DEVELOPMENT OF PRIMARY CELL CULTURE FROM TAIL EPIDERMAL TISSUE OF KOI CARP (*Cyprinus carpio koi*)

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ABSTRACT

Primary cell culture from tail epidermal tissue of koi carp (*Cyprinus carpio koi*) was developed. Cells were grown in Leibovits-15 medium supplemented with 20% fetal bovine serum and antibiotics (Penicillin/Streptomycin and Kanamycin). Cell growth was observed in a range of incubation temperature (17°C±2°C, 22°C±2°C, 27°C±2°C, and 32°C±2°C) in order to determine the optimum temperature. The cells were able to grow at a range of temperature between 17°C to 32°C with optimal growth at 22°C. Primary cells infected with koi herpes virus produced typical cytopathic effects characterized by severe vacuolation and deformation of nuclei, which is consistent with those of previous reports. Artificial injection experiment by using supernatant koi herpes virus SKBM-1 isolate revealed that it could cause 90% mortality in infected fish within two weeks. PCR test with Sph I-5 specific primers carried out with DNA template from supernatant virus, pellet cell, and gills of infected fish showed positive results in all samples (molecular weight of DNA target 290 bp). The cells were found to be susceptible to koi herpes virus and can be used for virus propagation.

KEYWORDS: koi herpes virus, primary cell culture, koi fish

INTRODUCTION

Koi herpes virus (KHV) that causes koi herpes virus disease (KHVD) is an emerging disease that contributes to severe financial and economic losses in both common carp and koi carp culture industries world-wide (Hedrick *et al*., 1999; Hedrick *et al*., 2000). This highly contagious and dreadful disease had caused mass mortality with high rates, between 50%-100% in Japan, USA, Israel, some European, African and Asian countries including Indonesia (Bretzinger *et al*., 1999). KHV only infect common carp (*Cyprinus carpio carpio*) and the koi carp (*Cyprinus carpio koi*) populations. KHV can invade and infect the fish of a wide range of ages (Oh *et al*., 2001) and fingerlings are more susceptible to the virus than adults (Perelberg *et al*., 2003).

The main clinical sign of KHVD is severe necrosis of the gills. Infected fish would experience appetite loss, uncoordinated movements and erratic swimming prior to death. Skin discolouration and increasing respiratory rate (Gray *et al*., 2002), skin lesions and pale, patchy, and swollen gills (Oh *et al*., 2001) also can be used as clinical sign of KHVD.

KHV disease outbreaks are strongly associated with environmental conditions. Water temperature is the main factor that affects virulence of KHV the most (Gilad *et al*., 2003). The optimum temperature for viral replication *in vitro* is at a range of 15°C-25°C and the virus is very difficult to grow at temperatures above and below the optimum temperature. According to Ronen *et al*. (2003) the virus induces a lethal disease and fish mortality when water

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temperature ranges between 18°C-25°C (permissive temperature).

Virus only lives and grows inside a living cells, so viral multiplication *in vitro* is highly dependent on the availability of cell culture. Cell cultures were derived from tissues or organs that mechanically or enzymatically separated into cell suspensions. Then cell suspensions were cultured and grown into a monolayer and can be renewed through sub culturing (passaging) in order to obtain sustainable cell/cell line (Freshney, 1994). Suitable cell lines are necessary tools for virus proliferation, purification, and further characterization. It can also be used as vaccine development of KHV.

KHV is not susceptible to the commonly used cell lines by fish virology laboratories. KF-1 is a cell line derived from tail epidermal tissue for koi herpes virus propagation. The cell lines are incubated at 25°C for cell growth and at 20°C after virus inoculation. Koi herpes virus isolation in KF-1 cell line is used to confirm the etiological agent. These methods are quite efficient in case of fish deaths, however sometimes virus isolation could be difficult if the tissue is frozen. Koi herpes virus isolation by using KF-1 cell line from the gills is less reliable than the kidney and spleen due to frequent bacterial contamination of gill tissue (Amita *et al*., 2002; Gilad *et al*., 2002).

