Animal cell culture, the concept of growth and maintenance of cells *in vitro* in a nutrient medium, started way back in 1907, when Harrison (Harrison, 1907) “Father of animal tissue culture”, succeeded in growing nerve tissue of frog in lymph clots. Three years after, Burrows (Burrows, 1910) cultured chick-embryo tissue, and in 1943 Earle *et al.* (1943) succeeded in developing primary cell culture from mouse fibroblast. All these success stories led to the development of the first continuous human cell line (HeLa) in 1952 (Gey *et al.*, 1952). These events generated a new wave of interest in cell and tissue culture research, and a new field of investigation was opened with an explosive expansion in biological sciences, during the second half of 20th century (Freshny, 2000). Today, human cell cultures, for that matter vertebrate cell lines, have emerged as one of the most powerful tools to address many fundamental questions in biology and medicine (Claydon, 2009). In contrast, development of *in vitro* models for invertebrates (especially marine invertebrates), which contribute to 95% of the animal kingdom (Ruppert and Barnes, 1994), is far less advanced due to elusive biological reasons. In this context, this chapter summarizes the advancements in cell culture development from crustaceans with the focus on the perception and orientation in shrimp cell culture, towards the attainment of stable cell lines.
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1.1. Crustacean cell culture

Crustacea constitute a class of animal species of biological interest for fundamental research and/or of high commercial value (Toullec, 1999). According to Johnson et al. (2008) a decapod crustacean, shrimp, represents one of the most economically important aquaculture species with a value of over 10 billion US $ annually. Unfortunately, in the mid 1990s the shrimp industry was struck with the white spot virus (WSV) leading to an economic loss of over 3 million US $ on annual basis (Lundin, 1995), and over 40% of world shrimp culture was affected (Lundin, 1995; Lotz, 1997). Even though enormous literature has been generated on WSV, understanding of the viral morphogenesis and development of an appropriate therapy and prophylaxis could not be accomplished due to the absence of a permanent cell line from shrimp or from a susceptible crustacean. Besides, such cell lines shall serve as excellent tools in toxicology as well. In addition, it can certainly be argued that there is a real need for development of in vitro techniques for aquatic invertebrate cell culture to ease pressures on wild stock to optimize growth condition (Mothersill and Austin, 2000). The published report on cell culture by Quiot et al. (1968) is considered as the first active long-term cell culture from a crustacean (Mothersill and Austin, 2000).

Despite the absolute requirement of a permanent in vitro model from this group of animals (Spann and Lester, 1997), till date, no authentic and reproducible cell culture system, from any aquatic invertebrate has been reported. Further, while comparing with mammalian cell culture techniques, culture methods available for invertebrates are under developed, even for the maintenance of primary cell cultures in vitro (Mothersill and Austin, 2000). This is mainly because specific culture media for crustacean cells have not been developed (Toullec, 1999), for the formulation of which it is imperative to consider biochemistry of various species and the requirement of each cell/tissue type in vitro.
1.2. Cell culture from shrimp: an economically important crustacean

Development of continuous shrimp cell lines has ever been a challenging task, for a long period of over 25 years (Jayesh et al., 2012). However, it still remains unattained presenting researchers more questions than answers (Chen et al., 1986; Owens and Smith, 1999). The in vitro cell culture system helps to analyze the interrelated environmental and pathogenic factors that interact with genetic and physiological traits of the cultured animals and to acquire knowledge for health protection and disease management in aquaculture (Villena, 2003). Moreover, primary cell cultures obtained from various organs / tissues represent the first step towards the establishment of cell lines and they provide useful information concerning the most suitable cell culture conditions involved in the survival and proliferative capacity of various tissues (Toullec, 1999). However, as on today, no permanent cell line could be made available from marine invertebrates in general (Rinkevich, 2011) and shrimp in particular (Jayesh et al., 2012). The major fall out of the situation is the impediment which it imposes on the isolation of crustacean viruses (Claydon, 2009; Jose, 2009; Claydon et al., 2010b). The fact is that the requirement of continuous cell lines is so high to investigate the radiating viral threats to shrimp aquaculture (Flegel, 2006; Walker and Winton, 2010; Zwart et al., 2010).

In the realm of cell line development, despite the current advancements in decoding the nutritional requirements of cells in vitro, molecular approaches at genomic level for transformation and immortalization of shrimp cells remain unknown and un-attempted. This might be due to the lack of information on the molecular mechanisms that inhibit neoplastic transformations in shrimp. Besides, tumours have only rarely been observed in the decapod crustaceans (Vogt, 2008). Therefore, a thread bear analysis on the very successful history of insect and mammalian cell line development might open up new vistas for focused research towards establishment of shrimp cell lines. Moreover, uncovering the underlying molecular and regulatory mechanisms of the absence of neoplasia and carcinoma in
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shrimps might provide new leads for the development of anti-ageing and anti-cancer interventions in humans as well (Vogt, 2011).

