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REVIEW ARTICLE

Establishment of Shrimp Cell Lines: Perception and Orientation

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Abstract Development of continuous shrimp cell lines for effective investigation on shrimp viruses remains elusive with an arduous history of over 25 years. Despite presenting challenges to researchers in developing a cell line, the billion dollar aquaculture industry is under viral threat. Advances in molecular biology and various gene transfer technologies for immortalization of cells have resulted in the development of hundreds of cell lines from insects and mammals, but yet not a single cell line has been developed from shrimp and other marine invertebrates. Though improved growth and longevity of shrimp cells in vitro could be achieved by using modified growth media this did not make any leap to spontaneous transformation; probably due to the fact that shrimp cells inhibited neoplastic transformations. Oncogenic induction and immortalization are considered as the possible ways, and an exclusive medium for shrimp cell culture and an appropriate mode of transformation are crucial. In this review status of shrimp cell line development and its future orientation are discussed.

Keywords Shrimp cell culture · Culture medium · Cell line · Transformation · Transduction · Immortalization

Introduction

Development of continuous shrimp cell lines has been a challenging task, for a long period of over 25 years. However, it still remains unattained presenting researchers more questions than answers [9, 65]. Till date, no permanent cell line could be made available from marine invertebrates in general [67] and shrimp in particular. The major fall out of the situation is the impediment which it imposes on the isolation of crustacean viruses [15, 16, 43]. The fact is that the requirement of continuous cell lines is so high to investigate the radiating viral threats to shrimp aquaculture [21, 88, 93].

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$ [20, 86]. This trend in production is unlikely to perpetuate, because there are more than 20 [6] among the 1,100 recognized invertebrate viruses [1] now known to occur in shrimps which include nine that pose serious threat to their culture [14], resulting in huge loss to shrimp industry [21, 50, 88]. This highlights the importance of developing shrimp cell lines for their isolation, and to bring out effective prophylactics.

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 [87]. 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 shrimps might provide new leads for the development of

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anti-ageing and anti-cancer interventions in humans as well [86].

In this review, we try to compile the current status and trends on shrimp cell line research and orient towards the prospects of development of continuous cell lines from penaeids.

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 [9]. 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 published an attempt [12] of shrimp cell culture development from *Penaeus penicillatus* and on the same year first report on the susceptibility of primary lymphoid cell culture to monodon-type baculovirus was published [8]. This is considered as the first report on in vitro cultivation of penaeid virus in shrimp cell culture. Although only limited success could be obtained, several researchers commenced attempting to develop cell cultures from various tissues and organs of different penaeid species [13, 19, 28, 35, 39, 44–46, 49, 57, 63, 70, 72, 80, 90], and this included test of their susceptibility to shrimp viruses as well [40, 51, 52, 56]. In 2000, report on the ultra structure of *white spot syndrome virus* (WSSV) grown in primary lymphoid cell culture was published [89], however, its morphogenesis could not be fully elucidated for want of certified shrimp cell lines. Although the morphology and ultrastructure of WSSV have not been fully understood, several characteristics of this virus have emerged in recent years [71]. 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' [29] in the process of cell culture development, in the year 1995 researchers attempted to induce transformation in shrimp cells by transfection with oncogene [78]. Accordingly, in 2000 first transgenic expression in shrimp cells could be accomplished [73] followed by the development of vesicular somatitis virus-glycoprotein (VSV-G) pseudotyped retroviral vectors [37] and their successful integration in shrimp primary cell culture genome [36]. 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 [15, 16], but, with little success. More recently, researchers have succeeded in viral gene expression [42], determinations of cytotoxicity and genotoxicity [41, 43], and viral multiplication [26] employing primary cell culture systems developed from different species of penaeids.

Cell Culture Medium: A Stepping Stone for Cell Line Development

Several hindrances stand in the way of the development of shrimp cell lines. One among them is the unsettling fact of an appropriate shrimp cell culture medium. 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 [60]. 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 [29–32] has brought about; ever since the publication of Grace's insect cell culture medium, over 500 insect cell lines could be established [53, 76]. Likewise, to formulate an exclusive shrimp cell culture medium, in-depth analysis of the biochemistry of body fluids [64, 74] 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.

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 [16, 43, 69]. 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 [2–4, 7–11, 13, 15, 18, 25, 37, 40–42, 46, 51, 56–58, 62–64, 69, 73, 74, 78, 80–83, 89], six (12 %) selected Grace's Insect Medium [26, 27, 49, 63, 82, 89], five (10 %) M199 [28, 39, 48, 74, 82], and three (6 %) MPS [19, 36, 80]. A couple of other media such as Pj-2 [55], NCTC 135 [89], MM Insect medium and TC 100 medium [63], were also tested for the development of cell lines from shrimp (Fig. 1). From the review it is rather inappropriate to point out any medium mentioned above as the most effective one as it has been a personal choice.

