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Medium optimization and characterization of cell culture system from *Penaeus vannamei* for adaptation of white spot syndrome virus (WSSV)

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ABSTRACT

The lack of shrimp cell lines and difficulty in establishing shrimp cell culture systems, with an appropriate medium is a major concern in the aquaculture sector. The present study attempts to address this issue by developing an *in vitro* cell culture system from various tissues (hemocytes, heart, lymphoid tissue, hepatopancreas, gill, eye stalk, and muscle) of *Penaeus vannamei* (*P.vannamei*) using commercially available L-15 medium. The cell culture medium was formulated using five different media such as HBSCM-1, HBSCM-2, HBSCM-3, HBSCM-4, and HBSCM-5 containing L-proline and glucose with fetal bovine serum (FBS) supplements. Among the different media used, the HBSCM-5 medium with supplements showed good attachment and proliferation of cells with fibroblast-like, epithelioid, round, and adherent cell morphology in hemocyte culture. The same medium was further screened using different tissues to enhance the cell growth. The hemocytes, heart, and lymphoid tissue cells were passaged five times and maintained up to 20 days. Hepatopancreas and gill cells initially showed good morphological features and survived for more than ten days following subculture cells. Eye stalks and muscle cells perished within five days and did not show any unique morphology. The primary hemocyte cells were subjected to species identification, using cytochrome oxidase subunit I (*COI*) gene. To assess the primary hemocyte cell culture, cells were used for *in vitro* propagation of white spot syndrome virus (WSSV) and confirmed by the conventional polymerase chain reaction (PCR). Similarly, the primary cells were treated with bacterial extracellular products (ECPs) from *Vibrio parahaemolyticus* and *Vibrio harveyi,* to evaluate the cytotoxicity.

1. INTRODUCTION

The development of a continuous crustacean cell line has been in a nascent stage for the past three decades (Jayesh et al., 2012). The *in vitro* cell culture technique is a powerful tool to study crustacean endocrinology and the replication of intracellular pathogens, such as viruses (Jiravanichpaisal et al., 2006; Toullec et al., 1996). In order to study the pathogenesis and mechanism of the virus, developing a crustacean cell line is vital (Jayesh et al., 2013; Rinkevich, 1999). Development of a cell culture system also helps to mimics the pathogenesis of virus which destroy aquaculture sector and cause the huge commercial loss to the farmers (Han et al., 2013; Toullec et al., 1996). Several studies have focused on the development of the cell culture system, but these have been hampered by the choice of tissues, media, and supplements (Toullec, 1999). Various factors impinge on the *in vitro* growth of cells and most importantly media composition affects cell growth (Mitsuhashi, 2001). To develop crustacean cell culture researcher has used various commercially available media by modifying the composition, that is, by the addition of numerous supplements, which may be hindering the development of crustacean cells (Jayesh et al., 2012). Commercially available media namely L-15, M199, Grace insect, DMEM, MEM, and the TC100, were screened for the development of crustacean cell culture (Goswami et al., 2010; Maeda et al., 2003; Toullec et al., 1996). Among these media, L-15 has been the most common medium subjected to modification, with one or more supplements, including growth factors, vitamins, amino acids, glucose, and

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the like (Jayesh et al., 2013). Development of the Grace medium has led to the discovery of more than 500 cell lines in insects, which attracted researchers to develop a medium specific for insects (Grace, 1962). This interest has led to a deeper examination of the composition of body fluids, as the primary need is to meet the nutritional supplement of the cells (Najafabadi et al., 1992; Shimizu et al., 2001).

Previous studies analyzed the biochemical constituents of the hemolymph of various crustaceans such as, Shrimp, Blue crab, and American lobster. Hemocytes are produced from hematopoietic tissues therefore hemocytes cells were processed (Grigorian and Hartenstein, 2013). This study shows that the variations in the concentration of constituents present in the hemolymph could be due to stress and diet (Najafabadi et al., 1992). In another study, the hemolymph composition of *P.stylirostris* was analyzed and a new medium was formulated according to the hemolymph components (Shimizu et al., 2001). Similarly, the hemolymph composition in *P.monodon* was evaluated and a new shrimp cell culture medium (SCCM), using sea water as the base, was formulated. In the present study we developed a new culture medium formulation based on the hemolymph composition of *P.vannamei* using L-15 medium as a base and the growth of various tissues was evaluated to determine the medium is adequate for the propagation of cells.

2. MATERIALS AND METHODS

2.1. Shrimp acclimation and tissue dissection

Apparently healthy shrimp of *P.vannamei* (White shrimp) weighing 5 g to10 g were purchased from local suppliers. They were acclimatized and maintained in a wet laboratory facility at the Central Institute of Brackishwater Aquaculture (ICAR-CIBA) and the Peninsular and Marine Fish Genetic Resources (PMFGR) Centre, Kochi, National Bureau of Fish Genetic Resources (ICAR-NBFGR). All animals were maintained hygienically in clean 250 L fiber reinforced plastic (FRP) tanks, with adequate aeration (salinity 28 ± 2 ppt; temperature 30 ± 2 °C). The shrimps were fed daily with commercially available pellets and maintained with 50% water exchange daily. Animals were cleaned thrice with sterile seawater and their surface disinfected by wiping with 70% alcohol. After surface sterilization, the hemolymphs were collected, dissected, and various tissues such as the heart, lymphoid tissue, hepatopancreas, gill, eyestalk, and muscle, were individually transferred to phosphate-buffered saline (PBS) containing 1x antibiotic mixture (Penicillin 1,000IU/ml, Streptomycin 1,000 µg/ml, Gentamicin 250 µg/ml and Amphotericin B250 µg/ml, Life Technologies) and incubated for 10-15 minutes.