1.2.1. The history of shrimp cell culture

The earliest attempts on shrimp cell culture development appeared as published document in 1986 by Chen and colleagues from National Taiwan University, Taiwan (Chen et al., 1986). They had chosen Penaeus monodon as the species of choice from which several cell culture systems could be generated using various tissues and organs. Three years after the first publication in shrimp cell culture, in 1989, researchers (Chen et al., 1989) published an attempt of shrimp cell culture from P. penicillatus and on the same year first report on the susceptibility of primary lymphoid cell culture to monodon-type baculovirus was published (Chen and Kou, 1989). This is considered as the first report on in vitro cultivation of penaeid virus in shrimp cell culture. Even though only limited success could be obtained, several researchers commenced attempting to develop cell cultures from various tissues and organs of different species of penaeids (Ke et al., 1990; Rosenthal and Diamant, 1990; Luedeman and Lightner, 1992; Nadala et al., 1993; Ghosh et al., 1995; Hsu et al., 1995; Tong and Miao, 1996; Sano, 1998; Itami et al., 1999; Kasorchandra et al., 1999; West et al., 1999; Kumar et al., 2001; Fan and Wang, 2002; Chun-Lei et al., 2003; Maeda et al., 2003), and this included test of their susceptibility to shrimp viruses as well (Lu et al., 1995a; Maeda et al., 2004; Jiang et al., 2005). In 2000, report on the ultra structure of white spot syndrome virus (WSSV) grown in primary lymphoid cell culture was published (Wang et al., 2000), however, its morphogenesis could not be fully elucidated for want of certified shrimp cell lines. To date the morphology and ultrastructure of WSSV have not been fully understood, however, several characteristics of this virus have emerged in recent years (Sa`nchez-Paz, 2010). In addition to the effort on spontaneous transformation and immortalization by continuous maintenance and repeated passage of the cells in vitro and the 'organized neglect' (Grace, 1982) in
the process of cell culture development, in the year 1995 researchers attempted to induce transformation in shrimp cells by transfection with oncogene (Tapay et al., 1995). Accordingly, in 2000 first transgenic expression in shrimp cells could be accomplished (Shike et al., 2000a) followed by the development of vesicular somatitis virus – glycoprotein (VSV-G) pseudotyped retroviral vectors (Hu et al., 2008) and their successful integration in shrimp primary cell culture genome (Hu et al., 2010). However, this also did not lead to immortalization of cell cultures. The lack of success in spontaneous and induced cell line development subsequently paved the way for the attempts on developing fusion cell line (Claydon, 2009; Claydon et al., 2010b) that too, with little success. More recently, researchers succeeded in viral gene expression (Jose et al., 2010), determinations of cytotoxicity and genotoxicity (Jose, 2009; Jose et al., 2011), viral multiplication (George et al., 2011), and immune gene expression (Jose et al., 2012) employing primary cell culture systems developed from different species of penaeids.

1.2.2. Animal species used in shrimp cell culture trials - a major concern

Since the first attempt on shrimp cell line development, performed in 1986 by Chen et al. (1986), P. monodon remained the best sought-after candidate species among all penaeids in the development of cell cultures; may be due to its availability in all South East Asian Countries and its popularity as the most widely cultured species. Of the 50 selected publications, 17 reported (34%) P. monodon (Chen et al., 1989; Hsu et al., 1995; Chen and Wang, 1999; Fraser and Hall, 1999; Kasornchandra et al., 1999; West et al., 1999; Wang et al., 2000; Manohar et al., 2001; Roper et al., 2001; Uma et al., 2002; Assavalapsakul et al., 2003, 2005, 2006; Catap and Nudo, 2008; Claydon et al., 2010b; Jose et al., 2010, 2011), as the species of choice, eight researchers (16%) used P. japonicus (Machii et al., 1988; Sano, 1998; Chen and Wang, 1999; Itami et al., 1999; Lang et al., 2002a, 2004; Maeda et al., 2003, 2004), seven (17%) selected P. chinensis (Tong and Miao, 1996; Huang et al., 1999; Fan and Wang, 2002; Chun-Lei et al., 2003; Jiang
et al., 2005; Hu et al., 2008, 2010), and P. vannamei (Ellender et al., 1992; Luedeman and Lightner, 1992; Nadala et al., 1993; Lu et al., 1995b; Toullec et al., 1996; George and Dhar, 2010; George et al., 2011). Moreover, six authors (16%) selected P. stylirostris (Luedeman and Lightner, 1992; Nadala et al., 1993; Tapay et al., 1995; Lu et al., 1995a; Shike et al., 2000a; Shimizu et al., 2001), as the donor animal of tissues and organs. Besides, in two publications (4%) P. indicus (Toullec et al., 1996; Kumar et al., 2001) and P. aztecs (Ellender et al., 1992; Najafabadi et al., 1992) were the species used. There is only one report (2%) of using P. penicillatus (Chen and Wang, 1999), for extracting tissues and organs for cell culture development (Fig. 1). This indicated that the species selection was based on availability and personal choice and not on the basis of any advantage which one might obtain by selecting a species.