Organic and Inorganic Supplements to Improve Growth of Shrimp Cell Cultures 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

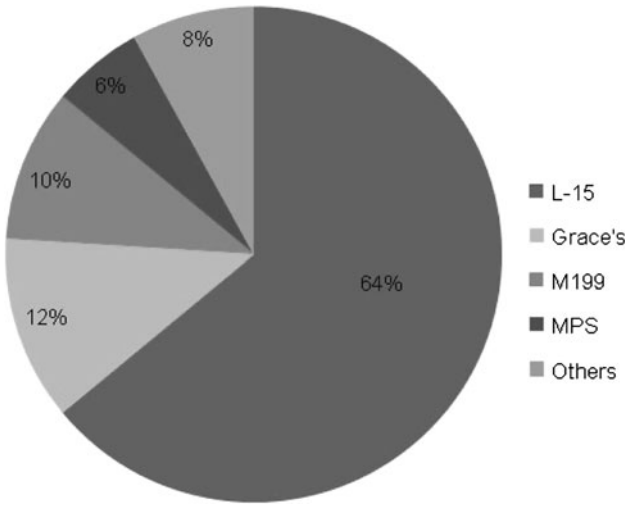

Fig. 1 Growth media used for shrimp cell culture (In % of the 50 selected publications)

multiples. Several investigators selected crustacean body fluids and extracts to improve the basal medium. Among them shrimp extract was the most popular one used in varying concentrations of 4 % [51], 8 % [63, 78], 10 % [8, 26, 27, 82], 27 % [46] and 30 % [9]. Hemolymph of lobsters at 10 % concentration [9] was also used. Moreover, ovary extracts [10] chitosan and nerve nodule extracts [19] were also incorporated in the medium as growth-promoting factors. Fetal bovine serum/fetal calf serum as the supplements with a concentration of 10 % [26, 27, 48, 49, 56, 57], 15 % [2–4], 18 % [9] and 20 % [19, 36, 40–42, 51, 55, 63, 73, 78, 89] were added as the source of minerals, proteins, lipids, hormones [24] and as the growth-promoting substances [59]. Considering the importance of inorganic salts for the maintenance of ionic balance and osmotic pressure [61], researchers have used KCl, $MgSO_4$, $MgCl_2$, and $CaCl_2$ at concentrations ranging from 0.9 to 3 g/l to supplement the required quantity in the growth medium [39, 48, 49]. To adjust osmolality, NaCl at a concentration ranging from 6 to 12 g/l [9, 19, 40, 49] has also been added besides the balanced salt solutions [40, 78].

Addition of vitamins [41, 42], proline [49, 56, 57, 82] and glutamine [28, 82] has been proven to be the choice of supplements in the growth media. In addition, lactalbumin hydrolizate at a concentration of 0.1–1 g/l [2–4, 39, 48, 55–57], tryptose phosphate broth at 2.95 mg/ml [41, 42] and TC Yeastolate at 1 g/l [56, 57] have also been used as the source of peptides, amino acids and carbohydrates. As the additional energy source 0.3–2 g/l glucose [40–42, 55–57] and 0.55 g/l sodium pyruvate [19] have also been supplemented. Buffering agents such as HEPS [28, 48, 82] and $NaHCO_3$ have been incorporated by many researchers [19, 28, 48, 49]. Growth factors such as epidermal growth factor at a concentration 20–30 ng/ml [51, 63, 78] and 10 units/ml of human recombinant interleukin-2 [78] 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 the establishment of any cell line.

Species of Choice: A Major Concern

Since the first attempt on shrimp cell line development, performed in 1986 by Chen et al. [9], *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* [2–4, 7, 8, 10, 15, 23, 35, 41, 42, 44, 58, 69, 83, 89, 90] as the species of choice, eight researchers (16 %) used *Penaeus japonicus* [10, 39, 47, 48, 55–57, 72], seven (17 %) selected *Penaeus chinensis* [13, 19, 36–38, 40, 80] and *Penaeus vannamei* [18, 26, 27, 49, 51, 63, 82]. Moreover, six authors (16 %) selected *Penaeus stylirostris* [49, 51, 63, 73, 74, 78] as the donor animal of tissues and organs. Besides, in two publications (4 %) *Penaeus indicus* [46, 82] and *Penaeus aztecus* [18, 64] were the species used. There is only one report (2 %) of using *P. penicillatus* [10] for extracting tissues and organs for cell culture development (Fig. 2). 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.

