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ORIGINAL RESEARCH

Susceptibility of testicular cell cultures of crab, *Scylla serrata* (Forskal) to white spot syndrome virus

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Abstract Testicular cell culture of crab, *Scylla serrata* (Forskal) was used to study the effects of White spot syndrome virus (WSSV). We are showing the susceptibility of cell culture of crabs to WSSV. The proliferating cell culture of testes were maintained for more than 4 months in a medium prepared from L15 and crab saline supplemented with epidermal growth factor. The cell cultures inoculated with different concentrations of virus showed distinct cytopathic effects such as change in cell appearance, shrinkage and cell lysis. WSSV infection of cultured cells was confirmed by Nested PCR technique. The incorporation of viral DNA in cultured cells was shown by RAPD profile generated using 10-mer primers. The controls that were not exposed to WSSV did not show cytopathic effects. This work shows the usefulness of proliferating testicular cell culture for studying WSSV infection using molecular tools. Thus, this report gains significance as it opens new vistas for diagnostics and drugs for WSSV.

Keywords White spot syndrome virus · *Scylla serrata* (Forskal) · Crab · Cell culture · Nested PCR · Pathogenesis

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Abbreviations

VS	Viral suspension
WSSV	White spot syndrome virus

Introduction

White spot syndrome is one of the major infectious viral diseases that cause large scale mortality of commercially cultivated crustacean species which include shrimp, prawn, crab and other arthropods (Wang et al. 1998; Chang et al. 1998; Chen et al. 2000). Numerous studies on morphology, histopathology and gene sequence of White Spot Syndrome Virus (WSSV) are reported but the mechanism of WSSV infection is not understood (Jiang et al. 2006). Although new information is available on WSSV, relatively less is known about host parasite interactions (Jiravanichpaisal et al. 2006). To understand and ultimately to control the viral diseases in crustacea, a specific and sensitive diagnostic tool to investigate this pathogen needs to be developed (Claydon and Owens 2008).

Tissue culture is an important tool employed for the study of pathogenic infections, especially for pathogens such as viruses that replicate intracellularly (Jiravanichpaisal et al. 2006). Claydon and Owens (2008) reported that due to the presence of dominant senescence genes, crustacean primary cell culture has limited proliferations. A few primary cultures of

crayfish and prawn tissues have been developed earlier for the diagnosis of viruses infecting crustaceans (Uma et al. 2002; Jiravanichpaisal et al. 2006; Li and Shields 2007; Seena et al. 2010) but long surviving and proliferating cell cultures were not available for evaluating viral infections. Since we reported long surviving proliferating testicular cell culture (cell line) of crab, *Scylla serrata* (Shashikumar and Desai 2011), it was decided to test our cell line of *S. serrata* (Forskal) as an in vitro system for studying pathogenesis of WSSV infection which is also known to infect crabs (Supamattaya et al.1998; Liu et al. 2011).

Materials and methods

Cell line preparation

Healthy crabs and their tissues which tested negative with Nested PCR WSSV technique were used for tissue culture purpose. Cell line from testes of *S. serrata* (Forskal) was established according to the method described by Shashikumar and Desai (2011). Briefly, the testes were removed, transferred to a flask containing sterile artificial sea water having 0.3 % antibiotics mixture (Antimycotic–A002A + Gentamysin–A005 + Amphotercin B–SD233, Himedia, Pune, India). The osmolality of seawater was adjusted to 1,050 mOsm/kg. After the final wash the tissues were cut into fragments and transferred to a 30 mm petridish containing sea water. The fragments were triturated and the dispersed cells were filtered through a 100 µm nylon mesh. The filtered cells were spun for 3 min at 1,000 rpm. The resulting pellet was resuspended in L15–crab saline (NaCl 440 mM, KCl 11.3 mM, $CaCl_2$ 13.3 mM, $MgCl_2$ 26 mM, Na_2SO_4 23 mM, HEPES 10 mM) medium. The cell viability was assessed by trypan blue dye exclusion test. The dissociated cells were then inoculated at a density of 1×10^6 cells in L15–crab saline medium supplemented with Epidermal growth factor (EGF—20 ng/ml, Sigma E4127) + antibiotics mixture (0.3 %). The osmolality of the medium was 1,050 mOsm/kg). The mitotic index of testicular cell culture was determined by counting mitoses in cultures as a proportion of the whole population. Stable proliferating cell cultures of testicular cells that underwent five passages were used as controls and for testing WSSV infection. The cell viability and cell count was recorded before exposure to WSSV. Cell size and shape was determined with Image analyzer (Image pro-Express US, Nikon microscope 102 Eclipse TS100, TI-SM Japan).