KHV has been cultured on FHM (fathead minnow) cell line in Korea (Oh *et al*., 2001) while cytopathic effect (CPE) is observed 3 to 5 days after virus inoculation and a complete cell lysis can be observed within 15 days. In China, a new cell line from the caudal fin of koi (KCF-1) has been established. KCF-1 cells predominantly consisted of short fibroblast-like cells, and grew well in DMEM medium supplement with 10% FBS (Dong *et al*., 2011). In Germany, KHV from kidney, liver, spleen have been cultured on brain (CCB) tissues of carp and cell line from gill (CCG), FHM, CHSE and EPC (Neukirch *et al*., 1999). CPE was observed in CCB cells 5 days after virus inoculation. In CCG cells, CPE developed since day 6 post-inoculation. Optimal growth of KHV occurs at temperatures from 15°C to 25°C, however the highest virus concentrations are observed at 20°C at 7 days post-inoculation, but there is no evidence of virus growth at 30°C or 37°C (Gilad *et al*., 2003).

In this study, we developed a new primary cell line from tail epidermal tissue of koi carp for culturing and propagation of KHV. We hope this primary cell culture is susceptible to KHV in Indonesia and can be used to obtain an effective vaccine to prevent koi herpes virus infection especially for koi carp.

MATERIAL AND METHODS

Primary Culture and Subculture

Healthy koi fishes (12 cm in total length) were collected from koi hatchery, Jakarta Provincial Government in 2011. Fish were maintained at room temperature for 24 hours in an aerated glass container containing sterile tap water with 500 IU/mL Penicillin/Streptomycin (Babu *et al*., 2011). Fish was killed by spinal damage, and then the skin was wiped out with 70% ethanol. The caudal fin was removed, washed in sterile tap water then soaked into a sterile glass beaker containing a concentrated chlorine solution (25 mL chlorine + 75 mL sterile tap water). Scales were exfoliated and keep soaked until the flesh become whitish, then transferred for washing in an Erlenmeyer containing 300 mL sterile tap water. Washing was done twice before retransfered into a sterile petri disk. The subsequent procedures were performed under sterile conditions inside Biosafety Cabinet Type A2 (Nuaire). Petri disk was added with 10 mL PBS^{++} solution (200 mL Phosphate Buffer Saline + 10 mL Penicillin/Streptomycin + 10 mL Kanamycin) before the washed fin was minced into approximately 1-2 mm tissue blocks, and then transferred into a sterile erlemeyer containing 100 mL PBS^{++} for washing. Washing was done twice while stirring until the color of the solution looks clear. The tissue pieces blocks then transferred into sterile bottle containing 100 mL of PBS^{++} solution and incubated at 28°C for 1.5 hours. The solution was removed then 20 mL of PBS^{++} Tsolution (50 mL PBS^{++} + 50 mL tripsin 0.25%) was added for washing. Washing was done twice before incubated with low striring in 20 mL PBS^{++} Tsolution at 4°C for all night. Tissue pieces were transferred into centrifuge tube with addition of 3 drops of serum before performing centrifugation at 3.000x g for 5 minutes at room temperature. Cell pellet was dissolved in 4 mL of PBS^{++} solution and then distributed into 2 culture flasks (size 25 cm^2) prewetted with 5 mL of primary culture medium (L-15 + 20% Serum + 10 mL P/S + 10 mL Kanamycin) then incubated at 25°C. Half of the growth medium was changed every three days. When the primary cells reached 80% confluence, the

medium was removed and the cells were rinsed with 5 mL of PBS solution. Then the cells were trypsinized with 1.5 mL of Trypsin-EDTA solution (Gibco) for 30 seconds. Detached cells were resuspended in the growth medium at a split ratio of 1:2. The cell suspension was distributed into two 25 cm^2 culture flasks containing 5 mL of growth medium and incubated at 25°C. After 3 passages, the concentration of FBS in the growth medium was decreased from 20% to 10%. Observation on cell growth and contamination were performed in everyday.

