tissue in 2X L -15 modified medium. Arrows show positive cells (40X)

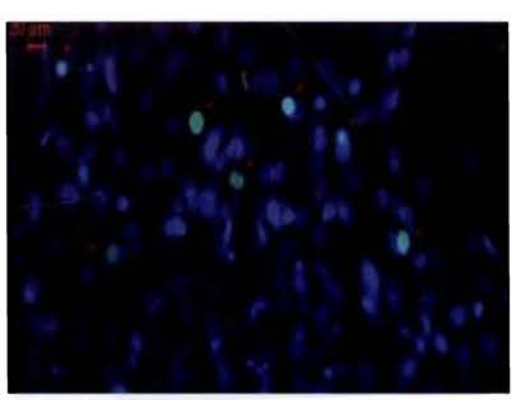


A B

Fig.17. Cytopathic effect of WSSV in lymphoid primary cell culture A) Cells before inoculation with WSSV, B) Cells 24 hours after inoculation with WSSV (40X)

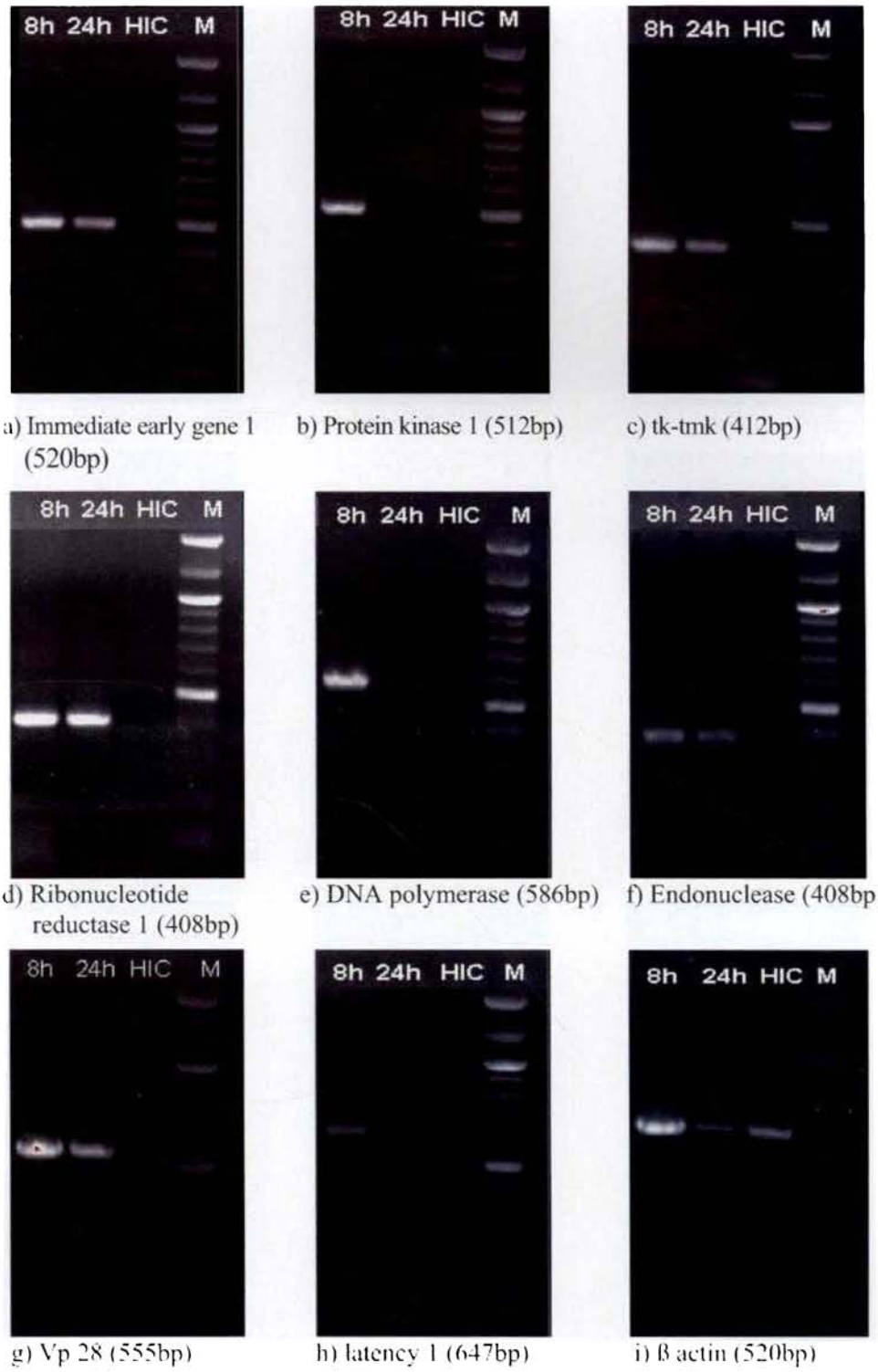


A B



C

Fig.18. Immunofluorescence detection of WSSV in primary lymphoid cell culture. A) Both infected and uninfected cells as seen under DAPI filter, B) Infected cells with green fluorescence under FITC filter, C) Merge of A and B showing green positive cells (red arrows) among DAPI stained uninfected cells



a) Immediate early gene 1 (520bp)
b) Protein kinase 1 (512bp)
c) tk-tmk (412bp)
d) Ribonucleotide reductase 1 (408bp)
e) DNA polymerase (586bp)
f) Endonuclease (408bp)
g) Vp 28 (555bp)
h) latency 1 (647bp)
i) ß actin (520bp)

Fig.19. Expression of WSSV genes in primary lymphoid culture at 8 and 24 hours of post infection (M-100bp ladder; HIC-heat inactivated control)

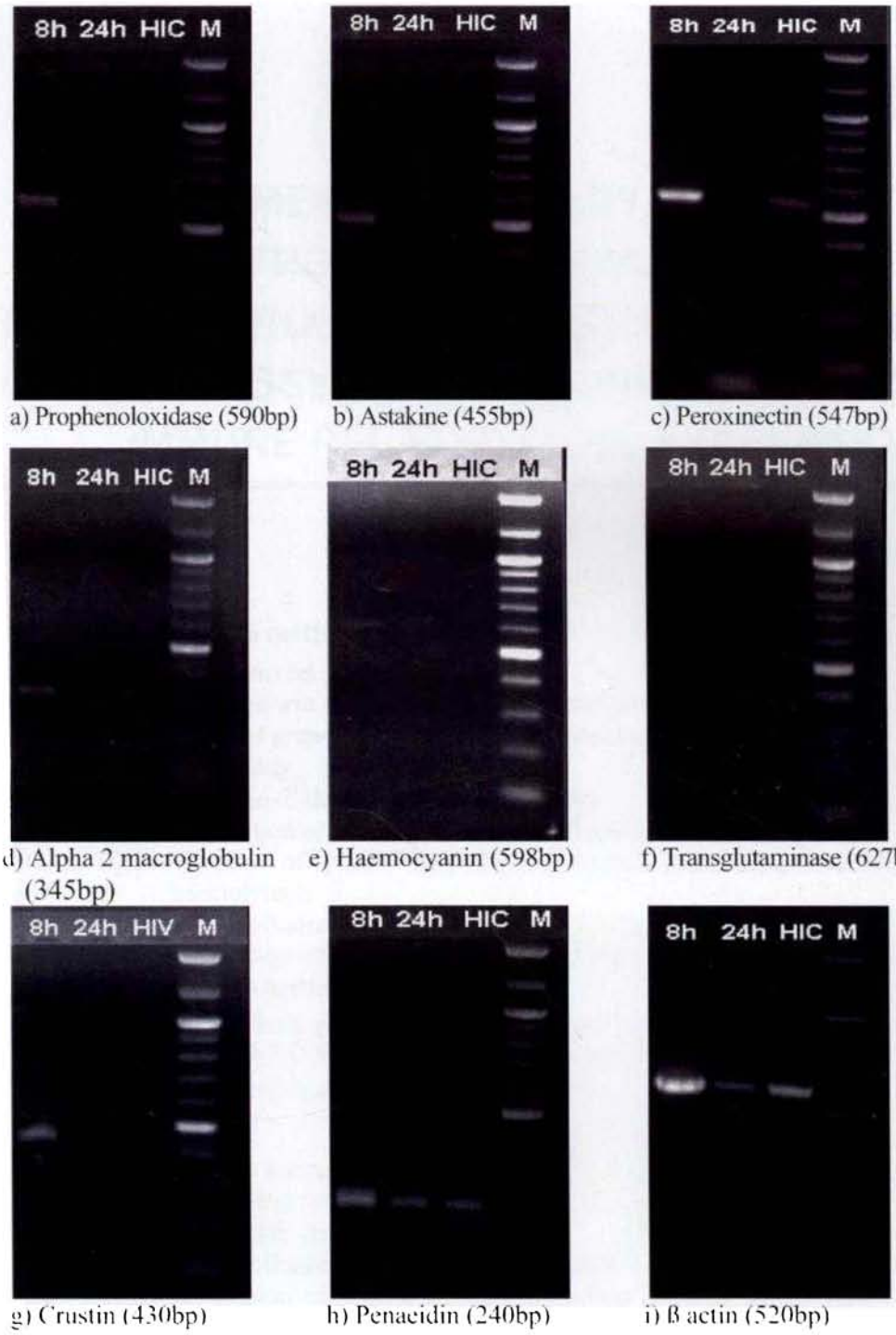


Fig.20. Expression of immune related genes in primary lymphoid culture at 8 and 24 hours of infection with WSSV (M-100bp ladder; HIC-heat inactivated control)

Chapter 3

DEVELOPMENT OF PRIMARY HAEMOCYTE CULTURE FROM *PENAEUS MONODON* AND ITS APPLICATION IN WHITE SPOT SYNDROME VIRUS (WSSV) TITRATION AND VIRAL AND IMMUNE RELATED GENE EXPRESSION

3.1. Introduction

Circulating haemocytes of crustaceans and other invertebrates play a crucial role in cellular defense mechanisms, performing functions such as phagocytosis, encapsulation and lysis of foreign cells (Smith and Soderhall, 1983; Ratcliffe et al., 1985; Johansson and Soderhall, 1989; Soderhall and Cerenius 1992). They are also responsible for releasing humoral defense molecules. Three morphologically different types of haemocytes are described including hyaline, semigranular and granular (Bauchau, 1981). In general hyaline cells are the smallest cell type with a high nucleus/ cytoplasmic ratio and no or few cytoplasmic granules. The granular cells are the largest cell type with a relatively smaller nucleus and fully packed with granules. The semigranular cells are intermediate between the hyaline and the granular cells. (Bachau, 1981; Soderhall and Cerenius, 1992). This classification is mainly based on the number of cytoplasmic granules in the haemocytes and different staining techniques, and to a much lesser extent, on density, functions and enzyme distribution (van de Braak et al., 2001[a]). Haemocytes are produced in the haematopoeitic tissue, existing as lobules, located in different areas in the cephalothorax, mainly at the dorsal side of the stomach in the epigastric region as a layer and in a substantial area in the onset of the maxillipeds and to a lesser extent towards antennal gland in *P. monodon.* (van de Braak et al., 2001[a,b]).

An essential step in the crustacean defense system is the recognition of invading microorganisms, which is mediated by the haemocytes and plasma proteins (Vargas-Albores and Yepiz-Plascencia, 2000). Different pattern recognition proteins in the haemolymph recognize and bind cell wall components of microorganisms (Soderhall et al., 1996; Vargas- Albores et al., 1996, 1997) and a second site becomes active for haemocyte binding. This operates as elicitor of defense responses following which the haemocytes degranulate and release off different proteins. Several proteins are pro-enzymes, others are substrates which

are involved in clotting (transglutaminase), prophenoloxidase activating system (prophenoloxidase activating enzyme, prophenol oxidase, phenol oxidase, peroxinectin) and in other cellular activation processes (proteinase inhibition and anti microbial peptide production), which are synthesized in haemocytes (Johansson and Söderhäll, 1992; Söderhäll et al., 1996; Sritunyalucksana and Söderhäll, 2000).

The proPO-activating system in crustaceans is also most extensively studied in the freshwater crayfish *P. leniusculus* (Söderhäll et al., 1996; Söderhäll and Cerenius, 1998). Proteins of the proPO system occupy a very prominent position in non-self recognition, haemocyte communication and the production of melanin. Upon activation and degranulation of the haemocytes, the inactive proPO is converted to the active phenoloxidase by prophenoloxidase activating enzyme. The phenol oxidase enzyme catalyses the stepwise oxidation of phenols to quinones, followed by several intermediate steps that lead to the formation of melanin, which is a dark brown pigment that sequesters the pathogens, thus preventing their contact with the host. During this formation also antimicrobial factors are formed (Söderhäll et al., 1996; Söderhäll and Cerenius, 1998). An important factor that is involved with the proPO system is peroxinectin, which is involved in cell adhesion and peroxidase activity. Transmembrane receptors of the integrin family on the haemocytes play an important role in the cell adhesion function of peroxinectin (Johansson, 1999). Alpha 2 macroglobulin, which is a broad spectrum protease binding protein, is stored in the haemocyte granules (Armstrong and Quigley, 1999). Transglutaminase and astakine were reported to be involved in cell proliferation and/or differentiation (Huang et al., 2004; Soderhall et al., 2005). Astakine is a cytokine which is directly involved in haemocyte differentiation and maturation in crayfish and is also present in *P. monodon* (Soderhall et al., 2005). Haemocyanine, a multifunctional molecule serves as an oxygen carrier for arthropods such as chelicerates and crustaceans (Decker and Terwilliger, 2000;

Decker and Tuczek, 2000). Among the crustacean antimicrobial peptides, crustins and penaeidins are reported in shrimp (Destoumieux et al., 1997; Relf et al., 1999; Gross et al., 2001).

White spot syndrome virus (WSSV), the major viral shrimp pathogen is an ellipsoid to bacilliform enveloped particle of about 275nm in length and 120nm in width, with a tail like appendage at one end. It is the type species of the genus whispovirus in the family Nimaviridae (Vlak et al., 2005) and it is unique with an infection strategy that does not match infection models of any other known viruses. Genes of most large dsDNA viruses infecting (in)vertebrates are expressed in a cascade fashion. In the cascade of viral regulatory events, successive stages of virus replication are dependant on the proper expression of the genes in the preceding stage. Immediate early and early genes are expressed before viral DNA replication, while expression of late genes occurs after replication of the viral genome (Honess and Roizman, 1974; Friesen and Miller, 1986; Blissard and Rohrmann, 1990; Blissard, 1996). Availability of the complete WSSV sequence facilitates the global molecular characterization of the virus by genomic and proteomic approaches and has recently led to the discovery of many important WSSV genes, including latency associated genes (Hossain et al., 2004; Khadijah et al., 2003), immediate early genes (Liu et al., 2005), many other non structural genes (Tsai et al., 2000[a, b]; Chen et al., 2002[a]; Tzeng et al., 2002) and more than 39 structural genes (Durand et al., 1997; Van Hulton et al., 2002; Zhang et al., 2004; Tsai et al., 2006).

Primary haemocyte culture of *P. japonicus* (Itami et al., 1999) and *P. chinensis* (Jiang et al., 2005) had already been reported. Itami et al. (1999) were able to culture granular haemocytes for 10 days while Jiang et al. (2005) cultured total haemocytes for 20 days. Jiang et al. (2005) proved the susceptibility of WSSV in haemocyte culture by immunocytochemical detection. Lack of shrimp cell lines is hampering the progress of research in WSSV (Marks et al., 2003; Han et al., 2007).

In the present study primary haemocyte culture was developed and its application in WSSV titration and in the expression studies of WSSV genes and haemocyte immune genes were explored.

3.2. Material and methods

3.2.1. Experimental animals

WSSV and Monodon Baculo virus (MBV) negative *P. monodon* larvae obtained from a local hatchery were stocked and reared in a Recirculating shrimp rearing system integrated with nitrifying bioreactors (Kumar et al., 2009) in sea water having a salinity of 15g l^{-1}. Water quality was maintained by the addition of a probiotic, Detrodigest ^{TM} (NCAAH, India), to manage detritus, and Enterotrophotic ^{TM} (NCAAH, India) to control *Vibrio*. The larvae were fed with commercially available pelleted feed (Higashimaru, India). They were confirmed WSSV negative by nested PCR (WSSV detection kit, Genei, India) when they grew to 8-12g, and used for all experiments.

3.2.2. Development of primary haemocyte culture

The animals were sacrificed by immersing in crushed ice and disinfected by maintaining in 800 mg l^{-1} sodium hypochlorite solution prepared in ice- cold sea water (salinity 15 g l^{-1}) for 10 minutes. Subsequently they were washed 5 times in sterile ice- cold sea water, dipped in 70% alcohol and rinsed in the former. Haemolymph was withdrawn asceptically from rostral sinus using capillary tubes containing 100µl anticoagulant (Tris HCl 0.01M, Sucrose 0.25M, Tri Sodium Citrate 0.1M) and diluted to obtain 5 x 10^5 cells ml^{-1} using modified L-15 (Leibovitz) medium, and aliquots of 200µl were dispensed into the wells of 96 well plates (Greiner Bio-One) and incubated at 25°C. For the immunofluorescence detection of WSSV, haemocyte cultures were prepared by adding 1ml of cell suspension (5 x 10^5 cells) prepared in growth medium into Leighton tubes with cover slips (10 x 22mm; Micro-Aid), and incubated at 25°C.

The medium used was Leibovitz's L-15 (Sigma) at double strength supplemented with 2% glucose, 20% FBS, 0.06µg ml^{-1} chloramphenicol, 100µg ml^{-1} streptomycin and 100 IU ml^{-1} penicillin. To prevent melanisation and subsequent cell death, N-phenylthiourea (0.2mM) was added. This served as the basal medium on which media supplements such as MEM Vitamins (1X), bovine embryonic fluid (5%) and tryptose phosphate broth (2.95g l^{-1}) were tested. Each of these additives was added to the basal medium individually and the haemocyte response was examined by measuring the metabolic activity through 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay for 6 days. Replicates were maintained for each combination of the medium.

3.2.3. Effect of growth factors on primary haemocyte culture

Growth factors tested were fibroblast growth factor-basic (bFGF, from bovine pituitary glands (Sigma, USA), epidermal growth factor (EGF, from mouse submaxillary glands (Sigma, USA), insulin like growth factor-1 (IGF-1, human, recombinant expressed in *E. coli* (Sigma, USA), insulin like growth factor-2 (IGF-2, human, recombinant expressed in *E. coli* (Sigma, USA) and transforming growth factor-ß1 (TGF- ß1, from porcine platelets ((Sigma, USA)). Primary stock solutions of bFGF and EGF were prepared in the medium while IGF-1 was prepared in water, IGF-2 in 10mM acetic acid containing 10µg ml^{-1} bovine srum albumin (BSA) and TGF- ß1 in 4mM HCl containing 1 µg ml^{-1} BSA. Primary stocks were diluted to required concentrations with the growth medium. 96 well plate cultures of haemocytes were prepared and growth factors were added to get a final concentration of 20 ng ml^{-1} and 40 ng ml^{-1} for bFGF, 10 ng ml^{-1} and 30 ng ml^{-1} for EGF and 25 ng ml^{-1} and 50 ng ml^{-1} for IGF-1, IGF-2 and TGF- ß1. MTT assay was done after 24 and 48 hours. Quadruplicates were kept for each concentration of growth factors and control wells without the addition of growth factors.

3.2.4. MTT assay

This assay is a colorimetric method based on the determination of cell viability utilizing the reaction of a tetrazolium salt (3-(4, 5-dimethylthiazol-2-yl)-2,

5-diphenyl tetrazolium bromide, MTT) with the mitochondria of metabolically active cells. Reduction of the tetrazolium salt by nicotinamide adenine dinucleotide dehydrogenase (NADH) and nicotinamide adenine dinucleotide phosphate dehydrogenase (NADPH) within the cells produces insoluble purple formazan crystals, which are later solubilized yielding a purple-colored solution (Mosmann, 1983).

After replacing the medium, a sample of 50µl of MTT (Sigma) solution (5mg ml^{-1} in PBS; 720mOsm) was added to each well and incubated for 5 hours in dark. Control consisted of medium alone with MTT added. After incubation, the medium was removed and MTT-formazan crystals were dissolved in 200µl dimethylsulfoxide. Absorbance was recorded immediately at 570nm in a micro plate reader (TECAN Infinite Tm, Austria).

3.2.5. 5- bromo-2'-deoxyuridine (BrdU) assay

A sample of 20µl of 10mM BrdU solution was added to each well of 96 well plate haemocyte cultures with 200µl medium and the detection done after 2, 24 and 48 hours. The medium was removed, cells were fixed with acidified ethanol for 30 minutes and blocked using 3% bovine serum albumin in PBS. Peroxidase conjugated anti BrdU solution (Amersham Biosciences, UK) of 100 µl was added to each well and incubated for 90 minutes at room temperature. Wells were rinsed thrice with PBS and diaminobenzidene (0.6mg ml^{-1} in 0.05M tris buffer with 0.03% hydrogen peroxide) substrate was added to the wells immediately. After 20 minutes wells were rinsed with PBS and counterstained with Meyer's Haematoxylin for 30s. Cells without BrdU were kept as control. Cells were observed under inverted microscope, and the total and differential counts (brown coloured positive cells) were taken.

3.2.6. Preparation of WSSV lysate from gill and haemolymph

A 500 mg sample of gill tissue from WSSV infected shrimps under laboratory conditions was macerated with mortar and pestle kept in ice bath in

10ml cell culture medium with glass fiber wool. The extract was centrifuged at 10,000g for 10minutes at 4°C and the supernatant passed through 0.22µm polyvinylidene fluoride (PVDF) membrane (Millipore). Frozen haemolymph from a WSSV infected shrimp was thawed, centrifuged at 10,000g for 10 minutes at 4 °C, diluted 10 times with cell culture medium and passed through 0.22µm PVDF membrane. In the same pattern extracts from healthy *P. monodon* adults were used for examining cytotoxicity of gill tissue and haemolymph.

3.2.7. Titration of WSSV suspension prepared from infected gill and haemolymph

The method of MTT_{50} was adopted here for the titration of WSSV in the suspension prepared. A 96 well culture of haemocytes was generated employing the modified L-15 medium. A double dilution series of WSSV suspension from gill and haemolymph extracts was prepared in a deep well plate (Axygen). The medium was removed from the wells and 200µl WSSV inoculum added to each well maintaining quadruplicate for each dilution. For comparison, uninfected controls were also maintained. On the third day the wells were observed under phase contrast microscope (Carl Zeiss, Germany) for CPE, and subsequently MTT assay was done. In the same manner gill tissue and haemolymph extracts from apparently healthy *P. monodon* were applied as controls to haemocyte culture to check the cytotoxicity.

The 50% infectious dose, termed MTT_{50} of the virus is defined as the titre of the virus at which the average well absorbance was 50% that of the uninfected cells. This was determined by plotting the optical density of each well versus the $-\log_{10}$ of the dilution factor. The optical density was defined as:

Optical density = (Blank absorbance −Well absorbance/ Blank absorbance) x 100%

The blank absorbance is the average absorbance of 12–48 uninfected wells and the well absorbance is the average absorbance of infected wells. From the plot of optical density versus $-\log_{10}$ of the dilution factor, the 50% optical density

was determined. This value was converted per milliliter basis and stated as the MTT_{50} titer (Heldt et al., 2006).

3.2.8. Immunofluorescence assay for detection of WSSV

Diluted WSSV suspension (300 times) was used for infecting haemocytes. The medium was removed and 1ml WSSV inoculum prepared in the growth medium was added to the Leighton tubes with cover slip cultures and incubated at 25°C. After 24, 48 and 72 hours cover slips were removed, washed twice in PBS (720mOsm) and immersed in 10% paraformaldehyde and maintained in 70% ethanol at -20°C until use. These fixed cover slip cultures were used for the immunofluorescence detection of WSSV. The cover slips were attached to a glass slide, free sites were blocked using 3% BSA in PBS and incubated in a humidified chamber for 1 hour. The slides were washed in PBS/Tween 20 (0.01%) and added WSSV monoclonal (C 38) antibody (Anil et al., 2002) on the slide, incubated for 1 hour and washed three times in wash buffer. It was again incubated for one hour after addition of rabbit anti mouse FITC conjugate, 1: 40 dilution (Sigma) and subjected for washing. After incubation with general nuclear stain DAPI (10 µl, 0.2µg ml^{-1}) for three minutes, the slides were rinsed with distilled water, air dried, mounted (Vectashield, USA), and observed under fluorescent microscope (Olympus, Germany). DAPI and FITC were viewed under filters with excitation wavelength 360-370nm and 470-490nm, respectively. The slides were compared with uninfected haemocytes (negative control). The images were processed and merged using the "Image pro-express" software (Media Cybernetics Inc, MD, USA).