Tissues and Organs used for Cell Culture Development

Ovary and the lymphoid 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 [3, 4, 7, 8, 10, 12, 35, 37, 39, 47, 48, 51, 52, 63, 64, 78–80, 89, 90] and 18 with ovary [9–11, 23, 27, 36, 48, 49, 56, 57, 62, 63, 73, 74, 80–82, 90]. Ten experiments were with hemocytes [10, 15, 18, 26–28, 39–42], four with eyestalk [27, 46, 62, 80]. Besides, testis [62, 81], heart [9, 10, 48, 62, 80], hepatopancreas [9, 28, 48, 55, 58, 62, 64, 81, 89], gill [9, 62], nerve [9, 13, 48, 62, 80, 81], muscle [9, 27, 48], hematopoietic tissue [11, 62, 90], embryonic tissue [19, 80, 82], epidermis [81, 82], gut [9, 62] and Y organ [81] were also widely used for cell culture development (Fig. 3). Among the tissues used the most advancement was obtained from lymphoid and ovarian tissues only.

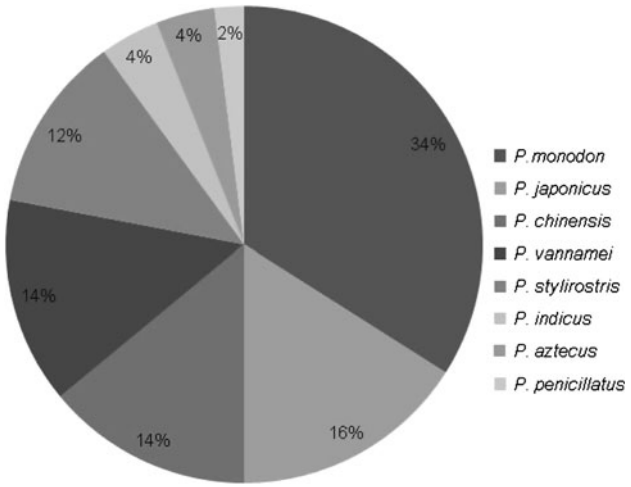

Fig. 2 Penaeid species used for cell culture development (In % of the 50 selected publications)

Longevity and Sub-culturing of the Cell Cultures

The ultimate objective of every shrimp cell culture development programme is the establishment of corresponding cell lines. However, this objective has not been achieved so far. Although unable to be sub cultured, various researchers could maintain cell cultures for different duration. Accordingly, researchers could maintain ovarian cell culture for 66 days [27], 45 days [57], 20 days [10], 10 days [49] and to several months [80, 82] along with single passage [62] and 3 passages [9, 10]. Lymphoid cell cultures were reported to be passaged 2 times [8], 3 times [10], and maintained for 54 days [39], 20 days [10] and for a period greater than 3 weeks [63] to 3 months [80]. However, Hsu et al. [35] claimed to have attained more than 90 passages for a lymphoid cell culture which was later reported as Thraustochytrid contamination by Rinkevich [68]. At the same time Tapay et al. [78] 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 [27], 3 months and attained 4 passages [46]. Besides, haemocyte cultures were maintained for 48 days [27], 20 days [40], 10 days [39], 8 days [41, 42], and 4 days [10]. Embryonic cell cultures could be maintained for several months [82] and attained 10 passages [19]. Moreover, researchers could maintain nerve cells from 15 days [13] and to up to 3 months [63], heart tissue for 4 days [10] and hepatopancreas for 30 days [27]. The striking observation was that there existed no consistency in the number of days which a cell culture could be maintained by different workers.

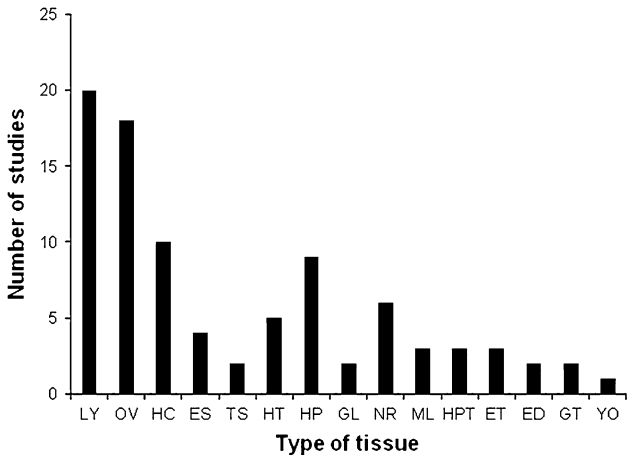

Fig. 3 Various tissues used for shrimp cell culture development. *LY* lymphoid, *OV* ovary, *HC* haemocytes, *ES* eye stalk, *TS* testis, *HT* heart, *HP* hepatopancreas, *GL* gill, *NR* nerve, *ML* muscle, *HPT* haematopoeitic tissue, *ET* embryonic, *ED* epidermis, *GT* gut, *YO* Y organ (results from 90 experiments)