Effect of Temperature on Cell Growth

The observation of temperature effect on the growth of cultured cells was carried out on the 3rd passage. Cells (1.3 x 10^5 cells/mL) were grown in 25 cm^2 flasks and incubated at 25°C for 30 minutes to optimize the attachment of the cells. Then cells were grown in 10% L-15^{++} medium on a four range incubation temperature (17°C±2°C, 22°C±2°C, 27°C±2°C, and 32°C±2°C). Five days later, cell counting were performed in duplicate at each of these temperatures by automatic cell counter (Countess, Invitrogen).

Koi Herpes Virus Isolation and Susceptibility Test

Diseased carp were obtained from common carp farm in Sukabumi (West Java, Indonesia). Molecular detection showed that the fish were positively KHV-infected. Supernatant homogenate was prepared from infected carp tissue homogenates (kidney, gill and spleen at the ratio 3:1:1 w/w). Approximate lyonegramof infected fish tissue was taken aseptically and then homogenized by using cooled sterile mortal and pestel in 9 mL of HBSS (Hank's Balance Salt Solution, Invitrogen). Centrifugation was then performed at 1.500x g for 15 minutes before removing cell debris and collecting the supernatant. To gain 50x total dilution of supernatant homogenate, HBSS was added to the supernatant at a ratio of 1:5 then filtered through 0.45 µm membranes. The supernatant homogenate should be used immediately for inoculating the primary cell culture. The primary cell cultures from 5th passage were used for the viral susceptibility test. Two culture flasks of primary cell culture (around 90% confluent) were used to perform viral inoculation while another culture flask was used as control. After discarding the growth medium, one milliliter of supernatant homogenate was added into the inoculated flask while HBSS was added into control flask. All the flasks then incubated at room temperature for 1 hour to optimize the absorption of the virus in the cell before addition of five mL of maintenance medium (2%L-15) into each inoculated and control flask. Cell cultures were maintained at 20°C. Observation of vacuolization formation inviral infected cells as the signs of cytophatic effects (CPE) were done daily.

Pathogenicity to Koi

Koi herpes virus of inoculated culture flask from susceptibility test were harvested and then kept at deep freezer -80°C in 1 mL aliquot. A total of 30 healthy koi fishes were distributed into 3 aquaria (volume 60 liters). The fish were fed with commercial diet once a day. Twenty fishes were intra peritoneally injected with 0.1 mL supernatant virus and 10 fishes were injected with 0.1 sterile HBSS as control. The injected fishes were observed daily and collected when they were moribund or death. The gills, kidney and spleen were collected for PCR analysis in order to detect the KHV.

Chromosome Analysis

Primary cell cultures were grown in 25 cm^2 flask and incubated at 25°C for 36-40 hours until it reaches 80%-90% confluences. Chromosome analysis was performed on cells which undergoing exponential growth at fourth passage according to the methods used by Dong *et al*. (2011). Cells were treated with colchicine at a concentration of 0.2 µg/mL and incubated for 3 hours at 25°C and then added 5 mL of 0.075 M KCl (hypotonic medium) for 25 minutes before 10 mL of Carnoy fixative (methanol : acetic acid = 3:1) was added for 5 minutes. After centrifugation for 5 minutes at 200x g at 4°C, the cells then resuspended in 2 mL of fresh Carnoy fixative solution and then dropped onto a cooled object glass. The cells then dispersed by air blowing and finally air-dried. Chromosomes were stained with 10% Giemsa solution for 20 minutes at room temperature for 30 minutes air-dried, mounted and documented.

***Polymerase Chain Reaction* (PCR) Analysis**

Extraction by DNAzol Reagent (Invitrogen)

500 mL of DNAzol reagent was added into 1.5 mL microtube containing approximately 25

mg tissue sampels and then homogenized by using pestle. Centrifugation was carried out at 12.000 rpm for 10 minutes, and then supernatant was poured into a new 1.5 mL microtube that already containing 500 mL of 100% ethanol. To ensure the homogenation, the solution was inverted up and down before performing centrifugation at 8.000 rpm for 2-3 minutes. After removing the supernatant, pellet was added 500 mL of 95% ethanol, homogenized by inverted up and down and then centrifuge at 8.000 rpm for 2-3 minutes. Supernatan then removed, and 100 µL TE buffer was directly added to resuspend the pellet. DNA was stored in -20°C to be used in amplification process.