3.2.9. Expression of WSSV genes and shrimp immune genes in WSSV infected haemocyte culture

3.2.9.1. RNA isolation from WSSV infected haemocyte culture

Twenty four well plate cultures of haemocytes were prepared in modified 2X L15 medium. One mL of cell suspension containing 8 $x10^5$ cells was added to each well. After 6 hours incubation, the medium was removed and 500 µl

WSSV inoculum added. RNA isolation was done after 2h, 12h, 24h, 36h, and 48h from 10 wells each. Control wells were maintained without WSSV and with heat inactivated (56°C for 1 hour) WSSV as inocula. RNA was isolated from control wells after 48 hours. For RNA isolation the medium was removed, wells washed with ice cold PBS and TRI reagent (Sigma) was added to each well. Complete lysis of cells was allowed to take place by repeated pipetting and the reagent was collected in 1.5 ml MCTs. The medium removed from the wells containing detached cells was centrifuged at 400g for 5 minutes, washed with ice cold PBS and pooled to each hour's sample. The samples were stored for 5 minutes at room temperature to ensure complete dissociation of nucleoprotein complexes. 0.2mL chloroform was added to 1ml TRI reagent, shaken vigorously for 15 seconds, and allowed to stand for 15 minutes. The resulting mixture was centrifuged at 12,000g for 15 minutes at 4°C. Colourless upper aqueous phase was separated carefully from the three layers formed and transferred to a fresh tube. 0.5ml isopropanol was added and stored for 10 minutes at RT and centrifuged at 12,000g for 10 min. RNA was precipitated on the sides and bottom of the tube after centrifugation at 12,000g for 10 min at 4°C. The supernatant was discarded and the pellet washed twice with 75% ethanol. The pelleted RNA pellet was air died and dissolved in 20 µl DEPC treated sterile water by repeated pipetting at 55°C. These RNA samples were subjected to DNase treatment with RNase-free DNase 1 (New England Biolabs). 0.2 units of the enzyme was added per µg of RNA and incubated at 37°C for 10 minutes. The enzyme was inactivated at 75°C for 10 minutes. Concentration and quality of RNA was measured by taking the absorbance at 260/280nm in a UV-Visible spectrophotometer.

3.2.9.2. RT-PCR of WSSV genes and haemocyte immune related genes

One µg of RNA was subjected to cDNA synthesis with 20µl of reaction mix containing M-MuLV reverse transcriptase (80U), RNase inibitor (8U), Oligo $(dT)_{12}$ primer(40pmoles), dNTP mix (1mM), RTase buffer (1X) and $MgCl_2$ (2mM) at 42°C for 1 hour. All reagents were purchased from New England

Biolabs. Subsequently, 8 WSSV genes and 8 immune related genes were amplified by PCR using 2µl cDNA with specific primer sets as given in Table 1 (Chapter 2). Shrimp ß actin gene was also amplified as a reference. 25µl PCR reactions contained 0.5U of Taq DNA polymerase, 200µM dNTP mix, 10pmoles of each forward and reverse primer and 1X PCR buffer. The hot start PCR programme used for WSSV genes was 94°C for 2min, followed by 35 cycles of 94°C for 30s, annealing for 30s, 68°C for 30s followed by final extention 68°C for 10 min. Annealing temperatures were 50°C for endonuclease, 53°C for latency 1 and ribonucleotide reductase (rr1), 54°C for DNA polymerase (dnapol), immediate early gene1 (ie1), Vp 28 and thymidine kinase and thymidilate kinase (tk-tmk), and 55°C for protein kinase 1(pk1) and ß actin. For immune related genes also hot start PCR was done with 94°C for 2min, followed by cycles of 94°C for 2 min, annealing for 1min, 72°C for 1min followed by final extension at 72°C for 10 min. Annealing temperature and number of cycles employed for PCR of Astakine and peroxinectin were 56°C and 30 cycles, for prophenoloxidase (Pro PO) and transglutaminase 56°C and 35 cycles, for crustin and penaeidin 55°C and 35cycles, for alpha 2 macroglobulin 65°C and 30cycles and for haemocyanin those were 56°C and 35cycles. 10µl of each PCR product were analysed by 1% agarose gel electrophoresis, stained with ethidium bromide and visualized under ultraviolet light and documented.

3.2.10. Statistical Analysis

The results in the figures are average values of 3-6 replicates ± standard deviation. The effects of treatments were statistically analyzed by single factor and two factor analysis of variance (ANOVA) wherever applicable. Differences were considered significant at $P \leq 0.05$.

3.3. Results

3.3.1. Primary haemocyte culture

Melanisation (blackening of the media and cell death) was observed in haemocyte culture with the basal medium from the first day of culture itself.

However, addition of N-phenylthiourea (0.2mM) could prevent this situation and enhance metabolic activity (Fig 1). Among the supplements tested addition of bovine embryonic fluid significantly reduced the activity, recorded as low MTT readings ($P<0.05$) (Fig 2). When the basal medium was supplemented with MEM Vitamins (1X) and tryptose phosphate broth independently, the differences observed were not significant, however, in combination the two ingredients could provide higher MTT values ($P<0.05$). Under such conditions haemocytes were viable up to 6-8 days. Accordingly, the modified medium was finalized to contain Leibovitz's L-15 (Sigma) at double strength supplemented with 2% glucose, MEM Vitamins (1X), tryptose phosphate broth (2.95 g l^{-1}), N-phenylthiourea (0.2mM), 20% FBS, 0.06µg ml^{-1} chloramphenicol, 100µg ml^{-1} streptomycin and 100 IU ml^{-1} penicillin. In this medium haemocytes appeared spherical or elliptical initially, and within a few hours two distinct morphological types such as round to elliptical and small and large fibroblastic cells, with granules, could be seen (Fig 3).

Growth factors such as bFGF, IGF-1, EGF and TGF ß did not have much effect on the haemocytes while IGF-2 at 50ng ml^{-1} had significant difference (Fig 4) from the control (P=0.05).

The results of BrdU assay showed that maximum labeling had taken place at 24 hours, recording 22±7.38 % positive cells (Fig 5, 6).

3.3.2. Titration of WSSV

Sigmoid curves were obtained on plotting optical density versus $-\log_{10}$ of the dilution factor, and 50% optical density was extrapolated from the curve and converted to per millilitre. The MTT titre of infected gill extract and haemolymph were 2.9 $\log_{10}$ (MTT_{50}/ml) and 3.2 $\log_{10}$ (MTT_{50} ml^{-1}), respectively (Fig 7).

No toxicity was observed for the gill extract from healthy animals whereas the haemolymph showed 5% inhibition (MTT values) at 10 times dilution (Fig 8) at which the cells appeared normal morphologically.

3.3.3. Cytopathic effect (CPE)

On inoculating with haemolymph and gill extracts from shrimp infected with WSSV (diluted 80 and 20 times, respectively) the haemocytes in culture were found disintegrated within a few hours. In lower dilutions the cells were round and in still higher dilutions they were both round and small spindle shaped; beyond which a mixture of round, small spindle and a few long spindle shaped cells could be noticed. In higher dilutions the cells appeared the same as that of the control. This sequence of morphological changes suggested that both types of cells were susceptible to WSSV; the spindle shaped cells were the most. Cytopathic effect (CPE) included shrinkage and necrosis of haemocytes (Fig 9), which was visible from 1st day itself in lower dilutions of WSSV and 2nd to 3rd day onwards in higher dilutions. Hypertrophy of the infected nucleus was also observed (Fig 10).

3.3.4. Immunofluorescence detection of WSSV

Blue fluorescence of DAPI was observed in the cell nuclei in all preparations. Green positive signals with FITC conjugated monoclonal antibodies against WSSV were observed from the nuclei of cells assayed after 72 hours of exposure to the virus. However, FITC signals were observed in the cytoplasm subsequent to 24 hours incubation, however, no positive signals could be obtained from the control slides (Fig 11).

3.3.5. Expression of WSSV genes and shrimp immune related genes in WSSV infected haemocyte culture

3.3.5.1. Expression of WSSV genes in WSSV infected haemocyte culture

Expression of an immediate early gene (ie1), 5 early genes (pk1, tk-tmk, rr1, dnapol, endonuclease), one of the late genes (Vp 28) and a latency gene (latency 1) was examined at 2h, 12h, 24h, 36h and 48h post infection (Fig 12). They were categorized in to two as follows:

1. Those which got expressed and maintained more or less uniformly through out the study period of 48h: ie1, pk1, tk-tmk,rr1, endonuclease.

2. Genes which were not expressed after 24th hour: dnapol, Vp28 and latency 1.

When Vp28 was found upregulated the latency 1 was down regulated. None of the WSSV genes was expressed in control haemocytes without WSSV and control cells with heat inactivated WSSV.

3.3.5.2. Expression of immune related genes in WSSV infected haemocyte culture

Expression of immune related genes examined at the event of WSSV infection included prophenoloxidase, astakine, peroxinectin, alpha 2-macroglobulin, haemocyanine, transglutaminase, crustin and penaeidin (Fig 13). Expression of these genes compared to that of the negative control could be categorized as follows:

1. Those which were up regulated at 2nd hour: prophenoloxidase, peroxinectin, penaeidin, alpha 2-macroglobulin, crustin and transglutaminase.
2. Those which were maintained upregulated in the same order at 12th hour: peroxinectin, penaeidin, and crustin.
3. Those which were maintained their expression in the same order at 24th hour: Astakine.
4. Those which were upregulated and maintained at 48th hour at the event of administering heat inactivated WSSV: prophenoloxidase, peroxinectin, alpha 2-macroglobulin and penaeidin.

Meanwhile haemocyanin and transglutaminase were found faintly expressed even though not detected in the controls.

3.4. Discussion

Leibovitz L-15 was chosen as the basal medium based on its strong buffering capacity contributed by the relatively high aminoacid concentration. Over the years L-15 has been employed by several for shrimp cell culture

development (Wang et al., 2000; Mulford et al., 2001; Maeda et al., 2003), however, none of the attempts including ours has culminated in establishment of a cell line. In this context it was necessary to improvise the medium to support the cell cultures for a longer duration, which might ultimately lead to immortalization. With this on focus several supplements were incorporated to the medium and response of haemocytes was assessed in terms of enhancement of their metabolic activity using MTT assay, a maiden attempt in shrimp cell culture development specifically for screening growth supplements. As on today the screening of media and media supplements were based on microscopic observation and not by quantitative assays. Lone deviation from this normal practice recorded in literature was the attempt of Shimizu et al. (2001) who opted for ^{3}H-thymidine uptake assay and ^{35}S-methionine uptake assay for analyzing performance of ovarian cells in different media. Considering the easiness of the protocols involved and the quantitative data generated on the metabolic activity of cells under study we have been choosing MTT assay in the screening of media supplements.

Among the supplements tested, addition of bovine embryonic fluid to the medium was detrimental to the cells. Even though the effects of tryptose phosphate broth and MEM vitamins (1X) were not significant when tested individually, in combination they contributed to the longevity of haemocytes for 6-8 days. However, animal- wise variation in the response of the cells with respect to their lifespan was experienced. Metabolic activity of haemocyte did not vary considerably for 3 days and the activity started decreasing only then onwards. Advantageously, with in this period WSSV titration and cytotoxicity assays could be completed. Among the types of cells developed half turned out to be fibroblastic within three hours of incubation matching with the findings of Roper et al. (2001). This could be ascribed to the specificity of L-15 medium which was known to support more the growth of fibroblastic cells (Fraser and Hall, 1999). The medium was found to support hyaline haemocytes from marine

decapode crustaceans such as *Liocarcinus depurator* (L) and *Carcinus maenas* (L) (Walton and Smith, 1999), hyaline, semigranular and granular haemocytes from Carribean spiny lobster, *Panulirus argus* (Li and Shields, 2007), and haemocytes from *Penaeus chinensis* (Jiang et al. 2005).

Among the vertebrate growth factors tested only IGF-2 at 50 ng ml^{-1} had a significant effect on the culture. Fan and Wang (2002) noticed enhanced growth and proliferation in embryonic cells of *P. chinensis* after the administration of IGF-2 and bFGF while Fraser and Hall (1999) reported no stimulation of growth after the application of EGF and bFGF.

To determine the proliferation of the cultured haemocytes, incorporation of 5- bromo-2'-deoxyuridine (BrdU) was accomplished (Gratzner, 1982). BrdU, a synthetic analogue of thymidine incorporated into deoxyribonucleic acid during the S-phase of the cell cycle, was used for the analysis of cell proliferation, as monitoring DNA synthesis happened to be an indirect method. Several workers have attempted to study the proliferation of circulating haemocytes in shrimp. Sequeira et al. (1996) observed that only about 0.6 percentage of circulating haemocytes in *P. japonicus* were in G2 or M phase, and the percentage increased to 3 with the injection of lipopolysaccharide or infection with the fungus *Fusarium,* however, with out any dividing cells under circulation. Subsequently, Gargionni and Baracco (1998) could observe mitotic figures just in less than 1% of circulating cells in *P. paulensis*. Soderhall et al. (2003) detected < 1-2.5% BrdU incorporation in circulating haemocytes of *Pacifastacus leniusculus* after 90 minutes of labeling. In our study BrdU incorporation was attempted in vitro where there were 22±7.38% positive cells detected at 24^{th} hour of incubation suggesting cell proliferation.

Because of the presence of ß- integrin receptor, which mediates WSSV infection (Li et al., 2007) shrimp haemocytes were selected for its titration. In our study, haemocyte culture was successfully demonstrated as in vitro system

for titrating WSSV. Researchers reported quantal assays for yellow head baculovirus (YBV) and non-occluded baculo like virus in primary lymphoid explant cultures (Lu et al., 1995; Tapay et al., 1997). The advantage of using haemocyte culture over explants as model for WSSV titration and cytotoxicity studies is the easiness in quantification of cells to be seeded to maintain uniform cell number in micro plates, a prerequisite for quantal assays, and the rapidity in obtaining monolayer of cells. .

Gill extract and haemolymph from WSSV infected shrimp were used for the virus titration. While doing so the observation made by Fraser and Hall (1999) on the cytotoxicity of haemolymph on shrimp cell culture was considered, and both gill extract and heamolymph from normal animals were proven non toxic to haemocyte culture prior to the experiment.

Determining viral titer of an infected tissue is of utmost importance in virology and a common method recommended is the endpoint titration assay, the number of infectious units expressed as $TCID_{50}$ ml^{-1}. Some of the major disadvantages of this assay are risk of erroneous results as they are done manually (Andersson et al., 2005), the need of an experienced person and a great deal of time when a large number of samples are analysed (Heldt et al., 2006). MTT assay employed in the present study is a high throughput assay which doesn't require manual assessment of cytopathic effect; instead metabolic activity of the cells is measured. Even though the MTT assay was successfully used in the titration of vertebrate viruses (Levi et al., 1995; Heldt et al., 2006; Andersson et al., 2005) this had not been employed for titration of WSSV so far.

MAb C38, which reacts strongly with the 28kDa but weakly with 18kDa envelope proteins of WSSV, has been used for immunofluorescence detection of the virus (Anil et al., 2002). Using these MAbs we could observe strong positive signals from the infected nucleus after 72 hours of virus administration. Nevertheless, localized signals could be seen from cytoplasm and nucleus as well

from 24hour onwards, a situation probably caused by the events such as phagocytosis and/or endocytosis(Wang et al., 2002).

Expression of viral genes and immune related genes at the event of WSSV infection was investigated along with that of negative control and heat inactivated haemocytes. Viral gene expression could be categorized in to two such as those which got expressed and maintained more or less uniformly through out the study period of 48h (iel, pkl, tk-tmk,rrl, endonuclease), and those genes which were not expressed after 24th hour (dnapol, Vp28 and latency 1). When Vp28 was found upregulated the latency 1 was down regulated. None of the WSSV genes was expressed in control haemocytes without WSSV and control cells with heat inactivated WSSV.

Classification of the viral gene expression in to two categories point out the regulatory events in the viral multiplication which take place in the shrimp haemocytes. WSSV early genes, which are regulatory genes, are transcribed at the early infection stage (Han et al., 2007). In the cycle of virus proliferation, the early genes initiate the synthesis and replication of the viral genome (Okano, 1999). In this process a few early genes can accelerate the expression of late genes as well (Jensen et al., 1996). In the present study amplification of the immediate early gene 1, tk-tmk and rrl from 2 h post infections and the other early genes such as protein kinase, DNA polymerase and endonuclease from 12 hour post infection suggested the sequence of events of viral DNA replication in the haemocytes. It has to be highlighted that Vp 28, a late gene, involved in the production of envelope protein could be expressed from 12-24 hours while the latency gene was down regulated. This is a clear indication of the virulence of WSSV to haemocytes by not having been entering in to a latent phase. It has been reported that envelop proteins such as Vp28 is highly expressed in infected shrimp relative to latency genes and the reverse is true in the case of specific pathogen free shrimp and/or asymptomatic carriers of WSSV (Khadijah et al., 2003). Use of in vitro crustacean cultures for analyzing the viral gene expression

has been rarely reported in literature. Among them the expression of Vp 28 gene demonstrated by Jiravanichpaisal et al. (2006) in crayfish haematopoietic stem cell culture by Rt-PCR is note worthy. In this context the detection of Vp28 by immunofluorescence in infected haemocytes accomplished in this study is a direct evidence of the viral multiplication in shrimp haemocytes. The study suggested that primary haemocyte culture could be used to delineate the infectious mechanisms of the virus and its morphogenesis.

Expression of immune related genes examined at the event of WSSV infection included prophenoloxidase, astakine, peroxinectin, alpha 2-macroglobulin, haemocyanin, transglutaminase, crustin and penaeidin. Expression of these genes compared to that of the negative control could be categorized in to four, such as those which were up regulated at 2^{nd} h (prophenoloxidase, peroxinectin, penaeidin, alpha 2-macroglobulin, crustin and transglutaminase), maintained upregulated in the same order at 12^{th} h (peroxinectin, penaeidin, and crustin), at $24th^{th}$ h (Astakine), and at 48^{th} hour when heat inactivated WSSV was administered (prophenoloxidase, peroxinectin, alpha 2-macroglobulin and penaeidin). Meanwhile haemocyanin and transglutaminase were found faintly expressed even though not detected in the controls.

Shrimps have no acquired adaptive immune system; their defense is believed to depend entirely on an innate, non adaptive mechanism to resist invasion by pathogens (Gross et al., 2001). Understanding the interaction between host and pathogen will be helpful in controlling infectious diseases in shrimp. The expression of immune related genes and other genes was reported in different larval stages of *P. monodon* (Jiravanichpaisal et al., 2007), in *P. japonicus* after exposure to peptidoglycan (Fagutao et al., 2008) and in *P. chinensis* after exposure to WSSV (Wang et al., 2006). The results suggested that immune related genes were triggered immediately after exposure to WSSV and the increase in expression might result in an enhanced immune response to ward off the pathogens. In contrast, the apparent decrease in expression of most

immune-related genes after initial hours of infection indicates that the heightened response is temporary or short lived in the event of WSSV infection in shrimp on considering the haemocyte culture as a model for WSSV infection. This pattern of expression was observed in *P. japonicus* following peptidoglycan stimulation. (Fagutao et al., 2008). Amplification of transglutaminase and haemocyanin was very weak and was detected only in the initial hours. Very low expression of haemocyanin gene in haemocytes could be explained on the basis of its production in hepatopancreas. (Gibson and Barker, 1979; Khayat et al., 1995; Lehnert and Johnson, 2002). The slight increase in the expression of prophenoloxidase, peroxinectin, alpha 2-macroglobulin and penaeidin in control cells with heat inactivated WSSV than the negative control cells has implications in vaccination of shrimp using inactivated WSSV. Singh et al. (2005) have demonstrated the efficacy of formalin inactivated WSSV in immunizing shrimp for 10 to 15 days. Crustin and penaeidins are antimicrobial peptides of which crustins act against gram positive bacteria (Rattanachai et al., 2004) while penaeidins have antibacterial and antifungal properties (Destoumieux et al., 1997). It has to be pointed out that expression of both genes decreased with increasing duration. But initial expression of crustin was higher than that of the control while initial penaeidin expression was similar to that of the control. This suggests the higher level of response of crustin to WSSV infection.

In conclusion, the haemocyte culture developed using the modified L-15 medium served as a convenient model for WSSV titration. The cell culture could be employed for investigating virus multiplication cycle and morphogenesis and also for the analysis of cellular responses to viral infection.

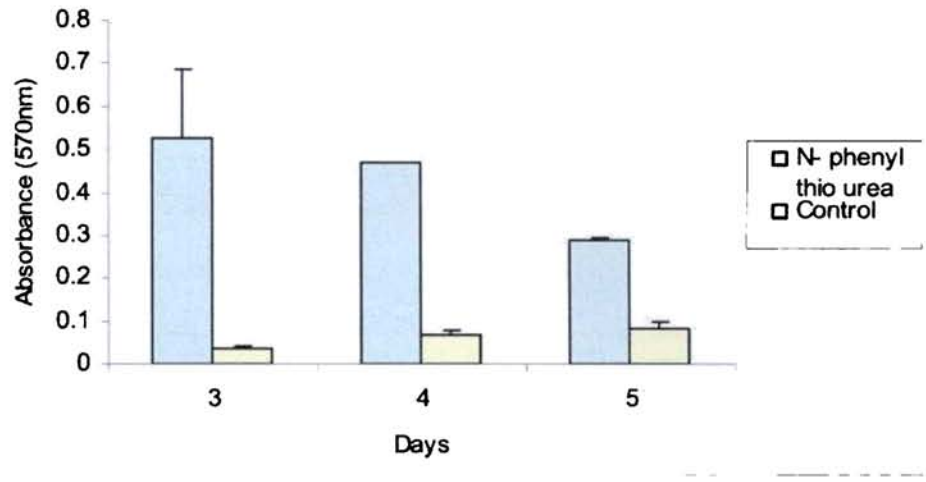


Fig.1. Effect of N- Phenyl thio urea on haemocyte culture based on MTT assay (n=4).

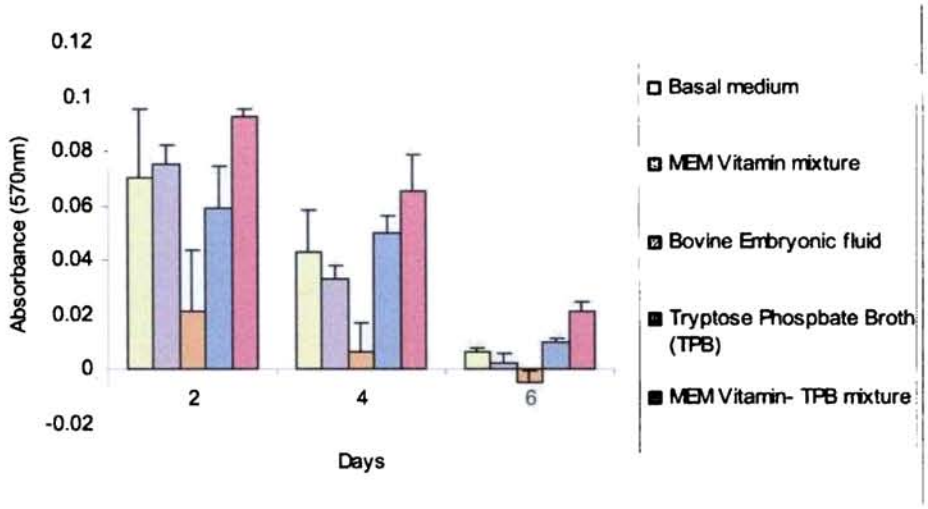
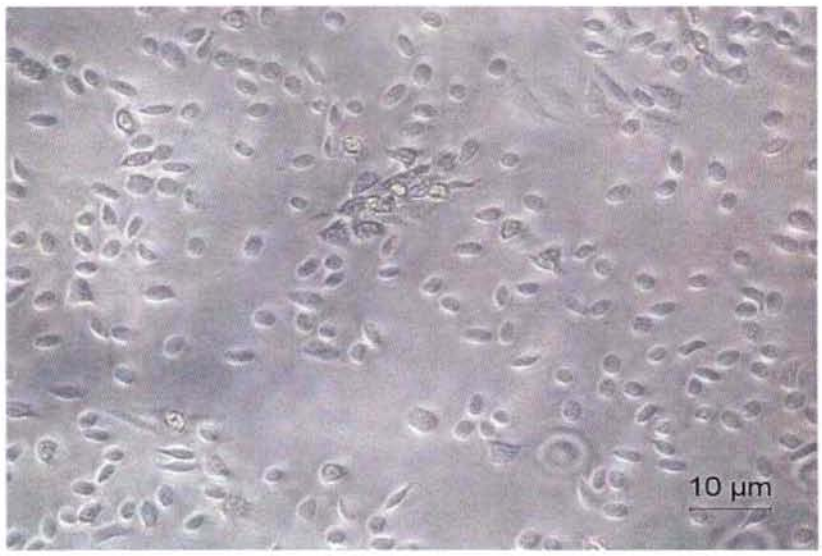
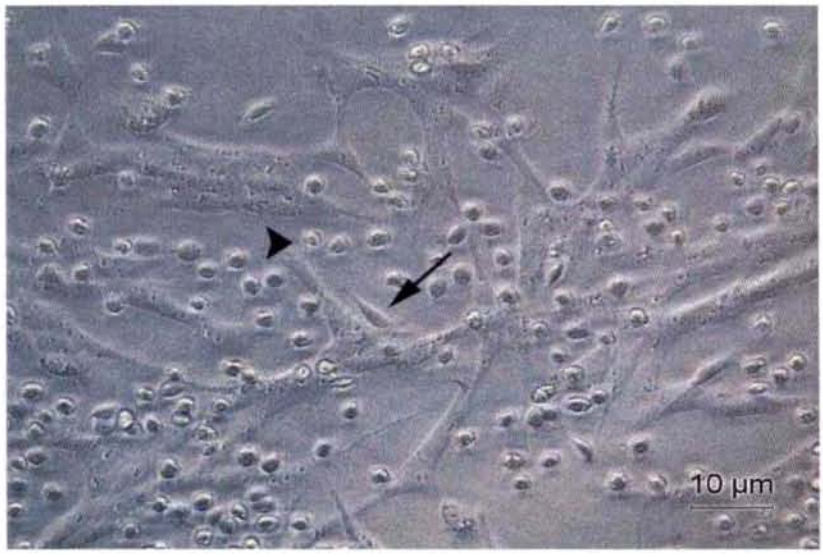