![Graphical representation of trends in selection of penaeid species used for cell culture development (% of the 50 selected publications)](image)

Fig. 1. Graphical representation of trends in selection of penaeid species used for cell culture development (% of the 50 selected publications)

1.2.3. *Penaeus monodon* an economically important penaeid shrimp

More than 360 species of finfish and shellfishes are being cultured worldwide and around 25 of them are of high value and traded globally (FAO, 2010). A successful harvest has always been very much encouraging, and this has
spurred the expansion of aquaculture production in terms of area and geographical range. Shrimp continues to be the largest single commodity in terms of value, accounting for 15 percent of the total fishery products traded internationally (FAO, 2006), and *P. monodon* (Fig. 2) is the highly preferred penaeid species (Pechmanee, 1997), in South East Asian countries including India (Sudheer, 2009). Meanwhile, intensive aquaculture practices globally, since 1990, paved the way for the spread of shrimp viral diseases resulting in severe damage to the industry (Bachère, 2000; Valderrama and Engle, 2004). However, according to ‘FAO Status of World Fisheries and Aquaculture, 2010,’ in the year 2008, the capture fisheries and aquaculture production of decapods was 10,230 tonnes, corresponding to 41 billion US $ (FAO, 2010; Vogt, 2011). This trend in production is unlikely to perpetuate, because there are more than 20 (Bonami, 2008) among the 1100 recognized invertebrate viruses (Adams, 1991) now known to occur in shrimps which include nine that pose serious threat to their culture (Flegel, 2006; Claydon *et al*., 2010a; Walker and Winton, 2010; Lightner, 2011). Altogether, considering the emerging viral threat on this economically important food commodity, development of a permanent shrimp cell line to bring out effective strategies to combat the viruses is pivotal.

*Fig. 2. Adult Penaeus monodon*
1.2.4. Most commonly used medium for shrimp cell culture

Despite the necessity of an exclusive medium for shrimp cell culture, several researchers, over decades, have been modifying commercially available media to suit the requirements of shrimp cells in vitro (Roper et al., 2001; Claydon, 2009; Jose, 2009). Among the commercial media used, Leibovitz’s – 15 (L-15) has been the most popular one for shrimp cell culture. Of the 50 selected publications 32 (64%), were based on L-15 as the basal medium (Chen et al., 1986, 1988, 1989; Fuerst et al., 1991; Ellender et al., 1992; Najafabadi et al., 1992; 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; Shike et al., 2000a; Wang et al., 2000; Kumar et al., 2001; Manohar et al., 2001; Roper et al., 2001; Shimizu et al., 2001; Uma et al., 2002; Chun-Lei et al., 2003; Jiang et al., 2005; Assavalapsakul et al., 2003, 2005, 2006; Catap and Nudo, 2008; Hu et al., 2008; Claydon et al., 2010b; Jose et al., 2010, 2011, 2012), six (12%) selected Grace’s Insect Medium (Luedeman and Lightner, 1992; Nadala et al., 1993; Toullec et al., 1996; Wang et al., 2000; George and Dhar, 2010; George et al., 2011), five (10%) M199 (Ghosh et al., 1995; Toullec et al., 1996; Itami et al., 1999; Shimizu et al., 2001; Lang et al., 2002b), and three (6%) MPS (Tong and Miao, 1996; Fan and Wang, 2002; Hu et al., 2010). A couple of other media such as Pj-2 (Machii et al., 1988), NCTC 135 (Wang et al., 2000), MM Insect medium and TC 100 medium (Nadala et al., 1993) were also tested for the development of cell lines from shrimp (Fig. 3). However, it was rather inappropriate to point out any medium mentioned above as the most effective one as it has been a personal choice. This highlights the importance of a new medium exclusively for shrimp cell culture.
1.2.5. Organic and inorganic supplements added to improve growth of shrimp cells *in vitro*