Random amplified polymorphic DNA (RAPD) analysis of cultured cells using primer specific for *S. serrata*

In order to confirm that the cultured cells were derived form parental tissue of *S. serrata*, RAPD analysis was done using primer OPD20, OPD08 (Yoon and Park 2006). The parental tissue and cultured cells genomic DNA was isolated using DNAzol® reagent (DN 127–Molecular Research Centre, Inc. Ohio) following the manufacturer's recommendation. The sequence of the primer was 5′ACCCGGTCAC′ (OPD20) and 5′ GT GTGCCCCA3′ (OPD08). Amplification reaction was carried out using biometra thermal cycler with 2.5 µl buffer (10X), 1 µl of each primer, 2.5 µl of 2.5 mM of each dNTP, 2.5 units of Taq DNA polymerase and 1 µl Template DNA and 9.5 µl nuclease free water. The PCR reaction was initiated by heating the mixture for 5 min at 94 °C followed by 35 cycles of 1 min at 94 °C, 1 min at 34 °C, 2 min at 72 °C and additional 1 cycle of 7 min at 72 °C. The resulting amplified products (12 µl) were loaded onto 1.4 % agarose gels, subsequently stained with ethidium bromide and viewed under UV-light.

Preparation of white spot syndrome virus inoculum

WSSV suspension was prepared from gills and cuticles of heavily infected *Penaeus monodon* stored at −80 °C following the protocol described by Jiang et al. (2006). Briefly 1 g of tissues, e.g. gills, cuticles were homogenized separately in 10 ml of artificial sea water (pH—7.4, osmolality—1,050 mOsm/kg) and then centrifuged at 600*g* for 20 min at 4 °C. The supernatants were passed through 0.2 µm sterile filter fitted to a syringe and the filtrates were labeled appropriately and stored at −80 °C until used. These virus suspensions were serially diluted (10^{-1}, 10^{-3} and 10^{-6}) with culture medium (L15–Crab saline). To evaluate the virus concentration that infects 50 % (ID_{50}) of the cell cultures, the viral titers were prepared from the gill and cuticle suspension by the end point dilution assay following the method of Reed and Muench (1938). In brief, the testicular cell cultures

(ten monolayers) were infected with each serial dilutions of viral stock as mentioned above. The number of cell cultures that were infected was then determined for each virus dilution, by looking for cytopathic effect.

Exposure of testicular cell line to WSSV

The cultured testicular cells were inoculated with different dilutions (10^{-1}, 10^{-3} and 10^{-6}) of WSSV suspension (Gill, Cuticle) following the protocol of Jiang et al. (2006). Control culture plates were inoculated with heat inactivated (at 95 °C for 5 min) viral suspension of 10^{-1}, 10^{-3} and 10^{-6} dilution as described by Maeda et al. (2004). The negative control culture plates received only plain medium used for dilution of virus in the identical volume. The extract of non infected *P. monodon* was used in the similar dilution as another control to check whether a factor other than the virus in a prawn is responsible for cytopathy. The virus inoculated culture plates were incubated at 28 °C for 24 h and then rinsed repeatedly (N = 5) with culture medium to divest the cultures of virus. Subsequently such cells were grown in chemically defined cultured medium (L15–crab saline medium osmolality—1,050 mOsm/kg supplemented with EGF (20 ng/ml) + antibiotics mixture 0.3 %) devoid of any viral contamination. The control and experimental cells were observed daily for cytopathic effects for a period of 1 month.

Exposure of primary cultures of *P. monodon* to WSSV extracted from infected testicular cell cultures

Penaues monodon of ~1 to 1.2 cm size were used for culture purpose; mechanical dissociation technique was employed to isolate cells form the muscle tissue of prawn. The tissue was triturated in L15 medium (osmolality—950 mOsm/kg), the cell suspension obtained was filtered through a nylon mesh (100 µm) and centrifuged at 1,000 rpm for a period of 3 min. The cells were inoculated at a density of 10^6/ml in L15 medium supplemented with Fibroblast growth factor (FGF 5 ng/ml). The infectivity of WSSV extracted from infected testicular cell cultures was tested on primary cell culture of *P. monodon* following the protocol described earlier for crab.