Primer and PCR Amplification

PCR analysis in this study used *Sph* I-5 specific primer (Yuasa *et al*., 2005) for koi herpes virus detection. Primer was synthesized by Alpha DNA (Montreal, Quebec) and sequence of the primers are *Sph* I-5 F: 5'-GAC ACC ACA TCT GCA AGG AG-3' and R: 5'-GAC ACA TGT TAC AAT GGT CGC-3'. PCR amplification were carried out in the reaction mixture in a total volume of 25 mL per sample as follows: 12.5 mL of GoTaq® Green master mix (Promega, Madison WI, USA), 8.5 mL of nuclease free water, 1 mL of each primer (reverse and forward) and 2 mL of DNA template. Amplification was performed using MJ Research thermal cycler and the cycling program condition as follows: after an initial denaturation step at 94°C for 30 seconds, then undergo 40 cycles of denaturation at 94°C for 30 seconds, annealing at 63°C for 30 seconds, and elongation at 72°C for 30 seconds. Lastly the final elongation was performed at 72°C for 7 minutes and RNAse-free water was used as negative control (No DNA Template).

Electrophoresis

Amplification product was detected by gel electrophoresis. 10 mL of each DNA amplicon were run into the well of 1.5% agarose gel in 1x Tris-acetate buffer-EDTA (220 V for 25 minutes). DNA staining was done by soaking the gel in a solution of ethidium bromide (0.5 mg per 100 mL of TAE buffer) for 15 minutes and the results were documented with Polaroid cameras.

RESULT AND DISCUSSION

Koi herpes virus was first isolated from outbreaks in Israel and USA (Hedrick *et al*., 2000). The virus that caused a highly contagious carp disease is not infectious to other cyprinid and non-cyprinid fish species. The fish morbidity and mortality are only restricted to koi and common carp populations (Perelberg *et al*., 2005). Several cell lines have been reported to be susceptible to koi herpes virus and currently being used in various studies. Some of them derived from koi caudal fin (Hedrick *et al*., 2000; Miwa *et al*., 2007; Pikarsky *et al*., 2004; Sunarto *et al*., 2010), common carp brain-derived cell line (CCB) (Neukirch *et al*., 1999), common carp fin-derived cell line (Neukirch *et al*., 1999), and a few other fish cell lines (Davidovich *et al*., 2007).

In this study, primary cells from the tail epidermal tissue of koi fish have been successfully developed. Primary cells culture at 5th passages stained by Giemsa showed predominated by fibroblasts-like cells although epithelial-like cells could be found in a quite amount. The primary cell cultures grew well in L-15 medium supplemented with 20% FBS. According to literature, other established cell lines derived from koi fin (KF-1 and KCF-1) predominantly consists of fibroblast-like cells and can grow well in different growth media (Pikarsky *et al*., 2004; Hedrick *et al*., 2000; Dong *et al*., 2011). We expected that with increasing number of passages in the future, the cells would be more uniform for easier cell identification and characterization.

The primary cells migrating from explants after 7 days of culture (Figure 1) and almost completely covered the bottom of the 25 cm^2 flask (90% confluences) within 7 weeks. The primary culture reached 50% confluences after 24 days of incubation at 25°C without any changing of medium. After that, the growth media was replaced gradually until it reached 90% confluences and the cells formed a monolayer. The old medium was removed and the monolayer was washed twice with 1x PBS (sterile) and EDTA to separate individual cells and split into two flasks with fresh media (subculture with split ratio of 1:2).

