Chapter 1

GENERAL INTRODUCTION

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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)
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 (IHNV). 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-Kei 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., 2000a)

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 (0.01 mg 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 IU ml⁻¹ penicillin and 3000 µg ml⁻¹ streptomycin for 5-10 minutes (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 (300-350 ppm) for 5 min, prepared in autoclaved sea water (20 g l⁻¹). Subsequently washed with sterile sea water and dipped in cold ethanol (70%) for 2-3 minutes, 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 ovigerous *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\(^{-1}\); 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°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 mm³. 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 0.012 µg/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 50 ml perfusion medium (144 mM KCl; 5 mM KH$_2$PO$_4$; 1.2 mM NaHCO$_3$; 33 mM EDTA at 7.5 pH) with 0.2 g l$^{-1}$ streptomycin sulphate at a flow rate of 12 ml/min. The tissue was then carefully removed and transferred to a beaker containing 20 ml 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 4 ml volumes of each enzyme with incubation for 15 min to 12 h 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) 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 (27g/l) 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 protists, 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 100 μm. 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

<table>
<thead>
<tr>
<th>S.No</th>
<th>Reference</th>
<th>Penicillin</th>
<th>Streptomycin</th>
<th>Amphotericin B</th>
<th>Gentamycin</th>
<th>Fungizone</th>
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<td>10 µg ml⁻¹</td>
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*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; Toulllec et al., 1996; Mulford and Austin, 1998; Chen and Wang, 1999; Wang et al., 2000; Kumar et al., 2001; Shike et al., 2000*; Maeda et al., 2003 & 2004; Jiang et al., 2005) M199 (Ghosh et al., 1995; Toulllec 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; Toulllec 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. (2000b) supplemented the medium with 20% FCS. Meanwhile, Lang et al. (2002) used 20% FBS along with NaCl-11 g l⁻¹, KCl- 0.4 g l⁻¹, MgSO₄·7H₂O- 3 g l⁻¹, MgCl₂·6H₂O- 3.3 g l⁻¹, CaCl₂·2H₂O - 0.9 g l⁻¹, Na₂HPO₄·12H₂O- 0.1 g l⁻¹, HEPES- 2.38 g, L-Glutamine- 0.15 g, Lactalbumin Hydrolysate- 0.1 g, NaHCO₃- 2.2 g. The medium used by Maeda et al. (2003, 2004) consisted of 10% FBS, glucose 1 g l⁻¹, proline- 0.1 g l⁻¹, TC Yeastolate- 1 g l⁻¹ and lactalbumin hydrolysate- 1 g l⁻¹. Chen et al. (1986) added 18% FCS, 30% muscle extract, 0.006 g ml⁻¹ NaCl and 10% lobster haemolymph to the growth medium. The additives provided by Machii et al. (1988) consisted of 300 mg l⁻¹ glucose, 100 mg l⁻¹ lactalbumin hydrolysate and 20% FCS. At the same time Fan and Wang (2002) incorporated 15-20% heat inactivated FBS, 0.55 g l⁻¹ sodium pyruvate, 0.75 g l⁻¹ NaHCO₃, 2.0 g l⁻¹ chitosan, 100 μl/flask of nerve nodule extracts, and 12 g 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, 2 g l⁻¹ glucose, 2.4% NaCl, GIT medium, AKN salt solution and inorganic acid of MPS. Itami et al. (1999) used 11 g NaCl, 0.4 g KCl, 3 g MgSO₄·7H₂O, 3 g MgCl₂·6H₂O, 0.9 g CaCl₂·2H₂O, 0.05 g NaH₂PO₄·2H₂O, 0.15 g l⁻¹ glutamine and 1 g lactalbumin hydrolysate. Mulford et al. (2001) added 10% heat inactivated FBS, 5% Nephrops serum, 5% Nephrops muscle extract, 0.06 g l⁻¹ of L-proline, 1 g l⁻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⁻¹ 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% CO₂ 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 β 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., 2000a). 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 Whisposivirus). 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 whisposivirus 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 haematopoietic 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 Moorhead, 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., 2004a). 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) 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 SV40 large 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. (2002b) 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; 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