Fig.2. Haemocyte response to various media supplements based on MTT assay (n=4)

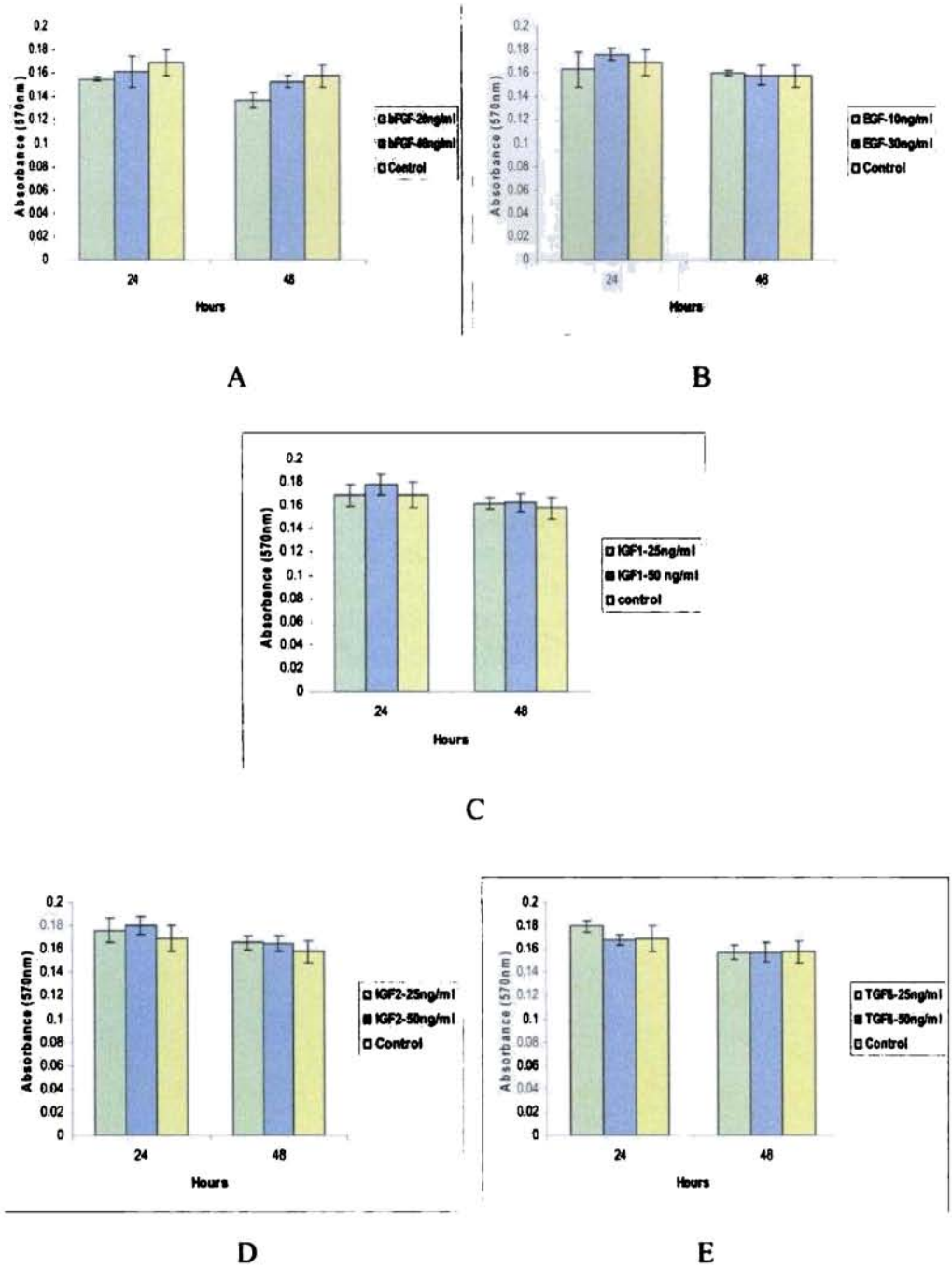


A



B

Fig.3. Morphology of primary haemocyte culture. (A) 0 hour, (B) 24 hour (arrow heads: rounded to elliptical cells; arrows: small to large fibroblastic cells)



A B

C

D E

Fig.4. Effect of various growth factors on haemocyte culture based on MTT assay A) bFGF B) EGF C) IGF-1 D) IGF-2 E) TGF-ß (n=4)

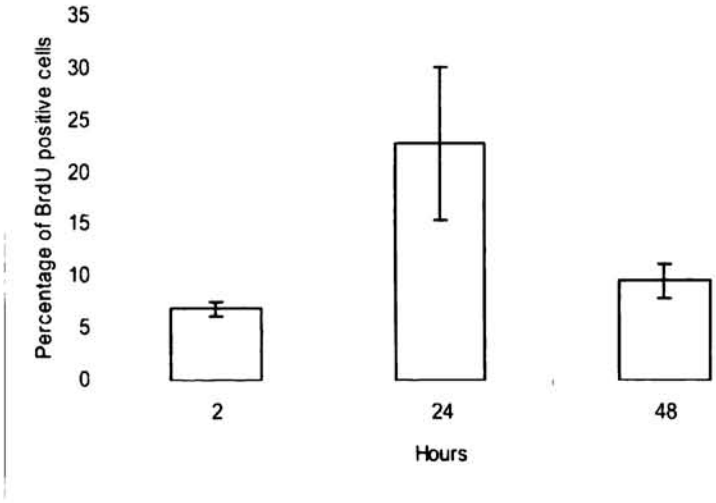


Fig. 5. Incorporation of BrdU in cultured haemocytes (n=6). 2 hours: 15.17±1.32 positive cells in a total count of 222.67±32.28; 24 hours: 27±11.38 positive cells in a total count of 118.64±14.58 cells; 48 hours: 11.33±4.18 positive cells in a total count of 115.67±24.23 cells

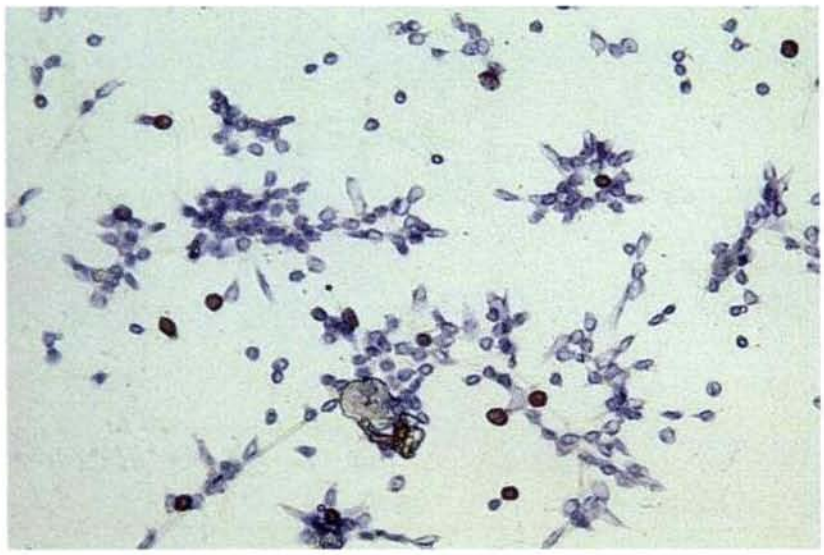
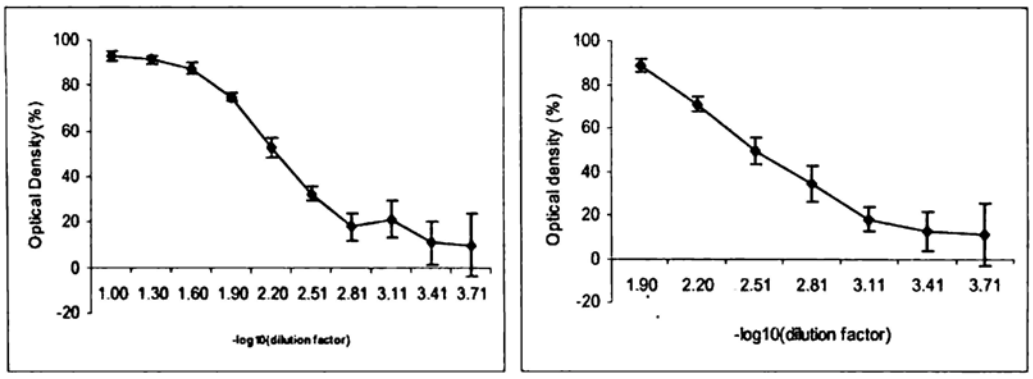


Fig. 6. BrdU labelled haemocytes (brown) among unlabelled (blue) at 24 hours of culture



A B

Fig.7. Determination of WSSV titre available in (A) gill extracts (B) haemolymph of infected shrimp in terms of MTT_{50} using primary haemocyte culture (n=4).

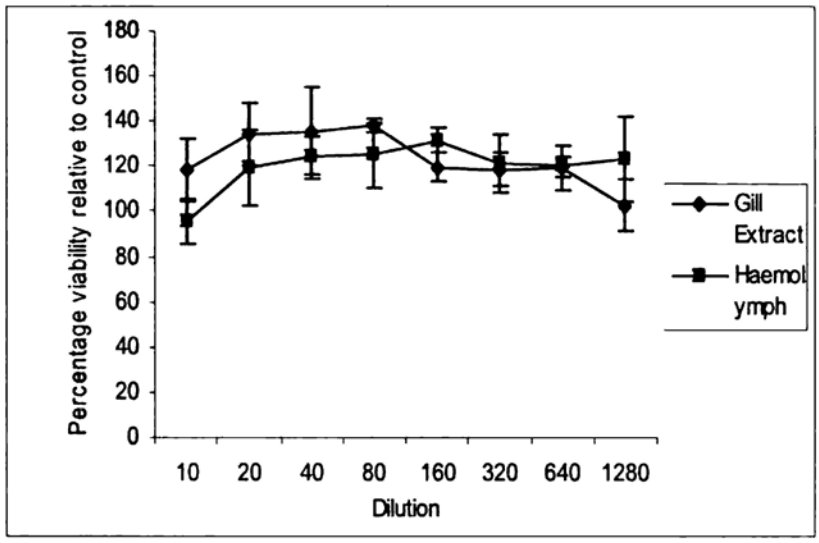


Fig.8. Toxicity (MTT assay) of gill extract and haemolymph of *P. monodon* in primary haemocyte culture (n=4).

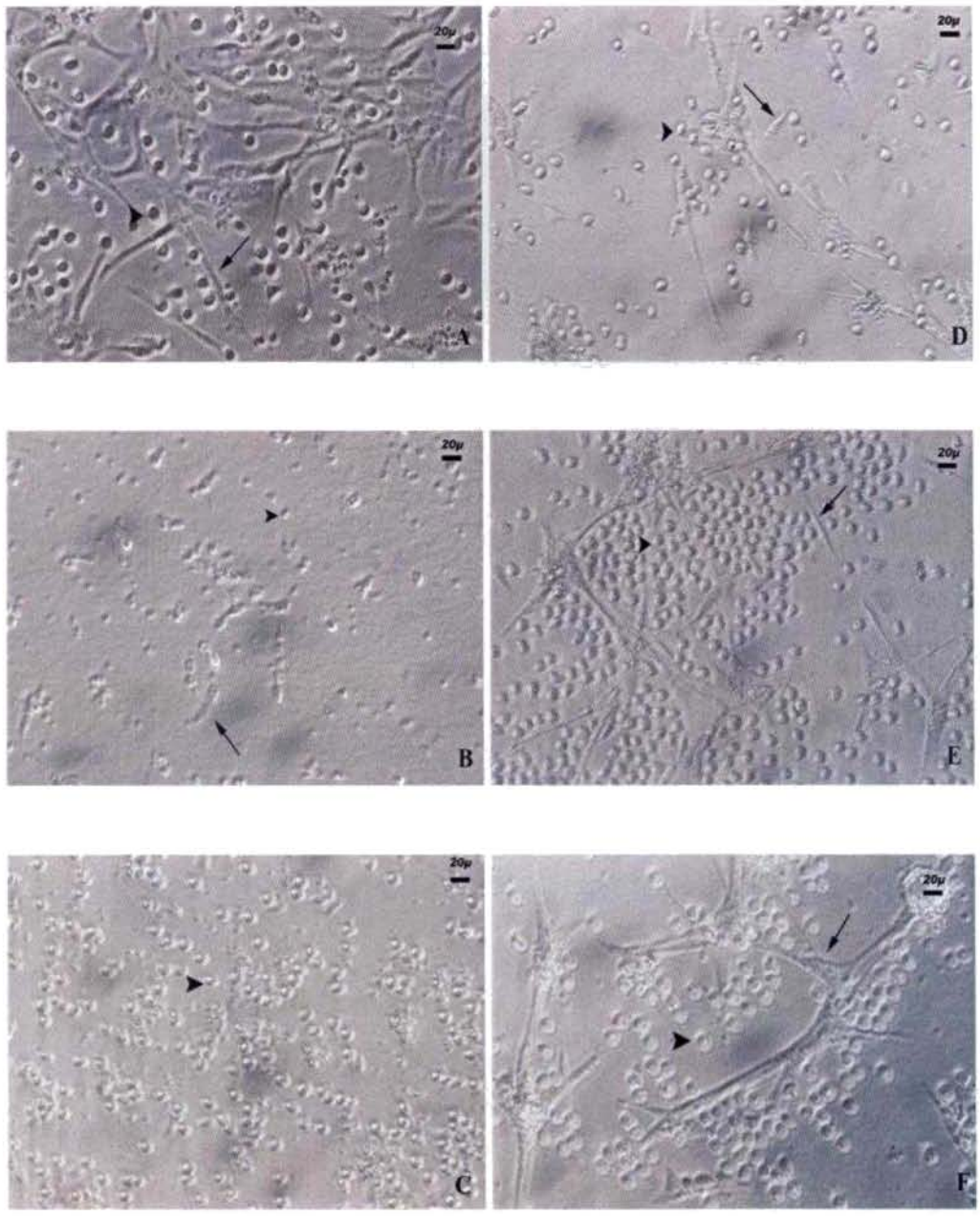
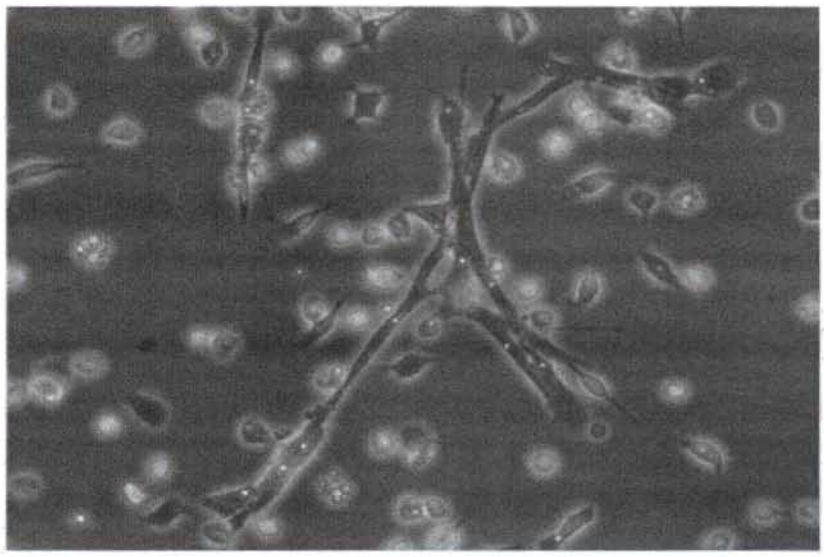
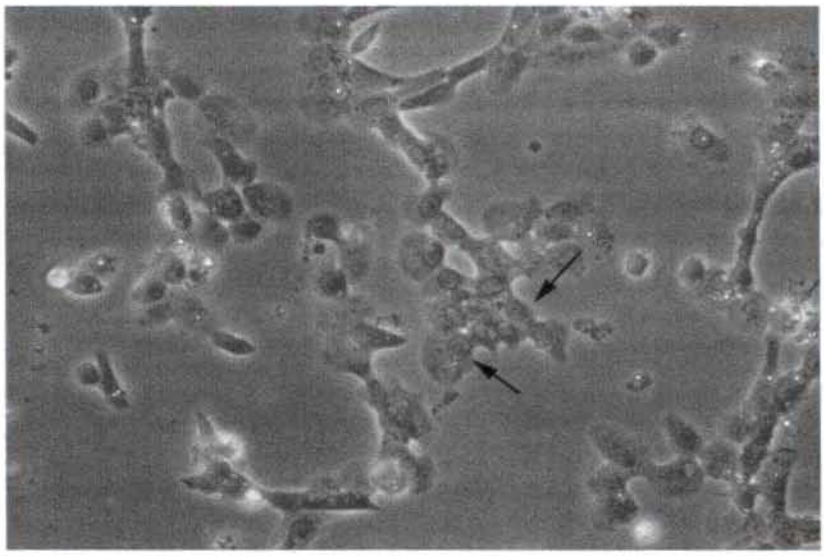


Fig.9. Cytopathic effect of WSSV in primary haemocyte culture. A) Control without WSSV, (B) 1/10 dilution: haemocytes were found disintegrated within a few hours of inoculation, (C) 1/40 dilution : only round to elliptical cells were present, (D) 1/160 dilution: round to elliptical and small to large fibroblastic cells were present, (E) 1/320 dilution: a mixture of larger number of round to elliptical and small to large fibroblastic cells noticed, (F) 1/2560 dilution: cells appeared the same as that of the control, (arrow heads: rounded to elliptical cells; arrows: small to large fibroblastic cells).



A



B

Fig.10. Nuclear hypertrophy in haemocytes infected with WSSV A) Control without WSSV B) WSSV infected haemocytes with hypertrophied nucleus (arrows)

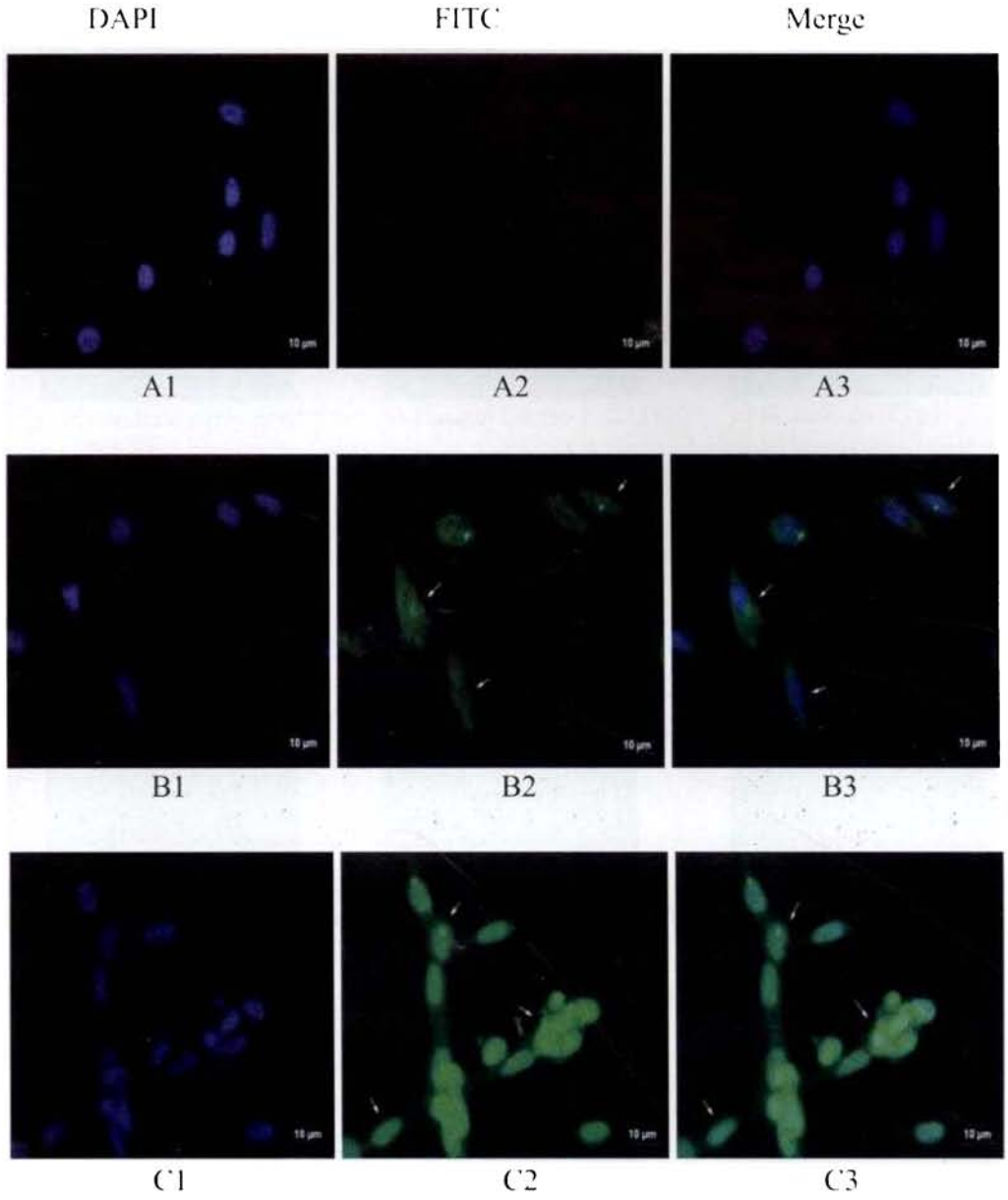


Fig.11. Immunofluorescence detection of WSSV in primary haemocyte culture. (A) Control (B) 24 hour post infection (C) 72 hour post infection. Fig. 9B2 & Fig 9B3: Positive signals in the cytoplasm after 24 hours incubation suggesting phagocytosis and/or endocytosis. Fig.9C2 & Fig. 9C3: Strong positive signals from nuclei of cells after 72 hours of infection. (Arrows: Infected cells).