2.2. Collection of hemolymph from P.vannamei for amino acid analysis

Live animals (*P.vannamei*) from 80 days of culture (DOC) weighing approximately ± 10 g were procured from shrimp farms located in Thiruvallur district, Tamil Nadu, India. The animals were cleaned with sterile seawater and surface disinfected with 70% alcohol, following which hemolymph was collected aseptically from the ventral sinus of five healthy animals of *P. vannamei,* located at the base of the first abdominal segment, using a 2 ml syringe. The collected hemolymph was pooled and hemolymph was transferred to sterile vials and lyophilized. The lyophilized vials were stored at room temperature until use.

2.3. Amino acid and biochemical analysis of the hemolymph

20 mg of lyophilized samples were analyzed for the quantification of essential and non-essential amino acids by hydrolyzing the sample in 6 N HCL for 24 hours at 110 °C. The acid was removed by using vacuum evaporation and the residue was diluted in 0.05 N HCL and analyzed using high-performance liquid chromatography (HPLC) (Kavitha et al., 2004). The hemolymph collected from ten healthy animals were pooled and centrifuged at 5000 rpm for 10 minutes. The supernatant was transferred to another vial and analyzed for various enzymes and biochemical parameters using an auto analyzer (Toshiba, TBA-25FR). The pH and electrolytes such as bicarbonate, chlorine, sodium, and potassium were measured using an electrolyte analyzer (ELITE, Trivitron healthcare) Table 1.

Table 1
Comparison of free amino acids (FAAs) in hemolymph with L-15 Medium. Electrolytes and biochemical analysis of hemolymph.

FAAs	Hemolymph mg/ml	L-15 mg/L	Electrolytes and biochemical analysis	
Arginine	11.9	500	pH	6.67
Lysine	12.9	93.7	Sodium	5520 mg/L
Leucine	7.5	125	Potassium	659.88 mg/L
Threonine	2.4	300	Bicarbonate	4.8 mmol/L
Histidine	5.7	250	Chlorine	9558 mg/L
Isoleucine	4.3	125	Phosphorus	10.27 mg/dl
Valine	3.5	100	Calcium	80 mg/dl
Phenyl alanine	5.9	125	ALP	995 mg/dl
Methionine	0.9	75	Magnesium	74 mg/dl
Alanine	3.7	225	Cholesterol	16.3 mg/dl
Glycine	4.1	200	Glucose	39.1 mg/dl
Aspartate	9.9	250	Protein	11.51
Serine	2.3	200	Urea	18.68 mg/dl
Glutamate	8.5	300	Creatinine	1.56 mg/dl
Proline	3.8	-	ALT	378 IU/L
Tyrosine	11.9	300	TGL	15.1 mg/dL
Cystine	ND	120	Amylase	2937 IU/L

2.4. Formulation of shrimp cell culture medium based on the hemolymph amino acid concentration

Commercially available Leibovitz's L-15 powder medium (L-15) was used in this study. The L-15 medium powder was prepared using five different media at concentrations of :0.025x, 0.25x, 0.5x, 1x and 2x (Table 2), with L-proline (0.1 g/L), 1 g/L of Glucose, 15% FBS (Sigma-Aldrich, USA), containing 1x antibiotic mixture (Penicillin 1,000IU/ml, Streptomycin 1,000 µg/ml, Gentamicin 250 µg/ml and Amphotericin B250 µg/ml, Life Technologies). All the media were supplemented with the same compounds. The pH and osmolality were adjusted to pH 7.2 and 730 ± 20 mOsm kg^{1}, the media were filtered using a 0.2 µm filter and a vacuum pump. All the five formulated media (HBSCM-1, HBSCM-2, HBSCM-3, HBSCM-4, and HBSCM-5) were individually screened for cell growth and viability of various tissues of the heart, lymphoid organ, gill, hepatopancreas, eyestalk, muscle, and hemocytes.

2.5. Mechanical method using explant cell culture

A healthy live animal was purchased from a local supplier and maintained in an aerated condition, as described above. The animal was anesthetized using MS-222 (Sigma-Aldrich) and wiped with 70% ethanol. Various tissues such as the lymphoid tissue, heart, gills, eye stalk, and muscle were dissected out aseptically and were washed thrice in phosphate buffer saline (PBS) containing the antibiotic–antimycotic solution (Penicillin 1,000IU/ml, Streptomycin 1,000 µg/ml, Gentamicin 250 µg/ml and Amphotericin B250 µg/ml, Life Technologies) and treated for 10-15 minutes. All the tissues were mechanically cut with a fine scalpel into smaller pieces and transferred onto a 25 cm^2 flask. The PBS with antibiotic–antimycotic solution was removed and attachment of the tissues to the surface was enabled by adding 200 µl of FBS. After six hours, about 5-7 ml of five different media containing 1x concentration of antibiotic–antimycotic solution was added to the culture flasks and incubated at 28 °C.

Animals were dissected aseptically and the hepatopancreas was removed carefully (Zeng et al., 2010). Special attention was given to

Table 2
Development of the primary hemocyte cell culture from *P.vannamei* using various media with FBS supplements. Hemolymph based shrimp culture medium (HBSCM).

