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Doctor Forum

Establishment and Characterization of a New Cell Line from the Kidney of Spotted Halibut *Verasper variegates*

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Abstract A cell line, SHK, was derived from the kidney of spotted halibut *Verasper variegates*. The cell line was subcultured more than 40 passages in minimum essential medium (MEM) supplemented with fetal bovine serum (FBS) and 10 ng ml^{-1} basic fibroblast growth factor (bFGF). Cell morphology from primary culture and subculture was observed continuously by microscopy. The SHK cell line consisted predominantly of fibroblast-like cells. The cell line was able to grow between 20℃ and 30℃ with the optimum growth at 24℃ and with a reduced growth between 12℃ and 20℃. The growth rate of the cells increased as the proportion of FBS increased from 10% to 20% at 28℃ with optimum growth at the concentration of 20%. The doubling time of the cells was determined to be 44.8 h. Chromosome analysis revealed that 52% of the SHK cells maintained a normal diploid chromosome number (2n=46). The cells were successfully transfected with green fluorescent protein (GFP) reporter plasmids and the expression of GFP gene in the cells indicated the possible utility of the cells in gene expression studies. The cells were infected by lymphosystis disease virus (LCDV) and found to be susceptible to the virus in cytopathic effect (CPE) observation. The infection was confirmed by PCR and electron microscopy experiments, which proved the existence of the viral particles in the cytoplasm of the virus-infected cells.

Key words cell line; spotted halibut Verasper variegates; SHK cell line; transfection; LCDV

1 Introduction

In vitro culture of fish cells provides an important tool for studying cellular physiology, molecular biology, functional genomics, toxicology and transgenic applications (Hightower and Renfro, 1988; Bejar *et al.*, 2005; Bahich and Borenfreund, 1991; Gagné and Blaise, 2000; Conception *et al.*, 2001; Oh *et al.*, 2001; Tiago and Laizé, 2008; Ma *et al.*, 2001; Pombinho *et al.*, 2004). By 1994, more than 150 cell lines have been established from teleost fishes, while relatively few cell lines were developed in marine fishes (Fryer and Lannan, 1994). In recent years, for the purpose of identifying fish viruses and researching functional genes in marine fish species, more and more cell lines were developed in marine fishes (Chi *et al.*, 1999; Chen *et al.*, 2003; Chen *et al.*, 2004; Chen *et al.*, 2007; Ye *et al.*, 2006; Parameswaran *et al.*, 2007a; Parameswaran *et al.*, 2007b; Zhou *et al.*, 2007).

In recent years, spotted halibut *Verasper variegatus* is becoming a commercially important marine teleost and has been widely farmed in China and other Asian countries (Yamada *et al.*, 1995). However, intensive aquaculture of this species has resulted in outbreak of viral diseases which brought high mortalities. By now, most researches about spotted halibut have emphasized on physiology, pathologic bacterium and breeding of spotted halibut (*V. variegatus*) (Wada *et al.*, 2004; Hirazawa *et al.*, 2004; Tian *et al.*, 2008) and little research has been done in the viruses that cause serious diseases in this species. Virus isolation and screening are inhibited due to lack of suitable host tool of the viruses. Thus, a susceptible cell line of spotted halibut is urgently needed for isolation and identification of viruses. At the same time, as the spotted halibut is a kind of commercially expensive fish in China, the research in vitro with a cell line of the fish will reduce the cost distinctly. In the present study, a kidney cell line from spotted halibut was developed. The efficiency of transfection and the susceptibility to the flounder lymphosystis disease virus (LCDV) were examined for further application of the cell line in gene operation and virus research.

2 Materials and Methods

2.1 Primary Cell Culture and Subculture

A healthy spotted halibut (*V. variegatus*) was obtained from Haiyang Marine Fish Hatchery (Qingdao, China).

