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Development and characterization of a cell line from the snout of koi (*Cyprinus carpio* L.) for detection of koi herpesvirus

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ABSTRACT

A permanent growing cell line, designated as koi snout (KS), was established from snout tissues of koi utilizing the trypsin method. The KS cell line was maintained in Leibovitz's L-15 medium supplemented with 10–20% fetal bovine serum (FBS) within incubation temperatures ranging from 22 to 27 °C. Maximum cell proliferation rates occurred at 27 °C in Leibovitz's L-15 medium containing 10% FBS. The KS cell line was subcultured more than 115 times, and karyotyping analysis indicated the modal chromosome number was 2n = 100. The results of virus isolation demonstrated that KS cells were susceptible to koi herpesvirus (KHV), which was demonstrated by the presence of obvious cytopathic effects and abundant virus particles with high virus titer of $10^{6.98}$ $TCID_{50}$/mL. Immunofluorescence and Western blot assays provided confirmation that KS could replicate KHV. The newly established KS cell line will provide a useful tool for KHV detection.

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1. Introduction

The koi herpesvirus (KHV) was first isolated in the US in 1998 following outbreaks in koi and common carp (*Cyprinus carpio* L.) in Israel and in the US (Hedrick et al., 2000). The disease was previously also known as "carp interstitial nephritis and gill necrosis" in Israel (Pikarsky et al., 2004; Ronen et al., 2003). Waltzek et al. (2005) coined the name cyprinid herpesvirus 3 (CyHV-3), following the nomenclature of other cyprinid herpesviruses, such as CyHV-1 (carp pox virus, fish papilloma virus) and CyHV-2 (goldfish hematopoietic necrosis virus). CyHV-3 is the etiologic agent of a lethal, highly contagious pathogen causing KHV disease (KHVD) with significant mortality rates in worldwide populations (Bergmann et al., 2006; Haenen et al., 2004) of common carp and koi (Bretzinger et al., 1999; Hedrick et al., 2000; Michel et al., 2010). KHVD causes a mortality of between 80% and 100%, which brings significant economic losses to carp aquaculture.

Since the first reported findings, the extent of the geographical range of KHVD spread to many countries worldwide, predominantly through the koi trade moving latently infected but healthy appearing fish. Nowadays, more is known about the disease and advancements have been made in detection methods (Bergmann et al., 2010b). KHVD is now known to occur in, or has been recorded in fish imported into at least 28 different countries. In Europe, KHV has been detected in numerous countries across the continent (Bergmann et al., 2006; Novotny et al., 2010).

Successive outbreaks of KHVD among numerous countries are indicative of the transboundary movement of this emerging viral pathogen (Lio-Po, 2007). The extensive international trade in live ornamental koi has largely contributed to the global spread of KHV. Hence, KHVD was recognized by the Office International des Epizooties, with all 5 notifiable fish diseases being of viral etiology (Taylor et al., 2010).

Infected fish exhibit typical clinical signs, including sunken eyes, pale patches on the body surface, reddening of the skin (together with a rough, sandpaper-like texture), focal or total loss of epidermis, over- or under-production of mucus on the skin and gills, and pale discoloration of the gills. Affected fish often swim on the surface and exhibit respiratory distress. However, some infected fish may be latent carriers and display no visible signs of the disease (Hedrick et al., 2000).

Presently, KHV diagnostic methods include cell culture isolation technology and identification by indirect fluorescent antibody techniques, polymerase chain reaction, enzyme-linked immunosorbent assay, in situ hybridization technology, and loop mediated isothermal

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amplification. Cell culture isolation is considered the gold standard for virus identification. Studies have shown that the virus has been isolated from KHV-infected common carp/koi in several cell lines obtained from carp tissues, including koi fin 1 (KF-1), koi fin cell (KF), common carp brain (CCB), koi caudal fin 1 (KCF-1) and koi tail 2 (KT-2) in the US, Israel, Germany, Indonesia, Japan, Singapore, and China (Hedrick et al., 2000; Neukirch and Kunz, 2001; Sano et al., 2004; Lio-Po and Orozco, 2005; Ilouze et al., 2006; NACA and FAO, 2008; Bergmann et al., 2010a,b; Dong et al., 2011). It is important to note that the virus has only been isolated in a limited number of cell lines, and that those cells can be difficult to handle.

KHVD has caused severe financial losses to fish breeders, and KHV isolation and identification is viewed as the key to preventing and controlling this viral disease. There is a strong antecedent associated between KHV infection and susceptible cell lines. Hence, establishing the cell lines derived from koi or common carp is essential for the separation and identification of KHV, vaccine preparation, and development of immunization technology.

The objectives of this study were to investigate the effects of different conditions for the culture of koi snout (KS) cells and the characteristics of subcultured cells to determine the optimum cultural conditions for these cells. In addition, the sensitivity of KS cells to KHV was investigated to determine whether KS cells are suitable for KHV vaccine development.