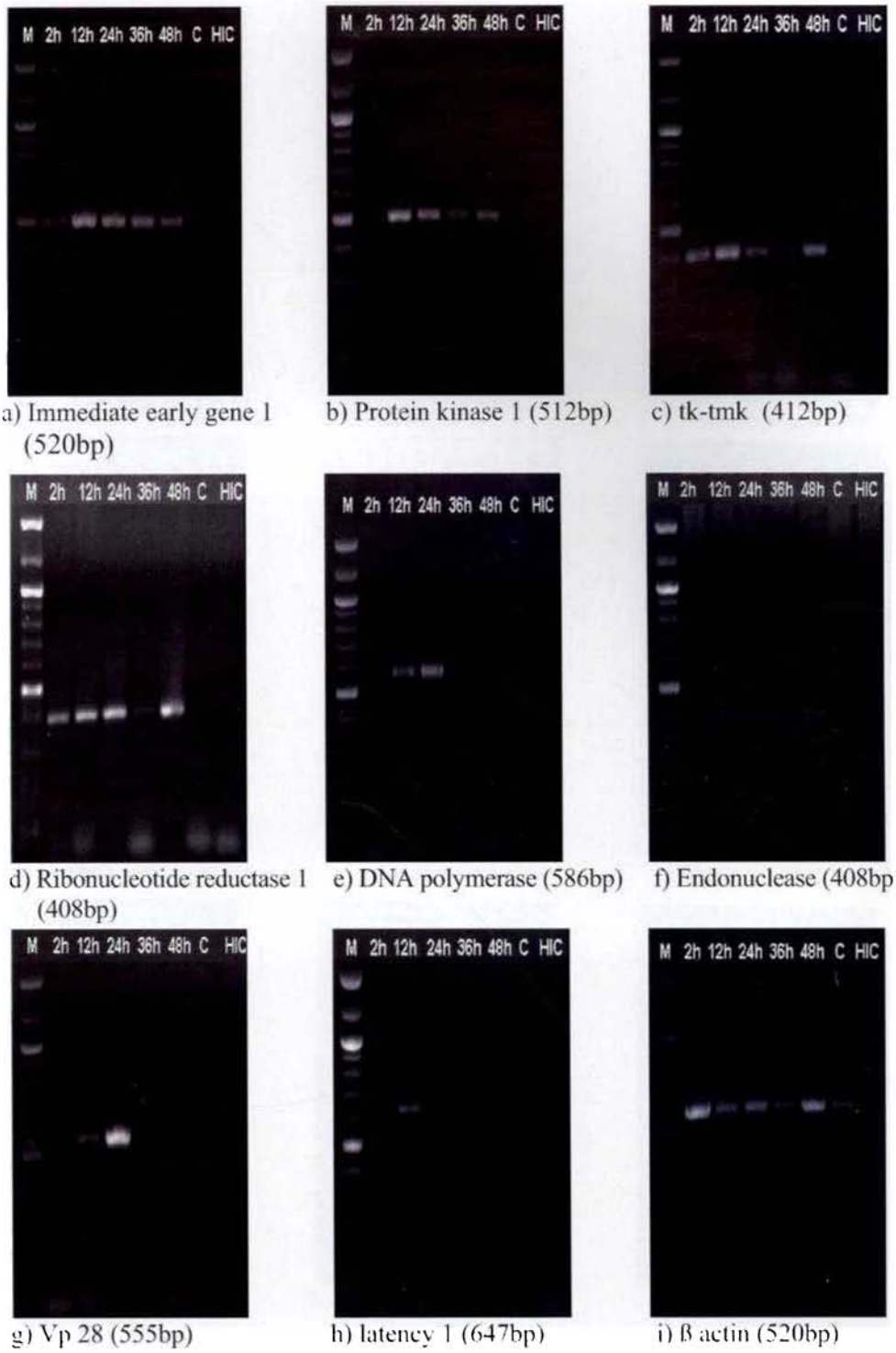


Fig.12. Expression of WSSV genes in primary haemocyte culture at 2, 12, 24, 36 and 48 hours of post infection (M-100bp ladder; C-Control, HIC-heat inactivated control)

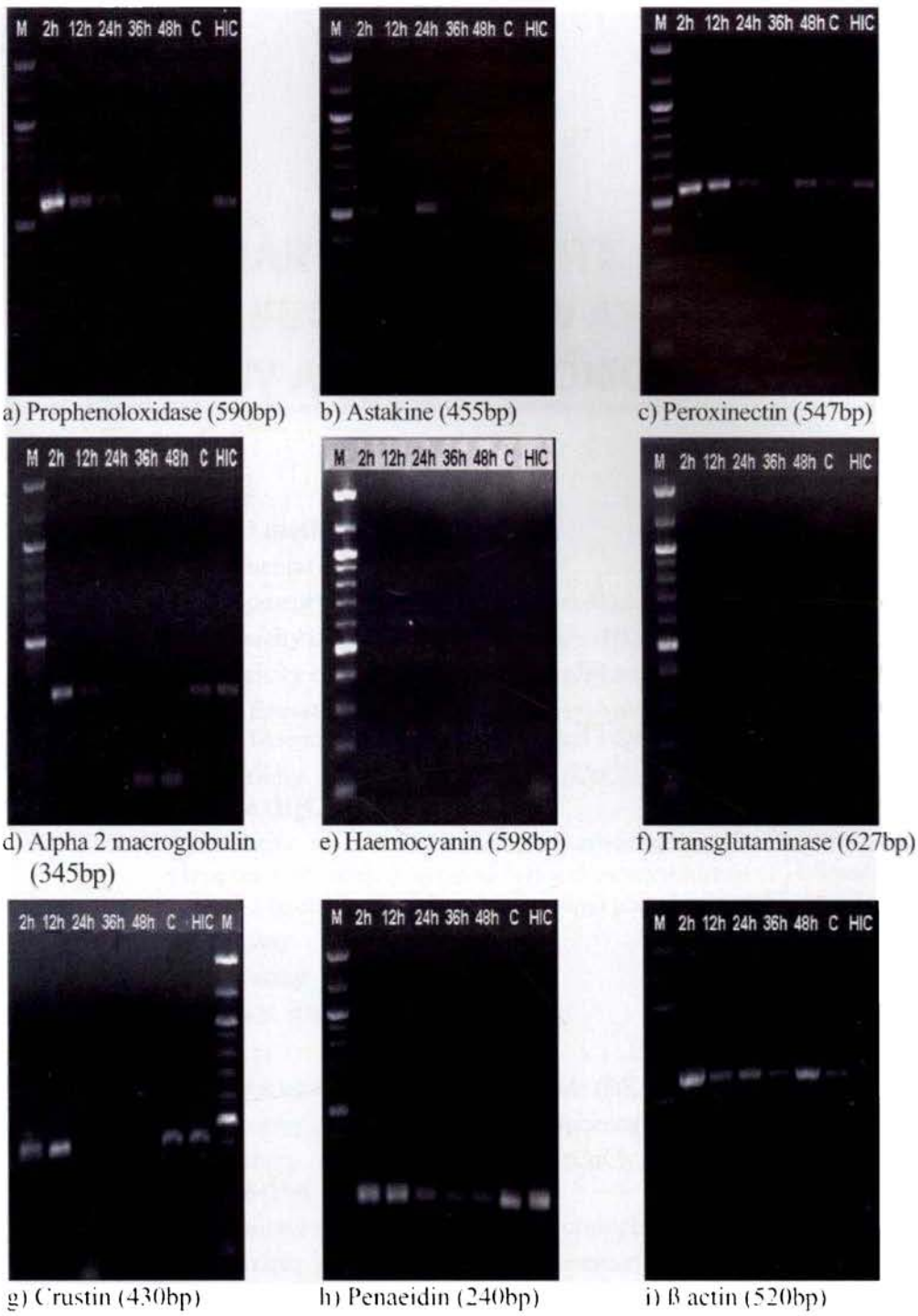


Fig.13. Expression of shrimp immune genes in primary haemocyte culture after 2, 12, 24, 36 and 48 hours of infection with WSSV (M-100bp ladder; C-Control, HIC-heat inactivated control)

Chapter 4

PRIMARY HAEMOCYTE CULTURE OF *PENAEUS MONODON* AS MODEL FOR CYTOTOXICITY AND GENOTOXICITY STUDIES

4.1. Introduction

Cytotoxicity is considered an important index for evaluating safety of antimicrobials and management chemicals prior to their administration in aquaculture. For its accomplishment cell lines can be used which out-rightly eliminates animal experimentation as part of the bioassays as is being followed now. Such an approach can forecast their *in vivo* effects as well as assisting their optimization for field level application. This is specifically because under field conditions realizing their negative impacts happens to be quite cumbersome (MacGowan et al., 2001).

Cell lines can also be used for assessing the toxicity of major aquatic pollutants such as heavy metals and pesticides. Effluents from industries and runoff from agricultural fields may contain such pollutants, which on reaching aquatic environments such as rivers, ponds, lakes, and estuaries lead to impairment of the ecosystem. Needless to say, the bio-accumulation and persistence of these pollutants in the aquatic environment constitute a serious threat to biological life and also to human beings indirectly by virtue of their involvement in the food chain (Binelli and Provini, 2004). Majority of these effluents/chemicals are mutagenic (Galloway et al., 1987; Garaj-Vrhovac and Zeljezic, 2000) linked either to the development of cancer (Leiss and Savitz, 1995) or to developmental deficits (Arbuckle and Server, 1998). This is apart from the chronic sublethal effects manifested in varied forms of physiological and metabolic abnormalities.

Among heavy metals, cadmium and mercury are the most highly toxic elements owing to their persistence in the environment. They are redox inactive which challenge antioxidant defense by binding to thiols in the cell such as reduced glutathione (GSH) (Stohs and Bagchi, 1995; Ercal et al., 2001) and induce apoptosis (Pulido and Parrish, 2003). Cell toxicity caused by heavy metal ions is attributed to oxidative and nitrosative stress (Stohs and Bagchi, 1995;

Ercal et al., 2001; Pompella et al., 2003), defined as an excess of oxidants over antioxidants. Macromolecules in cells are damaged by metal- induced production of oxygen and nitrogen containing free radicals (oxidants) and/or metal-induced depletion of the cell's antioxidant defenses. Apoptosis or necrotic cell deaths are the usual consequences (Pulido and Parrish, 2003).

Toxic effects of cadmium have been well documented in a wide range of organisms: teratogenesis in *Xenopus laevis* (Mouchet et al., 2006, 2007), carcinogenesis in Syrian hamsters (Waalkes and Rehm, 1998) and apoptosis in *Paracentrotus lividus* larvae are useful examples of its toxicity. In addition, cadmium is irreversibly accumulated in cells, as demonstrated in rats and marine mollusks (Roesijadi et al., 1996; Haouem et al., 2007). Experimental evidences suggest that the metal crosses the plasma membrane as bivalent ion, Cd^{2+}, exerting an agonistic role against calcium ionic channels (Viarengo and Nicotera, 1991; Rainbow et al., 2005). Cadmium has been associated with blockage of oxidative phosphorylation, glutathione depletion and antioxidant enzymatic activity inhibition, production of reactive oxygen species, DNA damage, inhibition of relative repair mechanisms, and a general reduction of protein synthesis coupled with an increase in stress protein synthesis (Kesseler et al., 1994; Shimizu et al., 1997; Ercal et al., 2001; Schroder et al., 1999, 2005; Roccheri et al., 2004; Lin et al., 2007[b]).

Mercury (Hg) is one of the most toxic metals described (Ayllon and Garcia-Vazquez, 2000), and its toxicity is widely studied in fishes where it accumulates mainly in the form of methyl mercury. Mercury nitrate and metallic mercury (Hg^0) were demonstrated to be micronucleus inducers in *Poecelia latipinna* (Ayllon and Garcia-Vazquez, 2000) and *Cyprinus carpio* (Nepomuceno et al., 1997), respectively. In addition, methyl mercury proved to be teratogenic as well as a micronucleus and chromosomal aberration inducer in *Fundulus heteroclitus* embryos (Weis and Weis, 1977; Perry et al., 1988). A chronic study of mercuric chloride exposure in rats and mice reported an

increased incidence of stomach and thyroid tumors in rats and renal tumors in mice (NTP, 1991). The most important biological effects of organic and Hg vapour exposure are neurological, while inorganic Hg is mostly nephrotoxic (Kerper et al., 1992).

Worldwide pesticide consumption in the year 2000 was estimated to the tune worth of *5.35 billion pounds (USEPA, 2001). Meanwhile in India, the pesticide consumption registered more than fourfold increase during the post-Green Revolution era (1966–1999). However, in later years, the pesticide consumption exhibited a steady decline (Prajneshu, 2002) probably due to awareness about their ill effects among the public. Although the consumption of pesticides in India is estimated to be the lowest (0.5 kg/ha) as against 17 kg/ha in Taiwan, 12 in Japan, 6.6 in Korea, 7 in the United States, and 2.5 in Europe, the food and agricultural products do contain substantial quantities of pesticide residues (Assocham, 2007), a situation which emerges due to their indiscriminate and non – judicious use as well as to the nonobservance of prescribed waiting/withdrawal periods.

Toxicity of pesticides on non- target organisms and ecosystems is of world wide concern (Pimentel et al., 1998). Among pesticides, organophosphate pesticides (Ops), as esters, amides, or simple derivatives of phosphoric or thiophosphoric acids, are synthetic chemicals designed to be toxic, some extend volatile, and stable enough to remain in their toxic form for relatively shorter periods of time (Poovala et al., 1998). Ops are groups of chemicals that act primarily by inhibiting the enzyme acetylcholine esterase (AchE) at cholinergic junctions of the nervous system (Gallo and Lawryk, 1991); as a result, acetylcholine levels accumulate in the synapse, followed by enhanced stimulation of cholinergic receptors on postsynaptic cells and subsequent alteration of receptor - mediated signaling pathways, e.g. alteration of intracellular cAMP levels. These cellular changes may lead to functional changes at the tissue/organism level (Pope et al., 2005).

Heavy metals and pesticides are known genotoxicants too, harmfully affecting aquatic biota. Deoxyribonucleic acid (DNA) being the carrier of inherited information in living organisms, any unprogrammed change in the structure of the DNA molecule may potentiate serious biological consequences. Certain environmental chemicals and/ or physical agents are classified as genotoxicants, as they have the capacity to interact with and damage the structure of the DNA molecule, often with a concomitant adverse effect on biological integrity (Shugart, 1995; 1998). Within the nucleus of a cell the DNA molecule is in a constant state of flux between a functionally stable, double-stranded entity without discontinuity and an intermediate, unstable state where the structural integrity of DNA is in a state of alterations (Shugart, 1990; 1998 and Shugart et al., 1992). Under normal cellular conditions the occurrence of the latter state is a transient phenomenon that can be triggered by physiological processes e.g., DNA replication, random thermal collision between molecules and cellular metabolism. The resulting structural changes that occur to the DNA molecule under these conditions are rapidly repaired (Elliott and Elliott, 1997) or become part of the new stable structure e.g., postreplication modifications. Under such situations exposure of an organism to a genotoxicant disrupts normal cellular processes and results in structural modifications to the DNA, which might cause subsequent negative impacts on the cells. An organism's inability, whether transient or permanent to cope up with this type of stress and to maintain its structural integrity provides the investigator an opportunity to test for the genotoxicity of the chemical and physical agents in the environment. Some of the more common DNA structural modifications caused by genotoxicants are summarized in Table 1. Two general classes of structural modifications result from exposure to a genotoxicant. Class 1 modifications are brought about by ultraviolet light in the 290-320 nm range (UV-B) which cause specific dimerization of pyrimidine bases within the DNA. Besides, several chemicals, such as the polycyclic aromatic hydrocarbon benzo[a]pyrene, become covalently attached to DNA and form adducts. In both the examples, the structural

modifications become a specific fingerprint of the responsible genotoxicant. The class 2 modifications are the structural changes not specific to a particular genotoxicant, however, this may suggest the potential for genotoxicant exposure. Meanwhile, many genotoxicants may cause DNA strand breakage directly. For example, the formation of free radicals or abasic sites by genotoxicants can result in the breakage of phosphodiester linkages within the DNA molecule. Further, genotoxicants can also interfere with normal DNA processing activities such as replication, methylation and repair which might result in mutations- e.g., base additions or deletions. The potential for genotoxicant exposure is implied when nonspecific structural modification such as strand breaks, abasic sites, hypomethylation and mutations occur in excess of what might be anticipated compared to controls (Shugart, 2000).

Table 1. DNA structural modifications caused by genotoxicants (Shugart, 2000)

Genotoxicant	Type of modification	Mechanism
Physical	Thymine-Thymine Dimer	Dimerization of Pyrimidine Bases by UV-B Light
	Strand Breakage	Formation of Free Radicals by Ionizing Radiation
Chemical	Adduct	Covalent Attachment of Genotoxicant
	Altered Bases	Chemical Modification of Existing Bases
	Abasic Site	Loss of Chemically Unstable Adduct or Damaged Base
	Strand Breaks	Breakage of Phosphodiester Linkages due to Formation of Free Radicals and Abasic Sites
	Hypomethylated DNA	Interference with Postreplication
	Mutation	Interference with DNA Repair

DNA strand breakage is not an uncommon occurrence in a cell. Heat energy causes thousands of abasic sites per cell per day which, however, are rapidly repaired under normal conditions (Elliott and Elliott, 1997). An abasic site is an example of an insult to DNA that indirectly results in strand breakage i.e., the initial damage is a loss of a base from the DNA chain and the repair of this damage results in a temporary gap in the DNA molecule. Ionizing radiation as well as free radicals can cause strand breakage directly, whereas other physical agents such as UV light and certain chemical agents that are genotoxic,

potentiate alterations to the DNA molecule that are candidates for repair (Table 1: photoproducts, adducts, altered bases, etc.) and thus for the occurrence of strand breaks (Shugart et al., 1992; Shugart, 1994; 1998) . Several of the DNA strand break assays currently in use are based on the general principle that, under in vitro denaturation conditions of high pH, the rate at which single-stranded DNA is released from the duplex DNA is proportional to the number of strand breaks in the DNA molecule (Rydberg, 1975). Alkaline labile sites in the DNA molecule (abasic sites for example) will also be detected because they are chemically converted to single strand breaks under these in vitro assay conditions.

The Comet Assay or single cell gel electrophoresis (SCGE) assay is a rapid, sensitive and relatively simple method for detecting DNA damage at the level of individual cells (Singh et al., 1988). It combines the simplicity of biochemical techniques for detecting DNA single strand breaks (strand breaks and incomplete excision repair sites), alkali labile sites and cross linking, with the single cell approach typical of cytogenetic assays. Several reviews have been published in recent years to highlight the procedures, advantages and limitations of this assay in genotoxicological, ecotoxicological and biomonitoring studies (Fairbairn et al., 1995; Dixon et al., 2002; Lee and Steinert, 2003; Collins, 2004). The main advantages of the Comet Assay include: (a) the collection of data at the level of the individual cell, allowing more robust statistical analyses, (b) the need for a small number of cells per sample (<10,000), (c) sensitivity for detecting DNA damage and (d) use of any eukaryote single cell population both in vitro and in vivo, including cells obtained from exposed human populations and aquatic organisms for eco-genotoxicological studies and environmental monitoring (Collins et al., 1997; Dixon et al., 2002; Lee and Steinert., 2003; Jha, 2004).

The Comet Assay is based on the ability of negatively charged loops/fragments of DNA to be drawn through an agarose gel in response to an

electric field. The extent of DNA migration depends directly on the DNA damage present in the cells. It should be noted that DNA lesions consisting of strand breaks after treatment with alkali either alone or in combination with certain enzymes (e.g. endonucleases) increase DNA migration, whereas DNA–DNA and DNA–protein cross-links result in retarded DNA migration compared to those in concurrent controls (Tice et al., 2000).

In this assay, a suspension of cells is mixed with low melting point agarose and spreads onto a microscope glass slide. After lysis of cells with detergent at high salt concentration, DNA unwinding and electrophoresis is carried out at a specific pH. Unwinding of the DNA and electrophoresis at neutral pH (7–8) predominantly facilitates the detection of double strand breaks and cross links; unwinding and electrophoresis at pH 12.1–12.4 facilitates the detection of single and double strand breaks, incomplete excision repair sites and cross-links; whereas unwinding and electrophoresis at a pH greater than 12.6 expresses alkali labile sites in addition to all types of lesions listed above (Miyamae et al., 1997). When subjected to an electric field, the DNA migrates out of the cell, in the direction of the anode, appearing like a 'comet'. The size and shape of the comet and the distribution of DNA within the comet correlate with the extent of DNA damage (Fairbairn et al., 1995).

In the study undertaken here primary haemocyte culture of *P. monodon* was utilized for studying cytotoxicity of benzalkonium chloride (BKC), a health management chemical in aquaculture, N-methyl-1-hydroxyphenazine, an antivibrio compound, two heavy metal compounds cadmium chloride ($CdCl_2.2\ ½\ H_2O$) and mercuric chloride ($HgCl_2$) and two organophosphate insecticides, malathion ($C_{10}H_{19}O_6PS_2$) and monocrotophos ($C_7H_{14}NO_5P$) which reach aquaculture systems through run off. Subsequently genotoxicity of the heavy metal compounds and insecticides was examined in haemocyte culture employing comet assay.

4.2. Material and methods

4.2.1. Experimental animals

WSSV and Monodon Baculo virus (MBV) negative *P. monodon* larvae obtained from a local hatchery were stocked and reared in a Recirculating shrimp rearing system integrated with nitrifying bioreactors (Kumar et al., 2009) in sea water having a salinity of 15g l^{-1}. Water quality was maintained by the addition of a probiotic Detrodigest ^{TM} (NCAAH, India) to manage detritus and Enterotrophotic ^{TM} (NCAAH, India) to control *Vibrio*. The larvae were fed with commercially available pelleted feed (Higashimaru, India). They were confirmed WSSV negative by nested PCR (WSSV detection kit, Genei, India) when they grew to 8-12g, and used for all experiments.

4.2.2. Development of primary haemocyte culture

The animals were sacrificed by immersing in crushed ice and disinfected by maintaining in 800 mg l^{-1} sodium hypochlorite solution prepared in ice cold - sea water (salinity 15 g l^{-1}) for 10 minutes. Subsequently, they were washed 5 times in sterile ice cold sea water, dipped in 70% alcohol and rinsed in ice- cold sea water. Haemolymph was withdrawn asceptically using capillary tubes containing 100µl anticoagulant (Tris HCl 0.01M, Sucrose 0.25M, Tri Sodium Citrate 0.1M) from rostral sinus and diluted to obtain 5 x 10^5 cells ml^{-1} using modified L-15 (Leibovitz) medium and aliquots of 200µl were dispensed into the wells of 96 well plates (Greiner Bio-One) and incubated at 25°C.

4.2.3. Cytotoxicity of benzalkonium chloride (BKC)

BKC is used here as a representative of health management chemical in aquaculture to assess the suitability of primary haemocyte culture for testing cytotoxicity. A 96 well plate culture of haemocytes was developed and incubated at 25°C for 12 hours. Different concentrations of BKC prepared in growth medium were added to the wells to attain final strength ranging from 0.012-25mg l^{-1} with replicates for each concentration. Cells with the growth

medium were kept as control. After 12 hours incubation wells were observed under phase contrast microscope (Leica, Germany) and MTT assay was performed. Percentage inhibition of cells at each concentration of BKC was calculated based on the formula, percentage inhibition of haemocytes = [100-(Average absorbance (MTT assay) of haemocytes at a particular concentration of the compound/Average absorbance of control haemocytes without the compound) x 100)].

4.2.4. Cytotoxicity of N-methyl-1-hydroxyphenazine (pyocyanin)

4.2.4.1. Extraction of N-methyl-1-hydroxyphenazine

N-methyl-1-hydroxyphenazine is used here as a representative of therapeutic chemical (antivibrio compound) used in aquaculture. *Pseudomonas* MCCB 102, previously described by Vjayan et al. (2006) and Jayaprakash (2005) and confirmed of its identity based on 16S rRNA gene sequence analysis (Genbank accession no. EF062514) was used for the production of the antivibrio molecule. This isolate formed a part of the culture collection of National Centre for Aquatic Animal Health (NCAAH), Cochin University of Science and Technology (CUSAT), India. The organism was grown in ZoBell's marine broth 2216 E (peptone: $10gl^{-1}$, yeast extract: $1gl^{-1}$, sodium chloride: 12.9 gl^{-1}, pH: 6.5 and incubation temperature: 25 °C). The cultures (3-5 days old) were centrifuged at 10,000 g at 4°C for twenty minutes, the supernatant filtered through a series of filters including glass microfiber filter (GF/C, Whatman), cellulose acetate membrane (0.45 µm; Millipore) and PVDF membrane (0.22 µm; Millipore), and used for further extraction. To extract the phenazine compound, 5 ml culture supernatant was treated with 2ml chloroform acidified with 1ml 0.1N HCl (Chang and Blackwood, 1969). The compound was quantified by measuring absorbance at 520 nm, multiplying with 17.072 and expressed in µg ml^{-1} (Essar et al., 1990). Resulting solution containing the compound was vacuum dried, dissolved in cell culture medium and pH adjusted to 7.5 with 1N NaOH.

4.2.4.2. Determination of IC_{50} of N-methyl-1-hydroxyphenazine

Different concentrations of the compound were added to the wells to attain a final strength ranging from 0.5-14 mg l^{-1} with replicates for each concentration. Cells with the growth medium were kept as control. After 12 hours incubation wells were observed under phase contrast microscope (Leica, Germany) and MTT assay was performed. Percentage inhibition of cells at each concentration of the compound was calculated based on the formula given above.

4.2.5. Cytotoxicity of cadmium chloride ($CdCl_2.2\ ½\ H_2O$) and mercuric chloride ($HgCl_2$)

Cadmium chloride and mercuric chloride were used as representatives of aquatic toxicants. Stock solutions of cadmium chloride (Qualigens) and mercuric chloride (Merck) were prepared in growth medium. Different concentrations of the compounds were added to the wells to get final concentrations ranging from 0.49 to 500 µM for cadmium chloride and 0.49 to 62.5 for mercuric chloride maintaining replicates for each concentration. Cells with the growth medium were kept as control. After 12 hours incubation wells were observed under phase contrast microscope (Leica, Germany) and MTT assay was performed. Percentage inhibition of cells at each concentration of the compounds was calculated based on the formula given above.

4.2.6. Cytotoxicity of malathion [S-(1, 2-dicarboethoxyethyl) O, O-dimethyl phosphorodithioate]; ($C_{10}H_{19}O_6PS_2$)) and monocrotophos (E)-(dimethyl1-methyl-3-(methylamino)-3-oxo-1-propenyl phosphate); ($C_7H_{14}NO_5P$))

Commercially available preparations of malathion (50% w/w) and monocrotophos (53% w/w) were used for toxicity studies. Insecticides were dissolved in DMSO and diluted with medium to get stock concentrations of 500 mg l^{-1} and 1000 mg l^{-1} of malathion and monocrotophos respectively. Different concentrations of the compounds were added to the wells to get final concentrations ranging from 0.76 to 185.2 mg l^{-1} for malathion and 0.98 to 1000 mg l^{-1} for monocrotophos maintaining replicates for each concentration. Cells

with the growth medium were kept as control. After 12 hours incubation wells were observed under phase contrast microscope (Leica, Germany) and MTT assay was performed. Percentage inhibition of cells at each concentration of the compounds was calculated.

4.2.7. MTT assay

The assay is a colorimetric method based on the determination of cell viability utilizing the reaction of a tetrazolium salt (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide, MTT) with the mitochondria of metabolically active cells. The reduction of the tetrazolium salt by nicotinamide adenine dinucleotide dehydrogenase (NADH) and nicotinamide adenine dinucleotide phosphate dehydrogenase (NADPH) within the cells produces insoluble purple formazan crystals, which are later solubilized yielding a purple-colored solution (Mosmann, 1983).

After replacing the medium, 50µl of MTT (Sigma) solution (5mg ml^{-1} in PBS; 720mOsm) were added to each well and incubated for 5 hours in dark. Blank consisted of medium alone with MTT added. After incubation, the medium was removed and MTT-formazan crystals were dissolved in 200µl dimethylsulfoxide. Absorbance was recorded immediately at 570nm in a micro plate reader (TECAN Infinite Tm, Austria).

4.2.8. Comet assay

Haemocytes were exposed to cadmium chloride (140µM), mercuric chloride (17µM), malathion (60 mg l^{-1}) and monocrotophos (186 mg l^{-1}) for 4 hours. These concentrations were chosen because more than 80% viability of cells was recorded after 4 hour exposure by trypan blue viability assay. After the exposure to compounds, cells were detached using a cell scrapper, pelletised, washed and suspended in PBS. Negative control consisted of cells without any treatment. Added 150µl of normal agarose solution (Sigma, USA) (0.75% in PBS) to the frosted slide, covered with a coverslip (22 x 50mm) to get a uniform

layer and allowed to solidify. To prepare the second layer 10µl of cell suspension were mixed with 70µl of low melting agarose solution (Sigma, USA) (0.75% in PBS) and the suspension was layered over the first layer after removing the coverslip and kept undisturbed until agarose solidified. A third layer of low melting agarose was made over the second layer. Coverslips were removed and the slides were treated with pre chilled lysis solution (2.5M NaCl, 100mM EDTA, 10mM Tris, 1.5M NaOH, pH 10 with 1% Triton X 100 and 10% DMSO) for 1 hour at 4°C. After lysis, slides were incubated in electrophoresis buffer (300mM NaOH and 1mM EDTA, pH>13) at 4°C for unwinding of DNA, and were electrophoresed for 40 minutes at 25V in the same buffer. Subsequently, the slides were neutralized in 0.4M Tris (pH 7.5), stained with ethidium bromide (2µg ml^{-1}) and observed under fluorescent microscope (Olympus, Germany). For each sample replicate slides were prepared and 25 cells from each slide were analysed for DNA damage using Comet Score™, a comet scoring software. Percentage of nuclei with tails, tail length, percentage DNA in the tail and tail moment (arbitrary units) were used to estimate DNA damage. Tail moment is the percentage DNA in the tail multiplied by tail length.