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The fish was wiped with 70% alcohol and killed by over-anaesthetizing with 20% urethane. The kidney was removed sterilely and washed three times with phosphate-buffer saline (PBS). The kidney was sterilized with 70% alcohol for 5 min and then washed three times with PBS. The tissue was minced thoroughly with scissors and transferred to tissue culture dishes containing 5 mL of 0.25% trypsin solution (0.25% trypsin and 0.2% EDTA in PBS) for trypsinization. After 15 min, the trypsinized solution was filtrated and the supernatant containing cells was centrifuged at 1000 g for 5 min. Then the cells were transferred into complete growth medium in 25 cm^2 tissue culture flasks and incubated at 24℃ in a normal atmosphere incubator. The complete growth medium was MEM supplemented with 20 $mmolL^{-1}$ HEPES pH 7.4, antibiotics (penicillin, 100 U mL^{-1}, streptomycin, 100 $mgmL^{-1}$), 10% –20% FBS (Gibco), 10 $ngmL^{-1}$ bFGF and 100 mgL^{-1} Phytohemagglutinin.

When the cells formed a monolayer, the old medium was removed and the cell sheets were washed with PBS twice and dispersed with 0.25% trypsin and distributed into two flasks. The primary cells were cultured in the complete growth medium with 20% FBS, and the subcultured cells were cultured in complete growth medium with 10% FBS.

2.2 Morphological Observation

The CKX31 inverted microscope (Olympus Optical Co., Ltd) equipped with phase optics was used to observe and photograph living cell cultures every 2–3 d for primary cell cultures and subcultures.

2.3 Cryopreservation and Thawing of Cells

For cryopreservation, SHK cells were harvested by centrifugation and suspended at a density of 10^6 cells ml^{-1} in complete growth medium with 10% dimethyl sulphoxide and 10% FBS. The cells were stored at 4℃ for 0.5 h and then at –70℃ for 4 h, followed by storing in liquid nitrogen. When thawed, the cryogenic vials were dissolved at 40℃ and centrifuged at 1000 g for 4 min. Then the cells were suspended in MEM and seeded into 25 cm^2 cell culture flasks.

2.4 Growth of Cells

For growth studies, SHK cells were seeded into 6-well plate at an initial density of 1.5×10^4 cells per well and incubated at 24℃ for 6 days. The cells were trypsinized and counted using a haemocytometer every day with the method described by Wang and Belosevic 1994. The experiment was repeated three times.

2.5 Effect of Temperature and FBS on Cells Proliferation

To determine the effect of temperature on the proliferation of the cells, the SHK cells were inoculated in 25 cm^2 cell culture flasks at an initial density of 1.5×10^5 cells mL^{-1} with 20% FBS in medium and incubated at 12℃, 20℃, 24℃ and 30℃, respectively. After 2, 4 and 6 d the cells were trypsinized and counted microscopically using a haemocytometer. To determine the effect of different FBS concentration on cell proliferation, 1.5×10^4 cells were inoculated in 6-well plate in medium containing 20% FBS, 10% FBS or FBS-free media, respectively. Six days later, the cells were trypsinized and counted. Each experiment was repeated three times. Data were expressed as mean ± S.D., with mean values from the three replicate samples.

2.6 Chromosome Analysis

For chromosome analysis the SHK cells at passage 25 were studied. The preparation of chromosome was by the method of Ye (Ye *et al.*, 2006). The cells were inoculated in colchicine (0.8 $\mu g mL^{-1}$) for 4 h in 25 cm^2 culture flask and harvested by centrifugation with 1000 g for 5 min. Single cells were suspended in hypotonic solution of 0.075 $mmolL^{-1}$ KCl for 30 min and fixed two times in cold Carnoy's fixative with 15 min each time. Slides were prepared using the conventional drop-splash technique (Freshney, 1994) and then air-dried. Chromosomes were stained with 5% Giemsa for 20 min. Finally, chromosomes were observed and counted microscopically. One hundred photographed cells at metaphase were counted, and chromosome karyotype was analyzed according to the reported method (Levan, 1964).