S.No	Different media	Media with supplements	Cell proliferation
1	HBSCM -1	0.025x L-15 medium with 15% FBS supplements	Cells attached and degranulation observed was observed.
2	HBSCM -2	0.25x L-15 medium with 15% FBS supplements	Cells attached and proliferation was not observed.
3	HBSCM -3	0.5x L-15 medium with 15% FBS supplements	Cells attached and cell proliferation was observed.
4	HBSCM- 4	1x L-15 medium with 15% FBS supplements	Cells attached, cell migration and Proliferation was observed.
5	HBSCM -5	2x L-15 medium with 15% FBS supplements	Cells attached, showed healthy, cell migration and proliferation was seen and cells could be maintained up to 20 days.

tissues of the hepatopancreas soon after harvesting, as these tissues were prone to liquefaction compared to other tissues (George and Dhar, 2010). The tissues were cut into small pieces and passed through a cell strainer to separate the individual cells. The cells were separated through a stainless steel mesh (190 µm pore size) followed by a nylon mesh cell strainer (40 µm pore size), to remove cell debris. Finally, the cells were centrifuged and resuspended in the culture medium in a 25 cm^2 culture flask. All the cell culture chemicals, media, antibiotics, buffers, and plastic ware were procured from Life Technologies. The culture flask was observed daily under an inverted light microscope (Nikon).

2.6. Development of the primary hemocyte culture

The hemolymph was collected aseptically from the *P.vannamei* ventral sinus located at the base of the first abdominal segment using a 2 ml syringe containing Modified Alsever's solution (27 mM sodium citrate, 336 mM sodium chloride (NaCl), 115 mM Glucose, and 9 mM ethylenediaminetetraacetic acid (EDTA) made up to 100 ml using sterile de-ionized water (George and Dhar, 2010). The collected hemocytes containing 1 ml of Modified Alsever's solution and 1 ml of hemolymph were mixed with L-15 medium and stored in ice, immediately, for a few minutes. The cells were pelleted by centrifugation at 200 x g for 10 minutes at 25 °C and resuspended in the culture medium and seeded in 25 cm^2 tissue culture flasks and incubated at 28 °C.

2.7. Subculture of cells

When a monolayer of cells was formed it was washed with PBS to remove the cellular debris and FBS. The cells were incubated with 0.25% of trypsin EDTA solution at room temperature for a few seconds and were observed under the inverted phase contrast microscope. When the cells began to detach, the trypsin EDTA solution was discarded and the culture flask was gently tapped by hand to completely dislodge the cells. The trypsinized cells were treated with 2x L-15 medium containing 20% FBS, which was added to inhibit the trypsin action. The cells were subcultured at a split ratio of 1:2 and observed periodically for confluency.

2.8. Molecular identification of cultured cells

The DNA was extracted from the primary monolayer cells of *P.vannamei* and PCR was carried out (Swaminathan et al., 2010). The fragments of the cytochrome c oxidase subunit I (*COI*) genes were amplified using universal primers F 5′- TCA ACC AAC CAC AAA GAC ATT GG CAC-3′ and R 5′TAG ACT TCT GGG TGG CCA AAG AAT CA-3′. The PCR products of the fragment (*COI*) gene were sequenced by an ABI 3730 DNA analyzer (Applied Biosystems). The sequences of the mtDNA gene fragments were compared with the published and known sequences in the National Center for Biotechnology database, using the basic local alignment search tool (BLAST).

2.9. In vitro replication of white spot syndrome virus in hemocyte culture

To confirm the presence of WSSV in infected *P. monodon* were collected from a local farm and confirmed with PCR methods for WSSV detection (Kimura et al. 1996). The tissues, namely, gills, pleopods, and hemolymph were pooled and homogenized with sterile PBS (10 ml, pH 7.2). The homogenized sample was centrifuged at 6000 x g for 20 minutes at 4 °C and the supernatant was collected in a sterile tube and centrifuged at 10,000 xg for 20 minutes at 4 °C. The collected supernatant was filtered through a 0.4 µm syringe filter (Millipore, India) and stored at -80 °C for further use (Jose et al., 2010). Control inoculums of gills, pleopods, and hemolymph were prepared, as described above.

When the hemocyte cell culture reached the confluent monolayer, the WSSV diluents (500 µl with ~1×10^6 copies in culture medium), prepared in maintenance medium (L-15 medium with 2% fetal bovine serum), were inoculated in the culture flask. After one hour of adsorption at room temperature, the media was replaced with WSSV-free maintenance medium and the infected cells were incubated at 28 °C. The cells were observed under an inverted phase contrast microscope, daily, for a Cytopathic effect, and the infected cells were collected for further use as described by Jiang et al., 2006; Maeda et al., 2004.

2.10. Confirmation of white spot syndrome virus infection in the hemocyte culture

The DNA was extracted from the WSSV-infected *in vitro* primary hemocytes and the control hemocyte cells, using the QIA amp DNA Mini Kit (Qiagen, USA). Approximately, 1 ml of the cell suspension was collected from the control and infected cells and centrifuged at 1000 rpm for 10 minutes and DNA was extracted according to the manufacturer's instruction. Total DNA was quantified by the Nanophotometer (Implen, Germany) and used for PCR, the presence of WSSV in the hemocyte cultures confirmed by Kimura et al. 1996. The PCR amplification was performed in 25 µl reaction mixture, as described. The reaction mixture comprised of 25 µl mixture containing both forward and reverse primers (25 ng/µl each), 2.5 µl of reaction buffer (10x), 0.5 µl of 10 mM dNTPs, 1.0 µl Taq DNA polymerase (1 U/µl), 1 µl template, and nuclease-free water (18.0 µl). The PCR profile was as follows: Initial denaturation at 95 °C for 5 minutes followed by 35 cycles of denaturation at 95 °C for 30 seconds, annealing at 55 °C for 30 seconds, extension at 72 °C for 1.5 minutes, and a final extension at 72 °C for 7 minutes. The thermocycling conditions were the same for both the first and second steps. The amplified products were mixed with 6x loading dye along with the buffer and electrophoresed through 1.5% of agarose gel (containing 0.5 µg/ml ethidium bromide) in the Tris/Borate/EDTA (TBE) buffer at 100 V for approximately one hour. The gel was visualized using a gel documentation system (Bio-Rad, USA).