Methyl thiazol tetrazolium (MTT) assay

The toxic effects of viral infection to the cultured cells were analyzed using MTT—In vitro toxicology assay kit (TOX–1, Sigma Aldrich, Bangalore, India). Before the assay the cells were rinsed five times with crab saline to remove WSSV from the medium. The assay mixture contained tetrazolium salt. Mitochondrial dehydrogenase of viable cells cleave tetrazolium (3-[4,5-dimethylthiazol-2-yl]-2 diphenyltetrazolium bromide) to yield formazan crystals. The formazan crystals thus obtained were dissolved in acidified isopropanol. The optical density (OD) of resulting solution was measured at 570 nm wavelength using NanoDrop Technologies, Wilmington, DE, USA). The drop in OD of the solution indicates loss of mitochondrial dehydrogenase reflecting cell damage (Chen et al. 1999).

Isolation of DNA

The control as well as WSSV exposed cells were rinsed repeatedly with crab saline and then treated with accutase (1 %) for detaching them from the substratum. The detached cells were once again rinsed five times, vortexed and centrifuged at 1,000 rpm. The cell pellet was used for isolation of DNA using DNAzol® reagent following the manufacturer's recommendation. DNA concentration was determined by measuring absorbance at 260 nm wavelength using Nanodrop spectrophotometer (ND-1000). DNA extracts were stored at −20 °C.

Nested PCR with WSSV specific primers

For Nested PCR assay, WSSV detection Kit was used (Bangalore GeNei 106070, Bangalore, India). The first step—PCR reactions were carried out in 25 µl of reaction mixture containing 1.0 µl cell DNA, 23 µl PCR pre mix and 1 µl Taq Polymerase enzyme. The PCR reaction was performed in a thermocycler with the following protocol: denaturation at 95 °C for 3 min followed by 28 cycles: 30 s at 95 °C, 30 s at 58 °C, 30 s at 72 °C and extension for 5 min at 72 °C. Second step—PCR reaction was initiated by using 1 µl of first PCR product, 23 µl Nested PCR pre mix, 1 µl Taq Polymerase enzyme. Thermocycle condition was same as first step—PCR reaction. PCR reaction

products were separated on 1 % agarose gel and stained with ethidium bromide for band visualization.

RAPD analysis for testing WSSV infection

Randomly amplified polymorphic DNA profiles were generated using 10-mer RAPD primers such as RPL 304, B2, B9, and B19. Under PCR conditions, the sequences of these primers were 5′- AAAGCTGCGG-3′ (RPL 304), 5′-ACCCAAGTGG-3′ (B2), 5′-ACC CTTGTGG-3′ (B9), 5′-AAAGCTGCGC-3′ (B19). Amplification reaction was carried out in 25 µl of reaction mixture that consisted of 1.0 µl of cell DNA, 1.0 µl dNTP mix (2.5 mM), 1.0 µl primer, 0.5 µl $MgCl_2$ (25 mM), 0.5 µl XT5 DNA polymerase assay buffer (10X), 3U XT5 DNA polymerase enzyme and the mixture was made up to 25 µl with sterile distilled water. The PCR reaction was initiated by heating the mixture at 94 °C for 4 min followed by eight cycles of 45 s at 94 °C, 60 s at 35 °C, 90 s at 72 °C and 35 cycles for 45 s at 94 °C, 60 s at 38 °C and 60 s at 72 °C with a final extension for 7 min at 72 °C. After amplification, 12 µl of the amplified products were loaded on to 1.5 % agarose gel and resolved by electrophoresis. The gel was stained with ethidium bromide and viewed under UV-light.

Results

Testicular cell culture

Testicular cells from *S. serrata* formed a monolayer, after 2 h of inoculation. The cells could be maintained for more than 4 months at 25 °C in L15–crab saline (osmolality—1,050 mOsm/kg) supplemented with epidermal growth factor (20 ng/ml). The cultured cells always remained attached to the substratum of culture plates. The cells were subcultured after 6 days of initial plating and subsequently sub-cultured at intervals of 2 weeks. The cell cultures remained healthy viable and stable for 4 months and underwent ten passages during this period when not exposed to WSSV. These cells were examined regularly under microscope for: morphological changes, contaminations, proliferation and mitosis. RAPD profile generated using primers OPD20 and OPD08 from parental tissue and cultured cells shared similar band size (Fig. 1) indicating that the culture cells were derived from parental tissue of *S. serrata*. The end point dilution assay shows 50 % cell infection with 10^{-3} dilution for gill and 10^{-1} dilution for cuticle, indicating 10^{-3} and 10^{-1} as ID_{50}/ml for gills and cuticle suspensions respectively .

Testicular test cells

The cultured cells (testicular) used as controls were round (size ~ 10–38 µm) and healthy (Fig. 3a). These cells were proliferating with a mitotic index equal to 26 owing to which the cells varied in size.