2.7 Cell Transfection with GFP Reporter Gene

The ability of the SHK cells to be transfected was determined using pEGFP-N1 plasmid (Clontech, Germany), which expresses a green fluorescent protein (GFP) under the control of human cytomegalovirus (CMV) promoter. The plasmid DNA was prepared according to the supplier's instructions (Qiagen). Transfection reagent lipofectamine 2000 (Invitrogen) was used for plasmid transfection. In brief, the cells were seeded at a density of 1.5×10^4 cells per well in 12-well plates individually. After monolayer was with 90% confluence, the cells were transfected with 1 μg pEGFP-N1 plasmid in 6 μl lipofectamine 2000 reagent and incubated at 24℃. After 6 h the medium was changed with normal medium. GPF expression in the cells was detected under a fluorescence microscopy (Nikon eclipse, TE2000-U). The green fluorescence images were obtained by using a microscopy camera on the microscope.

To extract total RNA, the transfected cells were trypsinized and harvested by centrifugation at 1000 g for 5 min. The cells were mixed with 1 mL of TRIzol reagent (Invitrogen) and RNA was extracted according to the protocol of the manufacturer. In brief, the solution was incubated for 5 min at room temperature, and then 0.2 mL of chloroform was added. The sample was vigorously shaken for 3 min and then centrifuged at 12000 g for 15 min at 4℃. Then the RNA was precipitated from the aqueous phase with isopropanol, washed with 75% ethanol, and dissolved in 20 mL of TE buffer (10 $mmolL^{-1}$ Tris-HCl, 1 $mmolL^{-1}$ EDTA, pH 7.5). The amount of nu-

cleic acid in the sample was quantified by measuring the absorbance at 260 nm. The purity of nucleic acid was checked by measuring the ratio of OD260nm/OD280nm. The cDNA synthesis was carried out using M-MLV Transcriptase (Promega). For RT-PCR, one pair of primers specific to GFP (accession number: EU716638) was designed. The sequences were 5'- AGCCGCTACCCCGA CCACAT-3' (forward) and 5'- TGTACA GCTCGTCCA TGCCGA (reverse)-3'. The size of the PCR fragment was 499 bp. Reactions followed the steps: One cycle denaturation at 95℃ for 5 min followed by 32 cycles of denaturation at 94℃ for 30 s, annealing at 56℃ for 30 s and elongation at 72℃ for 45 min, ending with an additional elongation step of 10 min at 72℃. Amplified products were characterized by agarose gel electrophoresis with 1.2% agarose at 90 V voltages.

2.8 Virus Susceptibility

Lymphosystis disease virus (LCDV) separated from the infected *Paralichthys olivaceus* was employed to detect the susceptibility of the SHK cells 2×10^5 cells mL^{-1} at passage 25 was seeded into 25 cm^2 cell culture flasks with 80% confluence. After removal of the medium, 1 mL of virus suspension was inoculated into the cell culture. After 1 h the virus solution was removed and 3 mL of maintenance medium containing 5% FBS was added. The cells were incubated at 24℃ and observed daily for the appearance of CPE up to 2 weeks.

Genomic DNA was extracted from the infected cells. Cells were homogenized with 1 mL extraction buffer (100 $mmolL^{-1}$ NaCl, 10 $mmolL^{-1}$ Tris pH8.0, 25 $mmolL^{-1}$ EDTA, 1% SDS, 3 μg proteinase K). The mixture were incubated at 55℃ for 30 min. Equal volume of phenol/choloroform was added and then the mixture were centrifuged at 13000 $rmin^{-1}$, 4℃ for 15 min. Supernatant liquids were collected and extracted twice with equal volume of phenol/choloroform. Subsequently, twice volume of anhydrous ethanol was used to precipitate DNA. Finally, DNA was collected and washed with 75% ethanol, and then dissolved in sterile water. For PCR, one pair of primers specific to LCDV (accession number: EF059991) was designed. The sequences were 5'- CCGTTGATTCCA-ATGGTCA-3' (forward) and 5'- CACCGTCAAAGA-TTACAGGAG (reverse). The size of the PCR fragment was 491bp. Reactions followed the steps: One cycle denaturation at 95℃ for 5 min followed by 30 cycles of denaturation at 94℃ for 40 s, annealing at 55℃ for 40 s and elongation at 72℃ for 1 min, ending with an additional elongation step of 10 min at 72℃.