Considering the inadequacy of the available growth media several attempts have been made to improvise the composition by adding supplements in isolation as well as in multiples. Several investigators selected crustacean body fluids and extracts for improving the basal medium. Among them shrimp extract was the popular one with varying concentrations such as 4% (Lu *et al*., 1995a), 8% (Nadala *et al*., 1993; Tapay *et al*., 1995), 10% (Chen *et al*., 1989; Toullec *et al*., 1996; George and Dhar, 2010; George *et al*., 2011), 27% (Kumar *et al*., 2001) and 30% (Chen *et al*., 1986). Haemolymph of lobsters at 10% (Chen *et al*., 1986) was also used. Moreover, ovary extracts (Chen and Wang, 1999), chitosan and nerve nodule extracts (Fan and Wang, 2002) were also incorporated in the medium as growth promoting factors. Fetal bovine serum (FBS) / fetal calf serum (FCS) as the supplements with a concentration 10% (Luedeman and Lightner, 1992; Lang *et al*., 2002a; Maeda *et al*., 2003, 2004; George and Dhar, 2010; George *et al*., 2011), 15% (Assavalapsakul *et al*., 2003, 2005, 2006), 18% (Chen *et al*., 1986) and 20%
(Machii et al., 1988; Nadala et al., 1993; Lu et al., 1995; Tapay et al., 1995; Shike et al., 2000a; Wang et al., 2000; Fan and Wang, 2002; Jiang et al., 2005; Hu et al., 2010; Jose et al., 2011, 2010) were added as the source of minerals, proteins, lipids, hormones (Freshney, 2000) and as the growth-promoting substances (Mitsuhasi, 2002). Considering the importance of inorganic salts for the maintenance of ionic balance and osmotic pressure (Mitsuhasi, 1989), researchers have used KCl, MgSO₄, MgCl₂, and CaCl₂ at concentrations ranging from 0.9 to 3 g l⁻¹ to supplement the required quantity in the growth medium (Luedeman and Lightner, 1992; Itami et al., 1999; Lang et al., 2002a). Moreover, to adjust osmolality, NaCl at a concentration ranging from 6 to 12 g l⁻¹ (Chen et al., 1986; Luedeman and Lightner, 1992; Fan and Wang, 2002; Jiang et al., 2005) has also been added besides the balanced salt solutions (Tapay et al., 1995; Jiang et al., 2005).

Addition of vitamins (Jose et al., 2010, 2011), proline (Luedeman and Lightner, 1992; Toullec et al., 1996; Maeda et al., 2003, 2004) and glutamine (Ghosh et al., 1995; Toullec et al., 1996) has been proven to be the choice of supplements in the growth media. In addition, lactalbumin hydrolyzate at a concentration of 0.1-1 g l⁻¹ (Machii et al., 1988; Itami et al., 1999; Lang et al., 2002b; Maeda et al., 2003, 2004; Assavalapsakul et al., 2003, 2005, 2006), tryptose phosphate broth at 2.95 mg ml⁻¹ (Jose et al., 2010, 2011) and TC Yeastolate at 1 g l⁻¹ (Maeda et al., 2003, 2004) have also been used as the source of peptides, amino acids and carbohydrates. As the additional energy source 0.3-2 g l⁻¹ glucose (Machii et al., 1988; Maeda et al., 2003, 2004; Jiang et al., 2005; Jose et al., 2010, 2011) and 0.55 g l⁻¹ sodium pyruvate (Fan and Wang, 2002) have also been supplemented. Buffering agents such as HEPES (Ghosh et al., 1995; Toullec et al., 1996; Lang et al., 2002a) and NaHCO₃ have been incorporated by many researchers (Luedeman and Lightner, 1992; Ghosh et al., 1995; Fan and Wang, 2002; Lang et al., 2002b). Growth factors such as epidermal growth factor (EGF)
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at a concentration 20-30 ng ml⁻¹ (Nadala et al., 1993; Lu et al., 1995a; Tapay et al., 1995) and 10 U ml⁻¹ of human recombinant interleukin-2 (Tapay et al., 1995) have been used to improve the proliferation of cells in vitro. All these modifications have led to improvisation of growth media with enhancement in growth and multiplication of primary cell cultures, but have never lead to establishment of any cell line.