WSSV effect on cells culture

Proliferating testicular cells inoculated with the virus showed cytopathic effects such as change in appearance, shrinkage, clumping, lysis and depletion of cell density. Table 1 shows the effect of viral suspension (WSSV titer) on cultured cells. After 4 h of inoculation with 10^{-1} diluted viral suspension (VS) prepared from the gills of infected *P. monodon*, the cells appeared dark under microscope (Fig. 2a, b), exhibited shrinkage followed by cell lysis and decline in population density after 24 h (Fig. 3d). Subsequently a clear area without cells was seen after 48 h of exposure to virus. The viral toxicity to cells depended on the dilution of viral suspension. At 10^{-3} dilution of VS cell shrinkage was less prominent but as the time progressed, the infected cells gradually detached from the substratum and formed aggregates or clumps leading to cell lysis within 5 days. The cells exposed to 10^{-6} dilution of VS appeared normal for about 1 month but subsequently became surrounded by cell debris (Fig. 3b). However, the cells exposed to VS

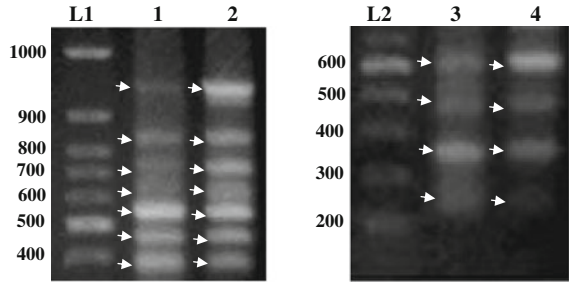

Fig. 1 RAPD profile of *S. serrata* (Forskal) generated using RAPD primers, OPD20 (*Lane L1* 100 bp ladder; *Lane 1* parental tissue DNA, *Lane 2* cultured cell DNA) and Primer OPD08—(*Lane L2* 100 bp ladder, *Lane 3* parental tissue DNA, *Lane 4* cultured cell DNA). *Arrowheads* indicate RAPD bands

prepared from cuticle at a concentration of 10^{-1} showed cell aggregation (Fig. 3c) at the end of 24 h followed by cell lysis on fourth day, but the cells treated with cuticular VS of 10^{-3} and 10^{-6} appeared nearly normal for first 20 days and then exhibited accumulation of debris around the clumps with a decline in cell proliferation depending upon the dilution of VS and exposure period. No WSSV effect was observed in the control cells exposed to heat inactivated VS of any dilution. The controls that were not exposed to WSSV showed no cytopathic effects, including the one prepared from the non infected (WSSV) prawn tissue. The cells of *P. monodon* showed cytopathic effect after exposure to virus at 10^{-1} and 10^{-3} dilution.

Methylthiazol tetrazolium (MTT) assay

MTT is a biochemical test for detecting changes in cell proliferation viz–viz cell toxicity. Cells infected with WSSV showed a significant decrease in cell viability as compared to controls. Viral suspension of 10^{-1} dilution prepared from gills and cuticles induced a prominent decrease in cell viability as indicated by drop in Optical density (OD) after 24 h of exposure (Fig. 4), whereas with VS of 10^{-3} and 10^{-6} dilution the toxicity effects were relatively less important. The toxic effects of VS of gills at 10^{-3} dilution was higher than that promoted by VS prepared from cuticles of the same strength (Fig. 4). The effects of VS of gills and cuticles at 10^{-6} dilutions were significantly less important than those exhibited by VS of 10^{-3} dilutions (Fig. 4). The MTT assay was meaningful for the first 24 h and subsequently was not useful as the cell lysis increased.