For electron microscopy observation, the SHK cells infected with LCDV were fixed with 2.5% glutaraldehyde in 0.2 $molL^{-1}$ sodium cacodylate buffer (pH 7.4) for 24 h at 4℃ and then post -fixed with 1% osmium tetroxide in 0.2 $molL^{-1}$ sodium cacodylate buffer for 1 h. The infected cells were then embedded in epoxy resin, sectioned and stained with 2% uranyl acetatelead citrate and examined under a Philips 201C electron microscopy.

3 Results

3.1 Primary Cell Culture and Subculture

The cells from kidney tissue completely covered the bottom of the flask within 2 weeks. Elongated fibroblastic cells formed the major component of the culture (Fig.1a, b). During the initial 8 subcultures, the cells grew slowly with a 15 d subculture interval. A combination of 50% of both new and old MEM media containing 20% FBS was used. Afterwards the cell proliferation was fast and subculture could be done every 6 or 7 d. To date, SHK cell line has been subcultured more than 40 passages.

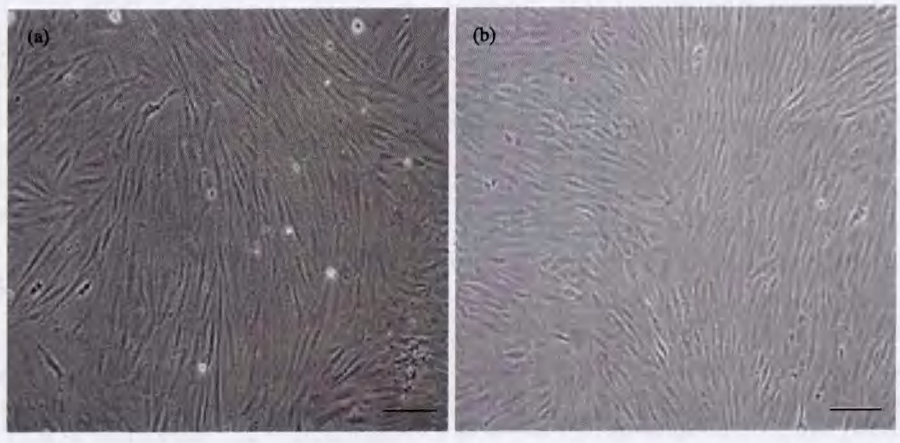

Fig.1 Morphology of the SHK cells: (a) after confluent monolayer, cells morphology being predominantly fibroblast-like cells at passage 8 (×100); (b) at passage 22 (×100). Bar: 100 μm.

3.2 Cryopreservetion and Thawing of Cells

The SHK cells were cryopreserved at different passages. After seeded into flasks the cells were recovered with a survival rate of 60%–70%. The cells could grow to confluence within 4–5 d and retained viability well.

3.3 Growth of Cells

The doubling time was determined to be 44.8 h for the SHK cells at an original density of 1.5×10^4 cells per well

at 24℃. The growth curves of SHK cells are shown in Fig.2. After 6 d, the cells number reached 3.5×10^5 cells mL^{-1}.

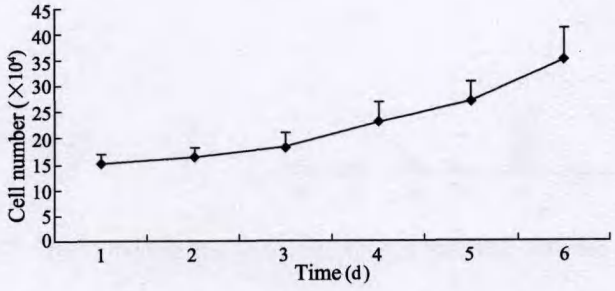

Fig.2 The growth curves of the SHK cells. The SHK cells were incubated at 24 ℃ at an initial density of 1.5×10^4 cells well-1 in 6-well plate. The cells were trypsinized and counted using a haemocytometer every day. Value are means ± S. E. (n=3).