4.2.9. Statistical analysis

The results in the figures are average values of 4-6 replicates ± standard deviation. The effects of treatments were statistically analyzed by analysis of variance (ANOVA). Differences were considered significant at $P < 0.05$. The results of the cytotoxicity assay were analyzed by probit analysis using the SPSS software (SPSS Inc., USA).

4.3. Results

4.3.1. Cytotoxicity of benzalkonium chloride (BKC)

Morphological changes in the cells such as clumping, shrinkage and necrosis were visualized microscopically after exposure to higher concentrations (above 3.13mg l^{-1}) of BKC, (Fig 1) with an IC_{50} of 0.49 ±0.13 mg l^{-1} (Fig 2).

4.3.2. Cytotoxicity of N-methyl-1-hydroxyphenazine (pyocyanin)

Necrosis was observed at higher concentrations of pyocyanin (Fig 3). IC_{50} value of the compound in primary haemocyte culture of *P. monodon* was found to be 1.4 ± 0.31 mg l^{-1} (Fig 4).

4.3.3. Cytotoxicity of cadmium chloride ($CdCl_2.2\ ½\ H_2O$) and mercuric chloride ($HgCl_2$)

Necrosis of cells was observed at higher concentrations (>125 µM for cadmium chloride and >15.6 µM for mercuric chloride) and cell rounding at lower concentrations (<15.6 µM for cadmium chloride and <1.95 µM for mercuric chloride) (Fig 5). IC_{50} values were 31.09±16.27 µM and 5.52±1.16 µM for cadmium chloride (Fig 6) and mercuric chloride respectively (Fig 7).

4.3.4. Cytotoxicity of malathion and monocrotophos

At higher concentrations of Malathion and Monocrotophos (>61.7 mg l^{-1} and >166mgl^{-1} respectively) necrosis was observed (Fig 5). IC_{50} value of Malathion (Fig 8) was found to be 59.94 ± 52.3 mg l^{-1} and of Monocrotophos (Fig 9) was 186.76 ± 76.995 mg l^{-1}. Malathion was found to be non toxic below 6.86mg l^{-1} and monocrotophos below 15.6mg l^{-1}.

4.3.5. Genotoxicity of cadmium chloride, mercuric chloride, malathion and monocrotophos

All pollutants tested induced DNA strand breakage and thereby comets in the haemocytes (Fig 10). In the negative control almost all nuclei were circular without any visible tail while short tails were observed in 6.73±4.83% of cells. Greater than 60% of the cells were with tails for cadmium chloride, mercury and malathion in the concentrations tested while for monocrotophos it was less than 60% (Fig 11). Tail length of the comets in mercuric chloride treatment was the lowest (23 ±6.83 pixels) while for cadmium chloride, malathion and monocrotophos length of the tail ranged from 26-42 pixels (Fig 12). Percentage tail DNA for cadmium chloride, mercuric chloride, malathion and monocrotophos were 46.76±8.37, 44.71±7.25, 40.81±9.10 and 36.04± 21.54,

respectively (Fig 13). Tail moment (Fig 14) was found to be lesser for mercuric chloride (11.82±5.5) when compared to the other three and the values were highest for cadmium chloride (23.07±14.99).

4.4. Discussion

P. monodon is one among the important cultured species in the world. Lack of shrimp cell lines has been hampering progress of research in testing the effects of drugs and management chemicals prior to their administration in aquaculture systems. This has also hindered the study of pollutants such as heavy metals and pesticides in the aquatic environment, which adversely affects shrimp health and its export value. Primary haemocyte culture is an alternative to bioassay systems, which involves animal experimentation to study the genotoxic and cytotoxic effects of chemicals and pollutants.

The cytotoxicity of Benzalkonium chloride (BKC), a quaternary ammonium compound widely used as broad spectrum disinfectant and at low concentration (0.1ppm) as immunostimulants and moderately higher (1ppm) to stimulate moulting in shrimp culture ponds, was assessed in primary haemocyte culture and found safe at 0.1mg l^{-1} level. The heamocyte culture was again applied for assessing cytotoxicity of N- methyl-1- hydroxyphenazine, a phenazine compound reported useful in managing *Vibrio* in aquaculture (Preetha et al., 2009). Cytotoxicity of aquaculture immunostimulants and 2, 4-dinitrophenol using neutral red uptake assay was investigated by Hauton and Smith (2004) in *Homarus gammarus* granulocyte primary culture while Sung et al. (2003) examined the effects and toxicity of phthalate esters on the isolated haemocytes of *Macrobrachium rosenbergii* utilizing haemocytic adhesion, pseudopodia formation, superoxide anion production and phenoloxidase activity.

Toxicity of cadmium chloride and/or mercuric chloride has been evaluated on different aquatic organisms such as fishes (Arabi et al., 2004; Pereira et al., 2009), sea urchin (Filosto et al., 2008), shrimp (Lee et al., 2000; Espericueta et al.,

2001) and crab (Botton, 2000). In our study 12 hour IC_{50} values for cadmium chloride and mercuric chloride were 31.09±16.27 µM (7.10±3.72 mgl^{-1}) and 5.52±1.16 µM (1.50±0.31 mgl^{-1}), respectively. Espericueta et al. (2001) reported the LC_{50} values as 2.49 and 1.23 mgl^{-1} for cadmium and mercury, respectively in post larvae of *Litopenaeus vannamei* after 96 hour exposure while in *Limulus polyphemus* embryos IC_{50} was reported to be >1000 mgl^{-1} and 12.8 mgl^{-1} for Cd and Hg after 24 hour exposure (Botton, 2000). At higher concentrations of Malathion and Monocrotophos (>61.7 mg l^{-1} and >166mgl^{-1} respectively) necrosis was observed and the IC_{50} value of Malathion was found to be 59.94 ± 52.3 mg l^{-1} and that of Monocrotophos was 186.76 ± 76.995 mg l^{-1}. Malathion was found to be non toxic below 6.86mg l^{-1} and monocrotophos below 15.6mg l^{-1}. It has to be pointed out that the information on toxicity generated using whole animal is often insufficient in the sense that several of the toxic effects go on unnoticed which might have long term implications in the biotic community.

Comet assay was widely employed by many researchers to assess strand breakage due to to genotoxicant exposure (Lee et al., 2000; 2008; Lee and Kim 2002; Kim and Lee, 2004; Arabi et al., 2004; Rank et al., 2005; Pereira et al., 2009; Bissett et al., 2009) in a wide variety of species such as *Cyprinus carpio* (Arabi et al., 2004), *Liza aurata* (Pereira et al., 2009), *Palaemonetes pugio* (Lee et al. 2000; 2008; Kim and Lee, 2004; Lee and Kim 2002) *Mytilus edulis* (Rank et al., 2005) and *Crassostrea virginica* (Bissett et al., 2009). Lee et al. (2000) studied the effect of genotoxicants such as chromium (III) chloride, sodium chromate, mercuric chloride, and 2-methyl-1,2-naphthoquinone (MNQ) on grass shrimp, *Palaemonetes pugio,* embryos employing comet assay. DNA damage in a variety of aquatic animals has been associated with reduced growth, abnormal development and reduced survival of embryos, larvae and adults (Shugart et al., 1992; Lee et al., 1999; Steinert et al., 1999). In grass shrimp comet assay was used to provide a sensitive measure of DNA strand breakage exposed to

genotoxicants, highway run off sediments, sediments with coal ash and phototoxicants (Lee et al., 2000; 2008; Lee and Kim 2002; Kim and Lee, 2004).

Tail length is the basic parameter used for quantifying DNA damage in the comet assay. However, the tail length levels off after migrating to a certain distance. After the maximum length is attained, the comet tail will not be extending in length, however grow in intensity as compared to that of with the head portion (Bowden et al., 2003). So, in addition to tail length, percentage tail DNA and tail moment were also calculated using comet scoring software.

Genotoxicity of cadmium was reported by Stefania et al (2002) in rat hemispheres and cerebellum, Yang et al. (2003) in rat Leydig cells, Pruski and Dixon (2002) and Emmanouil et al. (2007) in *Mytilus edulis* and Hook and Lee (2004) in *Paleomonetes pugio* embryos, and Chang et al. (2009) in *Litopenaeus vannamei*. The present study proved that cadmium chloride induced DNA damage in primary haemocyte culture.

Genotoxicity of mercury was reported in *Cyprinus carpio* gill cells (Arabi et al., 2004), blood cells of *Liza aurata* (Pereira et al., 2009), human salivary gland tissue cells and lymphocytes (Schmid et al., 2007) and *Palaemonetes pugio* embryo (Lee et al., 2000). Arabi et al. (2004) found that $HgCl_2$ have a deleterious effect on the membrane integrity and glutathione (GSH) content of gill cell suspensions of carp, *Cyprinus carpio* L. and mercury caused DNA strand breaks at 3000μM. Mercuric chloride has been reported to cause DNA damage in *P. pugio* embryos at concentrations as low as 0.003 μM after a 12 hour exposure period (Lee et al., 2000). DNA damage was reported by Pereira et al. (2009) in goden grey mullet, *Liza aurata* due to environmental exposure of mercury. The present study proved that mercuric chloride induced DNA damage in primary haemocyte culture.

Jamil et al. (2005) reported genotoxic effect of monocrotophos in human lymphocytes in vitro employing comet assay. Giri et al. (2002) evaluated the

genotoxicity of malathion using chromosome aberration, sister chromatid exchange (SCE) and sperm abnormality assays in mice. Effect of pesticides such as DDT, azinphosmethyl, permethrine, parathion, chlorpyrifos, malathion, endosulfan, and carbaryl was studied on DNA and protein of *Litopenaeus stylirostris* larvae (Reyes et al., 2002). The results indicated reductions in protein and DNA in larvae exposed to these pesticides, and in those exposed to DDT, breaks and/or adducts were registered. The study suggested pesticide pollution as a possible cause of reduced growth rate in larvae due to protein reduction and other disease conditions. Malathion and monocrotophos, which are two commonly used insecticides, have proved to be genotoxic to *P. monodon* primary haemocyte culture in the present study.

In conclusion, the study has demonstrated the use of primary shrimp haemocyte culture for testing the cytotoxicity of a shrimp health management chemical, an anti *Vibrio* compound, and aquatic pollutants such as heavy metals and pesticides employing MTT assay. The primary cell culture system could also be employed to assess the DNA damage induced by genotoxic pollutants in the aquatic environment. The present study forms the first report of comet assay carried out to record DNA strand breakage as demonstration of genotoxicity of pollutants using shrimp cell culture as a model system.

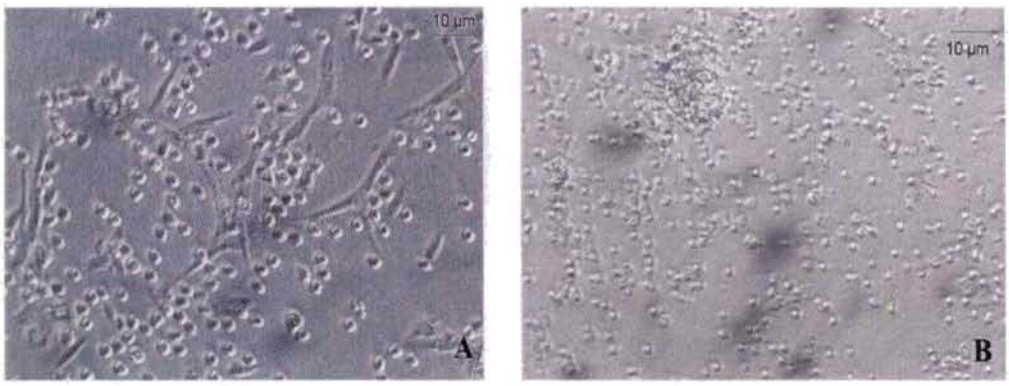


Fig.1. Cytoxicity of BKC in primary haemocyte culture. A) Control B) Necrosis and shrinkage of haemocytes seen at 25 mg l^{-1} (40x).

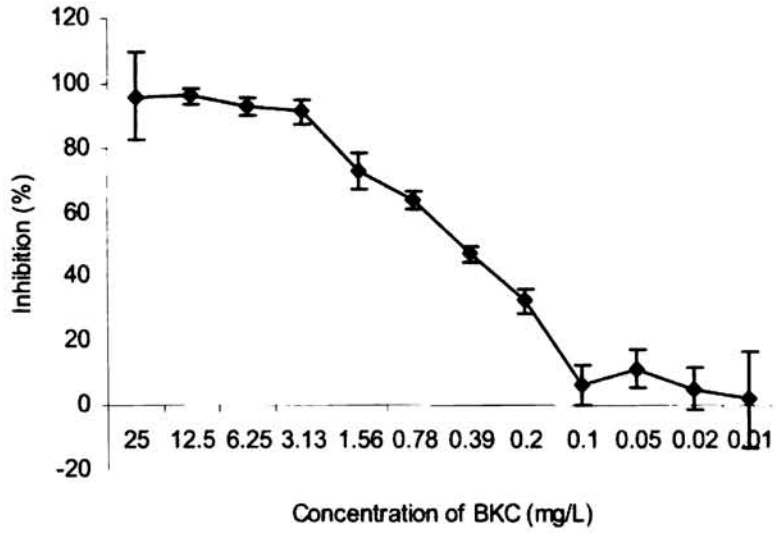
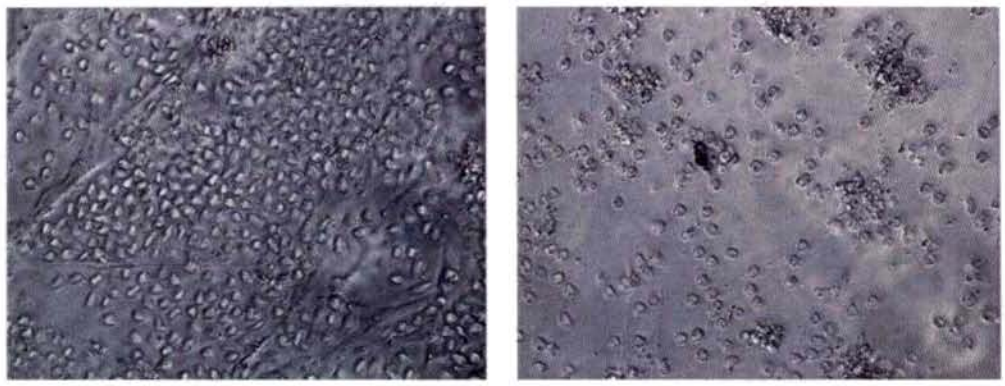


Fig.2. Toxicity of benzalkonium chloride in primary haemocyte culture in terms of percentage inhibition of cells determined through MTT assay (n=4).



A B

Fig.3. Toxicity of N-methyl-1-hydroxyphenazine in primary haemocyte culture. A) Control, B) Cells after exposure to N-methyl-1-hydroxyphenazine (10mg l^{-1}) (40X)

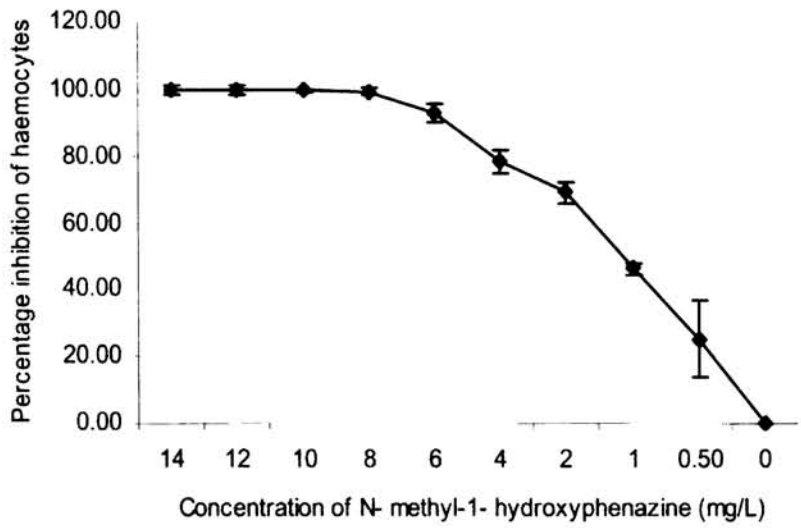


Fig.4. Toxicity of N-methyl-1-hydroxyphenazine in primary haemocyte culture in terms of percentage inhibition of cells determined through MTT assay (n=3).

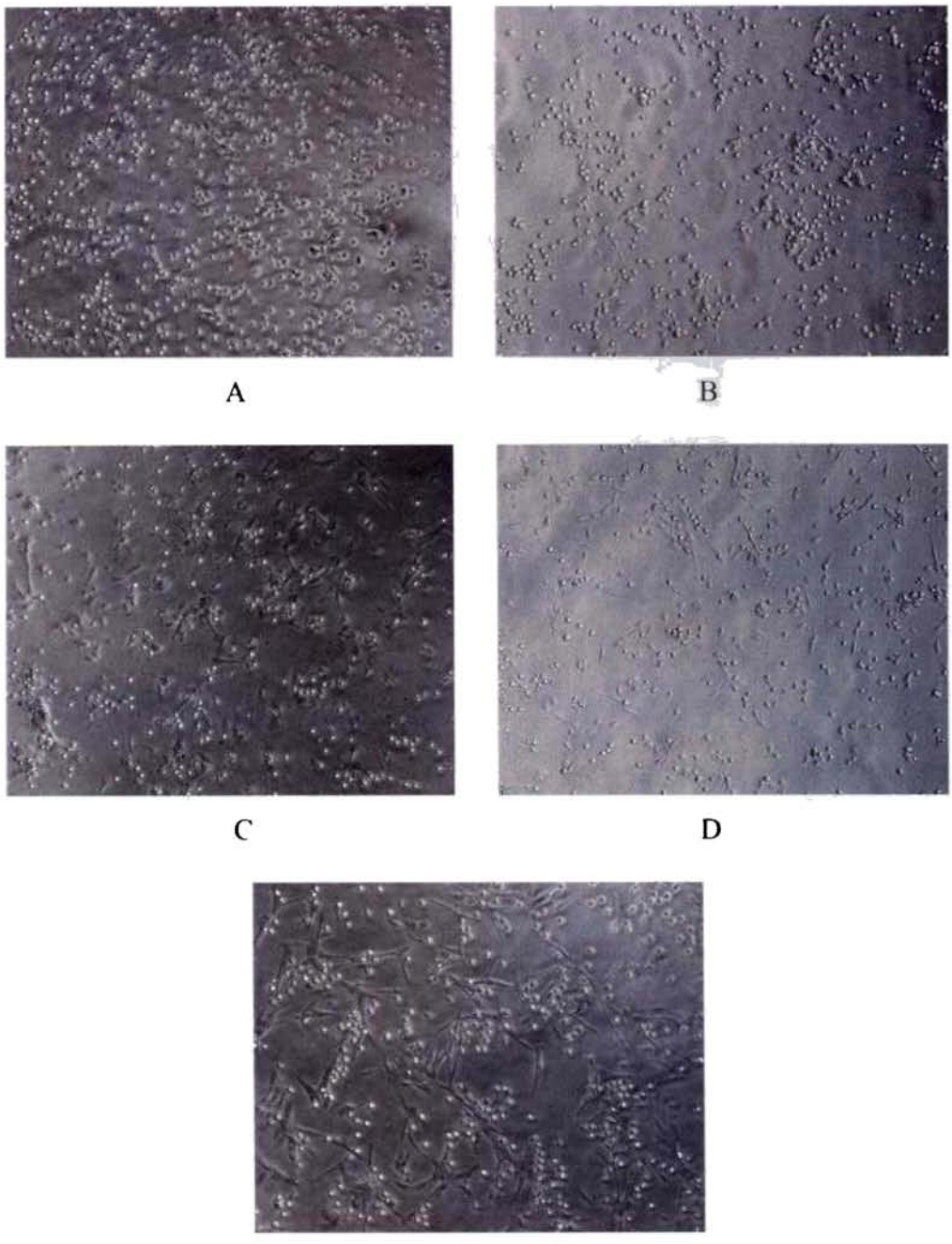


Fig.5. Primary haemocytes after 12 hour exposure to A) Cadmium chloride (15.63μM), B) Mercuric chloride (1.95μM), C) Malathion (61.7mgl^{-1}), D) Monocrotophos (100 mgl^{-1}) and E) Control (20X).

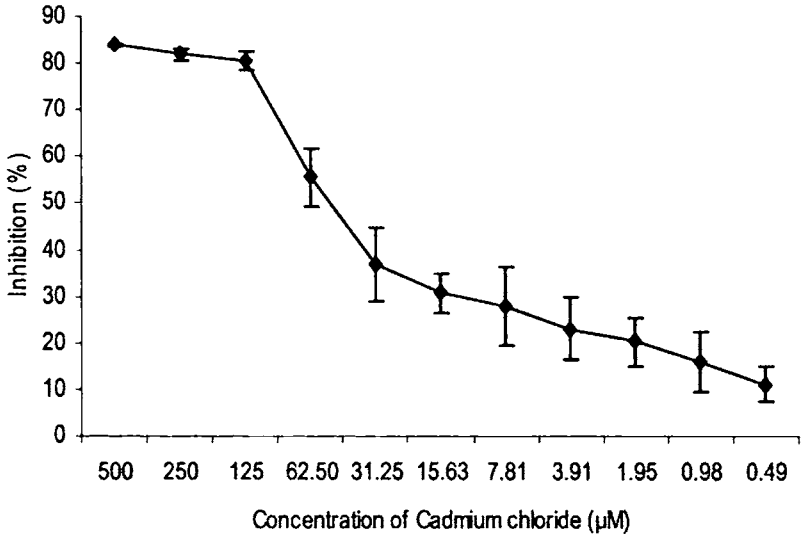


Fig.6. Toxicity of cadmium chloride in primary haemocyte culture in terms of percentage inhibition of cells determined through MTT assay (n=4).

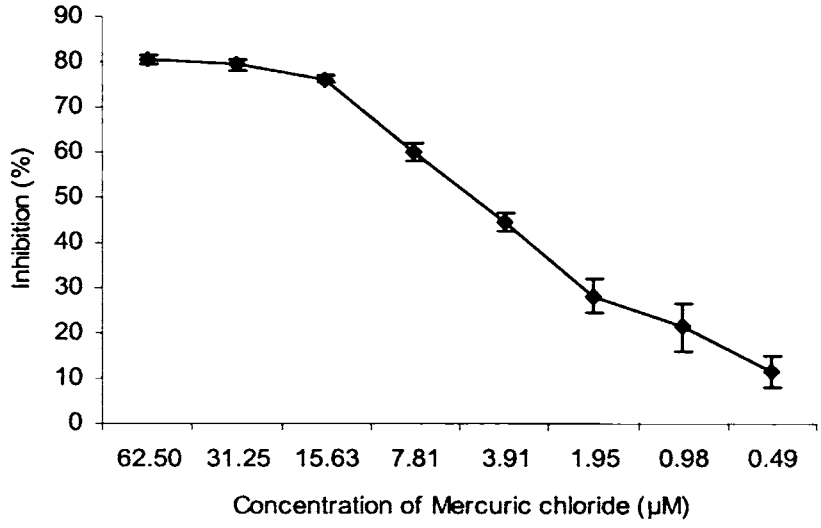


Fig.7. Toxicity of mercuric chloride in primary haemocyte culture in terms of percentage inhibition of cells determined through MTT assay (n=4).

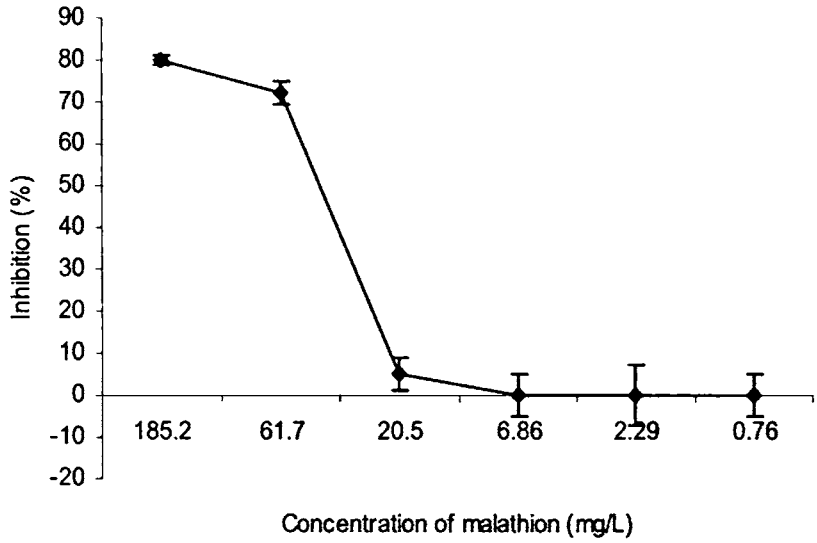


Fig.8. Toxicity of malathion in primary haemocyte culture in terms of percentage inhibition of cells determined through MTT assay (n=4).