Virus Susceptibility in Various Cell Culture Systems Developed from Shrimp

Penaeid lymphoid organ cell culture system 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 *P. monodon* [7, 8]. Susceptibility of Yellow head virus in lymphoid cell culture from *P. monodon* [2, 4, 10, 79], *P. japonicus* and *P. penicillatus* [10], and from *P. vannamei* [51, 52] have been reported. Moreover, Lu et al. [52] 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 WSSV in lymphoid cell culture from *P. monodon* [89], from *P. monodon, P. japonicus* and *P. penicillatus* [10], ovarian cell culture from *P. japonicus* [56], hepatopancreatic cell culture from *P. monodon* [83] haemocytes from *P. chinensis* [40] have also been reported. Recently, Jose et al. [42] conducted a detailed investigation on the viral titration and viral gene expression in *P. monodon* haemocyte culture. Still more recently, George et al. [26] investigated the multiplication of taura syndrome virus in haemocytes from *P. vannamei*. Inspite 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 uses 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.

Molecular Approaches for Shrimp Cell 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 [17] 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 [33, 34]. 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 [86]. The high regeneration capability 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 [66], the technique which has not yet been fully applied to crustacean and aquatic invertebrate cells [17]. 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 [86].

To date, oncogenic mammalian virus gene, simian virus 40 large T antigen [36, 37, 78] 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 [78] with the pSV-3 neo plasmid vector encoding SV40-T antigen gene from Simian virus-40 by lipofection. Further, retroviral vectors pseudotyped with the envelop glycoprotein of VSV was proved to be infective to primary cell cultures from *P. stylirostris* [73], however, without any direct evidence of integration. Even though, researchers [36, 37] proved the use of VSV-G pseudotyped pantropic retroviral vectors by confirming the stable expression of SV40T gene in post transfected cells, the attempts failed to induce in vitro transformation. Moreover, Claydon and Owens [17] transfected human papillomaviruses (HPV) E6 and E7 genes into the *Cherax 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 [77] in combination with activation of a telomere maintenance mechanism is suggested to be necessary for immortalization of somatic cells [5, 84]. 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 [22]. Moreover, researchers proved that the introduction of telomerase activity in normal human cells caused an extension of replicative life span [5, 75, 85]. In our study (un-published data) we could not find any telomerase activity in primary lymphoid cell culture using telomeric repeat amplification protocol assay. Even though, this is contradictory to the reported active telomerase activity in cultured lymphoid organ cells for up to 30 days [47], 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 [15], 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 made 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.

25 Years of Futile History of Cell Line Development: What Went Wrong and What Could be Done?

The 'futile attempts' in shrimp cell line development might be the outcome of the neglect on 'know your animal' [54] 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 [91, 92]. Despite the modification of commercially available medium based on hemolymph analysis [18, 74] an exclusive medium for the growth and development of shrimp cells in vitro has not been accomplished. Even though, Wyatt [91] was not totally successful, her contribution was essential to Grace's ultimate success in the development of Grace's insect cell culture medium [30–32] which resulted in the development of over 500 insect cell lines [53, 54]. 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. 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.