3.4 Effect of Temperature and FBS on Cell Proliferation

At 2, 4 and 6 d the SHK cells at different temperatures were trypsinized and cell numbers were counted. The result showed that the SHK cells were able to grow between 12℃ and 30℃ (Fig.3). The highest growth rate was obtained at 24℃ with the cell number reaching 5.2×10^5 cells mL^{-1} after 6 d. The lowest growth rate was at 12℃ with the cell number reaching 2.1×10^5 cells mL^{-1} after 6 d. The growth of SHK cells was highly dependent on FBS concentration in the culture media. No obvious cell proliferation could be observed when there was no FBS in the media. Cell proliferation increased with the increase of FBS concentration in the media and the highest growth rate could be observed in medium containing 20% FBS (Fig.4).

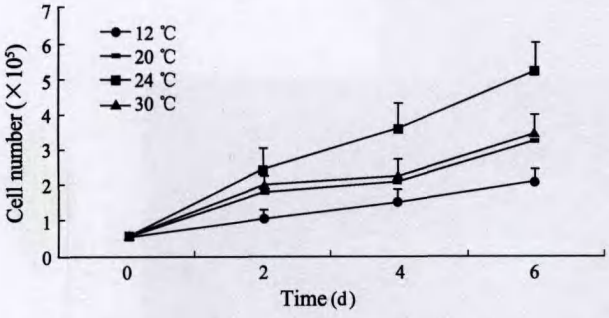

Fig.3. Effect of temperature on the growth and proliferation of the SHK cells. The SHK cells were inoculated in medium with 20% FBS at an initial density of 1.5×10^5 cells mL^{-1} in 25 cm^2 cell culture flasks. The cells were treated at 12℃, 20℃, 24℃ and 30℃, respectively. After 2, 4 and 6 d the cells were trypsinized and counted. Value are means ± S. E. (n=3).

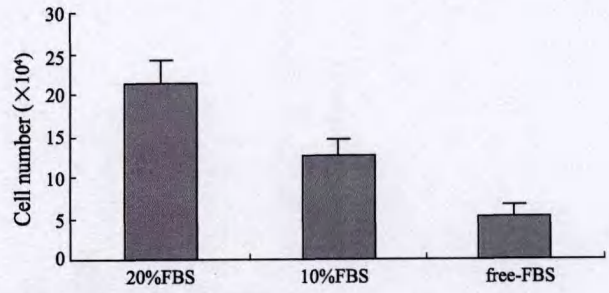

Fig.4 Effects of FBS on the growth cells and proliferation of the SHK. 1.5×10^4 cells were inoculated at 24 ℃ in 6-well plate in medium containing 20% FBS, 10% FBS or FBS-free medium, respectively. 6 days later, the cells were trypsinized and counted. Value are means ± S. E. (n=3).

3.5 Chromosome Analysis

Chromosome morphology of SHK cells is shown in Fig.5a and all chromosomes from the cells are telocentric (Fig.5b). The chromosome counts of 100 metaphase plates revealed that the number of chromosomes in SHK cells varied from 24 to 90. The modal number of chromosomes for the cell line was 46 (Fig.6) and 52% of the cells contained 46 chromosomes. Both heteroploidy and aneuploidy were observed in the cell line though the proportion is very low.