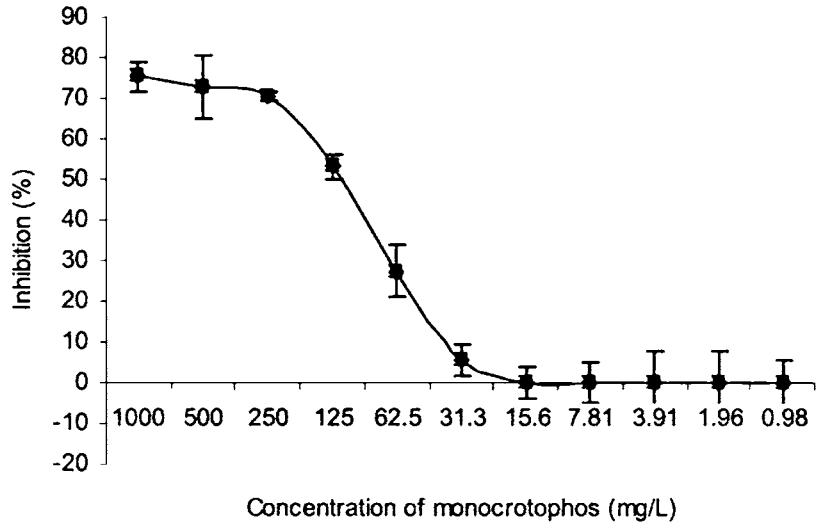


Fig.9. Toxicity of monocrotophos in primary haemocyte culture in terms of percentage inhibition of cells determined through MTT assay (n=4).

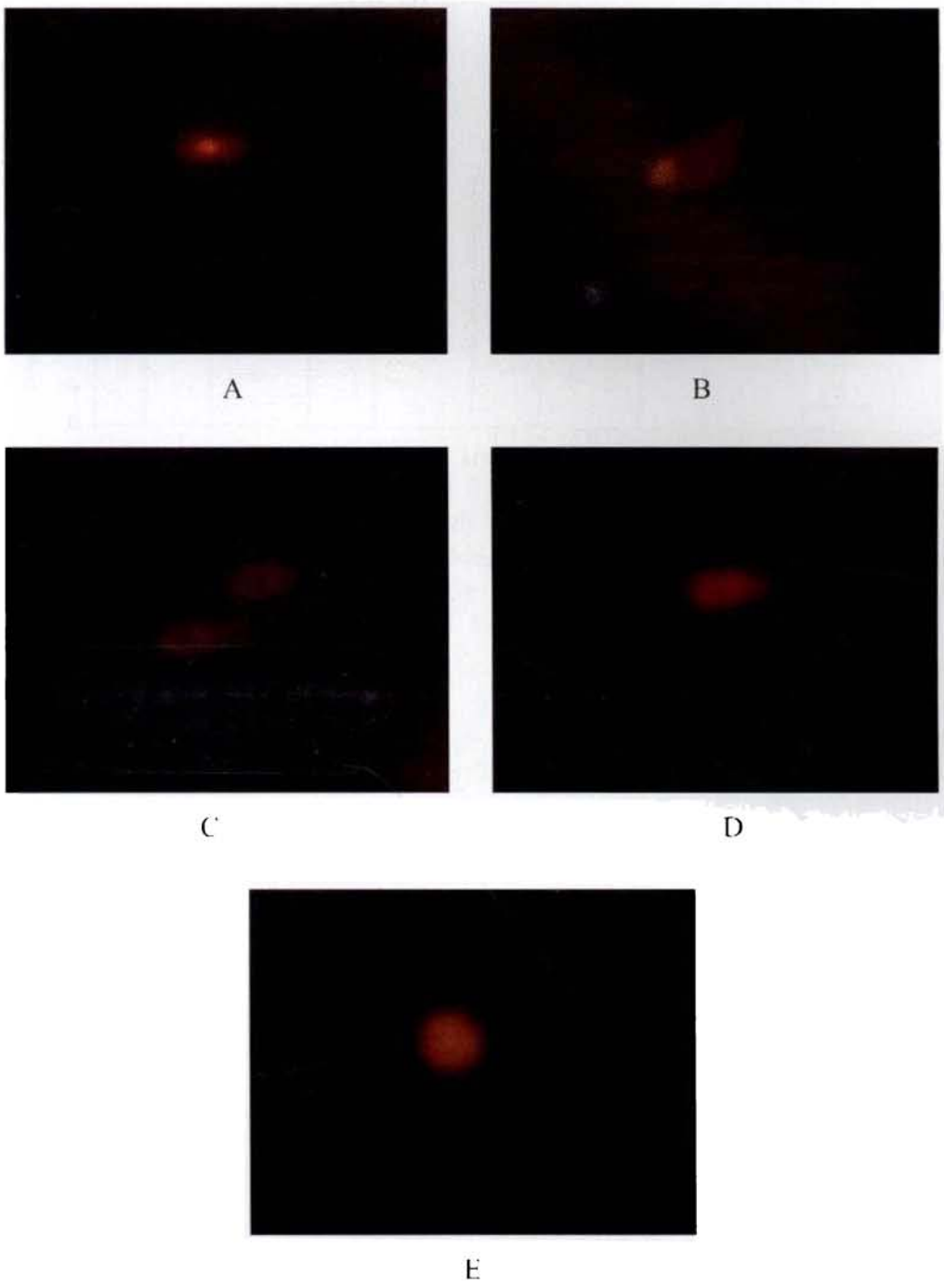


Fig.10. Comets formed by haemocytes after exposure to A) Cadmium chloride, B) Mercuric chloride, C) Malathion, D) Monocrotophos and E) Control (100X).

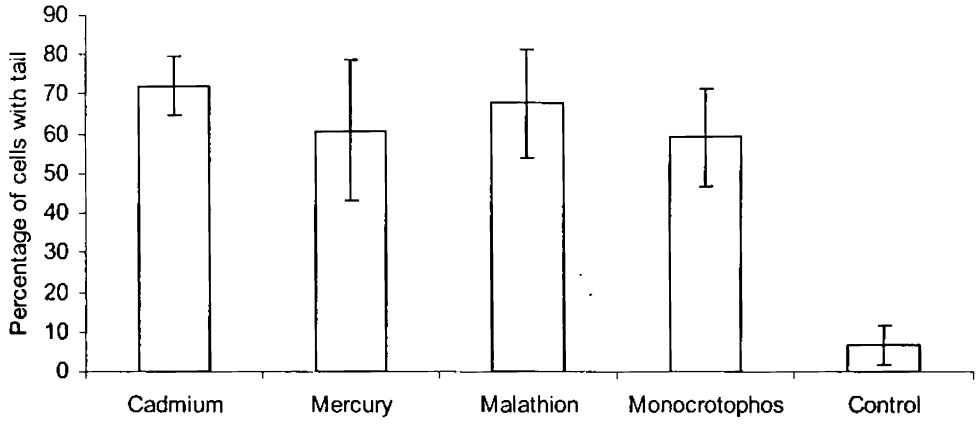


Fig.11. Percentage of nuclei with tail after exposure to cadmium chloride, mercuric chloride, malathion and monocrotophos (Cells counted around 100).

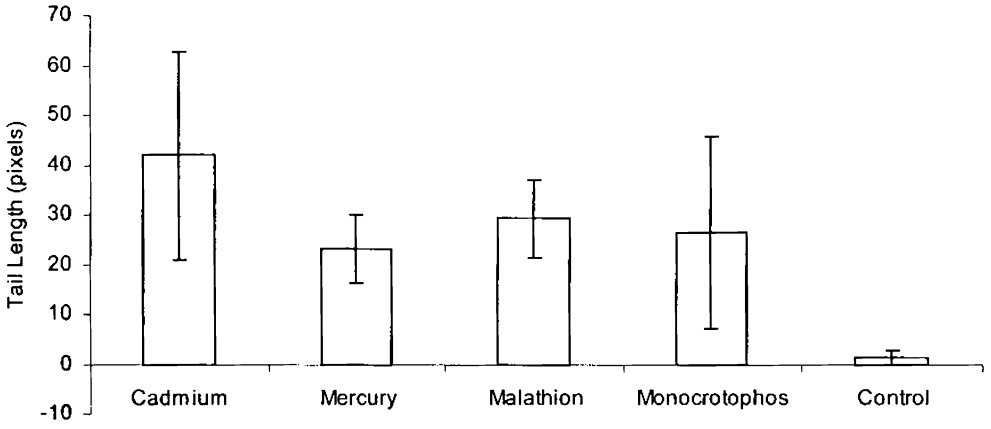


Fig.12. Mean tail length of the comets formed by exposure to cadmium chloride, mercuric chloride, malathion and monocrotophos (Cell count around 100).

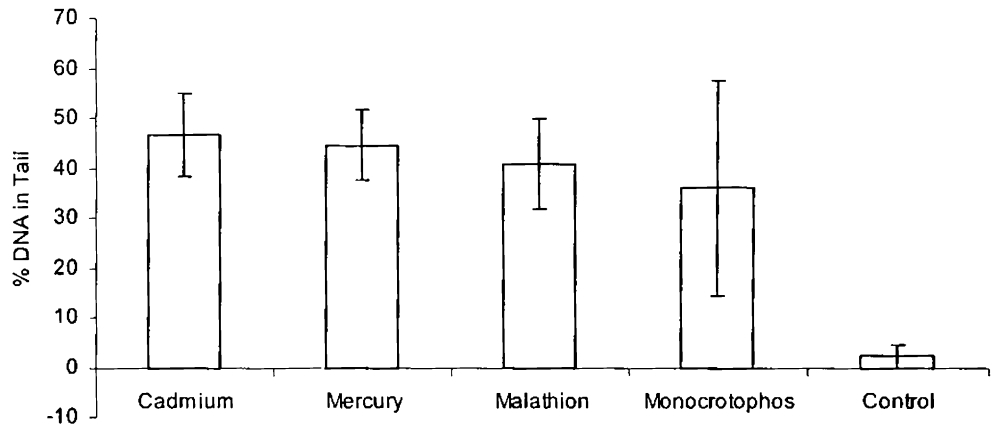


Fig.13. Percentage DNA in the tail of comets after exposure to cadmium chloride, mercuric chloride, malathion and monocrotophos (Cells counted around 100).

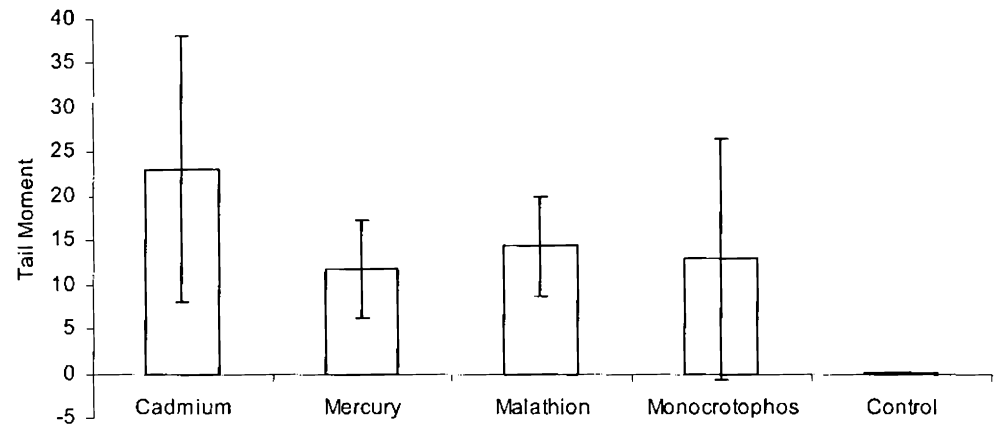


Fig.14. Mean tail moments of the comets formed by exposure to cadmium chloride, mercuric chloride, malathion and monocrotophos (Cells counted around 100).

Chapter 5

TRANSFECTION OF *PENAEUS MONODON* PRIMARY CELL CULTURES, PRIMARY OOCYTES AND SPERM CELLS IN VITRO

5.1. Introduction

Finite cell lines have a life span of 20-100 generations after which they undergo senescence, but some cells undergo spontaneous transformation and produce continuous cell lines with an infinite life span. In vitro transformation may not take place by mere passage of the cell culture continuously in most of the cases. In such instances when the cells are unable to produce cell lines by themselves they are subjected to transfection of foreign genes, especially oncogenes employing methods such as calcium phosphate precipitation, lipid-mediated transfections and electroporation.

The method of calcium phosphate-DNA co-precipitation was first introduced by Graham and Van der Eb in 1973. Principle of this technique is the formation of insoluble calcium phosphate-DNA complexes in a supersaturated solution achieved by adding a solution containing DNA and calcium chloride to a buffered saline solution containing phosphate and incubating the mixture for a period of time. DNA molecules enter the cells by endocytosis of the calcium phosphate-DNA co precipitates (Hafezparast, 2000) resulting in an expression of the foreign gene. Factors influencing the success rate include the quality and concentration of DNA, pH, and duration of incubation time.

The use of cationic lipids for DNA transfection has become widespread since in its introduction by Felgner et al. (1987). Various formulations of lipid reagents for transfection are available in the market. They normally contain a positively charged moiety attached to a neutral lipid component (Gao and Huang, 1995). On mixing these reagents with DNA, the charged head groups are drawn towards the phosphate backbone of DNA and form lipid-DNA complexes. When the suspension is added to the cells, the positively charged head groups of lipid are attracted to the negatively charged cell membrane. The end result is that the lipid-DNA complex is either fused to the cell membrane or enters the cell by endocytosis, transferring its DNA load into the cell. Factors influencing the

efficiency of lipofection are cell type, chemical and physical structure of the cationic liposomes, lipid: DNA ratio, their concentrations, DNA quality, cell culture density and the duration of exposure of cells to the lipid. The advantages of lipofection over other methods include higher efficiency, ability to transfect a wide variety of cells including cells which are refractile to other transfection procedures, relatively low cell toxicity and delivery of large DNA fragments upto several megabase pairs (Freshny, 2000)

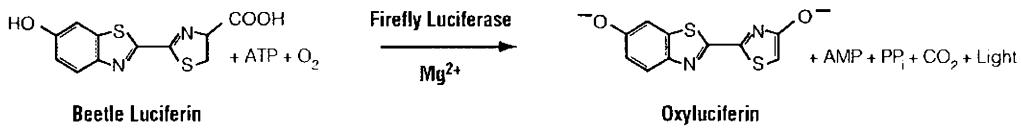
DNA can be introduced into cells by electroporation, when a high cell concentration is briefly exposed to a high-voltage electric field in the presence of DNA to be transfected (Chu et al., 1987). Small holes are generated transiently in the cell membrane (Zimmerman and Vienkin, 1982), and the DNA is allowed to enter the cell. Removal of the electric field results in spontaneous sealing of the pores, as long as the field strength and duration of the pulse are within tolerable range of the cells (Zimmerman, 1982). Most cells refractory to chemical methods of gene transfer are successfully transfected by electroporation (Andreason and Evans, 1988; Chu et al., 1987). Chemical methods of transfection usually result in the integration of large concatamers, which may inherently interfere with cell function and obscure investigations involving specific gene expression (Robins et al., 1981; Kucherlapati and Skoultchi, 1984). Suspension cells are more easily transfected by electroporation than are adherent cells, since adherent cells must be detached from the culture vessel (Freshny, 2000). Parameters affecting the transfection efficiency of electroporation are voltage, time constant, composition of electroporation buffer and temperature. The drawbacks of electroporation include its requirement for more cells and DNA than chemical methods of gene transfer and its variability in optimal parameters between cell types. Other techniques of gene delivery include DEAE dextran neutralization, microinjection of DNA directly into the cells and the use of viral vectors.

Following transfection the exogenous DNA remains in the nucleus for a limited time unless it is integrated into the host genome. However, even in the

non integrated form, the transfected DNA is subject to the regulatory mechanisms that control gene expression and therefore its genes can be expressed transiently. Integration of the transfected gene into the host genome, on the other hand, gives rise to stable retention of the exogenous gene in the cells, providing constitutive expression.

In most transfection experiments the cDNA of the gene of interest is inserted into an expression vector that carries an expression cassette constituting of a strong promoter, a multiple cloning site and a polyadenylation sequence. The vector also contains a bacterial origin of replication and a bacterial selectable marker for propagation and selection of the vector in bacterial cells. In order to isolate the few transfected cells from non transfected cells, a dominant selectable marker is also utilized. Some vectors have reporter genes which are screenable marker genes. These differ from selectable marker genes in that they do not confer a property that allows transformed cells to survive under selective conditions. Instead, they encode a product that can be detected using a simple and inexpensive assay. When controlled by a strong constitutive promoter, reporter genes are often used as markers to confirm transient or stable transformation, since only cells containing reporter-gene construct can express the corresponding protein. Importantly, the assays used to detect reporter gene activity are quantitative, so they can also be used to measure transformation efficiency.

The vector P2completete Fluc pGL3 basic has been used in the present study, having firefly (*Photinus pyralis*) luciferase (*luc*) gene as the reporter. The functional enzyme is created immediately after translation. Firefly luciferase is a 61kDa monomer that catalyzes the mono-oxygenation of beetle luciferin. The enzyme uses ATP as a co-factor, although most of the energy for photon production comes from molecular oxygen.



Beetle Luciferin **Oxyluciferin**

When the substrate, luciferin and ATP are added to the lysate of the cells containing the enzyme luciferase photons are produced. This is measured using a luminometer.

Transfection of shrimp primary cultures was reported by Tapay et al. (1995) and Hu et al. (2008). Tapay et al. (1995) employed lipofection and Hu et al. (2008) employed reteroviral infection for transfection of lymphoid organ cultures. Gene transfer to shrimp zygotes and embryos was reported by Preston et al. (2000), Tseng et al. (2000), Arenal et al. (2004), Sun et al. (2005), Lu and Sun (2005) and Arenal et al. (2008). Transfection methods were polyethylenimine mediated (Sun et al., 2005; Lu and Sun, 2005), electroporation (Tseng et al., 2000; Arenal et al., 2004) and microinjection (Preston et al., 2000).

The present study deals with the transfection of shrimp primary haemocyte culture, lymphoid organ culture and primary oocytes and sperm cells maintained in vitro.

5.2. Material and methods

5.2.1. Experimental animals

WSSV and Monodon Baculo virus (MBV) negative *P. monodon* larvae obtained from a local hatchery were stocked and reared in a recirculating shrimp rearing system integrated with nitrifying bioreactors (Kumar et al., 2009) in sea water having salinity 15g l^{-1}. Water quality was maintained by the addition of probiotics Detrodigest ^{TM} (NCAAH, India) to manage detritus, and Enterotrophotic ^{TM} (NCAAH, India) to control *Vibrio*. The larvae were fed with commercially available pelleted feed (Higashimaru, India). They were confirmed WSSV negative by nested PCR (WSSV detection kit, Genei, India) when they grew to 8-12g, and used for all experiments. For isolation of ovary and testes male and female animals weighing 60-120g obtained from local hatcheries were used.

5.2.2. Surface sterilization of the animals

The animals were sacrificed by immersing in crushed ice and disinfected by maintaining in 800 mg l^{-1} sodium hypochlorite solution prepared in ice cold sea water (salinity 15 g l^{-1}) for 10 minutes. Subsequently, they were washed 5 times in sterile ice cold sea water, dipped in 70% alcohol and rinsed in ice cold sea water.

5.2.3. Development of primary haemocyte and lymphoid cell cultures

Haemolymph was withdrawn asceptically from rostral sinus using capillary tubes containing 100µl anticoagulant (Tris HCl 0.01M, Sucrose 0.25M, Tri Sodium Citrate 0.1M) and diluted to obtain 5 x 10^5 cells ml^{-1} using modified 2XL-15 (Leibovitz) medium and aliquots of 200µl were dispensed into the wells of 96 well plates (Greiner Bio-One, USA) and incubated at 25°C for 6 hours before using for transfection experiments. Media used was composed of Leibovitz's 2XL-15 (Sigma) at double strength supplemented with 2% glucose, MEM Vitamins (1X), tryptose phosphate broth (2.95 g l^{-1}), 20% FBS, N-phenylthiourea (0.2mM), 0.06µg ml^{-1} chloramphenicol, 100µg ml^{-1} streptomycin and 100 IU ml^{-1} penicillin.

Surface sterilized shrimps were dissected to remove the lymphoid organ and the tissue pieces were immediately immersed in HBSS. Before seeding, tissue pieces were washed thrice, cut into 1mm^3 pieces, pipetted vigorously and 200µl media containing explants were aliquoted into 96 well plate. Media used was 2X L15 modified with the same composition as that of haemocyte culture but devoid of N-phenylthiourea. The culture was allowed to grow for 48 hours before using for transfection experiments.

5.2.4. In vitro maintenance of primary oocytes and sperm cells of *P. monodon*

Ovary and testes were removed from surface sterilized animals, cut into 1mm^3 pieces and seeded into 25 cm^2 flasks, incubated at 25°C in modified 2X L 15 medium. Primary oocytes and sperm cells migrated from the explants were maintained in the growth medium in the flask until transfection experiment.

5.2.5. Isolation and purification of the plasmids

5.2.5.1. Transformation of P2completete Fluc pGL3 basic vector into Escherichia coli

P2completete Fluc pGL3 basic vector (Dhar et al., 2007), is a modified pGL3-Basic vector (Promega, USA) containing a modified *luc* coding region of the firefly (*Photinus pyralis*) luciferase that has been optimized for evaluating transcriptional activity in transfected eukaryotic cells. The P2 promoter region of the infectious hypodermal and hematopoietic necrosis virus (IHHNV) was inserted between *Sac I* and *Xma I* sites of pGL3-Basic upstream of the luciferase coding sequence (Fig 1).

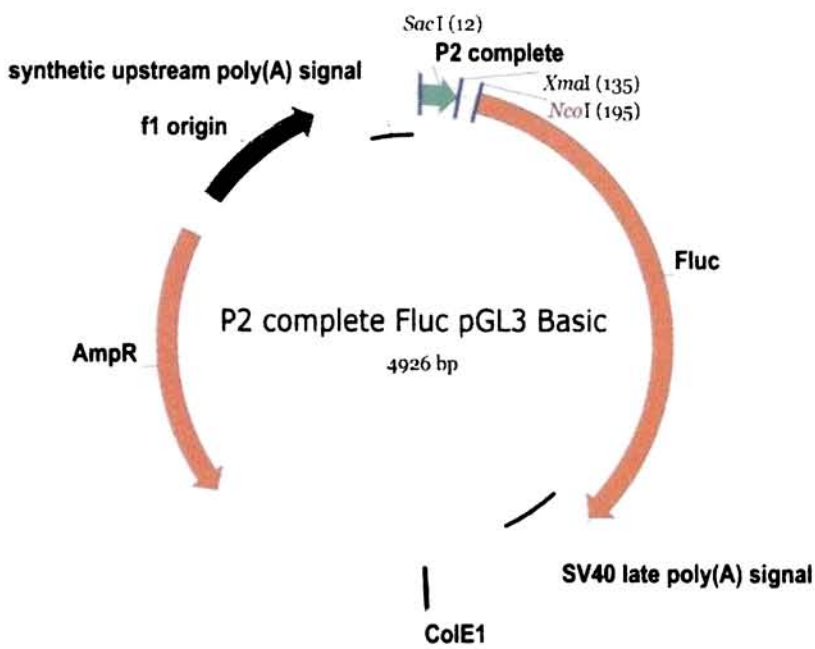


Fig.1. Vector map of P2completete Fluc pGL3 basic vector

The P2completete Fluc pGL3 basic vector spotted on to the blotting paper was soaked for 15 minutes in 100µl Tris EDTA buffer in a microcentrifuge tube for 15 minutes and centrifuged at 10,000 xg for 10 minutes. 5µl of the solution obtained was used to transform *E. coli* strain JM109 (Invitrogen).

To the 5µl plasmid in a screw cap tube, added 50µl competent JM109 cells, mixed gently and incubated in ice for 20 minutes. Heat shock was given for

90 seconds at exactly 42°C. The tube was returned to ice for 2 minutes. Added 600µl Super Optimal broth with Catabolite repression (SOC; Composition for 10ml: Tryptone-0.2 g; yeast exytract-0.1g; NaCl-0.005g, 250mM KCl- 100µl; 2M $MgCl_2$-50µl; 1M glucose-200µl. $MgCl_2$ and glucose was added just before transformation) and incubated for 2 hours at 37°C with shaking. After incubation, 200µl each was plated into LB ampicillin plates (LB medium-2g/100ml; Agar- 2g/100ml). After autoclaving added 100µl of ampicillin (100mg ml^{-1}) and incubated for 16 hours at 37°C. Individual colonies developed were inoculated into 10 ml LB ampicillin broth and incubated at 37°C with shaking for plasmid extraction.

5.2.5.2. Propagation of E. coli HB101 containing the plasmid vector, pSV3-neo

pSV3-neo vector with the simian virus 40 (SV40) large T antigen was purchased from ATCC (catalog No. 37150). Vector provides dominant selectable marker for resistance to antibiotic G418 (Fig. 2) in mammalian cell lines and to ampicillin in *E. coli*. E. *coli* was propagated in LB ampicillin (50 µg $µl^{-1}$) at 37 °C.