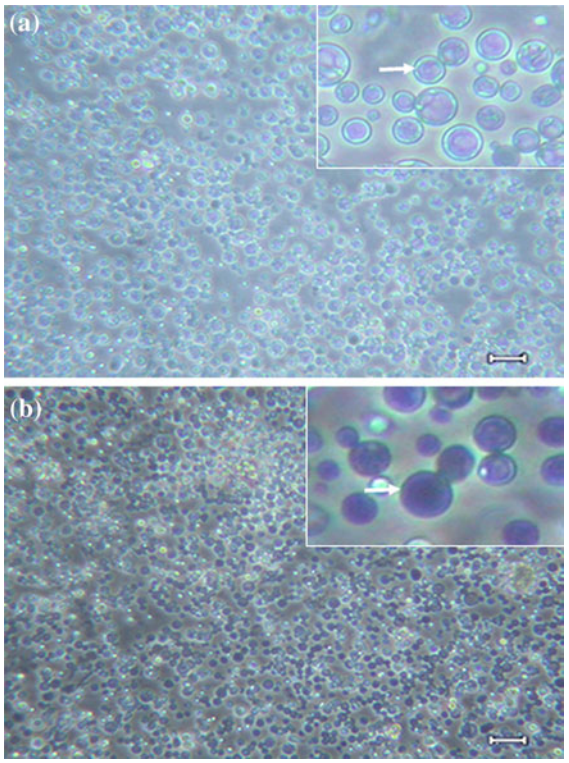

Fig. 2 Cell culture of testis inoculated with WSSV suspension (10^{-1} dilution). **a** Control culture **b** Experimental cells, showing change in cell appearance after 4 h of exposure to viral suspension. *Scale bar* 20 μm. In the insert in **a** arrow head indicates normal cell with prominent nucleus, and in **b** arrow head indicates change in appearance due to WSSV infection

Table 1 Effects of WSSV inoculums on cultured testicular cells of *S. serrata* (Forskal) at different viral concentrations

Inoculation days	Gill VS				Cuticle VS			
	10^{-1}	10^{-3}	10^{-6}	Control	10^{-1}	10^{-3}	10^{-6}	Control
2 h	+	–	–	–	–	–	–	–
1	+++	+	–	–	+	–	–	–
2	+++	+	–	–	++	–	–	–
3	+++	++	–	–	+++	–	–	–
4	+++	++	–	–	+++	–	–	–
5	+++	+++	–	–	+++	–	–	–
6	+++	+++	–	–	+++	–	–	–
7	+++	+++	–	–	+++	–	–	–
8	+++	+++	–	–	+++	–	–	–
9	+++	+++	–	–	+++	–	–	–
10	+++	+++	–	–	+++	–	–	–
Survival days without obvious change				11–60		11–30	11–60	11–60