Cytophatic effect (CPE) was not observed during the initial incubation of inoculated primary cells with tissue homogenates from pooled gills, kidney, and spleen of KHV-infected samples. CPE was observed after 14 days post-inoculation and medium contained koi herpes virus (isolate SKBM-1) was harvested at 20 days post-inoculation. Three consecutive passages of the original supernatant virus produced the same CPE at 2 days post-inoculation. Supernatant virus was harvested at 6

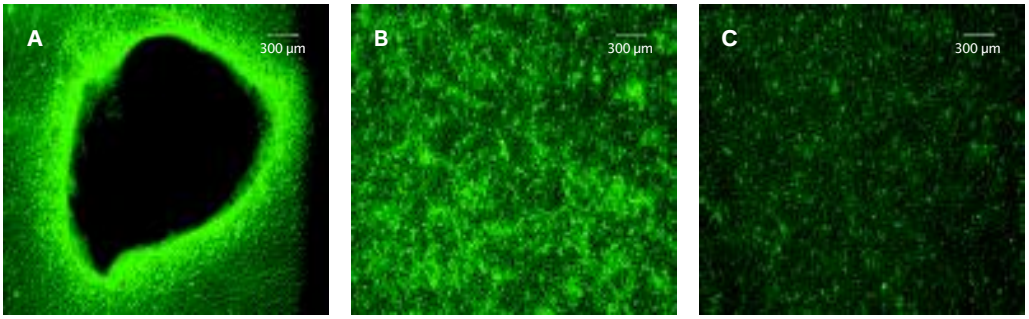


Figure 1. Primary cell cultures with migrating cells from an explant (A), primary cell culture at passage-1 (P1) (B), and passage-5 (P5) (C)

days post-inoculation, aliquot aliqouted in 1.5 mL cryovials and kept at deep freezer -86°C for stocking virus and another test.

SKBM-1 isolate was used to viral susceptibility of the primary cell culture. CPE caused by koi herpes virus was observed in primary cells at 2 days post-inoculation. A lot of small foci occurred inside the cells with cell lysis and round-cell aggregations were observed (Figure 2). As CPE development progressed, multiple vacuolization and round-cell aggregations became more extensive prior to detachment from the monolayer at 14 days post-inoculation. These infected cells showed similar CPE to that reported by Sunarto *et al*. (2000) and Dong *et al*. (2011) in KF-1 cell line and KCF-1 cell line, respectively. Using this primary cell, a strain of koi herpes virus (SKBM-1 isolate) was isolated from infected koi.

Fish began to die at 7 days post-infection (data not shown), while mass mortality occurred at 8-11 days post-infection. The highest mortality occurred at 8 days post infection (8 koi died). The clinical signs observed as gill expansion and pallor, slow swimming, lethargy, and loss of appetite were quite similar to those in previous report (Hedrick *et al*., 2000; Pikarsky *et al*., 2004). In an experiment performed by Hedrick *et al*. (2005), they conducted inoculation of koi herpes virus into KF-1 cell line. The koi herpes virus induced cell fusion and intense cytoplasmic vacuolation in KF-1 cells within 5 days after inoculation at 20°C. More complete CPE or cell lysis was evident at 7-10 days and progressed to involve all the cells after 14 days.

In this experiment, almost all the fish died on the 14th day post-infection. Cumulative mortality (Figure 3) continued to increased until 90% of the fish died in a short period of time (2 weeks). These results showed similar data with Ronen *et al*. (2003) and Dong *et al*. (2011) that virus harvested from KFC cell could cause 75%-