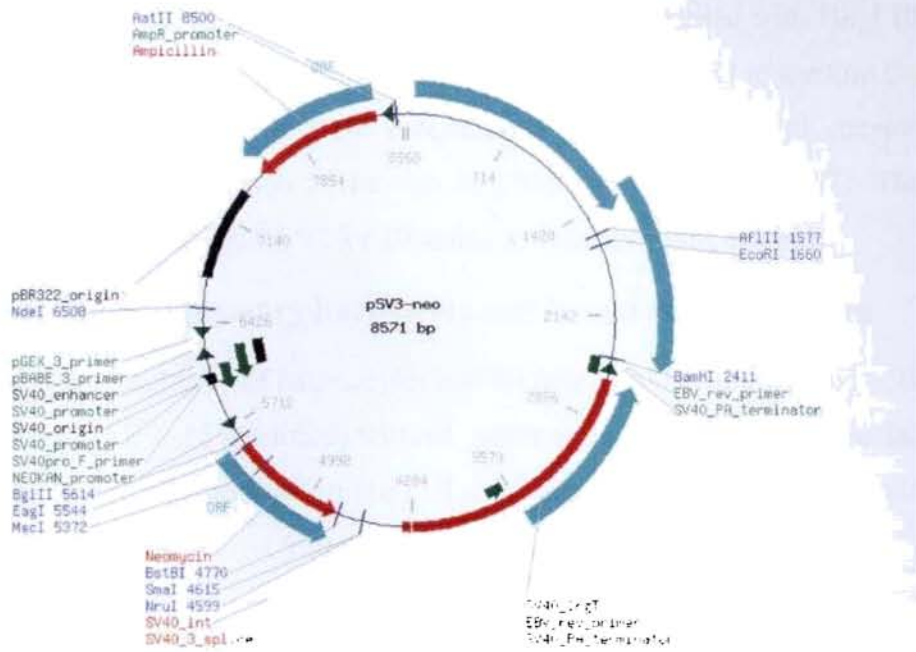


Fig.2. Vector map of pSV3-neo

5.2.5.3. Plasmid extraction

Plasmid extraction was done using GenElute HP Plasmid Miniprep kit (Sigma). 5ml culture after overnight incubation was pelletised at 12,000 x g for 1 miunte. The pellet was resuspended in 200µl resuspension solution containing RNase A and lysed by adding 200µl lysis buffer. 350µl neutralization solution was added and centrifuged at 12,000 x g for 10 minutes to remove the cell debris. Lysate was loaded into GenElute HP Miniprep. Binding column inserted in a microcentrifuge tube and centifuged at 12,000 x g for 1 minute. Plasmid DNA bound to the column was washed twice to remove the endotoxins, salt and other contaminants. To elute the plasmid DNA, the column was transferred to a fresh collection tube, added 100 µl molecular biology grade water and centrifuged at 12,000 x g for 1min. Purity of the plasmid DNA obtained was analysed by agarose gel electrophoresis and by determining the ratio of the absorbance reading at 260nm/280nm in a UV-VIS spectrophotometre (Shimadzu).

5.2.5.4. Restiction digestion

P2completete Fluc pGL3 basic vector was restriction digested with Hind III (New England Biolabs) enzyme and pSV3-neo plasmid using Eco R1 to confirm the plasmid size. 20 µl reaction mixture containing 5 µl plasmid, 0.5 µl enzyme (20,000U ml^{-1}) and 1X reaction buffer was incubated for 1 hour at 37 °C. The mixture was then incubated at 65 °C for 20 minutes to inactivate the enzyme.

5.2.6. Lipofection of primary haemocyte and lymphoid cell cultures

96 well plate cultures of haemocytes and lymphoid cells were washed with growth medium (2X L-15 modified) without serum and antibiotics and subjected to transfection with P2completete Fluc pGL3 basic vector. Composition of the transfection suspension was as follows:

Solution A

Plasmid DNA : 4µg

2X L-15 modified medium with out serum and antibiotics : 200µl

Solution B

Cellfectin (Invitrogen)	: 30µl
2X L-15 modified medium without serum and antibiotics	: 200µl

Solution B was added to solution A, mixed gently and incubated at room temperature (25°C±2) for 35 minutes. This lipid-DNA mixture was diluted to 2ml with serum free, antibiotic free media and subsequently a sample of 0.2ml of the transfection suspension was added to each well. After 24 hours of incubation transfection medium was replaced with normal medium with serum and antibiotics and the cells were subjected to luciferase assay after another 24 hours.

5.2.7. Lipofection of primary oocytes and sperm cells

For the transfection of oocytes and sperms, cells suspended in the medium were collected by centrifugation, washed with serum free, antibiotic free 2X L-15 modified medium, and 250 µl of cells at a concentration of 1 x 10^6 cells ml^{-1} were added to each well of the 24 well plate. Oocytes were transfected with P2completete Fluc pGL3 basic vector and sperm cells were transfected with both P2completete Fluc pGL3 basic vector and pSV3-neo vector. Composition of the transfection suspension was as follows:

Solution A

Plasmid DNA	: 8µg
2X L-15 modified medium without serum and antibiotics	: 200µl

Solution B

Cellfectin (Invitrogen)	: 60µl
2X L-15 modified medium without serum and antibiotics	: 200µl

Solution B was added to solution A, mixed gently and incubated at room temperature (25°C±2) for 35 minutes. This lipid-DNA mixture was diluted to 2ml with serum free, antibiotic free medium and subsequently 0.25ml of the

transfection suspension was added to each well. After 24 hour incubation in transfection medium half of the medium was removed from each well without disturbing the cells and normal growth medium was added. After another 24 hours primary oocytes and sperm cells transfected with P2completete Fluc pGL3 basic vector was subjected to luciferase assay and sperm cells transfected with pSV3-neo vector was subjected to immunofluorescence assay with anti SV40 T antigen

5.2.8. Lipofection of Sf9 cells with P2completete Fluc pGL3 basic vector

As a positive control *Spodoptera frugiperda* Sf9 cells were included. Cells in log phase were subjected to transfection. Microwell plate culture of the cell line was prepared by adding 0.2 ml of 5 $x10^5$ cells ml^{-1}. The medium used was TnM-FH (Sigma) with 10% FBS, the growth medium recommended for Sf9 cells. After 24 hours, the medium was removed, washed with serum free, antibiotic free TnM-FH and subjected to transfection. Composition of the transfection suspension was as follows:

Solution A	
Plasmid DNA	: 4µg
TnM-FH medium without serum and antibiotics	: 200µl
Solution B	
Cellfectin (Invitrogen)	: 30µl
TnM-FH medium without serum and antibiotics	: 200µl

Solution B was added to solution A, mixed gently and incubated at room temperature for 35 minutes. This lipid-DNA mixture was diluted to 2ml with serum free, antibiotic free medium and subsequently 0.2ml of the transfection suspension was added to each well. Transfection mixture prepared in serum free TnM-FH was added. After 24 hours transfection medium was replaced with normal growth medium and luciferase assay was done after another 24 hours.

5.2.9. Electroporation of sperm cells with P2completete Fluc pGL3 basic vector and pSV3-neo vector

5.2.9.1. Selection of the hypoosmolar buffer and determination of the cell diameter in hypoosmolar buffer

Osmolality of the electroporation buffer (25mM KCl, 0.3mM KH_2PO_4, 0.85mM K_2HPO_4) was adjusted to 280, 400,600 and 720mOsm by the addition of myo-inositol. Sperm cells were incubated in each of the buffers for 30 minutes and viability was determined by trypan blue staining. Cells incubated in modified 2XL 15 medium were kept as control. Cells after incubation in the selected electroporation buffer for 15 minutes were observed under phase contrast microscope (Leica, Germany) and the diameter of the cell was determined using "Leica Application Suite" software (Leica Microsystems, Switzerland).

5.2.9.2. Calculation of optimum pulse voltage for electroporation of sperm cells

Critical field strength necessary for the electro permeation of the cell membrane can be calculated approximately using the following formula:

$E_c = V_c / (0.75 \times d)$, where E_c= critical field strength (V/cm), V_c= permeation voltage of the membrane (1 V at 20 °C) and d= cell diameter (cm).

To calculate the voltage to be set on the Multiporator (Eppendorf, Germany) the critical field strength (E_c) was multiplied with the gap width of the cuvette (0.2cm). The calculated voltage was applied at different pulse lengths of 40, 70 and 100µs. Pore formation at the selected pulse voltage was checked immediately after pulsing by propidium iodide staining. The stain was added to the cells at a final concentration of 5µg ml^{-1} and observed under fluorescent microscope (Olympus, Germany).

Electroporation of sperm cells was done with both P2completete Fluc pGL3 basic vector and pSV3-neo vector separately. For electroporation an aliquot of 400 µl (1 x 10^6 cells) cell suspension was aliquoted into microcentrifuge tubes, plasmid DNA added to a final concentration of 5 µg ml^{-1},

and transferred the suspension to electroporation cuvettes of 2 mm gap width (Eppendorf, Germany). Control consisted of cells without the addition of the plasmid. After pulsing in the eukaryotic mode of the equipment, cells were allowed to remain in the cuvette for 10 minutes, transferred to 24 well plates with 1ml growth medium, replaced half of the medium at 24 hours. After 24 hours cells transfected with P2completete Fluc pGL3 basic vector and pSV3-neo vector was subjected to luciferase assay and immunofluorescence assay respectively.

5.2.10. Luciferase assay of cells transfected with P2completete Fluc pGL3 basic vector

Cells transfected with P2completete Fluc pGL3 basic vector (both electroporation and lipofection) was subjected to luciferase assay. Assay was done with Bright-Glo Luciferase Assay System (Promega) according to the manufacturer's protocol. From the haemocyte and lymphoid cell culture whole medium was removed and 100µl of the reagent added to each well. Half (250µl) of the medium was removed from the oocytes and sperm cells and 250µl reagent was added. Duration of 2 minutes was given for complete cell lysis, then the samples were transferred to microcentrifuge tubes and measurement was taken in luminometer (Turner Biosystems, USA) immediately. Normal cells without transfection were also subjected to Luciferase assay. This served as the control.

5.2.11. Immunofluorescence assay of sperm cells transfected with pSV3- neo

Sperm cells transfected with pSV3-neo vector (both electroporation and lipofection) subjected to immunofluorescence assay with anti SV40 T antigen. After transfection cells were pelletised by centrifugation at 400g for 5 minutes, washed with PBS (720mOsm) and fixed in 10% paraformaldehyde, and subsequently with 70% ethanol. Cells were sedimented on to glass slides, pre coated with poly-L-lysine (0.01%) using a cytocentrifuge (Wescor, USA). Subsequently the free sites were blocked using 3% BSA in PBS and incubated in a humidified chamber for 1 hour. The slides were washed in PBS/Tween 20 (0.01%) and added 100µl anti SV40T

antigen (5μg ml^{-1}) (Calbiochem) on the slide, incubated for 1 hour and washed three times in wash buffer. It was again incubated for an hour after addition of 100μl rabbit anti mouse FITC conjugate, 1: 50 dilution (Sigma) and subjected for washing. After incubation with general nuclear stain DAPI (10 μl, 0.2μg ml^{-1}) for three minutes, the slides were rinsed with distilled water, air dried, mounted (Vectashield, USA) and observed under fluorescent microscope (Olympus, Germany). DAPI and FITC were viewed under filters with excitation wavelength 360-370nm and 470-490nm respectively. The slides were compared with untransfected control cells. The images were processed and merged using the "Image pro-express" software (Media Cybernetics Inc, MD, USA).

5.2.12. Statistical analysis

The results in the figures are average values of 3-4 replicates ± standard deviation. The effects of treatments were statistically analyzed by Student's-t test. Differences were considered significant at $P < 0.05$

5.3. Results

5.3.1. Development and maintenance of primary haemocyte and lymphoid cell cultures

Primary haemocyte cultures formed a monolayer within 4 hours. Round to elliptical and small and large spindle shaped cells, with granules, could be seen. In lymphoid cell culture explants attached to the culture vessel within few hours and fibroblastic and round cells migrated from the explant.

5.3.2. In vitro maintenance of primary oocytes and sperm cells

Primary oocytes cells and sperm cells migrated from the gonadal tissues once the tissue pieces were seeded onto growth medium. The primary oocytes were large and round (Fig 7), however cells of different sizes could also been obtained. Oocytes were maintained for 1 week in the growth medium. Sperm cells on the other hand were small and non motile with anterior spike, central cap region and posterior main body (Fig 8B). These cells were maintained in modified L 15 medium for 1 month.

5.3.3. Restriction digestion of the P2completete Fluc pGL3 basic and pSV3-neo plasmids

Extracted plasmids P2completete Fluc pGL3 basic (Fig 3A) and pSV3-neo (Fig 4) were analyzed by agarose gel electrophoresis. Restriction digestion of P2completete Fluc pGL3 basic with Hind III (Fig 3B) and that of pSV3-neo with Eco RI linearised the plasmids (Fig 4).

5.3.4. Lipofection of haemocyte culture, lymphoid cell culture, primary oocytes, sperm cells and Sf9 with P2completete Fluc pGL3 basic vector

On performing lipofection on haemocyte (Fig 5) and lymphoid cell cultures (Fig 6) with P2completete Fluc pGL3 basic vector, most of the cells were found dead. On the other hand primary oocytes (Fig 7) and sperm cells (Fig 8A) after transfection were viable, however, with less percentage of dead cells. Luciferase assay did not show any significant difference between transfected cells and control cells in primary oocytes, however, in sperm cells there was significant difference ($P<0.05$) (Table 1). Meanwhile, transfected Sf9 cells showed highly significant difference from that of the control (Table 2). For sperm cells luminometer readings were in the range of 162-215 relative light units (RLU) meanwhile for Sf9 cells the readings ranged from 12,110-17,038 RLU.

5.3.5. Transfection of sperm cells by electroporation with P2completete Fluc pGL3 basic vector

Percentage viability of sperm cells in electroporation buffers with different osmolality was examined to select the hypo osmolar buffer for further experiments. Buffer with an osmolality of 600 mOsm was found suitable with 86% viability. In the isoosmotic control with 720 mOsm viability was 89% (Table 3). Diameter of the cells was 6µm on 15 minutes incubation in the hypoosmolar buffer (600 mOsm) and 5µm in the normal medium (modified 2XL 15). Using the formula, $E_c = V_c / (0.75 \times d)$ critical field strength was calculated to be 2222.22 V cm^{-1} which, on using a 2 mm electroporation cuvette, was set at 444.44 V. Optimum voltage was selected to be 440 V. Among the different pulse lengths tested 40 µs was found to be better with greater

viability than 70 and 100 µs. When checked for pore formation at 440V/40 µs by propidium iodide staining almost all the cell nuclei were stained (Fig 9). Optimum conditions for eletroporation of sperm cells were summarized in Table 4. After electroporation most of the cells were live in the control without plasmid and in the test with added plasmid. But when subjected to luciferase assay after 48 hours only a slight increase (Table 5) in the readings was observed when compared to that of the control but it was not significant ($P>0.05$).

5.3.6. Lipofection and electroporation of sperm cells with pSV3 neo vector

Sperm cells transfected with pSV3 neo vector were subjected to immunofluorescence assay to detect the SV 40 T antigen. Positive signals of green fluorescence were found in a few cells (Fig 10) demonstrating the expression of SV 40 T antigen in lipofected cells having no fluorescence in the control.

5.4. Discussion

P. monodon sperms, as in various other shrimp species, are of natantian form (Brown et al., 1976; Kleve et al., 1980; Clark Jr. et al., 1981; Rios and Barros, 1997; Hall et al., 1999; Pongtipatee et al., 2007) which are non motile and composed of a single spike originating from the acrosomal region, a central cap with an acrosome and a subacrosomal region and a nucleus of decondensed chromatin structures (Pongtipatee et al., 2007). In the present study non motile sperm cells with anterior spike, central cap region and posterior main body were observed and maintained in modified 2XL15 medium for 1 month.

P2completete Fluc pGL3 basic vector (Dhar et al., 2007), is a pantropic modified luciferase reporter vector containing a modified coding region for firefly (*Photinus pyralis*) luciferase (*luc*) that has been optimized for monitoring transcriptional activity in transfected eukaryotic cells. The P2 promoter region of the IHHNV which causes "Runt Deformity Syndome" in penaeid shrimp (Lightner et al., 1983; Kalagayan et al., 1991; Lightner et al., 1996; Primavera and Quinitio, 2000) is cloned upstream of the luc gene. In IHHNV, the P2 promoter drives the expression of the nonstructural gene, where as P61 promoter drives the expression

of the structural gene (Shike et al., 2000[b]; Dhar et al., 2007). Promoters for viral capsid genes are generally stronger than those for non-structural genes. However, in insect and fish cell lines the luciferase expression was higher when the gene was under the control of the nonstructural gene promoter, P2 compared to its expression under the structural gene promoter, P61 (Dhar et al., 2007). In shrimp tail muscle the difference in expression between P2 and P61 promoters were not significant. In shrimp, P2 driven luciferase expression was less than 10,000 fold compared to the luciferase expression in insect cells and fish cells. Dhar et al. (2007) suggested that this was likely due to difference in transfection efficiencies in vitro and in vivo. The present study tested the transfection efficiency of the vector in shrimp primary cell cultures, oocytes and sperms maintained in vitro. Cell death was evident in primary haemocyte and lymphoid cell cultures, which might be due to the toxicity of lipofection reagent, cellfectin. (1:1.5 (M/M) liposome formulation of the cationic lipid N, N^{I}, N^{II}, N^{III}-Tetramethyl- N, N^{I}, N^{II}, N^{III}-tetrapalmityl-spermine (TM-TPS), and dioleoyl phosphatidylethanolamine (DOPE) in membrane-filtered water). Sperm cells and oocytes withstood lipofection but luciferase expression was not significant in oocytes. Meanwhile, sperm cells exhibited significant expression, but it was 300 fold lesser than that of the insect cell line, Sf9.

Primary lymphoid organ culture of *P. stylirostris* was reportedly transfected using pSV-3 neo shuttle vector containing the tumor (T) antigen gene from Simian virus 40. Transfected cells were passaged upto 44 times (Tapay et al., 1995). The frequency of SV-40 induced immortalization is approximately 100% in rodent cells, whereas in human cells the occurrence is dramatically lower (10^{-8} to 10^{-5}) (Shay and Wright, 1989; Radna et al., 1989). Hu et al. (2008) attempted immortalization of lymphoid organ cultures of *P. chinensis* using a pantropic retroviral vector in which SV40 large T antigen was expressed from a Moloney murine leukemia virus promoter (MoMLV). The authors were able to passage the cells upto 21 times. In the present study sperm cells were transfected with pSV3-neo vector and the expression of T antigen was detected in a few cells.

Electroporation conditions for sperm cells could be standardized in the present study. Better expression of the luciferase gene was observed in lipofected sperm cells than in electroporated cells. Electroporation of penaeid shrimp zygote/embryo have been reported by Preston et al. (2000), Tseng et al. (2000), Arenal et al. (2004), Sun et al. (2005) and Arenal et al. (2008). Most of the workers (Tseng et al., 2000; Arenal et al., 2004; Arenal et al., 2008) have obtained better results by electroporation while Preston et al. (2000) and Sun et al. (2005) observed that microinjection and poyethylenimine mediated transfection worked better in shrimp zygotes than electroporation. Transgenic shrimp was developed by gene transfer in to zygote by various researchers (Tseng et al., 2000; Lu and Sun, 2005; Arenal et al., 2008). Taura syndrome resistance and growth enhancement were conferred in transgenic shrimp by transfection of antisense Taura syndrome virus coat protein (Lu and Sun, 2005) and tilapia growth hormone gene (Arenal et al., 2008), respectively.

Transfection of gametes is a method to generate transgenic animals (Esponda, 2005). The first experiment reporting transfection of male gamete was in rabbit (Brackett et al., 1971). Arezo (1989) demonstrated transfection of sea urchin spermatozoa, and Lavitrano et al. (1989) produced transgenic mice employing transfected spermatozoa. Transfection of spermatozoa was reported in abalone (Tsai et al., 1997), bivalved mollusks (Guerra et al., 2005), mussel (Kuznetsov et al., 2001) salmon (Sin et al., 1993; Symonds et al., 1994), zebrafish (Khoo et al., 1992; Patil and Khoo, 1996) and carp (Kang, 1999). Transfected sperm cells can be injected into the thelycum of mature female shrimp, which during spawning might form transgenic zygotes.

Further studies are required to confirm the viability and fertility of transfected spermatozoa and the development of transgenic animals. Gene transfer to sperm cells is only an initial step in the formation of transgenic shrimps.

Table 1. Luciferase assay of sperm cells 48 hours after lipofection with P2completete Fluc pGL3 basic

	Control (RLU)	Test (RLU)
	148	215
	123	162
	144	179
	124	164
Average	134.75	180
Standard deviation	13.10	24.54

Table 2. Luciferase assay of sf9 cells 48 hours after transfection with P2completete Fluc pGL3 basic

	Control (RLU)	Test (RLU)
	113	12,110
	141	14,096
	154	17,038
	137	14,660
Average	136.25	14,476.00
Standard deviation	17.11	2028.14

Table 3. Percentage viability of sperm cells in electroporation buffer with different osmolality

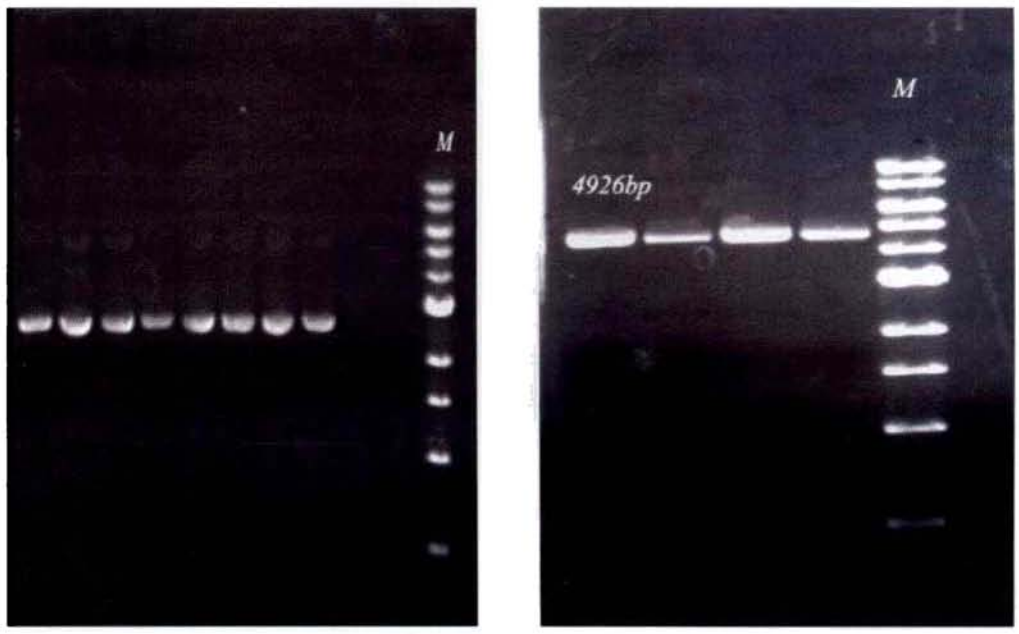
Osmolality of the buffer (mOsm)	Viability (%)
280	60
400	50.8
600	86
720	89

Table 4. Optimal conditions for electroporation of *P.monodon* sperm cells

Sl. No.	Conditions	Optimum
1	Osmolality of the buffer	600mOsm
	Cell density	
2	DNA	1×10^6
3	Cuvette gap width	$5\mu g\ ml^{-1}$
4	Voltage	2mm
5	Pulse length	440V
6	No. of pulses	40μs
7		1

Table 5. Luciferase assay of sperm cells transfected using P2completete Fluc pGL3 basic vector by electroporation

	Control (RLU)	Test (RLU)
	96	128
	102	118
	158	178
Average	118.67	141.33
Standard deviation	34.20	32.15



A B

Fig.3. A) 0.8% agarose gel showing the vector P2completete Fluc pGL3 basic isolated from *E.coli* strain JM109 B) Agarose gel showing the linearised plasmid digested with Hind III (M-1kb ladder)

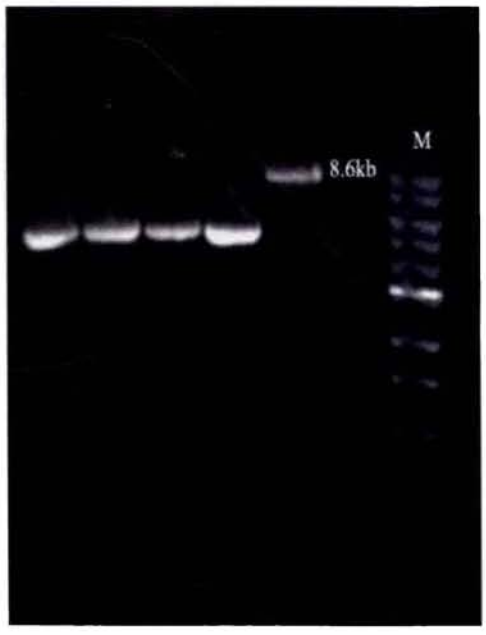
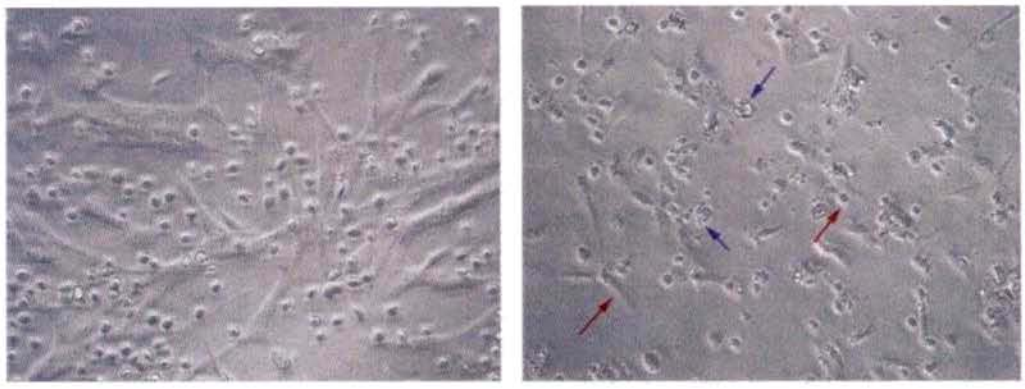
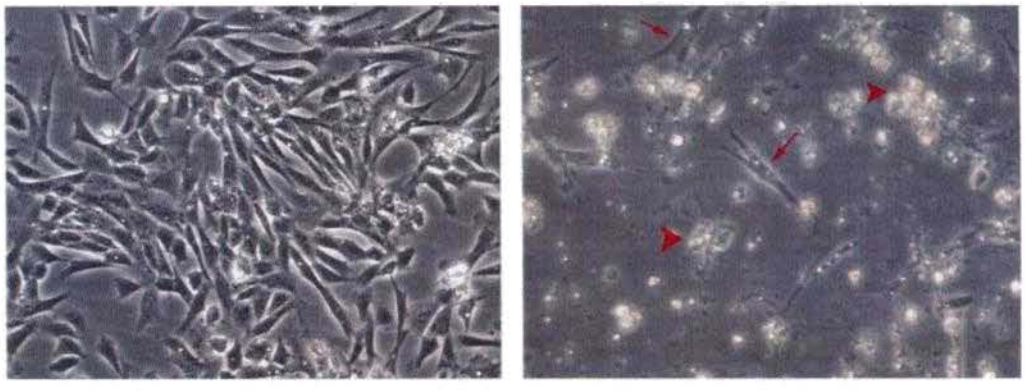