2.11. In vitro cytotoxicity of bacterial extracellular products used for primary hemocyte cell culture

The cytotoxicity of the primary hemocyte cell culture against

bacterial ECPs from *Vibrio parahaemolyticus* and *Vibrio harveyi* were evaluated. Bacterial ECP was isolated using the method as described by Swaminathan et al., 2010. To assess the toxicity, primary hemocyte cells were developed from *P.vannamei,* by using formulated HBSCM-5. Upon reaching the confluent monolayer, the cells were inoculated with 0.1 ml serial dilutions of ECPs of *V.parahaemolyticus* and *V.harveyi*. For negative controls, the cells were inoculated with sterile saline and incubated at 28 °C. The effects of ECPs on the cells were observed after 12–24 hours, to evaluate the toxicity.

3. RESULTS

In vitro cell culture was developed from the hemocytes of *P.vannamei* and various tissues of the heart, lymphoid tissue, hepatopancreas, gill, eyestalk, and muscle, using the newly formulated medium. The medium was formulated based on the amino acid contents of the hemolymph. The amino acid content in the hemolymph was lower than the amino acid content of L-15 medium. Therefore, five different mediums (HBSCM-1, HBSCM-2, HBSCM-3, HBSCM-4, and HBSCM-5) were prepared, with supplements, including proline, glucose, and 15% FBS. All these media were individually screened for proliferation of cells from hemocytes and various tissues of *P.vannamei.* Among the culture media, the HBSCM-5 medium was found to be the most prominent culture medium. The cell growth and rapid monolayer formation with typical morphological characteristic features such as fibroblasts, epithelial-like cells, and round cells were observed in the HBSCM-5 medium, when compared with other media.

3.1. Formulation of new cell culture medium based on the hemolymph amino acid composition of P.vannamei

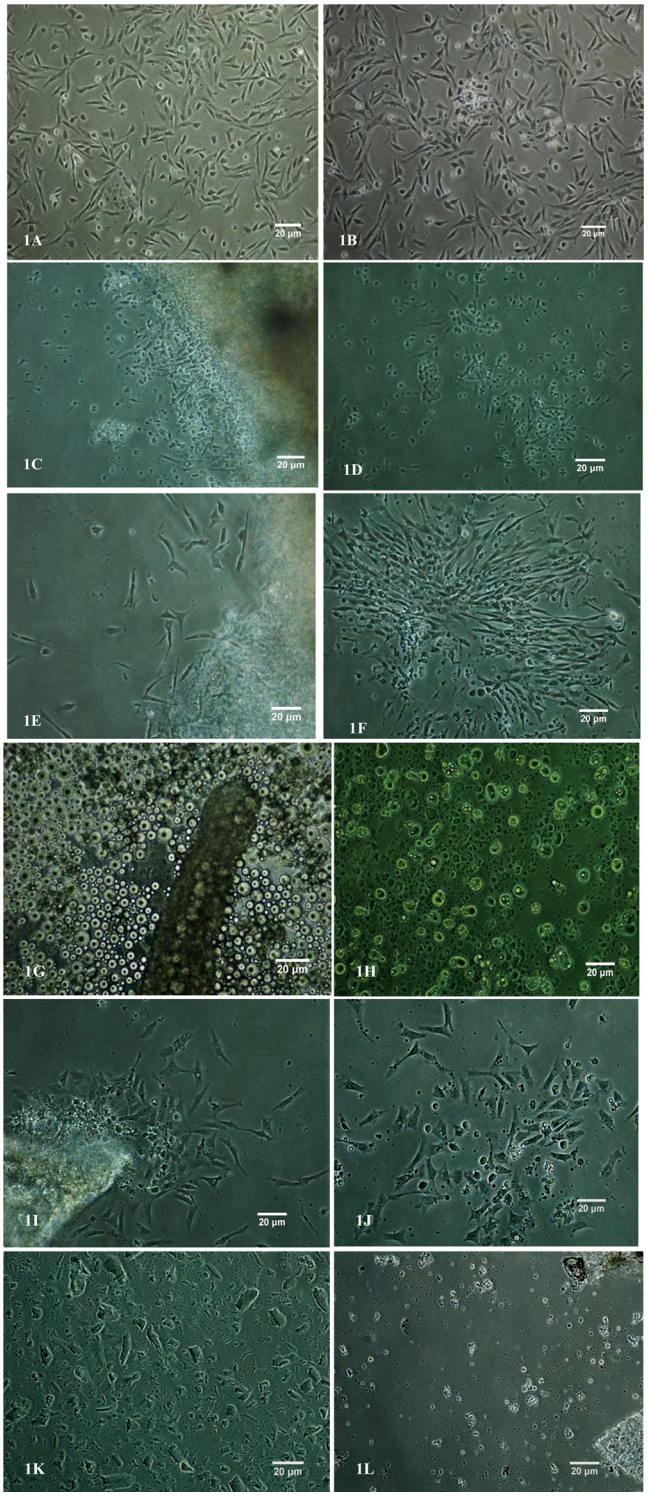
The presence of essential and non-essential amino acids in the hemolymph of *P. vannamei* was quantified using the HPLC method (Table 1). Cysteine was absent in the hemolymph. The amino acid content in the hemolymph was compared with that of the L-15 medium, which revealed a high concentration of amino acids in the L-15 medium. The biochemical parameters and electrolytes present in the hemolymph were estimated. The pH of the hemolymph was 6.8. The glucose concentration in the hemolymph ranged from 35–40 mg/dl.