1.2.6. Tissues and organs used for shrimp cell culture development

Ovary and the lymphoid tissue were the most commonly used donor tissues for cell culture development. Of the 90 selected experiments with 15 different tissues, 20 were conducted with lymphoid tissue (Chen et al., 1986, 1989; Najafabadi et al., 1992; Nadala et al., 1993; Hsu et al., 1995; Lu et al., 1995a, 1995b; Tapay et al., 1995; Tong and Miao, 1996; Chen and Wang, 1999; Itami et al., 1999; West et al., 1999; Wang et al., 2000; Lang et al., 2002a, 2004; Assavalapsakul et al., 2003, 2005; Catap and Nudo, 2008; Hu et al., 2008; Jose et al., 2012) and 18 with ovary (Chen et al., 1986, 1988, 1989; Luedeman and Lightner, 1992; Nadala et al., 1993; Tong and Miao, 1996; Mulford and Austin, 1998; Chen and Wang, 1999; Itami et al., 1999; Toullec, 1999; West et al., 1999; Shike et al., 2000; Shimizu et al., 2001; Lang et al., 2002a; Maeda et al., 2003, 2004; Hu et al., 2010; George and Dhar, 2010). Ten experiments were with haemocytes (Ellender et al., 1992; Ghosh et al., 1995; Chen and Wang, 1999; Itami et al., 1999; Jiang et al., 2005; Claydon et al., 2010b; George and Dhar, 2010; Jose et al., 2010, 2011; George et al., 2011), four with eyestalk (Tong and Miao, 1996; Mulford and Austin, 1998; Kumar et al., 2001; George and Dhar, 2010). Besides, testis (Mulford and Austin, 1998; Toullec, 1999), heart (Chen et al., 1986; Mulford and Austin, 1998; Tong and Miao, 1996; Chen and Wang, 1999; Lang et al., 2002a), hepatopancreas (Chen et al., 1986, Machii et al., 1988; Najafabadi et al., 1992; Ghosh et al., 1995; Mulford and Austin, 1998; Toullec, 1999; Wang et al., 2000; Manohar et al., 2001; Lang et al., 2002a), 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., 2002a; Chun-Lei et al., 2003), muscle (Chen et al., 1986; Lang et al., 2002; George and Dhar, 2010), hematopoietic tissue (Chen et al., 1988; West et al., 1999; Mulford et al., 2001), embryonic tissue (Tong and Miao, 1996; Toullec et al., 1996; Fan and Wang, 2002); epidermis (Toullec et al., 1996; Toullec, 1999) gut (Chen et al., 1986; Mulford and Austin, 1998) and Y organ (Toullec, 1999) were also widely used for cell culture development (Fig. 4). Among the tissues used, the most advancement was obtained from lymphoid and ovarian tissue only.

**Fig. 4.** Trends in various tissues used for shrimp cell culture development. LY-Lymphoid, OV-Ovary, HC-Haemocytes, ES-Eye stalk, TS-Testis, HT-Heart, HP-Hepatopancreas, GL-Gill, NR-Nerve, ML-Muscle, HPT-Haematopoietic tissue, ET-Epidermis, GT-Gut, YO-Y organ (results from 90 experiments)
1.2.7. Longevity and sub-culturing of the shrimp cell culture

The ultimate objective of every shrimp cell culture development programme was the establishment of corresponding cell lines. However, this objective has not been achieved so far. Even though not able to be sub cultured, various researchers could maintain cell cultures for different duration. Accordingly, researchers could maintain ovarian cell culture for 66 days (George and Dhar, 2010), 45 days (Maeda et al., 2003), 20 days (Chen and Wang, 1999), 10 days (Luedeman and Lightner, 1992) and to several months (Tong and Miao, 1996; Toullec et al., 1996) along with single passage (Mulford and Austin, 1998), and 3 passages (Chen et al., 1986; Chen and Wang, 1999). Lymphoid cell cultures were reported to be passaged 2 times (Chen et al., 1989) 3 times (Chen and Wang, 1999), and maintained for 54 days (Itami et al., 1999), 20 days (Chen and Wang, 1999), and for a period greater than 3 weeks (Nadala et al., 1993) to 3 months (Tong and Miao, 1996). However, Hsu et al. (1995) claimed to have attained more than 90 passages for a lymphoid organ cell culture which was later reported as Thraustochytrid contamination by Rinkevich (1999). At the same time Tapay et al. (1995) reported to have attained even 44 passages of lymphoid cell culture. With eye stalk cell culture, several workers reported to have maintained them for 12 days (George and Dhar, 2010), 3 months and attained 4 passages (Kumar et al., 2001). Besides, haemocyte cultures were maintained for 48 days (George and Dhar, 2010), 20 days (Jiang et al., 2005), 10 days (Itami et al., 1999), 8 days (Jose et al., 2010, 2011), and 4 days (Chen and Wang, 1999). Embryonic cell cultures could be maintained for several months (Toullec et al., 1996) and attained 10 passages (Fan and Wang, 2002). Moreover, researchers could maintain nerve cells for 15 days (Chun-Lei et al., 2003) and up to 3 months (Nadala et al., 1993), heart tissue for 4 days (Chen and Wang, 1999) and hepatopancreas for 30 days (George and Dhar, 2010). The striking observation was that there existed no consistency in the number of days which a cell culture could be maintained by different workers.
1.2.8. Virus susceptibility tests in various shrimp cell culture system.