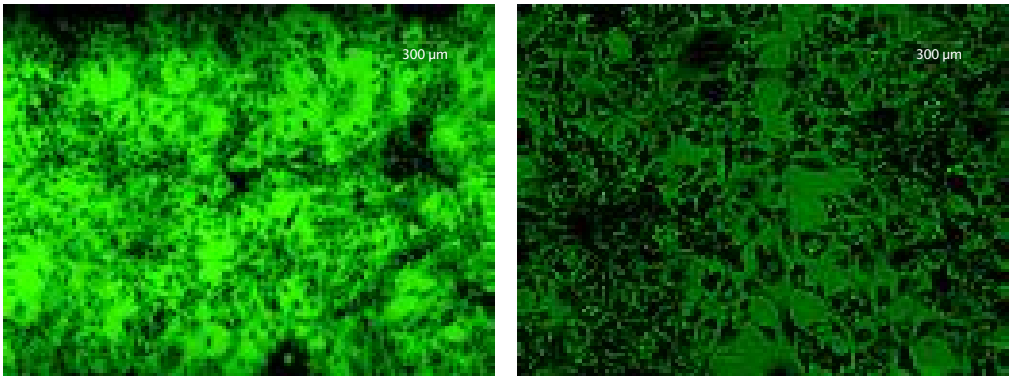


Figure 2. Early (left) and advanced (right) cytophatic effect on koi herpes virus-inoculated primary cell cultures at passage-4

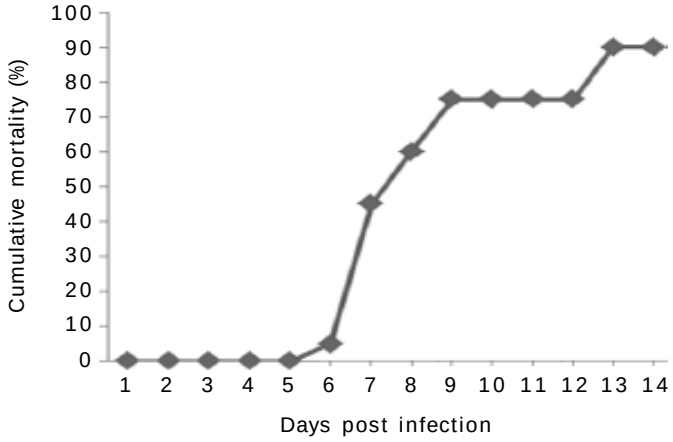
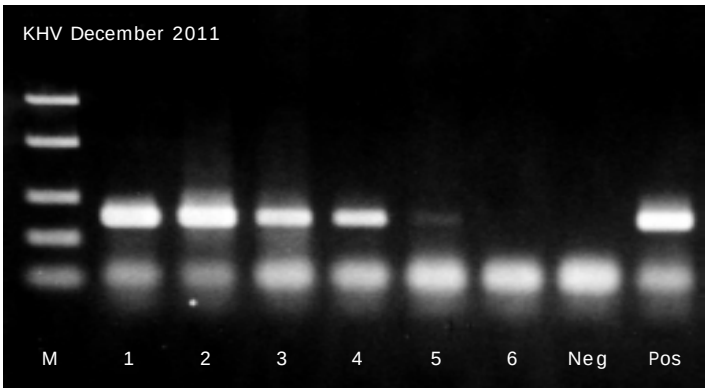


Figure 3. Pathogenicity of the primary cell cultured with KHV SKBM-1 isolate. Average cumulative mortality of KHV injected koi fish reached 90% in two weeks

100% mortality and virus harvested from KCF-1 cell could cause 100% mortality in infected koi. All dead fish from injected group were PCR positive (Figure 4) while there were none of fish died from control group and PCR test negative (Figure 4, Line 6).



Line	Samples	Result
M	Marker low range	
1	Koi fish of supernatant-injected group's death at 7th day post injection	+
2	Koi fish of supernatant-injected group's death at 8th day post injection	+
3	Koi fish of supernatant-injected group's death at 9th day post injection	+
4	Koi fish of supernatant-injected group's death at 11th day post injection	+
5	Koi fish of supernatant-injected group's death at 14th day post injection	+
6	Koi fish of control group	–
Negative	Negative control	–
Positive	Positive control	+

Figure 4. PCR detection of the dead fish challenged with KHV SKBM-1 isolate. The positive result shows a bright and clear band at its target molecular weight (290 base pair)