3.2. Screening of a newly formulated cell culture medium for developing a cell culture system from different tissues

The explant of heart, lymphoid tissue, hepatopancreas, gill, eyestalk, muscle, and hemocyte cells from *P.vannamei* were seeded in the formulated media with various supplements (HBSCM-1, HBSCM-2, HBSCM-3, HBSCM-4, and HBSCM-5). The medium formulated according to the hemolymph amino acid contents of the HBSCM-1 medium showed very low cell attachment and viability when compared to the other media. The HBSCM-2 and HBSCM-3 media showed moderate attachment and viability of cells. However, significant healthy cells were not observed when compared to HBSCM-4 and HBSCM-5 media. Comparison between HBSCM-4 and HBSCM-5 media revealed that the latter was the best medium for growth and attachment of cells. Culture duration and viability difference were not observed between HBSCM-4 and HBSCM-5 media, but prominent morphological changes were seen in the HBSCM-5 medium.

3.3. Development of primary hemocyte cell culture

The rapid multiplication and proliferation of cells was high in the hemocyte culture when compared to other tissues. The hemocyte cells adhered to the surface within six hours and a confluent monolayer was attained within 24–72 hours. Most of the cells were fibroblasts and a round cell morphology was observed (Fig. 1 1a and 1b). The monolayer cells were maintained for 20 days. The cells were subcultured up to five passages, using trypsin. After subculturing, the cells were seen to be attached, but a gradual decrease in the number of viable cells was found and a complete monolayer was not attained after trypsinization.



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3.4. Development of primary heart cell culture systems

Rapid cell migration and adherence was observed in the heart explant culture. From the explant culture, individual cells and clumps of cells migrated and attached firmly in the culture flask. The adhered

Fig. 1. Inverted phase contrast micrograph by using various tissue culture derived from the *P.vannamei* cells showing at (20x). (1A & 1B) Primary hemocytes cell culture showed fibroblast like morphology and complete monolayer cells was observed in the HBSCM-5 medium for 5 & 7 days respectively. (1C) Heart explants cell culture from clumps initially and cells migrated and attached firmly in the culture flask. (1D) Heart primary cells showed most intact, adherent cells, the cells were round and with epithelioid-like cells morphology. (1E) Lymphoid explant tissue culture showed rapid cell attachment and migration. (1F) Lymphoid cells attached to the surface of the culture flask, most of the cells were fibroblasts with a round like cell morphology. (1G) Primary cells of hepatopancreas form a complete monolayer of cells was observed. (1H) after sub cultured cells showed partially attachment and lead to cell lysis was observed. (1I) Gill explants tissue of the cells migrated and moved away from the initial site and showed fibroblast like morphology. (1J) Gill primary cells predominantly showed fibroblast-like and epithelioid cell morphology were observed. (1K) Muscle explant cells also were attached in the culture flak but these cells not seen unique morphology of the cells. (1L) Eyestalk explant cell culture did not showed unique cell morphology.

cells and cell masses proliferated rapidly and a monolayer was formed within four to five days. The cells were able to maintain maximum viability for 25 days in the HBSCM-5 medium. In heart cell culture, the cells were round, with epithelioid-like cells (Fig. 1 1c and 1d). The cells became black, detached, and the original cell morphology was lost. Initially, the cells were able to subculture for three passages, following which complete lysis of the cells was observed.

3.5. Development of primary lymphoid tissue cell culture

In the HBSCM-5 medium, rapid cell attachment and migration was observed in the lymphoid tissue culture. Within a few hours, the cells began to migrate from the explant and develop a round morphology. After 12–24 hours the cells attached to the surface of the culture flask, most of the cells were fibroblasts with a round cell morphology. The HBSCM-5 medium was found to support cell growth and formed a complete monolayer. When the primary cell culture attained a confluent monolayer, they were subcultured using trypsin. The cells got attached and predominantly showed fibroblast-like morphology (Fig. 1 1e and 1f). These cells were subcultured to a maximum of four passages in the HBSCM-5 medium with supplement, following which they lost their original morphology. Following the subculture, the cells were found to be partially attached, but cell replication could not be observed. The monolayer of cells could be maintained to a maximum of 27 days.

3.6. Development of primary hepatopancreas, gill, eyestalk, and muscle cell culture

The hepatopancreas cell culture revealed varied morphological features (Fig. 1 1 g and 1 h). The HBSCM-5 medium supported rapid migration and attachment of cells. Cell viability started declining after seven days, but the cells remained viable up to 10 days. Cells of the Hepatopancreas could be subcultured twice in this medium, but passaging lead to cell lysis. In the gill explant culture, the cells were partially attached and proliferation of cells in the HBSCM-5 medium was observed. From the explant tissue, the cells migrated and moved away from the initial site. They predominantly showed fibroblast-like and epithelioid cell morphology (Fig. 1. 1i and 1 j). The attached cells were able to maintain their integrity for 15 days. The cells could be subcultured, but appeared blackened, detached, and had lost their original morphology. After subculturing, the cells were found to be partially attached, without any proliferation. Within 48 hours of introduction of the explants into the culture flask containing the HBSCM-5 medium, very few cells of the eye stalk and muscle were seen to be attached (Fig. 1 1k and 1 l). The eyestalk and muscle cells did not show any unique morphology and only a few cells had attached in this medium.

3.7. In vitro cytotoxicity of hemocytes cells

This study revealed the susceptibility of primary hemocyte cells from *P.vannamei* to various concentrations of ECPs used. The hemocyte culture showed cytotoxicity against the bacterial ECPs isolated from *V.parahaemolyticus* and *V.harveyi*. After 24 to 48 hours of treatment, the hemocyte culture became round, detached, and the monolayer was destroyed. The cells lost their original spindle-shaped morphology and became round (Fig. 2. b and d). The normal hemocyte culture without treated cells showed fibroblast-like morphology was observed in (Fig. 2. a and c).

