SUSCEPTIBILITY OF CYPRINID AND NON-CYPRINID FISH CELL LINES TO KOI HERPESVIRUS (KHV)

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ABSTRACT

Koi herpesvirus (KHV) is an emerging virus that infects koi and common carp (*Cyprinus carpio*) with mortality up to 95% within 7 days. The disease is rapidly spreading worldwide including to Indonesia. However, it has only been documented in koi and common carp. The aim of this research was to evaluate the susceptibility of fish cell cultures originated from cyprinid and non-cyprinid fish to KHV. Koi Fin (KF-1) and Koi Tail (KT-2) cell lines derived from koi carp and SSN-1 cells originated from fry of striped snakehead were used in this study. The cells were inoculated with tissue extract of KHV-infected koi carp (experiment 1) and virus stock of KHV (experiment 2). The cultures were incubated at 22°C and the onset and type of cytophatic effect (CPE) were observed for 21 days post inoculation. The results of experiment 1 showed that CPE was observed in KT-2 at day 6 post inoculation. In the experiment 2, however CPE was observed in KF-1 and KT-2 cells at day 4 post infection. CPE was not observed in SSN-1 of either experiment 1 or experiment 2. CPE was characterized by extensive vacuolization of the infected cells. Polymerase chain reaction (PCR) assay of cell and tissue culture supernatants confirmed that KF-1 and KT-2 showing CPE were indeed infected with KHV. The results indicated that KF-1 and KT-2 cells were susceptible and SSN-1 was resistant to KHV. The implication of these findings was also discussed in the paper.

KEYWORDS: susceptibility, fish cell culture, KHV, cytophatic effect, PCR

INTRODUCTION

Koi herpesvirus (KHV) is an emerging virus that infects koi and common carp (*Cyprinus carpio*). The mortality rate was 80%–95% in fry as well as in adult fish (Perelberg *et al.*, 2003). The disease is spreading rapidly worldwide and causing huge economic losses in the USA, UK, Israel, Eastern European countries, Asian and Africans countries since 1998 (Hedrick *et al.*, 2000; Neukrich & Kunz, 2001; Hoffman *et al.*, 2002; Way *et al.*, 2002; Perelberg *et al.*, 2003; Ronen *et al.*, 2003; Haenen *et al.*, 2004; Sano *et al.*, 2004; Tu *et al.*, 2004; Sunarto *et al.*, 2005[a]).

KHV (Hedrick *et al.*, 2000) is also known as carp nephritis and gill necrosis virus (CNGV) (Ronen *et al.*, 2005) and Cyprinid herpesvirus-3 (CyHV-3) (Waltzek *et al.*, 2005). The virus is an enveloped virus with icosahedral nucleocapsid of 100–110 nm in diameter, and belongs to the family of Alloherpesviridae. The viral genome is linear double stranded DNA molecule of 295 Kbp, the biggest genome in the order of Herpesvirales (Hedrick *et al.*, 2005; Hutoran *et al.*, 2005).

No drugs or pharmaceuticals have been found to be effective to combat the viral disease. However, applying good aquaculture

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practices (GAPs) such as biosecurity and vaccination programme may prevent the outbreak and reduce the mortality rate caused by the disease (Sunarto *et al*., 2005[a]; Sunarto *et al*., 2005[c]). The epidemiology and control of KHV in Indonesia has been extensively studied (Sunarto *et al*., 2005[a]; Sunarto *et al*., 2005[c]; Sunarto & Cameron, 2005; Sunarto & Cameron, 2006). The reports showed that KHV only affects koi and carp, but not other fish species. Tilapia, giant gouramy, and other freshwater fishes reared in the same pond with diseased carp showed no signs of the disease or suffered mortality from it. However, scientific information on whether the virus is able to infect non-cyprinid fish cells is limited. Therefore, the aim of this study was to investigate the susceptibility of fish cell cultures originated from cyprinid (KF-1 and KT-2 cells) and non-cyprinid fish (SSN-1 cells) to KHV infection.

METHODS

Cell culture preparation. Three cell lines originated from cyprinid and non-cyprinid fish were used in this study. Koi fin cell line (KF-1), established from fin of koi (*Cyprinus carpio*) (Hedrick *et al*., 2000), was kindly donated by Prof Ronald Hedrick (University of California at Davis, USA). Koi tail cell line (KT -2), developed from tail of koi, was a newly developed cell line in our laboratory (Sunarto *et al*., 2005[b]). Striped snakehead cell (SSN-1) was initiated from the whole fry tissue of *Channa striatus* of the family of Channidae. The cell was imported from the University of Stirling, UK. All cell lines were maintained in Leibovitz L-15 medium supplemented with antibiotics and 10% serum. Only cell cultures showed confluency of more than 80% were used for the study. The cells for experiment 1 and 2 were prepared using the same methods. In the experiment 1, the cell lines were inoculated with tissue extract (TE) from KHV-infected fish and in the experiment 2, the cells were inoculated with virus stock of KHV.

Tissue extract preparation

Tissue extract was prepared from diseased fish according to the standard procedures (Freshney, 1994) with minor modifications. Pool of kidneys, spleens and gills were homogenized and diluted 1:10 with Hanks Balanced Salts Solution (HBSS) supplemented with 2% Fetal Bovine Serum (FBS). Tissue suspension was centrifuged at 1,500 g for 15 minutes at 4°C to deposit tissue debris. Clarified supernatant was removed and diluted 1:5 with HBSS supplemented with 2% FBS. Diluted tissue extract was filtered through 0.45 µm membrane filter into sterile container. A hundred microliter of filtered TE was separately subjected to PCR assay.