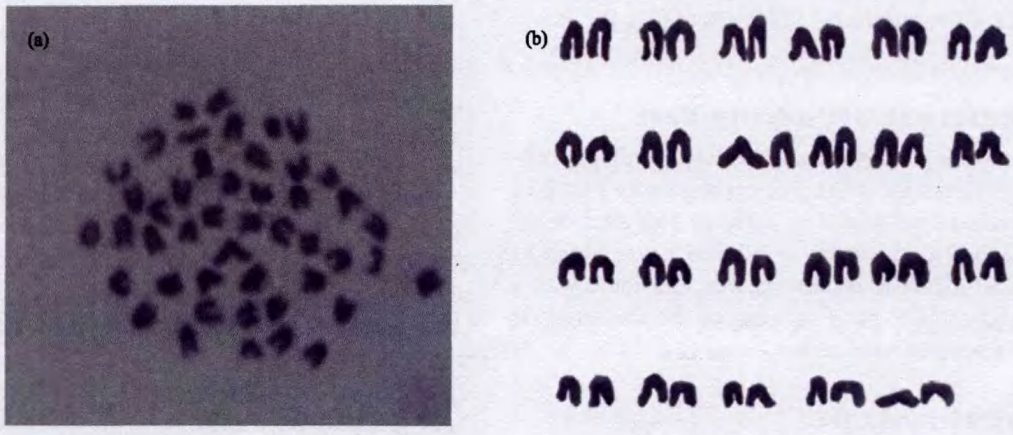

Fig.5 Photomicrographs of chromosomes (a) (×1000) and karyotype analysis (b) of the SHK cells. The chromosome morphologies of the cells were telocentric.

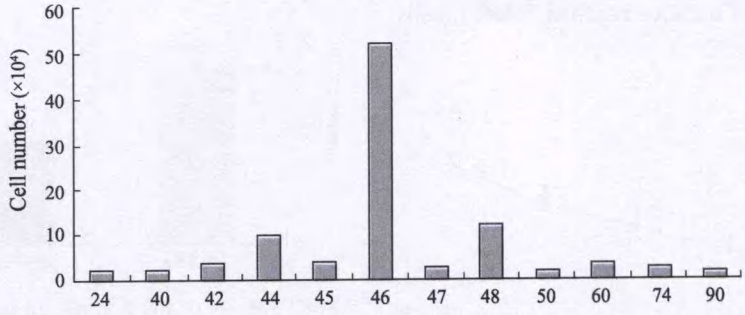

Fig.6 Chromosome number distribution, 100 metaphases being counted. SHK had a modal chromosomes number of 46.