References

1. Adams JR. Introduction and classification of viruses of invertebrates. In: Adams JR, Bonami JR, editors. Atlas of invertebrate viruses. Florida: CRC Press Inc.; 1991. p. 1–8.
2. Assavalapsakul W, Smith DR, Panyim S. Identification and characterization of a *Penaeus monodon* lymphoid cell-expressed receptor for the yellow head virus. J Virol. 2006;80:262–9.
3. Assavalapsakul W, Tirasophon W, Panyim S. Antiserum to the gp116 glycoprotein of yellow head virus neutralizes infectivity in primary lymphoid organ cells of *Penaeus monodon*. Dis Aquat Organ. 2005;63:85–8.
4. Assavalapsakul W, Smith DR, Panyim S. Propagation of yellow head virus particles prior to cytopathic effect in primary lymphoid cell cultures of *Penaeus monodon*. Dis Aquat Organ. 2003;55:253–8.
5. Bodnar AG, Ouelette M, Frolkis M, Holt SE, Chiu C, Morin GB, Harley CB, Shay JW, Lichtsteiner S, Wright WE. Extension of life span by introduction of telomerase into normal human cells. Science. 1998;279:349–52.
6. Bonami JR. Shrimp viruses. In: Mahy BWJ, Regenmortel MHVv, editors. Encyclopedia of virology. Oxford: Academic; 2008. p. 567–76.
7. Catap ES, Nudo LP. Susceptibility of primary cultured cells from the lymphoid organ of *Penaeus monodon* to monodon baculovirus (MBV) and white spot syndrome virus (WSSV). Philipp Agric Sci. 2008;91:450–8.
8. Chen S, Kou GH. Infection of cultured cells from lymphoid organ of *Penaeus monodon* fabricius by monodon-type baculovirus (MBV). J Fish Dis. 1989;12:73–6.
9. Chen S, Chi SC, Kou GH, Liao IC. Cell culture from tissues of grass prawn, *Penaeus monodon*. Fish Pathol. 1986;21:161–6.
10. Chen SN, Wang CS. Establishment of cell culture systems from penaeid shrimp and their susceptibility to white spot disease and yellow head viruses. Methods Cell Sci. 1999;21:199–206.
11. Chen SN, Jong KJ, Kou GH. Cell cultures from hematopoietic tissue and ovary of penaeid shrimp, *Penaeus monodon*. In: Kuroda Y, Kurstak E, Maramorosch K, editors. Invertebrate and fish tissue culture. Berlin: Springer-Verlag; 1988. p. 195–8.
12. Chen SN, Jong KJ, Kou GH. Cell cultures derived from tissues of penaeid shrimp, *Penaeus penicillatus*, hard clam/*Meretrix lusoria*. In: Mitsuhashi J, editor. Invertebrate cell system applications. Florida: CRC Press Inc.; 1989. p. 253–62.
13. Chun-Lei G, Jin-Sheng S, Jian-Hai X. Primary culture and characteristic morphologies of medulla terminalis neurons in the eyestalks of Chinese shrimp, *Fenneropenaeus chinensis*. J Exp Mar Biol Ecol. 2003;290:71–80.
14. Claydon K, Tahir RAH, Said HM, Lakim MH, Tamat W. Prevalence of shrimp viruses in wild *Penaeus monodon* from Brunei Darussalam. Aquaculture. 2010;308:71–4.
15. Claydon K, Roper KG, Owens L. Attempts at producing a hybridised *Penaeus mondon* cell line by cellular fusion. Fish Shellfish Immunol. 2010;29:539–43.
16. Claydon K. Advances in crustacean cell culture. Ph.D. Thesis, Townsville: James Cook University; 2009.
17. Claydon K, Owens L. Attempts at immortalization of crustacean primary cell cultures using human cancer genes. In Vitro Cell Dev Biol Anim. 2008;44:451–7.
18. Ellender RD, Najafabadi AK, Middlebrooks BL. Observations on the primary culture of hemocytes of *Penaeus*. J Crustac Biol. 1992;12:178–85.
19. Fan TJ, Wang XF. *In vitro* culture of embryonic cells from the shrimp, *P chinensis*. J Exp Mar Biol Ecol. 2002;267:175–84.
20. FAO. The state of world fisheries and aquaculture 2010. Rome: Food and Agriculture Organization of the United Nations; 2010.
21. Flegel TW. Detection of major penaeid shrimp viruses in Asia, a historical perspective with emphasis on Thailand. Aquaculture. 2006;258:1–33.
22. Forsyth NR, Morales CP, Damlez S, Bomanz B, Wrightz WE, Kopelovich L, Shay JW. Spontaneous immortalization of clinically normal colon-derived fibroblasts from a familial adenomatous polyposis patient. Neoplasia. 2004;6:258–65.
23. Fraser CA, Hall MR. Studies on primary cell cultures derived from ovarian tissue of *Penaeus monodon*. Methods Cell Sci. 1999;21:213–8.
24. Freshney RI. Culture of animal cells: a manual of basic technique. New York: Wiley-Liss; 2000.
25. Fuerst JA, Sambhi SK, Paynter JL, Hawkins JA, Atherton JG. Isolation of a bacterium resembling *Pirellula* species from primary tissue culture of the giant tiger prawn (*Penaeus monodon*). Appl Environ Microbiol. 1991;57:3127–34.
26. George SK, Kaizer KN, Betz YM, Dhar AK. Multiplication of Taura syndrome virus in primary hemocyte culture of shrimp (*Penaeus vannamei*). J Virol Methods. 2011;172:54–9.
27. George SK, Dhar AK. An improved method of cell culture system from eye stalk, hepatopancreas, muscle, ovary, and hemocytes of *Penaeus vannamei*. In Vitro Cell Dev Biol Anim. 2010;46:801–10.
28. Ghosh D, Ray AK, Dasmahapatra AK. Primary culture of prawn hepatocytes in serum free media. In Vitro Cell Dev Biol Anim. 1995;31:811–3.
29. Grace TDC. Development of insect cell culture. In: Maramorosch K, Mitsuhashi J, editors. Invertebrate cell culture applications. London: Academic; 1982. p. 1–8.
30. Grace TDC, Brzostowski HM. Analysis of the amino acids and sugars in an insect cell culture medium during cell growth. J Insect Physiol. 1966;12:625–33.
31. Grace TDC. Establishment of four strains of cells from insect tissues grown in vitro. Nature. 1962;195:788–9.
32. Grace TDC. Effect of various substances on growth of silkworm tissues in vitro. Aust J Biol Sci. 1958;11:407–17.
33. Hayflick L. The limited in vitro lifetime of human diploid cell strains. Exp Cell Res. 1965;37:614–36.
34. Hayflick L, Moorhead PS. The serial cultivation of human diploid cell strains. Exp Cell Res. 1961;25:585–621.
35. Hsu Y, Yang YH, Chen YC, Tung MC, Wu JL, Engelking MH, Leong JC. Development of an in vitro subculture system for the oka organ (Lymphoid tissue) of *Penaeus monodon*. Aquaculture. 1995;136:43–55.
36. Hu GB, Wang D, Ji-Xiang Chen. Retroviral delivery of simian virus 40 large T antigen gene into primary cultured ovary cells of the penaeid shrimp, *Penaeus chinensis*: indirect evidence of retroviral integration. J World Aquac Soc. 2010;41:137–43.
37. Hu GB, Wang D, Wang CH, Yang KF. A novel immortalization vector for the establishment of penaeid shrimp cell lines. In Vitro Cell Dev Biol Anim. 2008;44:51–6.