Growth studies were carried out to determine the optimum temperature for maximum growth of the cells. The primary cells were well adapted and grow well in L-15 medium supplemented with FBS. Fernandez-Puentes *et al*. (1993) have reported the suitability of L-15 cell culture medium for growth of fish cells among other available cell culture media. The primary cell culture at 3rd passage were exposed to different temperatures (Figure 5). Cells (1.3 x 10^5 cell/mL) were grown in 25 cm^2 flasksunder 10% L-15medium and incubated at 17°C±2°C, 22°C±2°C, 27°C±2°C, and 32°C±2°C. Five days later, cell were trypsinized and counted using an automatic cell counter (Countess, Invitrogen). The experiment was conducted in duplicate at each of these temperatures. The results of counting the cell number for optimum temperature can be seen in Table 1. Cells were able to grow quite well at all incubation tem-

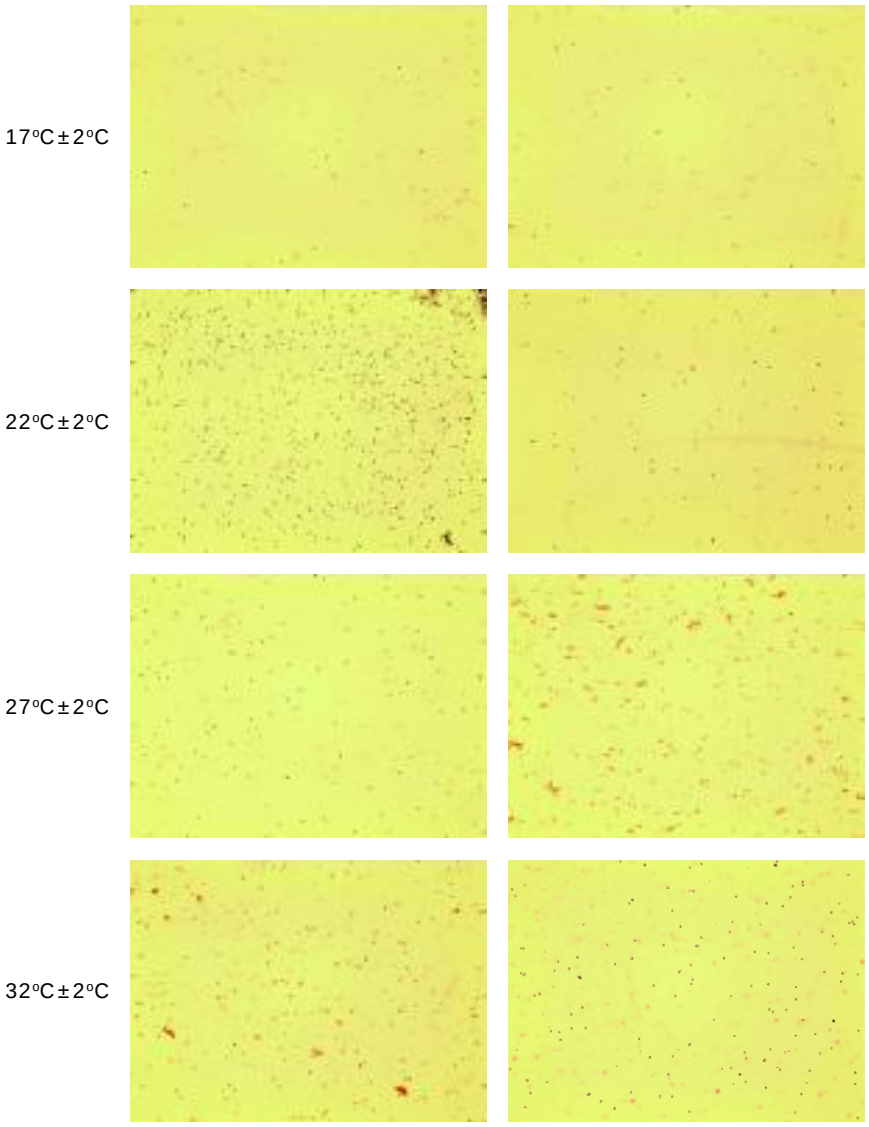


Figure 5. Photograph of cell number calculation of temperature optimization test (duplicates) for primary cell culture (cell/mL) using automatic cell counter (Countess, Invitrogen). Blue dots indicate the live cells which absorbed triphan blue dye