Lymphoid organ cell culture system from penaeid shrimp has been claimed as the best option for *in vitro* growth of several pathogenic viruses. Many researchers claimed the *in vitro* growth of monodon-type baculovirus in lymphoid cell culture from *Penaeus monodon* (Chen and Kou, 1989; Catap and Nudo, 2008). Susceptibility of Yellow head virus in lymphoid cell culture from *P. monodon* (Chen and Wang, 1999; Assavalapsakul *et al.*, 2003, 2006; Tirasophon *et al.*, 2005), *P. japonicus* and *P. penicillatus* (Chen and Wang, 1999), and from *P. vannamei* (Lu *et al.*, 1995a, 1995b) have been reported. Moreover, Lu *et al.* (1995b) suggested the *in vitro* growth of yellow head virus in cell culture from nine different tissues and organs including gill, hepatopancreas, head soft tissue, abdominal muscle, eyestalk, lymphoid organ, heart, nerve cord and midgut. Susceptibility of white spot syndrome virus (WSSV) in lymphoid cell culture from *P. monodon* (Wang *et al.*, 2000; Jose *et al.*, 2012) from *P. monodon, P. japonicus* and *P. penicillatus* (Chen and Wang, 1999), ovarian cell culture from *P. japonicus* (Maeda *et al.*, 2003), hepatopancreatic cell culture from *P. monodon* (Uma *et al.*, 2002) haemocytes from *P. chinensis* (Jiang *et al.*, 2005) have also been reported. Recently, Jose *et al.* (2010) conducted a detailed investigation on the viral titration and viral gene expression in *P. monodon* haemocyte culture. Still more recently, George *et al.* (2011) investigated the multiplication of Taura Syndrome Virus (TSV) in haemocytes from *P. vannamei*. In spite of the successful attempts by several researchers to grow a few shrimp viruses in cell culture systems from penaeids, strangely enough there has not been any attempt by other laboratories either to validate the methodology or to use them as the protocol for shrimp virus cultivation. However, with the available techniques it is possible to generate and maintain primary cell cultures from shrimp and use them for virus titration and viral gene expression.
1.3. Lymphoid organ cell culture- a promising *in vitro* system

Lymphoid organ was first described in *P. orientalis* by Oka (Oka, 1969) and found exclusively in penaeid prawns, and do not possess in other crustaceans such as crabs, lobsters and crayfish (Rusaini and Owens, 2010). Lymphoid organ consisted with two distinct lobes located dorso-anterior to the ventral hepatopancreas (Bell and Lightner, 1988) in the cephalothoracic region of the shrimp (Fig. 5a & 5b). Each lobe is composed of two parts: lymphoid tubules and interstitial spaces, permeated with haemal sinuses filled with large numbers of haemocytes (Duangsuwan *et al.*, 2008). Histology (Fig. 6a & 6b) and three dimensional organization of lymphoid organ were also well studied (Duangsuwan *et al.*, 2008).

![Fig.5a. Cephalothoracic region of the P. monodon showing lymphoid organ (arrow) and Hep-hepatopancreas (Jose, 2009).](image-url)
Fig. 5b. Lymphoid organ removed from *P. monodon*. A: Two lobes of lymphoid organ on standard glass slide; B: lymphoid organ under light microscope (4x magnification).

Fig. 6a. Overall longitudinal view of the lymphoid organ and surrounding tissue of *P. monodon* female, H & E stain, scale bar = 200 µm. Ag: antennal gland; Gs: gastric sieve; Hp: hepatopancreas; Mus: muscle; Ov: ovary and LO: Lymphoid organ (Rusaini, 2006).
Lymphoid cells were found to be susceptible to most of the viruses such as; Lymphoidal parvo like-virus (Owens et al., 1991), Monodon-type baculovirus (Chen and Kou, 1989; Catap and Nudo, 2008), Spawner-isolated mortality virus (Fraser and Owens, 1996), White spot syndrome virus (Chen and Wang, 1999; Wang et al., 2000; Rodriguez et al., 2003; Jose et al., 2012), Yellow head virus (Chantanachookin et al., 1993; Lu et al., 1995a, 1995b; Chen and Wang, 1999; Assavalapsakul et al., 2003, 2006; Tirasophon et al., 2005), Lymphoid organ virus (Spann et al., 1995), Taura syndrome virus (Hasson et al., 1999), Infectious myonecrosis virus (Tang et al., 2005), Mourilyan virus (Rajendran et al., 2006), Laem-Singh virus (Sritunyalucksana et al., 2006), Rhabdovirus of penaeid shrimp (Nadala et al., 1992), and Lymphoid organ vacuolization virus (Bonami et al., 2006).
In addition, viral proteins in infected lymphoid cells were also successfully detected by immunofluorescence using specific antibodies (Wang et al., 2000; Jose et al., 2012). Jose et al. (2012) used lymphoid cell culture system from *P. monodon* for studying WSSV mediated viral and immune gene expression. The same system was also used for studying the BrdU incorporation and for determining the metabolic activity using MTT assay. Lang et al. (2002a, 2002b) confirmed and recorded the mitotic division in lymphoid cells *in vitro*. Shike et al. (2000a) confirmed that lymphoid cell culture system could be used for foreign gene expression. Altogether, as the lymphoid organ probably was a prime target and site for replication of most systemic viruses (Rusaini and Owens, 2010) and confirmed to be useful for cellular and molecular studies, the development of an immortal cell line as a ‘model *in vitro* system’ from lymphoid organ will provide more acceptance than any other cell type from *P. monodon*.