38. Huang J, Song XL, Yu J, Zhang LJ. The components of an inorganic physiological buffer for *Penaeus chinensis*. Methods Cell Sci. 1999;21:223–5.
39. Itami T, Maeda M, Kondo M, Takahashi Y. Primary culture of lymphoid organ cells and haemocytes of kuruma shrimp, *Penaeus japonicus*. Methods Cell Sci. 1999;21:237–44.
40. Jiang YS, Zhan WB, Wang SB, Xing J. Development of primary shrimp hemocyte cultures of *Penaeus chinensis* to study white spot syndrome virus (WSSV) infection. Aquaculture. 2005;253: 114–9.
41. Jose S, Jayesh P, Mohandas A, Philip R, Singh ISB. Application of primary haemocyte culture of *Penaeus monodon* in the assessment of cytotoxicity and genotoxicity of heavy metals and pesticides. Mar Environ Res. 2011;71:169–77.
42. Jose S, Mohandas A, Philip R, Singh ISB. Primary hemocyte culture of *Penaeus monodon* as an in vitro model for white spot syndrome virus titration, viral and immune related gene expression and cytotoxicity assays. J Invertebr Pathol. 2010;105: 312–21.
43. Jose S. Cell culture from *Penaeus monodon*: development and application. Ph.D. Thesis, Cochin: University of Science and Technology; 2009.
44. Kasornchandra J, Khongpradit R, Ekpanithanpong U, Boonyaratpalin S. Progress in the development of shrimp cell cultures in Thailand. Methods Cell Sci. 1999;21:231–5.
45. Ke H, Liping W, Yumei D, Shuji Z. Studies on a cell culture from the hepatopancreas of the oriental shrimp, *Penaeus orientalis* kishinouye. Asian Fish Sci. 1990;3:299–307.
46. Kumar SG, Singh ISB, Philip R. Cell culture systems from the eye stalk of *Penaeus indicus*. In: Lindner-Olsson E, Chatzissavidou N, Lullau E, editors. Animal cell technology: from target to market. Kluwer: London; 2001. p. 261–5.
47. Lang GH, Wang Y, Nomura N, Matsumura M. Detection of telomerase activity in tissues and primary cultured lymphoid cells of *Penaeus japonicus*. Mar Biotechnol. 2004;6:347–54.
48. Lang GH, Nomura N, Matsumura M. Growth by cell division in shrimp (*Penaeus japonicas*) cell culture. Aquaculture. 2002;213: 73–83.
49. Leudeman R, Lightner DV. Development of an in vitro primary cell system from the penaeid shrimp, *Penaeus stylirostris* and *Penaeus vannamei*. Aquaculture. 1992;101:205–11.
50. Lightner DV. Virus diseases of farmed shrimp in the Western Hemisphere (the Americas): a review. J Invertebr Pathol. 2011; 106:110–30.
51. Lu Y, Tapay LM, Loh PC, Brock JA, Gose RB. Development of a quantal assay in primary shrimp cell culture for yellow head baculovirus (YBV) of penaeid shrimp. J Virol Methods. 1995;52: 231–6.
52. Lu Y, Tapay LM, Loh PC, Brock JA, Gose RB. Distribution of yellow-head virus in selected tissues and organs of penaeid shrimp *Penaeus vannamei*. Dis Aquat Organ. 1995;23:67–70.
53. Lynn DE. Novel techniques to establish new insect cell lines. In Vitro Cell Dev Biol Anim. 2001;37:319–21.
54. Lynn DE. Development of insect cell lines: virus susceptibility and applicability to prawn cell culture. Methods Cell Sci. 1999; 21:173–81.
55. Machii A, Wada KT, Awaji M, Nakamura HK, Townsley SJ. Some characters of cells of the midgut gland and chytrids from primary cultures of the prawn *Penaeus japonicus*. In: Kuroda Y, Kurstak E, Mararnorosch K, editors. Invertebrate and fish tissue culture. Tokyo: Japanese Scientific Societies Press; 1988. p. 11–4.
56. Maeda MHS, Mizuki E, Itami T, Ohba M. Replication of white spot syndrome virus in ovarian primary cultures from the kuruma shrimp, *Marsupenaeus japonicus*. J Virol Methods. 2004;116: 89–94.