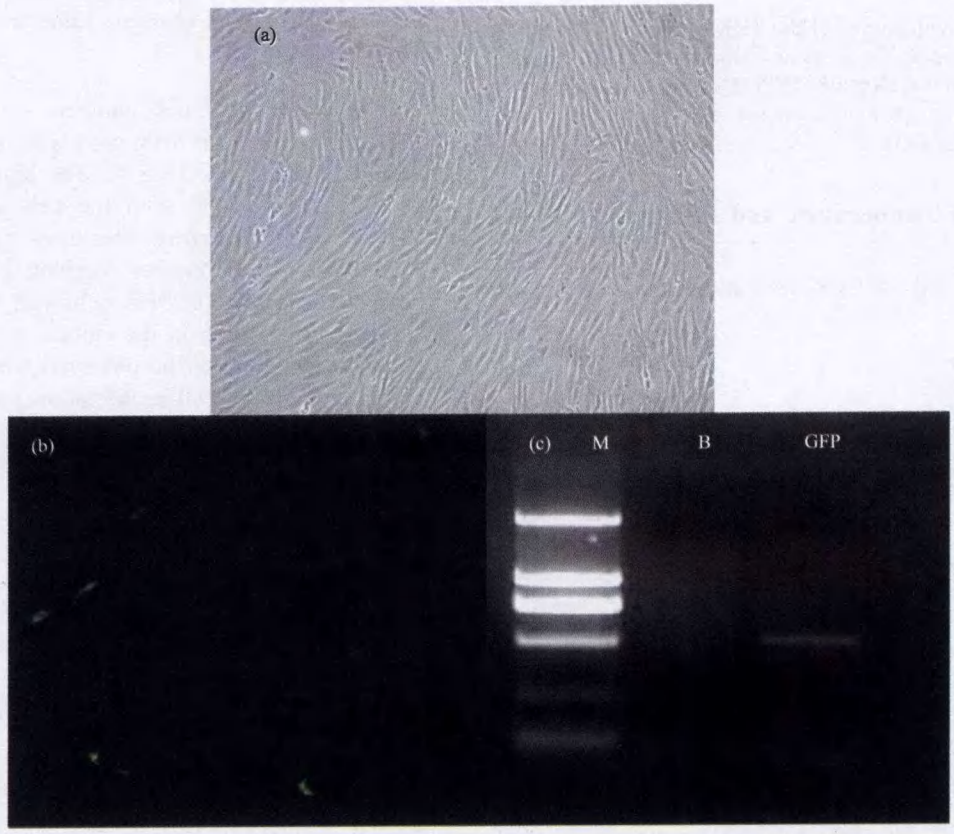

Fig.7 Green fluorescent protein (GFP) expression in transfected SHK cells transfected with pEGFP-N1. Control cell (a); Green fluorescent signals was found in the cells (b). EGFP was expressed in the SHK cells by RT-PCR (c) [M: marker; B: control cells; GFP: transfected SHK cells (499 bp)].

3.6 Transfection with GFP Reporter Gene

The cells were successfully transfected with pEGFP-N1 using lipofectamine 2000. The expression of EGFP in SHK cells could be detected as early as 16h after transfection (Fig.7). The transfection efficiency was found to be 10%–15%. RT-PCR results showed that there was a positive band of GFP gene segment in the transcription products of the transfected cells.

3.7 Susceptibility to LCDV

Cytopathic effect (CPE) in the cells was observed to evaluate the susceptibility of the SHK cells to LCDV. CPE appeared in the cells at 72h after infection of virus. Initially, the specific CPE developed as localized areas of rounded and refractile cells. Then the CPE with typical multiple vacuolation was observed and finally the monolayer was completely disintegrated after 10d (Fig.8b).

The CPE due to LCDV observed in SHK cells was confirmed by PCR (Fig.8c). The result of agarose gel electrophoresis showed that prominent band of PCR- amplified product of LCDV (491bp) appeared in the infected cells and no band appeared in the uninfected cells. Electron microscopy observation showed viral particles in the cytoplasm of virus-infected SHK cells (Fig.8d). Intracellular granules were distributed in the cytoplasm of virus-infected cells, which demonstrated viral infection. Virus with enveloped, hexahedral morphology were scattered throughout the cytoplasm of cells.