2. Materials and methods

2.1. Primary cell culture and subculture

Healthy koi 10 ± 0.5 cm in length were obtained from Pearl River Fisheries Research Institute, Guangzhou, China. The fish were disinfected by dipping into 75% ethanol. The snout tissue was removed aseptically, and washed with phosphate-buffered saline (PBS) containing 200 IU mL^{-1} penicillin, 200 μg mL^{-1} streptomycin, and 200 μg mL^{-1} nystatin. After washing, the tissue was minced with ophthalmic scissors and dispersed into single cells using 0.25% trypsin solution for approximately 30 min. The suspension was filtered through a 200-mesh sieve and then centrifuged at 160 *g* for 10 min. After several washes with PBS, the cells were transferred into 25 cm^2 tissue culture flasks and grown at 28 °C. Medium 199 (M199) supplemented with 20% fetal bovine serum (FBS), 100 IU mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, 1 ng mL^{-1} epidermal growth factor, and 20 ng L^{-1} basic fibroblast growth factor were used to culture KS cells. The cells were transferred into 25-cm^2 tissue culture flasks, and 50% of the medium was replaced every 4 days. Once the primary cell cultures grew to confluence, cells were subcultured at a ratio of 1:2 according to the standard trypsinization method. Confluent cells were trypsinized with 0.25% trypsin–EDTA solution, and half of them were maintained in M199 growth medium with 20% FBS. After the initial 20 passages, the concentration of FBS was reduced to 10% in an antibiotic-free medium.

2.2. Cryopreservation

KS cells were cryopreserved at different passage levels. Briefly, cell cultures were suspended in complete medium with 10% dimethyl sulfoxide (DMSO) and 20% FBS, placed in cryovials at −80 °C overnight and stored in liquid nitrogen for long-term storage.

2.3. Effects of culture conditions on cell growth

The effects of medium, incubation temperature, and FBS concentration on cell growth were investigated at the 50th passage level. Cells (5×10^4 cells mL^{-1}) were inoculated into 6-well plates with either Dulbecco's minimal Eagle's medium (DMEM), Leibovitz's L-15, M199, or minimum essential medium (MEM) containing 10% FBS, and were incubated at 28 °C. On alternate days, cells from duplicate wells with each medium were trypsinized, and counted by cell counter (Countstar, Beijing,China). The effect of different incubation temperatures on cell growth was also examined. Cells (5×10^4 cells mL^{-1}) were incubated in optimal medium determined as L-15, with 10% FBS at selected incubation temperatures of 15, 22, 28 and 33 °C. Cell growth was also determined in L-15 with different concentrations of FBS (5%, 10%, 15% and 20%) at 28 °C. Experiments were carried out for 7 d, temperatures assay was carried out for 8 d, and cell number was represented as mean ± standard deviation (SD).

2.4. Chromosome analysis

KS cells at passage 65 were used for chromosome analysis. Exponentially growing cells were treated with 20 μg mL^{-1} colchicine (Sigma, Germany). After about 4 h, the cells were harvested, treated with 5 mL of hypotonic solution of 0.075 mol L^{-1} KCl for 25 min. Cells were then centrifuged again at 1000 *g* for 5 min. The supernatants were discarded and cells resuspended. Freshly prepared, cold methanol–acetic acid (3:1) fixative called Carnoy's fixative was added slowly while aspirating the cell suspension gently. The fixed cells were then washed three times with fresh Carnoy's fixative, and then resuspended in a small amount of Carnoy's fixative. The suspension was placed on glass slides, air dried and stained with 5% Giemsa (pH 6.8) for 15–20 min. Chromosome counts were undertaken in more than 100 metaphase plates.

2.5. Viral susceptibility and confirmation

The susceptibility of KS cells to KHV was determined using KHV-T HP832 and KHV-E HP Z8Z viruses obtained from the Friedrich-Loeffler-Institut (Greifswald, Germany). Cells (2×10^5 cells mL^{-1}) at passage 60 were seeded into 25 cm^2 cell culture flasks when they reached 80% confluence. After removal of the medium, 1 mL of virus suspension with the viral titer of 10^3 $TCID_{50}$/0.1 mL was added to the cell culture. After1 h, the virus solution was removed and 5 mL of maintenance medium containing 3% FBS was added to the flasks. The cells were incubated at 22 °C and observed daily for up to 7 d for the appearance of cytopathic effect (CPE).

For electron microscopy observations, KS cells infected with KHV were fixed with 2.5% glutaraldehyde in 0.2 mol L^{-1} sodium cacodylate buffer (pH 7.4) for 24 h at 4 °C, and then post fixed with 1% osmium tetroxide in 0.2 mol L^{-1} sodium cacodylate buffer for 1 h. Infected cells were then embedded in epoxy resin, sectioned, stained with 2% uranyl acetate lead citrate, and examined under JEM-1400/1011 electron microscope (Tokyo, Japan).

2.6. The viral titer determination

For infection, KS cells of different densities (2×10^3, 2×10^4, 2×10^5, 2×10^6 cells mL^{-1}) were inoculated in a 24-well plate and incubated for 24 h at 28 °C to 90% confluence. After removing the medium, 0.2 mL of KHV-T HP832 virus suspension with a titer of 10^3 $TCID_{50}$/mL was inoculated onto the cell culture in the 24-well plate and allowed to adsorb for 1 h. Then, all of the wells were emptied carefully and 100 μL of maintenance medium containing 5% FBS was added to all the test and control wells. The cells were incubated at 22 °C and examined daily for the appearance of cytopathic effect (CPE) for up to 7 days. The virus was harvested from the infected cultures and processed by three cycles of freezing and thawing. The virus harvest was clarified by low-speed centrifugation (3000 ×*g*) at 4 °C, and the viral titer was then determined in a 50% tissue culture infective dose ($TCID_{50}$) assay in 96-well tissue culture plates based on the procedure described by Reed and Muench (1938) to find the most suitable cell density.

To determine the best time for KHV virus proliferation in the optimum cell density. KS cells were exposed to KHV-T HP832 virus as described above, the samples collected at different time intervals were then assayed to determine virus titres ($TCID_{50}$ mL^{-1}) using the KS cell

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