3.8. In vitro replication of white spot syndrome virus infection using primary hemocyte cells

The monolayer culture of hemocytes was maintained in the maintenance medium with 2% FBS. Normal hemocytes showed mostly fibroblast-like morphology after 24 hours. Infected cells showed cytopathic effects (CPE) after 24–72 hours of WSSV infection as seen in Fig. 2 (f). Cells were found to shrink and undergo lysis after 48 hours of treatment. The control cells appeared healthy until the completion of the experiment (Fig. 2. e). To confirm the presence of the WSSV virus in the hemocyte culture, conventional PCR was performed after three days of treatment. The polymerase chain reaction confirmed the presence of viral DNA in the cells, as was evident by the presence of prominent bands in the cells infected with WSSV and the positive control (Fig. 3).

3.9. Molecular identification of the cells

Molecular level identification of cell origin was performed by amplification of the *COI* mitochondrial DNA. A five-day-old confluent monolayer of hemocyte cells was used to confirm the origin of cells through PCR. DNA sequencing and comparative analysis shows a 99% match of the identified sequences of *COI* to mitochondrial DNA sequences of known *P.vannamei*. These sequences confirmed that primary hemocyte cells were derived from *P.vannamei*. These sequences were submitted to the GenBank, with accession numbers KY564433. This study confirms that the primary cell culture was originated from *P.vannamei*.

4. DISCUSSION

The present study is an attempt to develop an *in vitro* cell culture system from various tissues and hemocytes of *P.vannamei* using five different culture media, based on the amino acid composition of the hemolymph. Extensive farming increases the growth of extracellular parasites, namely, viruses, bacteria, and parasites that cause epizootic diseases in culture ponds and marine ecosystems. Therefore, this current scenario requires the need for the establishment of a cell line from a crustacean, to investigate the propagation of these viral pathogens (Wen et al., 1993). The limiting factors in the development of crustacean cell culture are the specific culture medium and preparation of cells to be cultured (Jayesh et al., 2013; Toullec, 1999).

Several researchers formulated the media by combining the commercially available medium with undefined solutions such as tissue extract, hemolymph, growth factor, sea water, sugar, and amino supplements, to augment continuous mitosis in cultured cells (Claydon and Owens, 2008; Najafabadi et al., 1992). These supplements supported mitotic cell division to some extent, but development of a continuous cell line remained unsuccessful. Various commercial available media namely L-15, M 199, DMEM, and Grace insect medium were used to develop cell culture systems, among which the L-15 medium supported the crustacean primary cell culture system (Cai and Zhang, 2014). Few investigators formulated the media based on the hemolymph composition and extended the longevity of cells (Jayesh et al., 2013; Najafabadi et al., 1992; Shimizu et al., 2001). The present study focused