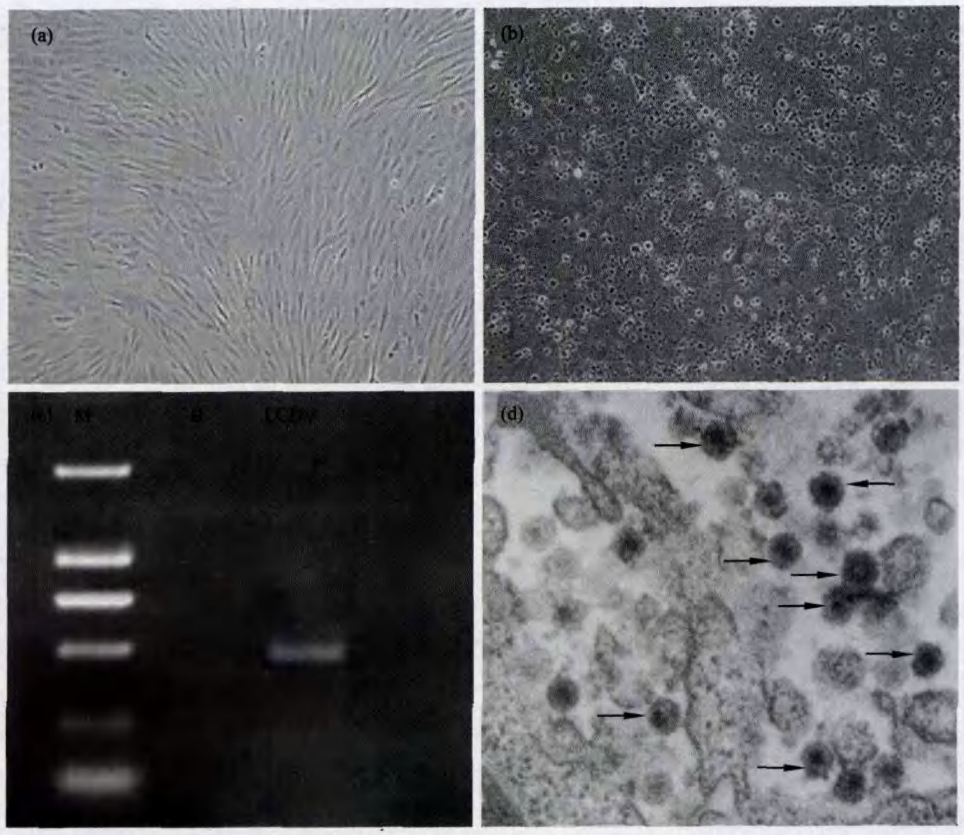

Fig.8 Susceptibility of infected SHK cells to LCDV at passage 25. (a) Confluent uninfected SHK cells; (b) The monolayer completely disintegrated after 10 days in LCDV-infected cells; (c) Confirmation of viral infection in SHK cells by PCR [M: marker; B: LCDV uninfected cells; LCDV: LCDV infected cells (491bp)]; (d) Electron micrograph of infected SHK cells. Enveloped hexagonal viral particles (thin arrow) were found in the cytoplasm of LCDV- infected SHK cells (×15 000).

4 Discussion

In recent years, many problems have been arisen in the aquaculture industry of the whole world. Virus and pollution have caused disease and death in marine fishes, entailing huge economic loss. Marine fish cell lines are increasingly established and have become important tools in toxicological and diagnostic studies of viruses (Parameswaran *et al.*, 2007a; Parameswaran *et al.*, 2007b; Zhou *et al.*, 2007). Because one fish species is usually susceptible to some specific viruses, developing more cell lines from different fish species is necessary to identify and characterize more viruses. Establishment of cell lines is also needed to develop cell models of cellular physiology, immunology and endocrinology and comparative biology in teleosts.

In the present study, a cell line from the kidney of spotted halibut (*V. variegatus*) was successfully established and characterized for the purpose of gene operation and virus research. The cell line was designated as spotted halibut kidney cell line (SHK cell line). The SHK cells maintained stable growth in MEM supplemented with growth factor bFGF. The cells have been subcultured more than 40 passages with a morphology of fibroblast-like cells and with elongated shapes.

The growth temperature range for the SHK cells was from 12 to 30℃ with an optimum growth at 24℃, which was identical to that for other fish cell lines reported previously (Chen *et al.*, 2004; Chen *et al.*, 2005). As the cells can grow over a wide temperature range they can be applied to isolate virus from both warm water and cold water fishes (Nicholson *et al.*, 1987).

The FBS is essential for survival and optimal growth of exogenous cells. In the primary cell culture, 20% FBS in medium was favorable for the SHK cell survival and proliferation.The proliferation of the subcultured cells increased as the FBS concentration increased from 10% to 20%, while the cells grew slowly in FBS-free medium. For reducing the cost, 10% FBS was applied in the SHK cell subculture.

Karyotype analysis revealed that the cells possessed a modal chromosome number of 2n=46, which was identical to the modal chromosome number of juvenile fish of spotted halibut (*V. variegatus*) (Sha *et al.*, 2007).

The transfection experiment using pEGFP showed that the SHK cells expressed the EGFP and produced green fluorescent signals. The result suggested that the SHK cells could serve as an *in vitro* system for exogenous gene manipulation study. It also showed that the CMV promoter could drive expression of GFP gene in the SHK cells. The result makes it possible to use the SHK cells in transplantation study.

Susceptibility to viral infection is necessary for a cell line in isolating and characterizing fish viruses. In the present study, the SHK cells were found to be susceptible to lymphosystis disease virus (LCDV). The observation was confirmed by PCR using specific primers for LCDV,

which could be propagated in the SHK cells. The results indicate the SHK cells can be employed for isolating and identifying LCDV from spotted halibut.

In conclusion, a new cell line was established from the kidney of spotted halibut. The cell line can be an important tool for studying exogenous gene manipulation, the infectious viruses of spotted halibut and in other research fields.

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