–, Cells remained without obvious change

+, Cell clumped, cell shrinkage, few cell debris

++, The piles of cell debris were large

+++, Cell death

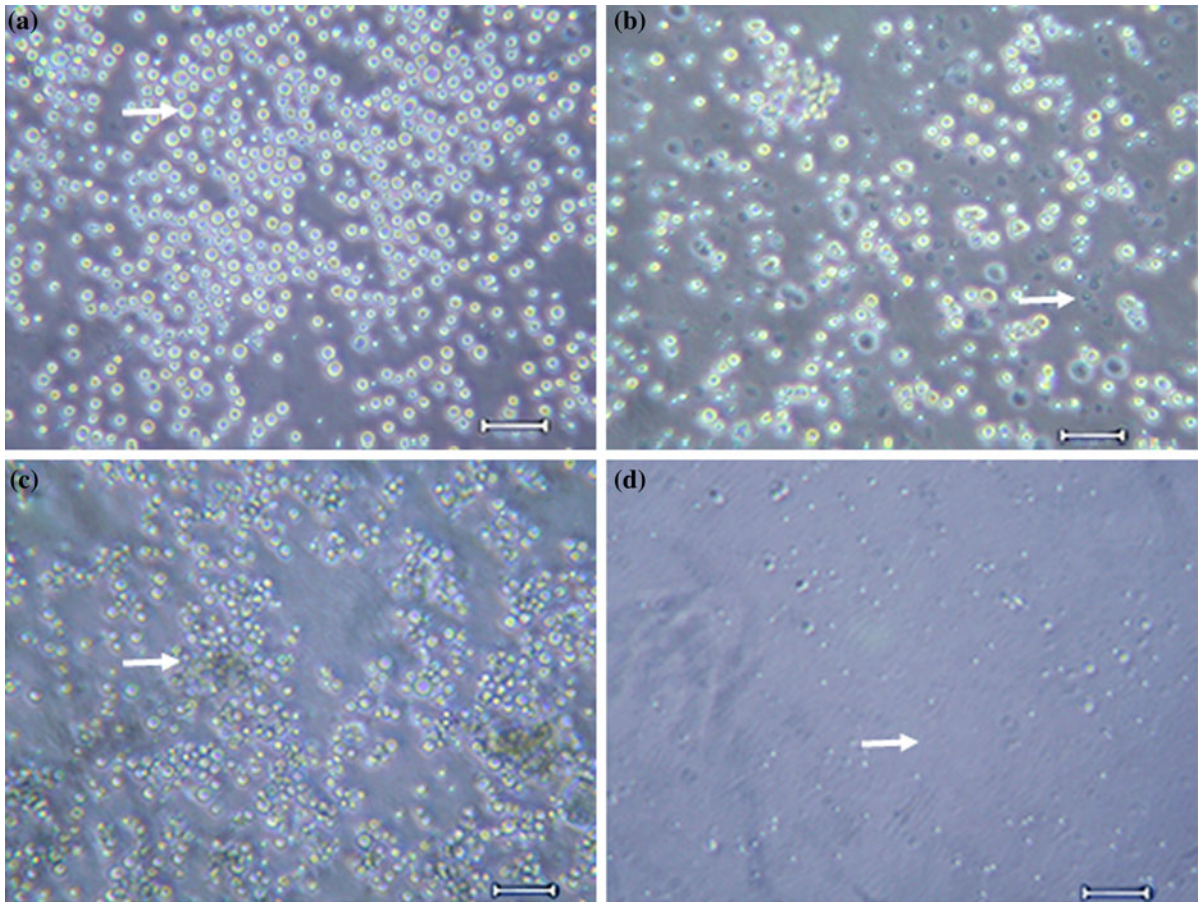

Fig. 3 Cell culture of testis showing cytopathic effects on exposure to WSSV–VS. **a** Control culture after 60 days of initiation of culture, the *arrow head* shows healthy cultured cells. **b** Experimental culture showing debris (Gill VS 10^{-6} dilution 60 days after initiation of culture), *arrow head* shows cell debris. **c** Experimental culture showing cell aggregation (Cuticle VS 10^{-3} dilution 3 days after initiation of culture), *arrow head* shows cell clumping. **d** Experimental culture showing cell lysis (Gill VS 10^{-1} dilution 24 h exposure), *arrow head* shows clear area divested of cells. *Scale bar* (**a**, **c**, **d**—20 μm), *Scale bar* (**b**—10 μm)

Nested PCR technique

The Nested PCR technique has clearly demonstrated the presence of viral DNA in the cells as evident by the presence of prominent bands at 275 bp for cells infected with WSSV and positive control (WSSV sample). However, the control cultured cells did not show any band (Fig. 5).

Random amplified polymorphic DNA (RAPD)

RAPD profile generated using primers RPL 304, B2, B9 shows presence of additional bands (of varying degrees of intensity) which were not present in the control cultured cells indicating the expression of these bands due to viral infection (Fig. 6). Primer B19 did not express any such additional bands.

Discussion

Cell cultures and established cell lines are regarded as better representatives of cells in vivo as they reflect the true activity and functions that they display in their natural environment (Morgan and Darling 1993). Primary cell cultures from different shrimp tissues such as lymphoid organ (Chen and Kou 1989; Chen and Wang 1999; Wang et al. 2000), heart (Chen and

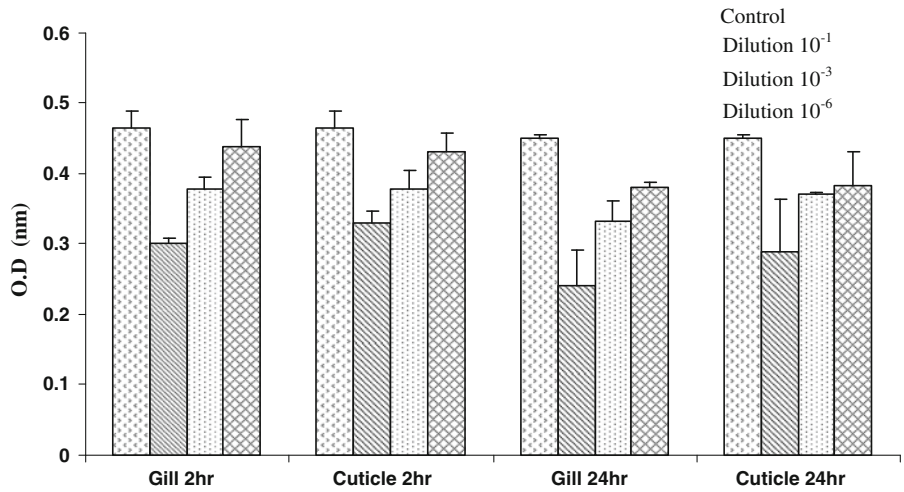

Fig. 4 MTT test on cultured cells inoculated with viral suspension (Gill, cuticle) diluted to 10^{-1}, 10^{-3}, and 10^{-6}, respectively, for 0–2, 2–48 h. All data represents mean ± standard error. Experiments were performed in triplicate