Fig.4. Agarose gel showing the vector pSV3-neo isolated from *E. coli* HB101 (lanes 1-4), vector linearised with Eco R1 (lane5) (M- 1kb ladder)



A B

Fig.5. Phase contrast micrographs showing lipofected haemocytes. (A) Control, (B) Live (red arrows) and dead (blue arrows) haemocytes 48 hours after transfection (40X)



A B

Fig.6. Primary lymphoid cell culture after lipofection. (A) Control, (B) Live (arrows) and dead (arrow heads) cells after lipofection (40X)

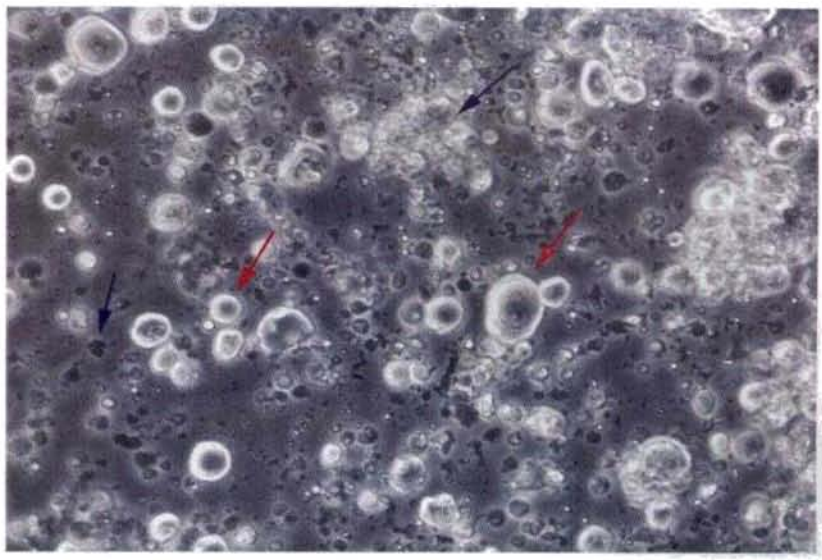
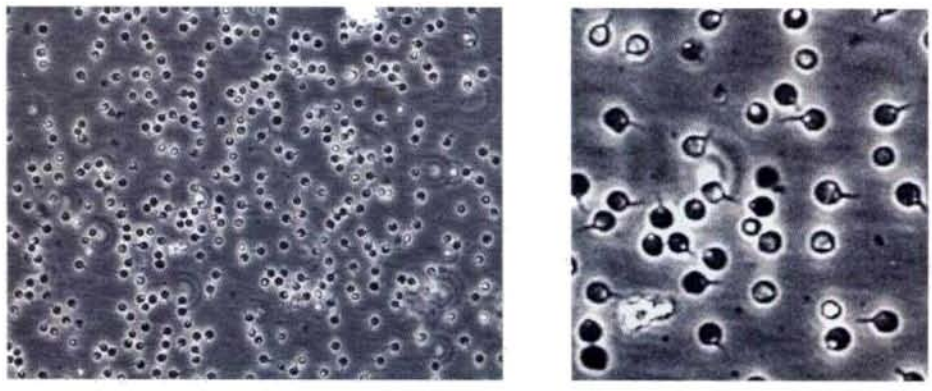


Fig.7. Live (red arrows) and dead (blue arrows) primary oocytes after lipofection with P2completete Fluc pGL3 basic vector (40X)



A B

Fig.8. A) Sperm cells of *Penaeus monodon* after transfection (20X), B) Enlarged image of the sperm cells showing spike, central cap region and posterior main body

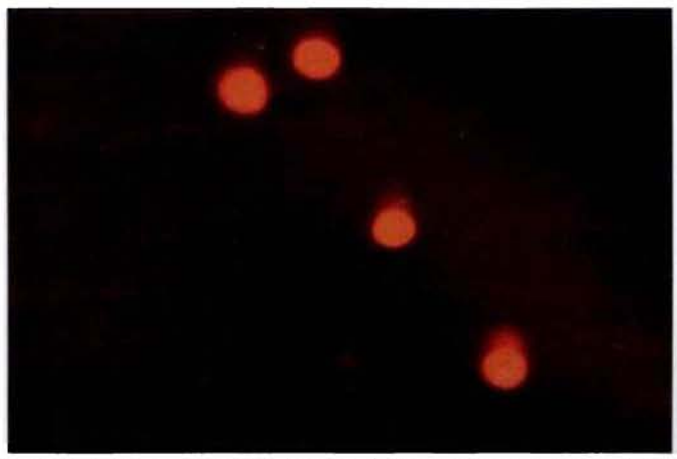


Fig.9. Sperm cells tested for "pore formation" by propidium iodide staining immediately after pulsing 440V/ 40µs (60X)

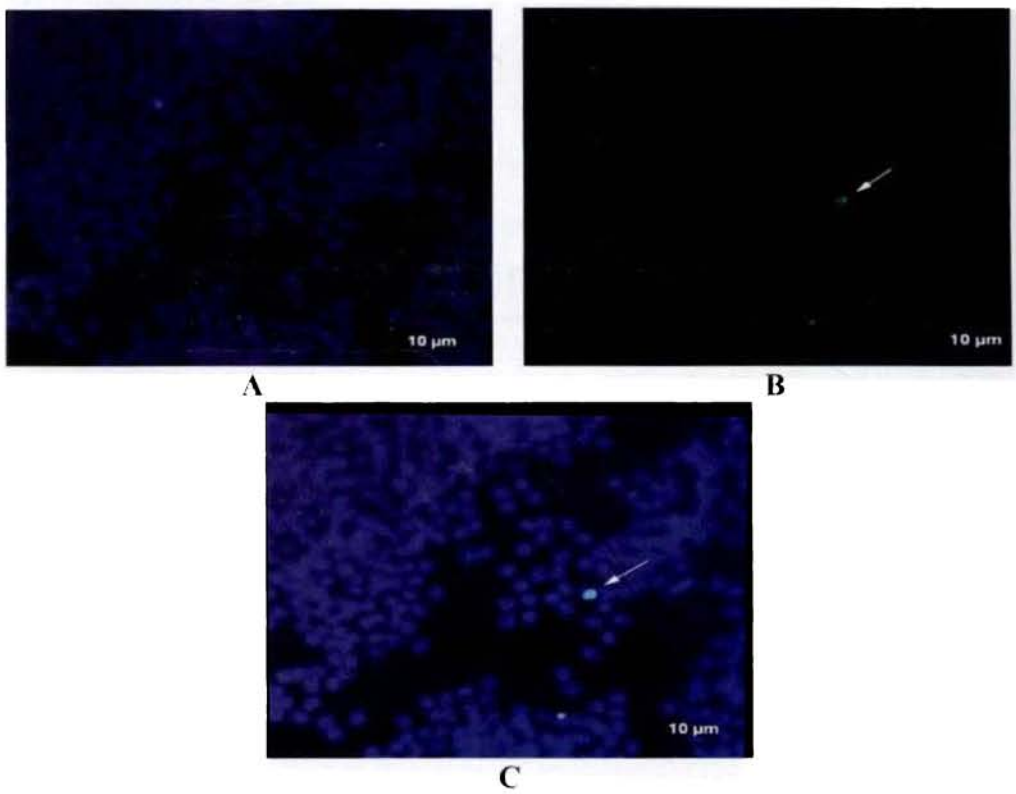


Fig.10. Immumunofluorescence detection of SV 40 large T antigen in pSV3-neo transfected sperm cells. A) Both tranfected and non transfected cells as seen under DAPI filter. B) Transfected cell with green fluorescence (arrow) as seen under FITC filter. C) Merge of A and B showing green positive cell (arrow) among nontransfected cells

Chapter 6

CONCLUSIONS AND SCOPE FOR FUTURE RESEARCH

Penaeid shrimp culture has taken up predominant position in aquaculture industry attaining globally the sixth position in terms of value among all taxonomic groups of animals cultivated (FAO, 2006). This is primarily due to the rapid growth of high density culture and its expansion in all shrimp growing Nations. Tragically, concomitant with this development has been the rapid spread of viral diseases over the shrimp farms worldwide causing severe financial losses. About 20 types of viruses have been reported in penaeid shrimp, among which the white spot syndrome virus (WSSV) has been responsible for the highest economic loss to the industry. Even though extensive investigations have been carried out on this virus, lack of a valid cell line has been hampering the progress of research in elucidating pathogenesis and morphogenesis as well as in the development of prophylactic and therapeutic measures. It is worth noticing that much before the onset of the white spot syndrome virus, there has been efforts in generating cell cultures from shrimp alike the attempts made in other animal groups. However, the difference has been that all such efforts made in shrimp cell line development were futile exercises, which, there by, has transformed the scenario most challenging.

The first chapter of the thesis deals with a detailed description of the efforts made world wide in establishing cell lines from shrimp in particular and crustacean in general. Ever since the first reported cell culture from *Penaeus monodon* by Chen et al. (1986), several researchers have been stepping in to the field and coming up with primary cell cultures from penaeid and non- penaeid species. In spite of these efforts no immortal cell line could be developed so far from shrimp and for that matter from any species of crustacean. It is in this background that the present study has been conceptualized and executed focusing on the development of cell culture systems from *Penaeus monodon* demonstrating their application in WSSV isolation and study, and also in using them as tools in testing cyto and genotoxicity of aquaculture management chemicals and environmental pollutants.

The subject matter in this thesis has been divided under the following heads:

1. Development of primary cell cultures from *Penaeus monodon* with special reference to lymphoid organ
2. Development of primary haemocyte culture from *Penaeus monodon* and its application in white spot syndrome virus (WSSV) titration and viral and immune related gene expression
3. Primary haemocyte culture of *Penaeus monodon* as model for cytotoxicity and genotoxicity studies
4. Transfection of *Penaeus monodon* primary cell cultures, primary oocytes and sperm cells in vitro

Overall achievements of this work are summarized as given below:

- Primary cell cultures from heart, ovary, muscle and lymphoid organ were developed from explants, which got attached to the culture vessel and cell migration started within a few hours. Superior performance of early stage immature ovaries was also noticed.
- Although explant cell cultures were obtained from various tissues and organs of penaeid shrimps, the cells from lymphoid organ formed monolayer more rapidly and remained stable for longer periods.
- Cell cultures from heart and lymphoid tissue were maintained for more than three weeks while ovarian and muscle primary cultures for two weeks and four days, respectively. Among them the epithelioid cells in lymphoid cell culture exhibited mitotic activity and were able to be subcultured once. However, this occurrence was sporadic and inconsistent.
- Among the various media tested the modified medium (L-15) containing Leibovitz's L-15 at double strength supplemented with 2% glucose, MEM Vitamins (1X), tryptose phosphate broth (2.95g l^{-1}),

20% FBS, 0.06µg ml^{-1} chloramphenicol, 100µg ml^{-1} streptomycin and 100 IU ml^{-1} penicillin was found to be the most suitable for the development and maintenance of lymphoid cell cultures.

- Among the cells generated from lymphoid, a very few fibroblastic cells were positive to BrdU assay demonstrating that DNA synthesis was limited to only such cells. This is suggestive of the fact that the monolayer formation from the explant of lymphoid tissue was mainly due to migration of cells from the explant, and not positively due to cell proliferation. However, the observed DNA synthesis has implications in their continued multiplication leading to immortalization.
- Poly-L-lysine coating of the culture vessel and addition of insulin like growth factor 1 (10ng ml^{-1}) were found to enhance the growth of lymphoid cell cultures.
- The lymphoid cell culture was susceptible to WSSV and as cytopathic effect shrinkage, rounding, accumulation of retractile granules and detachment of infected cells were the common features in the event. This was further confirmed by immunofluorescence detection assay employing monoclonal antibodies against WSSV.
- In the WSSV infected lymphoid cell cultures expression of WSSV early and late genes such as immediate early gene 1, protein kinase, thymidine kinase and thymidylate kinase, ribonucleotide reductase 1, endonuclease, DNA polymerase, Vp 28 and latency 1 gene could be detected. This has demonstrated the suitability of lymphoid cell cultures for WSSV isolation and the study on viral morphogenesis, as the cell culture supported the expression of virus' structural and functional genes.
- All the immune related genes (prophenoloxidase, astakine, peroxinectin, alpha 2-macroglobulin, haemocyanine, transglutaminase, crustin and penaeidin) studied except transglutaminase were expressed

in lymphoid cell culture. Haemocyanin, even though expressed in hepatopancreas and gets processed under acidic conditions to produce an antibacterial peptide, astacidine1, specifically during bacterial infection, was found to be weakly expressed in the WSSV infected *P. monodon* lymphoid cell culture.

- For the development of primary haemocyte culture, and its application in WSSV studies modified Leibovitz's L-15 medium containing L-15 at double strength supplemented with 2% glucose, MEM Vitamins (1X), tryptose phosphate broth (2.95 g l^{-1}), N-phenylthiourea (0.2mM), 20% FBS, 0.06µg ml^{-1} chloramphenicol, 100µg ml^{-1} streptomycin and 100 IU ml^{-1} penicillin was found to be suitable.
- Animal- wise variation was experienced in the response of the cells with respect to their lifespan. Metabolic activity of haemocyte did not vary considerably for 3 days and the activity started decreasing only then onwards. Advantageously, with in this period WSSV titration and cytotoxicity assays could be completed. Among the types of cells developed half turned out to be fibroblastic within three hours of incubation. This could be ascribed to the specificity of L-15 medium, which was known to support more the growth of fibroblastic cells. In this medium haemocytes appeared round to elliptical and small and large fibroblastic, with granules, which were viable upto 6-8 days.
- Several growth factors were tested on the heamocytes to investigate the efficacy in enhancing the longevity of the cells. Among them the vertebrate growth factor (IGF-2 at 50 ng ml^{-1}) alone had a significant effect on the culture.
- To determine the proliferation of the cultured haemocytes, incorporation of 5- bromo-2'-deoxyuridine (BrdU) was accomplished. At 24 hours 22±7.38

% of cells were found 5-bromodeoxyuridine (BrdU) positive suggesting DNA synthesis.

- In this study, haemocyte culture was successfully demonstrated as in vitro system for titrating WSSV by employing MTT_{50} method. The advantage of using haemocyte culture over explants as model for WSSV titration and cytotoxicity studies is the easiness in quantification of cells to be seeded to maintain uniform cell number in micro plates, a prerequisite for quantal assays, and the rapidity in obtaining monolayer of cells.
- MTT assay employed in the present study is a high throughput assay, which doesn't require manual assessment of cytopathic effect such as shrinkage and necrosis; instead metabolic activity of the cells is measured. Even though the MTT assay was successfully used in the titration of vertebrate viruses this had not been employed for titration of WSSV so far.
- Using MAbs, strong positive signals from the infected nucleus could be obtained after 72 hours of virus administration. Nevertheless, localized signals could be seen from cytoplasm and nucleus as well from 24hour onwards, a situation probably caused by the events such as phagocytosis and/or endocytosis.
- Expression of viral genes and immune related genes at the event of WSSV infection was investigated along with that of negative control and heat inactivated haemocytes.
- Viral gene expression could be categorized in to two such as those which got expressed and maintained more or less uniformly through out the study period of 48h (iel, pkl, tk-tmk,rrl, endonuclease), and those genes which were not expressed after 24^{th} hour (dnapol, Vp28 and latency 1). When Vp28 was found upregulated, the latency 1 was down regulated. None of the WSSV genes was expressed in control haemocytes without WSSV, and control cells with heat inactivated WSSV.

- Classification of the viral gene expression in to two categories points out the regulatory events in the viral multiplication which take place in the shrimp haemocytes. WSSV early genes, which are regulatory genes, are transcribed at the early infection stage.
- In the present study amplification of the immediate early gene 1, tk-tmk and rr1 from 2 h post infections and the other early genes such as protein kinase, DNA polymerase and endonuclease from 12 hour post infection suggested the sequence of events of viral DNA replication in the haemocytes. It has to be highlighted that Vp 28, a late gene, involved in the production of envelope protein could be expressed from 12-24 hours while the latency gene was down regulated. This is a clear indication of the virulence of WSSV to haemocytes by not having been entering in to a latent phase. In this context the detection of Vp28 by immunofluorescence in infected haemocytes accomplished in this study is a direct evidence of the viral multiplication in shrimp haemocytes.
- The study has suggested that primary haemocyte culture could be used to delineate the infectious mechanisms of the virus and its morphogenesis.
- Expression of immune genes was examined at the event of WSSV infection, and this included prophenoloxidase, astakine, peroxinectin, alpha 2-macroglobulin, haemocyanin, transglutaminase, crustin and penaeidin.
- Expression of these genes compared to that of the negative control could be categorized in to four, such as those which were up regulated at 2^{nd} h (prophenoloxidase, peroxinectin, penaeidin, alpha 2-macroglobulin, crustin and transglutaminase), maintained upregulated in the same order at 12^{th}h (peroxinectin, penaeidin, and crustin), at $24th^{th}$ h (Astakine), and at 48^{th} hour when heat inactivated WSSV was administered (prophenoloxidase, peroxinectin, alpha 2-macroglobulin

and penaeidin). Meanwhile haemocyanin and transglutaminase were found faintly expressed even though not detected in the controls.

- Immune response of haemocytes to heat inactivated WSSV is note worthy and supplements to the observation that shrimp can be protected by administering inactivated WSSV.
- The results have suggested that immune related genes were triggered immediately after exposure to WSSV and the increase in expression might result in an enhanced immune response to ward off the pathogens. However, subsequently, there was an apparent decrease in expression of most immune-related genes after initial hours of infection indicating that the heightened response is temporary or short lived in the event of WSSV infection in shrimp on considering the haemocyte culture as a model for WSSV infection. There was higher level of response of crustin to WSSV infection.
- This study demonstrates the potential of haemocyte culture as a tool for studying WSSV multiplication and morphogenesis, screening of antiviral formulations and its mode of action and the effectiveness of vaccination.
- Haemocyte cultures were demonstrated as tools for cytotoxixcity and genotoxicity determinations.
- Cytotoxicity is considered an important index for evaluating safety of antimicrobials and management chemicals prior to their administration in aquaculture. For its accomplishment primary haemocyte cultures have been demonstrated useful which out-rightly eliminates animal experimentation as part of the bioassays, as being followed now.
- Estimation of cytotoxicity of a shrimp health management chemical (benzalkonium chloride), an anti vibrio compound (N-methyl-1-hydroxyphenazine), and aquatic pollutants such as heavy metals

(cadmium chloride and mercuric chloride) and pesticides (malathion and monocrotophos) was done in primary haemocyte culture employing MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) assay.

- Benzalkonium chloride (BKC), a quaternary ammonium compound, widely used as broad spectrum disinfectant and at low concentration (0.1ppm) as immunostimulants and moderately higher (1ppm) to stimulate moulting in shrimp culture ponds, was assessed in primary haemocyte culture and found safe at 0.1mg l^{-1} level.

- The heamocyte culture was again applied for assessing cytotoxicity of N- methyl-1- hydroxyphenazine, a phenazine compound reported useful in managing *Vibrio* in aquaculture, the IC_{50} value of the compound was found to be 1.4 ± 0.31 mg L^{-1} and safe at <0.5 mg L^{-1}

- On undertaking a 12-hour exposure to heamocyte culture IC_{50} values for cadmium chloride and mercuric chloride were 31.09±16.27 µM (7.10± 3.72 mgL^{-1}) and 5.52±1.16 µM (1.5± 0.31mgL-1), respectively.

- At higher concentrations of Malathion and Monocrotophos (>61.7 mg l^{-1} and >166mgl^{-1} respectively) necrosis was observed having the IC_{50} value of Malathion being 59.94 ± 52.3 mg l^{-1} and that of Monocrotophos 186.76 ± 76.995 mg l^{-1}.

- Malathion was found to be non toxic below 6.86mg l^{-1}, and monocrotophos below 15.6mgL^{-1}.

- It has to be pointed out that the information on toxicity generated using whole animal is often insufficient in the sense that several of the toxic effects go on unnoticed which might have long term implications in the biotic community. In this context cytotoxicity studies help gather accurate information on the safe level of the toxicants/drugs which can be either discharged to the natural environment or to be administered in the health management of the aquatic animals.

- To demonstrate the application of haemocytes for determining genotoxicity of selected pollutants the cells were exposed to cadmium chloride (140μM), mercuric chloride (17μM), malathion (60 mg l^{-1}) and monocrotophos (186 mg l^{-1}) for 4 hours. These concentrations were chosen because more than 80% viability of cells was recorded after 4 hour exposure by trypan blue viability assay. In this experiment all compounds were found genotoxic and were found to induce DNA strand breakage and there by the formation of comets, assessed using the comet scoring software.
- Primary haemocyte and lymphoid cell cultures, primary oocytes and sperm cells were subjected for transfection to assess their suitability for in vitro transformation and for trangenesis.
- Transfection was attempted using a lipofection reagent, cellfectin with a luciferase gene reporter vector P2completete Fluc pGL3 basic vector, a pantropic modified luciferase reporter vector containing a modified coding region for firefly (*Photinus pyralis*) luciferase (*luc*) that has been optimized for monitoring transcriptional activity in transfected eukaryotic cells. The P2 promoter region of the IHHNV which causes "Runt Deformity Syndome" in penaeid shrimp.
- During this process cell death was evident in primary haemocyte and lymphoid cell cultures, which might be due to the toxicity of lipofection reagent, cellfectin. (1:1.5 (M/M) liposome formulation of the cationic lipid N, N^{I}, N^{II}, N^{III}-Tetramethyl- N, N^{I}, N^{II}, N^{III}-tetrapalmityl-spermine (TM-TPS), and dioleoyl phosphatidylethanolamine (DOPE) in membrane-filtered water).
- Sperm cells and oocytes withstood lipofection but luciferase expression was not significant in oocytes. Meanwhile, sperm cells exhibited significant expression, but 300 fold lesser than that of the insect cell line, Sf9.

- Subsequently, lipofection and electroporation of sperm cells with pSV3 neo vector was attempted and the sperm cells transfected were subjected to immunofluorescence assay to detect the SV 40 T antigen.
- Positive signals of green fluorescence were found in a few cells demonstrating the expression of SV 40 T antigen in lipofected cells having no fluorescence in the control.
- Gene transfer to sperm cells mediated by electroporation and lipofection employing P2completete Fluc pGL3 basic vector and pSV3-neo vector with SV 40 T antigen gene stands out to be the initial step towards development of transgenic shrimp.

Scope for future research

1. The Primary haemocyte culture can be used as the WSSV infection model to study the entire events starting from virus adsorption on to cell membrane, entry, and morphogenesis. Coupled with the expression of viral genes and immune related genes this model can be effectively employed for screening antiviral molecules and for studying their dose depended action.
2. Response of the heamocyte culture to inactivated WSSV in terms of expression of immune related genes points to the possibility of using this model for unraveling the cellular level response to inactivated WSSV as 'vaccine'.
3. As the haemocyte cultures and the lymphoid cell cultures were susceptible to WSSV, the same model can be used for isolating other shrimp viruses as well.
4. Cellular receptors involved in viral pathogenesis and the response of cells to viral infections can be analyzed using the haemocyte and lymphoid cell cultures.

5. Using the shrimp haemocyte model, cytototoxicity and genotoxicity of drugs, pesticides and other chemicals which are either used in aquaculture or passively enter in to the system can be evaluated.
6. Among all the tissues tested the cell cultures derived from lymphoid tissue showed at least a limited extend of cell proliferation, which can be exploited in attempting for immortalizing the cell cultures as cell lines.
7. Transfection of haemocyte cultures and lymphoid cell cultures with two vectors turned out to be failures suggesting that refined methods have to be evolved for accomplishment as a method for immortalizing the cell cultures.
8. Transfection of sperm cells could be accomplished in this study, which in turn stands out to be the initial step towards development of transgenic shrimp with disease resistance, faster growth and tolerance to varying environmental factors; the development of which would have far reaching positive implications in shrimp industry.

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T55