Table 1. Cell number calculation of temperature optimization test for primary cell culture (cell/mL) using automatic cell counter (Countess, Invitrogen)

Incubation temperature	Total of cells	Live cells	Average of live cells	Death cells	Viability (%)
17°C (Well-1)	1.5 x 10^5/mL	1.2 x 10^5/mL	2.4 x 10^5/mL	4.0 x 10^4/mL	77
17°C (Well-2)	1.8 x 10^5/mL	1.6 x 10^5/mL		2.0 x 10^4/mL	89
22°C (Well-1)	3.6 x 10^6/mL	3.5 x 10^6/mL	19.8 x 10^5/mL	1.4 x 10^5/mL	96
22°C (Well-2)	5.5 x 10^5/mL	4.7 x 10^5/mL		9.0 x 10^4/mL	85
27°C (Well-1)	7.4 x 10^5/mL	7.0 x 10^5/mL	6.7 x 10^5/mL	4.0 x 10^4/mL	95
27°C (Well-2)	2.0 x 10^6/mL	6.3 x 10^5/mL		1.3 x 10^6/mL	32
32°C (Well-1)	1.4 x 10^6/mL	6.5 x 10^5/mL	6.9 x 10^5/mL	7.3 x 10^5/mL	47
32°C (Well-2)	7.8 x 10^5/mL	7.3 x 10^5/mL		5.0 x 10^4/mL	94

perature (between 17°C and 31°C), however, maximum growth was obtained at 22°C±2°C with saturation density of the cells around 19.8x10^5 cells/mL. The primary cells were less grew at temperature 17°C±2°C with low saturation density of 2.4 x 10^5 cells/mL.

Chromosome analysis was conducted for primary cell at passage-4 and a total of 100 chromosome spreads were counted (Figure 6). These result was identical to others reports as described previously in koi and common carp cells. Chromosome analysis of KCF-1 (a cell line derived from caudal fin of koi) revealed that the cells maintained normal diploid chromosome number 2n = 100 (Dong *et al*., 2011). The diploid chromosome number of common carp specimens from Vietnam was found to be 2n = 100 (Anjum, 2005).

CONCLUSION

A new primary cell culture from tail epidermal tissue of koi carp has been developed and sub-cultured for more than 5 passages. Primary cells grow predominantly consisted of fibroblast-like cells and grew well in Leibovits-15 medium supplemented with 10% Fetal Bovine Serum (FBS). The primary cell culture appears to be suitable cell for isolation and culture of KHV. So, the cell can be used as a cultured medium to propagate koi herpes virus to obtain an effective vaccine to prevent the viral infection.

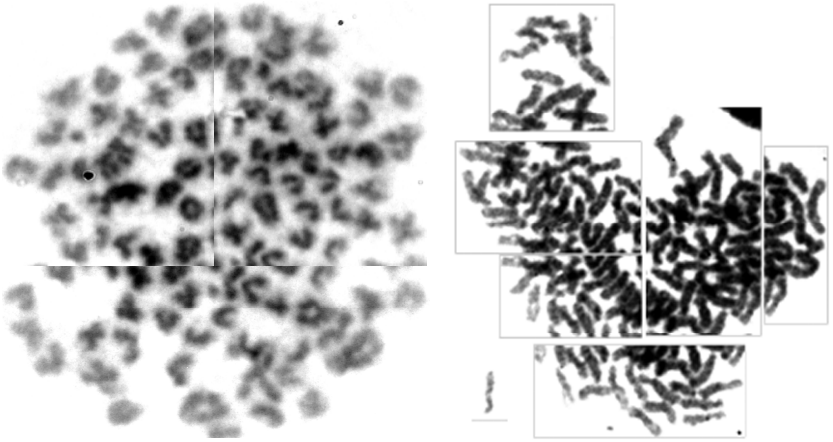


Figure 6. Photomicrography of chromosome number of koi primary cell. The diploid chromosome number (2n) was 100

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