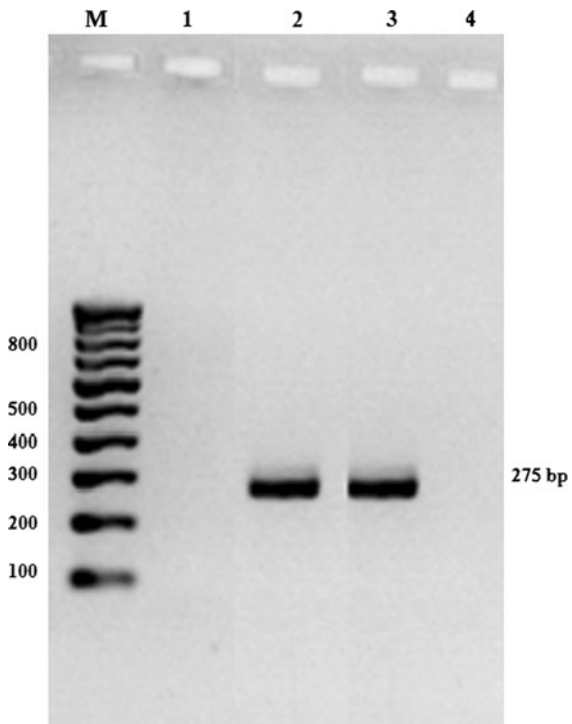

Fig. 5 Detection of WSSV by Nested PCR technique in cultured testicular cells inoculated with VS. *Lane M* StepUpTM 100 bp Ladder; *Lane 1* Control cultured cells, *Lane 2* Cells experimentally infected with WSSV, *Lane 3* Positive control (WSSV sample), *Lane 4* Negative control (Sample without WSSV)

Wang 1999; Owens and Smith 1999), gut (Nadala et al. 1993), ovaries (Maeda et al. 2004) have been used to study various viral infections and replications but long surviving and proliferating cell cultures were not then available for evaluating viral infections. Though, few researchers have attempted primary cell cultures of crabs (Ballard et al. 1993; Walton and Smith 1999; Sashikumar and Desai 2008; Zeng et al. 2009) none have used crabs' cell culture for studying WSSV pathogenicity.

The present study, probably the first of its kind, shows WSSV pathogenicity to the testicular cell line of crab (*S. serrata*). Microphotographs of infected cells clearly show: change in appearance, shrinkage, clumping, detachment from the substratum, cell lyses and declined cell population. But the control cells (normal, treated with heat inactivated WSSV with various dilution and the extract of non infected *P. monodon*) appeared normal and unaffected. Since the cell cultures exposed to WSSV were rinsed with culture medium (L15–crab saline) after 4 h to avoid further viral exposure of cells, the cytopathic effects obtained are due to the viruses which entered the cells. These results are similar to those reported for crayfish haematopoietic stem cells (Jiravanichpaisal et al. 2006; Uma et al. 2002). Maeda et al. (2004) reported rounding of primary cell cultures exposed to WSSV but in the present study WSSV infection did not promote rounding of cells.