### 1.4. Importance of ‘specific’ medium for shrimp cell culture - a stepping stone for cell line development

Several hindrances stand on the way of the development of shrimp cell lines. One among them is the unsettling fact of an appropriate shrimp cell culture medium, that the media used for shrimp cell culture development have been mostly the modified commercially available preparations, despite the fact that the media composition happens to be the most important factor which determines the success of any cell line development (Mitsuhashi, 2001). To date, a medium exclusively for *in vitro* growth of shrimp cell cultures has not been designed, and the fact that an appropriate medium is required to establish shrimp cell lines in tune with the quantum change which the Grace’s insect cell culture medium (Grace, 1958, 1962, 1982, Grace and Brzostowski, 1966) has brought about; ever since the publication of Grace’s insect cell culture medium, over 500 insect cell lines could be established (Lynn, 2001; Smagghe et al., 2009). Likewise, to formulate an exclusive shrimp cell culture medium, in-depth analysis of the biochemistry of
body fluids (Najafabadi et al., 1992; Shimizu et al., 2001) is the prime requirement. Moreover, to tide over the difficulties in developing a complete medium for shrimp cell culture, attention must be directed towards satisfying the nutritional requirements of each cell type.

1.5. Molecular approaches for in vitro transformation of shrimp cells and its immortalization

Given the tremendous advancements in human and veterinary virology thanks to the availability of a variety of cell lines, any radical change in crustacean virology would be possible only if appropriate cell lines for in vitro cultivation of intracellular pathogenic agents (Claydon and Owens, 2008) could be made available. Considering the past experience in this realm more focus should be on the molecular approaches to immortalize shrimp cells by disrupting cell cycle regulator genes and the telomere maintenance. Usually somatic cells do not spontaneously immortalize in culture, but instead enter replicative senescence after a finite number of population doublings (Hayflick and Moorhead, 1961; Hayflick, 1965). In contrast to mammals and most insects, decapod crustaceans can enlarge their organs in the adult life period and regenerate lost appendages, organs with indeterminate growth (Vogt, 2011). The high regeneration capabilities of the crustacean cells (including shrimp) do not show neoplastic transformation and thus it prevents spontaneous immortalization. Neoplastic transformation can be achieved by transfection with active oncogenes (Ratner et al., 1985), the technique which has not yet been fully applied to crustacean and aquatic invertebrate cells (Claydon and Owens, 2008). Moreover, unveiling the molecular and regulatory mechanisms that prevent neoplastic transformation in shrimp cells (decapod crustaceans) might provide new leads for the development of anti-ageing and anti-cancer interventions in humans (Vogt, 2011).

To date, oncogenic mammalian virus gene, simian virus 40 large T (SV40-T) antigen (Tapay et al., 1995; Hu et al., 2008, 2010) has only been used for
transformation of primary shrimp cell culture. The first transformation attempt in lymphoid organ primary cell culture of _P. stylirostris_ was made in 1995 (Tapay _et al._, 1995) with the pSV-3 neo plasmid vector encoding SV40-T antigen gene from Simian virus-40 by lipofection. Although, Tapay _et al._ (1995) claimed three transformed cells (OKTr-1, OKTr-23 and OKTr-25) with enhanced cell proliferation, extended life span, altered growth and morphology, and passed 44, 18 and 3 times for OKTr-1, OKTr-23 and OKTr-25 respectively and were to stain positively (OKTr-1, OKTr-23) with mouse-anti- SV40-T antigen, further improvement has not been reported, indicating the failure of the stable transformation. Further, retroviral vectors pseudotyped with the envelop glycoprotein of vesicular somatitis virus was proved to be infective to primary cell cultures from _P. stylirostris_ (Shike _et al._, 2000a), however, without any direct evidence of integration. Even though, researchers (Hu _et al._, 2008, 2010) proved the use of VSV-G pseudotyped pantropic retroviral vectors by confirming the stable expression of SV40-T gene in post transfected cells, the attempts failed to induce _in vitro_ transformation. Moreover, Claydon and Owens (Claydon and Owens, 2008), transfected human papilloma viruses (HPV) E6 and E7 genes into the _C. quadricarinatus_ cells by lipofection and the successful transfection was demonstrated by the presence of oncogene mRNA by RT- PCR. While transfection of the oncogenes was successful and transfected cells survived more than 150 days, cell proliferation was stagnant due to the lack of telomere maintenance.