57. Maeda MEM, Itami T, Ohba M. Ovarian primary tissue culture of the kuruma shrimp *Marsupenaeus japonicus*. In Vitro Cell Dev Biol Anim. 2003;39:208–12.
58. Manohar BM, Vivek KV, Kumanan K. Studies on a cell culture derived from hepatopancreas of tiger prawn *Penaeus monodon*. Indian J Fish. 2001;48:183–8.
59. Mitsuhashi J. Invertebrate tissue culture methods. Tokyo: Springer-Verlag; 2002.
60. Mitsuhashi J. Development of highly nutritive culture media. In Vitro Cell Dev Biol Anim. 2001;37:330–7.
61. Mitsuhashi J. Nutritional requirements of insect cells in vitro. In: Mitsuhashi J, editor. Invertebrate cell system applications. Florida: CRC Press; 1989. p. 3–20.
62. Mulford AL, Austin B. Development of primary cell culture from *Nephrops norvegicus*. Methods Cell Sci. 1998;19:269–75.
63. Nadala E, Loh PC, Lu Y. Primary culture of lymphoid, nerve and ovary cells from *Penaeus stylirostris* and *Penaeus vannamei*. In Vitro Cell Dev Biol Anim. 1993;29:620–2.
64. Najafabadi AK, Ellender RD, Middlebrooks BL. Analysis of shrimp hemolymph and ionic modification of a *Penaeus* cell culture formulation. J Aquat Anim Health. 1992;4:143–8.
65. Owens L, Smith J. Early attempts at production of prawn cell lines. Methods Cell Sci. 1999;21:207–11.
66. Ratner L, Josephs SF, Wong-Staal F. Oncogenes: their role in neoplastic transformation. Annu Rev Microbiol. 1985;39:419–49.
67. Rinkevich B. Cell cultures from marine invertebrates: new insights for capturing endless stemness. Mar Biotechnol. 2011;13:345–54.
68. Rinkevich B. Cell cultures from marine invertebrates: obstacles, new approaches and recent improvements. J Biotechnol. 1999; 70:133–53.
69. Roper KG, Owens L, West L. The media used in primary cell cultures of prawn tissues: a review and a comparative study. Asian Fish Sci. 2001;14:61–75.
70. Rosenthal J, Diamant A. In vitro primary cell culture from *Penaeus semisulcatus*. In: Perkins FO, Chen TC, editors. Pathology in marine science. San Diego: Academic; 1990. p. 7–13.
71. Sa'nchez-Paz A. White spot syndrome virus: an overview on an emergent concern. Vet Res. 2010;41:43.
72. Sano T. A novel tissue organized in the primary hemolymph culture of *Penaeus japonicus* bate. Aquaculture. 1998;164:289–96.
73. Shike HS, Klimpel KS, Burns JC. Expression of foreign genes in primary cultured cells of the blue shrimp *Penaeus stylirostris*. Mar Biol. 2000;137:605–11.
74. Shimizu C, Shike H, Klimpel KR, Burns JC. Hemolymph analysis and evaluation of newly formulated media for culture of shrimp cells (*Penaeus stylirostris*). In Vitro Cell Dev Biol Anim. 2001;37:322–9.
75. Simons JWIM. A theory on cellular aging and immortalization. In: Macieira-Coelho A, editor. Progress in molecular and subcellular biology—cell immortalization. Berlin: Spinger-Verlag; 1999. p. 1–21.
76. Smagghe G, Goodman CL, Stanley D. Insect cell culture and applications to research and pest management. In Vitro Cell Dev Biol Anim. 2009;45:93–105.
77. Smeets SJ, van der Plas M, SchaaijVisser TB, van Veen EA, van Meerloo J, Braakhuis BJ, Steenbergen RD, Brakenhoff RH. Immortalization of oral keratinocytes by functional inactivation of the p53 and pRb pathways. Int J Cancer. 2011;128:1596–605.
78. Tapay L, Lu Y, Brock JA, Nadala ECB, Loh PC. Transformation of primary cultures of shrimp (*Penaeus stylirostris*) lymphoid (Oka) organ with simian virus-40 (T) antigen. Proc Soc Exp Biol Med. 1995;209:73–8.
79. Tirasophon W, Roshorm Y, Panyim S. Silencing of yellow head virus replication in penaeid shrimp cells by dsRNA. Biochem Biophys Res Commun. 2005;334:102–7.