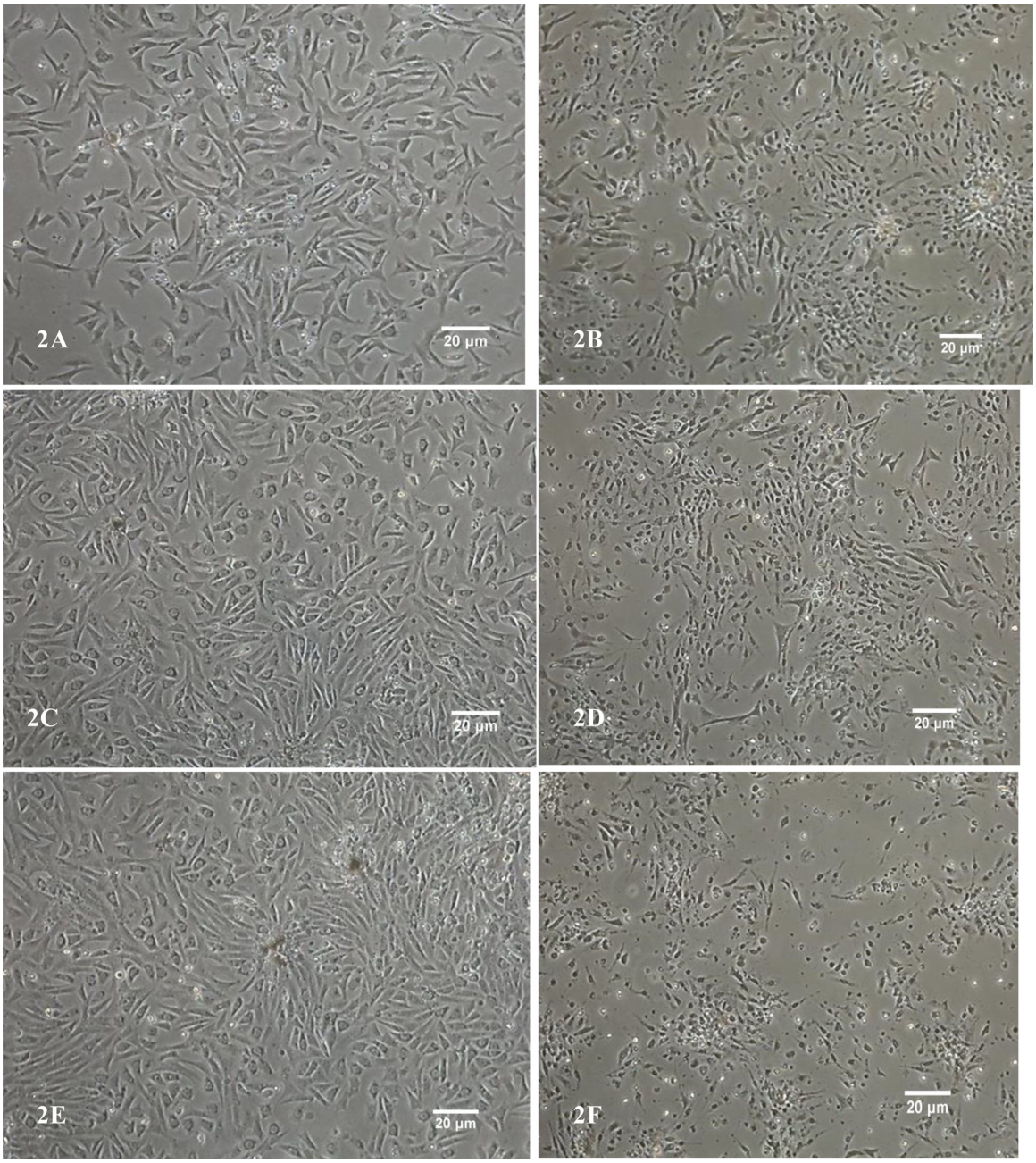

Fig. 2. Inverted phase contrast micrograph showing hemocyte culture derived from the *P.vannamei* cells at 20x. 2A, 2C & 2E showed normal hemocyte cells form a complete monolayer formation of cells and fibroblast-like morphology were observed. (2B & 2D) Primary hemocyte cells treated with *V.parahaemolyticus* and *V.harveyi* used for bacterial toxicity after 24-48 hours respectively and treated cells showed round, detached, and the monolayer was destroyed. The cells lost their original spindle-shaped morphology. (2F) Primary hemocyte cells treated with WSSV infected cells (48-72 hours) showed cells shrinkage, rounding and cell lysis was observed.

on a media formulation that was based on the hemolymph amino acid composition of *P.vannamei* and preparation of five different media according to the hemolymph contents.

The amino acid analysis of the hemolymph reveals the presence of various essential and non-essential amino acids and absence of cystine in the hemolymph. Amino acid proline is present in the hemolymph, which is absent in the L-15 medium. A similar result has been observed by Shimizu et al., 2001. Proline is one of the essential amino acids in crustaceans, which has two vital functions, namely, as osmotic effectors and for production of energy during starvation (Smith and Dall, 1991). Previous studies have also reported that proline enhances cell growth in the *in vitro* cell culture (Maeda et al., 2003). The same result has been observed in the present study, where addition of proline in the HBSCM-5 medium has enhanced cell growth. Glucose, sucrose, and trehalose can be used as energy sources for cultured shrimp cells (Alava and Pascual, 1987). The glucose level in the hemolymph varies according to species, diet, molting stage, and also varies from sample to sample, species to species, and season to season (Najafabadi et al., 1992; Shimizu et al., 2001). In the present study, the amount of glucose present in the hemolymph has been measured and was found to be 39.1 mg/dl. Glucose is the predominant carbohydrate in shrimp hemolymph, but the only carbohydrate source in the L-15 medium is galactose. Therefore, addition of glucose is vital for better growth of cells. Previous studies have modified the 2x L-15 medium with 2% glucose, which has enhanced cell growth (Jose et al., 2010; Kasornchandra et al., 1999). The present study also reveals the same

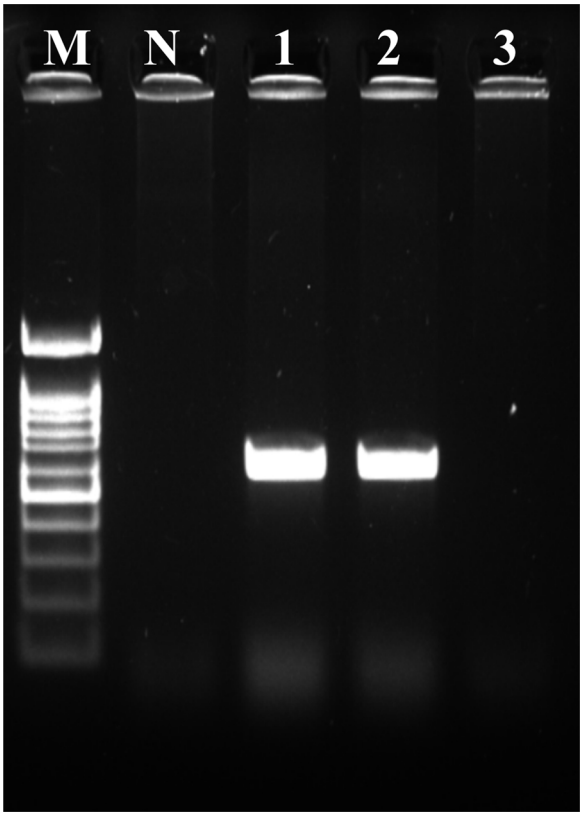

Fig. 3. The presence of WSSV was confirmed by conventional PCR method in the hemocyte culture (M) marker, (N) Negative, (1) WSSV positive control, (2) WSSV infected sample after 72 hours and (3) Negative control used for the hemocyte cells without WSSV.

result, where addition of 1% glucose enhances cell growth.