Telomerase activity in cultured cells is a limiting proliferating factor, as inactivation of pRb and p53 pathways (Smeets _et al._, 2011) in combination with activation of a telomere maintenance mechanism is suggested to be necessary for immortalization of somatic cells (Bodnar _et al._, 1998; Vaziri and Bachimol, 1999). Ablation of cell cycle checkpoint genes through mutation or viral oncogene expression is necessary to lead escape from senescence, additional doublings, and entrance into crisis phase, and finally the emergence of immortal clones. In the vast
majority of cases, telomerase is reactivated and telomeres are stabilized (Forsyth et al., 2004). Moreover, researchers proved that the introduction of telomerase activity in normal human cells caused an extension of replicative life span (Bodnar et al., 1998; Vaziri and Bachimol, 1998; Simons, 1999). In our study, we could not find any telomerase activity in primary lymphoid cell culture using telomeric repeat amplification protocol (TRAP). Even though, this is contradictory to the reported active telomerase activity in cultured lymphoid organ cells for up to 30 days (Lang et al., 2004), till date, no additional report has been seen in literature to confirm the telomerase activity in the cultured shrimp cells.

As spontaneous and induced transformation of somatic penaeid cells has not taken place (Claydon et al., 2010b) attempts to create hybrid cells by fusing cells from an immortal cell line of insects (Epithelioma papulosum cyprinid and Spodoptera frugiperda) with haemocytes from P. monodon were attempted and accordingly three fusion-cells could be produced (F11, F12 and F13). However, shrimp genes and viral susceptibility could not be observed in the fusion-cells; this happens to be the first attempt to produce hybrid cells from shrimp cells.

1.6. Critical analysis on shrimp cell line development and significance in this study

The ‘futile attempts’ in shrimp cell line development might be the outcome of the neglect on ‘know your animal’ (Lynn, 1999) philosophy, as the successful history of insect cell lines started from the in-depth knowledge gained on the insect biochemistry with which an appropriate and exclusive insect cell culture medium could be developed (Wyatt et al., 1956; Wyatt, 1956). Despite the modification of commercially available medium based on haemolymph analysis (Ellender et al., 1992; Shimizu et al., 2001) an exclusive medium for the growth and development of shrimp cells in vitro has not been accomplished. Even though Wyatt (Wyatt et al., 1956) was not totally successful, her contribution was essential to Grace’s ultimate success in the
development of Grace’s insect cell culture medium (Grace, 1958, 1962; Grace and Brzostowski, 1966) which resulted in the development of over 500 insect cell lines (Lynn, 1999, 2001). Such a scientific temper should be imbibed in the shrimp cell culture research for successful development of a continuous cell line. Moreover, lack of third party validation and confirmation of results achieved by researchers in sister institutions has also hampered the progress of research in shrimp cell culture development with a diminishing output. The transformation studies include identification of a putative promoter system to construct transformation and transduction vectors specific to shrimp. For the safe and stable transduction and immortalization, recombinant baculovirus with specific promoter for transcription initiation in shrimp cells are to be addressed. Development of hybrid cell line might also will pave way for the development of penaeid virus susceptible fusion cell lines. Moreover transgenic expression of oncogene and telomerase reverse transcriptase might also lead to a successful outcome of a valid shrimp cell line. With these concepts, the present study was undertaken with the following objectives:

1. A novel medium for the development of \textit{in vitro} cell culture system from \textit{Penaeus monodon}

2. Screening and optimization of growth factors and their potential impacts on lymphoid cell culture: Cellular activity and viral susceptibility

3. Differential expression of telomerase in various tissues and primary lymphoid cell culture, and identification and partial sequencing of telomerase reverse transcriptase (\textit{TERT}) gene in \textit{Penaeus monodon}

4. Construction and evaluation of the versatile recombinant baculoviral vector systems with hybrid promoters designed for the expression of foreign gene in shrimp cells

5. Transfection and transduction mediated oncogene expression in lymphoid cell cultures from \textit{Penaeus monodon} for its \textit{in vitro} transformation