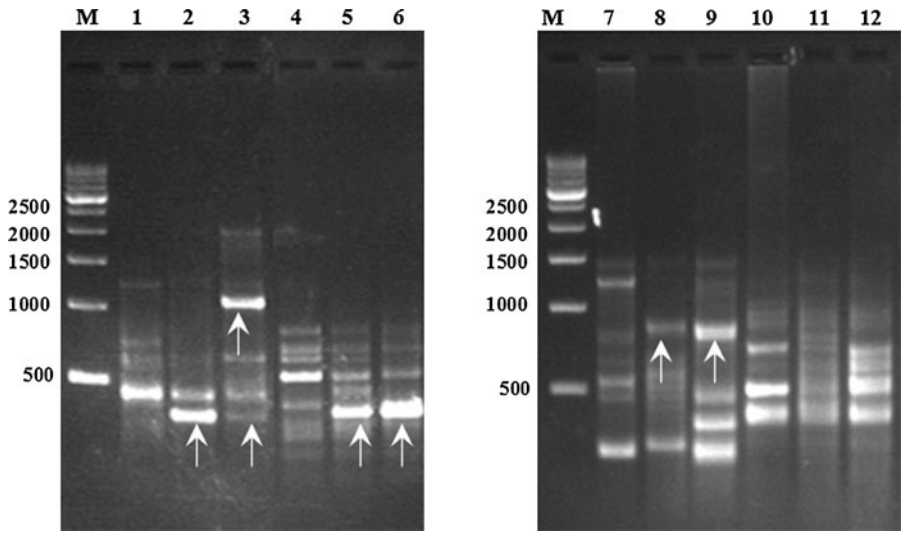

Fig. 6 RAPD Profile was generated from RAPD–polymerase chain reaction using 10-mers primers RPL 304, B2, B9 and B19. *Lane M* StepUpTM 500 bp Ladder, Primer RPL 304 [*Lane 1* Control cultured cells, *Lane 2* Cultured cells experimentally infected with WSSV (gill VS), *Lane 3* Cultured cells experimentally infected with WSSV (cuticle VS)], Primer B2 [*Lane 4* Control cultured cells, *Lane 5* Cultured cells experimentally infected with WSSV (gill VS), *Lane 6* Cultured cells experimentally infected with WSSV (cuticle VS). Primer B9 [*Lane 7* Control cultured cell, *Lane 8* Cultured cells experimentally infected with WSSV (gill VS), *Lane 9* Cultured cells experimentally infected with WSSV (cuticle VS)], Primer B19 [*Lane 10* Control cultured cells, *Lane 11* Cultured cells experimentally infected with WSSV (gill VS), *Lane 12* Cultured cells experimentally infected with WSSV (cuticle VS)]

Mosmann (1983) reported utility of MTT test to assess antiviral compounds' efficacy against a variety of viruses. However, this assay cannot be utilized for viruses that do not replicate well in cell cultures or for cells with low mitochondrial enzyme activity (Watanabe et al. 1995). The present study clearly demonstrates the utility of MTT test in detecting WSSV mediated cell damage and possibility of replication of WSSV in testicular cells as it does not show proper results for viruses that do not replicate in cell cultures (Watanabe et al. 1995). Besides, we also report the limitations of MTT test in case of very low infection of viruses in the cultured cells as those exposed to virus suspension of 10^{-3} and 10^{-6} dilution didn't significantly respond to MTT test. Nevertheless, MTT test significantly demonstrated the ability of WSSV (in 10^{-1} dilution) to infect the testicular cell causing cell lysis.

Boucard et al. (2010) have shown WSSV infection in oysters using PCR based molecular probes. PCR is the most sensitive molecular technique employed for the detection of WSSV infectivity. Currently, PCR techniques for WSSV detection use either a conventional amplification with a single sense/antisense primer set (Kanchanaphum et al. 1998; Kiatpathomchai et al. 2001) or a nested amplification (Lo et al. 1996). Nested PCR provides an increased level of sensitivity compared with conventional single primer-pair PCR. We have reported here WSSV infection in testicular cell cultures of *S. serrata* using PCR based molecular probes. The Nested PCR test and RAPD techniques clearly indicate that the morphological changes of testicular cells and subsequent decline in population density are due to WSSV infection.

Nested PCR is an effective tool in detecting early infection of WSSV and prognosis of it has been reported earlier by Otta et al. (1999); Hameed et al. (2003) and Uma et al. (2007). Chakraborty et al. (2002) successfully employed Nested-PCR for screening 89 tissue samples obtained from shrimps, crabs and squilla. The use of Nested PCR technique in the present work is in conformity of the reports with aforementioned scientists with reference to detection of WSSV infection and pathogenesis.

In addition to Nested-PCR technique we have used RAPD technique, using RPL 304, B2, B9 and B19 primers for ascertaining the infection. The RAPD technique has shown that primer RPL 304, primer B2 and B9 are suitable for detection of WSSV virus as the

cultured cells infected with the WSSV distinctly showed bands which were not reflected in controls.

Since the cultured cells were rinsed repeatedly (N = 5) after removal of virus containing medium, there was no chance for the virus to remain in the replenished medium or even attached to the cells. Had the toxicity been due to toxic substances released by the virus the results of the Nested PCR and RAPD analysis would have been negative, in the absence of the viral DNA. As the Nested PCR results are positive it clearly indicates the presence of viral DNA in the cells. Therefore, when the DNA was extracted from the cells the viral DNA accompanied the host DNA. The Nested PCR is known to be a highly sensitive test used in the detection of viral infections. Our current observations based on the positive Nested PCR test of infected cell line of crab, indicates the utility of cultured cells of crab for testing pathogenicity of WSSV.

There is hardly any report on establishment of a cell line from the crustacean species. This has resulted in lack of diagnostic techniques for detection of WSSV. Besides, non-availability of either cell lines or long lasting viable primary cell cultures has posed difficulties in studying the mechanism of viral infection, its replication and treatment as well as development of new drugs to contain WSSV. Our technique of culturing testicular cells has opened new vistas for development of drugs for WSSV and other crustacean diseases. As the testicular cells can be maintained in a viable and proliferating state for 4 months with susceptibility to WSSV, they could be a good diagnostic tool.

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