The present study has formulated the medium according to the contents of amino acid in the hemolymph. As the amino acid content in the hemolymph is considerably less when compared to the L-15 medium, the five different media have been formulated with supplementation of proline, glucose, and FBS. Though the media is chosen according to the amino acid content in the hemolymph, the cells are not able to survive in the HBSCM-1 medium, which may be due to insufficient nutrients. Metal ions such as strontium and bromine are present in natural seawater and absent in the L-15 medium, which can have a negative effect on the growth of shrimp cells (Shimizu et al., 2001). Among the five formulated media used in this study, the HBSCM-5 medium showed the best cell growth, when compared with the other formulated media. The medium formulated according to the hemolymph amino acid contents of the HBSCM-1 medium showed very low cell attachment and viability when compared to the other media. The HBSCM-2 and HBSCM-3 media showed moderate attachment and viability of cells. The HBSCM-1 and HBSCM-2 medium cells proliferation was not observed. When compared to the HBSCM-4 medium, HBSCM-5 showed better cell growth, cell morphology, and rapid proliferation, therefore, further studies were conducted with the HBSCM-5 medium. This was in agreement with the previous study by Han et al. (2013), in which the primary cell culture was developed from *Metapenaeus ensis* in different concentrations of the L-15 medium (0.2, 1.5, and 2x). Among these different concentrations, the cells were healthy and proliferated in 1.5 and 2x concentrations. Several studies reported that the 2x L-15 medium supported the cell growth and enhanced the proliferation of cells (Deepika et al., 2014; Jose et al., 2012).

Hemocytes play a vital role in defense mechanism by performing functions like phagocytosis, encapsulation and lysis of foreign cells and release of humoral defense molecules (Jose et al., 2010). In the present study hemocyte culture system was developed from *P.vannamei* showed rapid growth in HBSCM-5 and the cells were rapidly attached and confluent monolayer was formed. The hemocyte cells remained viable for 20 days and could be subcultured successfully for five times. Several researchers claimed to develop hemocyte culture in 2x L-15 medium and reported the same to be viable respectively for 30 days; 20 days; 8 days (Ellender et al., 1992; Jiang et al., 2006; Jose et al., 2010). Heart cell culture from *P.vannamei* also showed rapid growth in HBSCM-5 and the cells could be maintained for 25 days and three subcultures were performed. Our result were similar with (Tong and Miao, 1996) in which heart cells were viable for 20-30 days in a newly designed (MPS) medium. The result was comparable with other studies. Heart cells were found to be attached but proliferation was not observed in shrimp cell culture medium (SCCM) with natural seawater (Jayesh et al., 2013). Similarly, the present study attempted to develop a cell culture system from lymphoid tissue. Cells were found to be attached rapidly in the HBSCM-5 and maintained for 27 days. Lymphoid tissue culture from penaeid shrimp were maintained for 7-11 days (Chen and Wang, 1999; Kasornchandra et al., 1999).

Primary cell culture system was developed from hepatopancreas of *P.vannamei* by using the HBSCM-5 showed rapid migration, attachment, proliferation of cells and could be maintained for 10 days and successfully sub cultured twice. Similar result was observed in hepatopancreas culture (Chen and Wang, 1999). Hepatopancreas cells were able to survive for short period and these cells were maintained for 10 days, 25 days respectively (Jayesh et al., 2013; Nathiga Nambi et al., 2012). In gill explant culture rapid cell migration and proliferation were observed in this study. Healthy cells were maintained for 15 days in the HBSCM-5 and subcultured twice successfully. Only limited numbers of studies have been attempted using gill tissue but very poor attachment and cell migration was observed in these studies (Chen et al., 1986; Mulford and Austin, 1998; Nadala et al., 1993). Similarly, gill explant cell culture from freshwater crab was developed and maintained for 15 days (Nathiga Nambi et al., 2012). Limited research has been carried out to develop primary cell culture from eyestalk and muscle. In the present findings, cells were not found to exhibit any unique morphology and failed to replicate in any of the media. Previous study also showed that cell dissociation was induced using enzyme treatment such as collagenase and trypsin. The muscle and eye stalk cells were not attached to the surface of the culture flask (George and Dhar, 2010). The intricate structure outgrowth of neurons is a complicate process which got injured easily during dissociation process leading to cell death (Gao et al., 2003). This may be the reason for the failure of eye stalk primary culture. Similar results were observed in previous studies. Neither adhesiveness nor growth of cells could be achieved in muscle cells (George and Dhar, 2010; Jayesh et al., 2013; Kasornchandra et al., 1999; Nadala et al., 1993)

Primary cell culture and continuous cells lines are considered to mimic *in vivo* conditions, as they reflect similar activity and functions that they perform in the natural environment (Shashikumar and Desai, 2013). The primary culture that has been developed from various species and organs of crustaceans has been studied for the replication and pathogenesis of virus (Deepika et al., 2014; Jiang et al., 2006; Maeda et al., 2004). The present study also shows the propagation of WSSV in hemocyte cells cultured in the HBSCM-5 medium, supplemented with glucose, proline and 15% FBS. Microphotographs of virus-infected cells show rounding of cells, shrinkage, clumping, and detachment of cells from the substratum after 48 hours. The results are concordant with the previous studies, where WSSV in cultured cells of *P.vannamei* has shown rounding and detachment (Han et al., 2013). Apart from CPE, the presence of virus has been confirmed by the conventional PCR method, a sensitive technique for the detection of WSSV (Shashikumar and Desai, 2013). In the present study, the replication of WSSV in the hemocyte culture has been confirmed by the conventional PCR technique.

5. Conclusion

The present study has focused on the development of the *in vitro* cell culture system from *P.vannamei,* using six tissues with five different media and supplements. Among these media, the HBSCM-5 medium showed promising results in the proliferation of cells. The cultured hemocyte cells were able to form a complete monolayer and survived for a sufficient period, for use in several applications. The hemocyte cells were used to evaluate the *in-vitro* replication of WSSV and toxicity of various bacterial ECPs. The primary cell culture developed in this study would be useful for further research and for developing a continuous cell line.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jviromet.2019.04.016.

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