80. Tong SL, Miao HZ. Attempts to initiate cell cultures from *Penaeus chinensis* tissues. Aquaculture. 1996;146:151–7.
81. Toullec JY. Crustacean primary cell culture: a technical approach. Methods Cell Sci. 1999;21:193–8.
82. Toullec JYC, Patrois J, Porcheron P. Development of primary cell cultures from the penaeid shrimps *Penaeus vannamei* and *P. indicus*. J Crustac Biol. 1996;16:643–9.
83. Uma A, Prabhakar TG, Koteswaran A, Ravi kumar G. Establishment of primary cell culture from hepatopancreas of *Penaeus monodon* for the study of white spot syndrome virus (WSSV). Asian Fish Sci. 2002;15:365–70.
84. Vaziri H, Benchimol S. Alternative pathways for the extension of cellular life span: inactivation of p53/pRb and expression of telomerase. Oncogene. 1999;18:7676–80.
85. Vaziri H, Benchimol S. Reconstitution of telomerase activity in normal human cells leads to elongation of telomeres and extended replicative life span. Curr Biol. 1998;8:279–82.
86. Vogt G. Hidden treasures in stem cells of indeterminately growing bilaterian invertebrates. Stem Cell Rev Rep. 2011. doi: 10.1007/s12015-011-9303-1.
87. Vogt G. How to minimize formation and growth of tumours: potential benefits of decapod crustaceans for cancer research. Int J Cancer. 2008;123:2727–34.
88. Walker PJ, Winton JR. Emerging viral diseases of fish and shrimp. Vet Res. 2010;41:51.
89. Wang CH, Yang HN, Tang CY, Lu CH, Kou GH, Lo CF. Ultrastructure of white spot syndrome virus development in primary lymphoid organ cell cultures. Dis Aquat Organ. 2000;41: 91–104.
90. West L, Mahony T, McCarthy F, Watanabe J, Hewitt D, Hansford S. Primary cell cultures isolated from *Penaeus monodon* prawns. Methods Cell Sci. 1999;21:219–23.
91. Wyatt GR, Loughheed TC, Wyatt SS. The chemistry of hemolymph- Organic components of the hemolymph of the silkworm, *Bombyx mori*, and two other species. J Gen Physiol. 1956;39: 853–65.
92. Wyatt SS. Culture in vitro of tissue from the silkworm, Bombyx mori L. J Gen Physiol. 1956;39:841–52.
93. Zwart MP, Dieu BTM, Hemerik L, Vlak JM. Evolutionary trajectory of white spot syndrome virus (WSSV) genome shrinkage during spread in Asia. PLoS One. 2010. doi:10.1371/journal.pone.0013400.