Inoculation of cell culture. Two replication of cell cultures were inoculated with TE in 1:50 dilution (experiment 1) at the rate of approximately 1 mL per 25 cm^2 of cell sheet. Control cell cultures were inoculated at the same time with an equal volume of HBSS. Following an adsorption period of 30–45 minutes, a fresh culture medium was added into the culture flask. The cultures monolayer were incubated at 22°C and observed daily for the evidence of Cytophatic Effect (CPE). The same procedure was used for the inoculation of cell culture with virus stock of KHV (experiment 2).

Passage of virus-infected cells

Following the appearance of CPE, a 100 µL aliquot of Tissue Culture Supernatant (TCSN) was used to inoculate a 25 cm^2 flask of KT-2 cells with 80% confluent. The cultures were incubated at 22°C and observed daily for the evidence of CPE. A hundred microliter of TCSN and the remaining cells were separately subjected to PCR assay.

Polymerase chain reaction

PCR test against KHV was conducted using commercially available PCR kits (IQ2000, Taiwan) according to the manufacturer's protocol. The IQ2000 PCR kits produced semi-quantitative results of 229 bp (light KHV infection) and/or 440 bp (severe KHV infection). Prior to virus isolation, tissue extract of pooled kidney, spleen and gills were tested against KHV using PCR to confirm that the fish was indeed infected with KHV. The cells and the cell-free TCSN of virus-infected cell cultures were also tested using PCR assay.

RESULT AND DISCUSSION

Koi was collected from fish farm experiencing mass mortality with clinical signs typical to KHV infection. PCR test against KHV using commercially available kit confirmed that the outbreak was caused by KHV. The results of this semi-quantitative PCR assay revealed that the koi had severe infection (Figure 1).

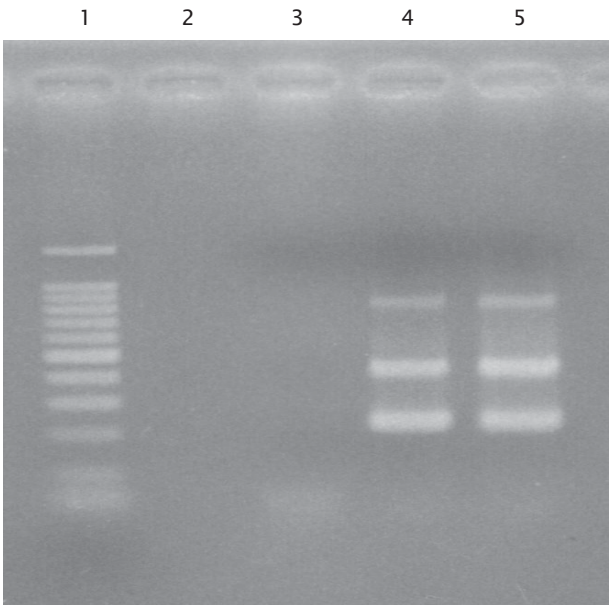

Figure 1. PCR detection against KHV on tissue extract of sick koi used for viral isolation indicated severe infection of KHV. Lane 1. DNA marker of 100bp, 2. blank, 3. negative control, 4. sick koi (positive), 5. positive control

Three koi samples with damaged gills signs varied from excess mucus production, white patches to severe necrosis were used for the tissue extract preparation. Pool of kidney, spleen and gills were used for viral isolation using KT-2, KF-1 and SSN-1 cell cultures. The development of cytophatic effect was first observed at day 6 post inoculation of the tissue extract in KT-2 cell line. The cells showed extensive vacuolization, retracted from the flask and completely detached after 2 weeks post inoculation. CPE was not observed in KF-1 and SSN-1 cell lines inoculated with the same tissue extract (Figure 2).

Primary virus and its three consecutive passages induced CPE at day 4 post inoculation. The CPE was characterized by an extensive vacuolization witihin the cells (Figure 3).

The PCR assay of cell and cell-free TCSN of the primary culture and its consecutive passages confirmed that the KT-2 cells were indeed infected with KHV (Table 1).

Culture fluid (TCSN) of the 3rd passage of KHV was used for inoculation of KT-2, KF-1 and SSN-1 cell cultures (experiment 2). In experiment 2, the development of cytophatic effect was first observed at day 4 post inoculation of the cultures. However, CPE was only observed in the KF-1 and KT-2 cell lines, but not in SSN-1 cell line (Figure 4). The infected cells showed extensive vacuolization typical to KHV-induced CPE, retracted and completely detached from the flask after two weeks post inoculation.

Table 2 summarized the susceptibility of fish cell cultures originated from cyprinid and non-cyprinid fish to Koi herpesvirus. The table showed that the tissue extract prepared from KHV-infected fish was only induced CPE in KT-2 cell line (experiment 1). However, in experiment 2 where the cell cultures were inoculated with virus stock of KHV, CPEs were observed in KT-2 and KF-1 cell lines. CPE was not observed in SSN-1 of either experiment 1 or experiment 2.

Koi herpesvirus was first isolated from outbreaks of carp suffering mass mortality in Israel and the USA (Hedrick *et al.*, 2000). Although the disease has spread to fish farm worldwide, it has presently only documented in koi and common carp. In Indonesia, KHV was first isolated from pool of kidney, spleen and

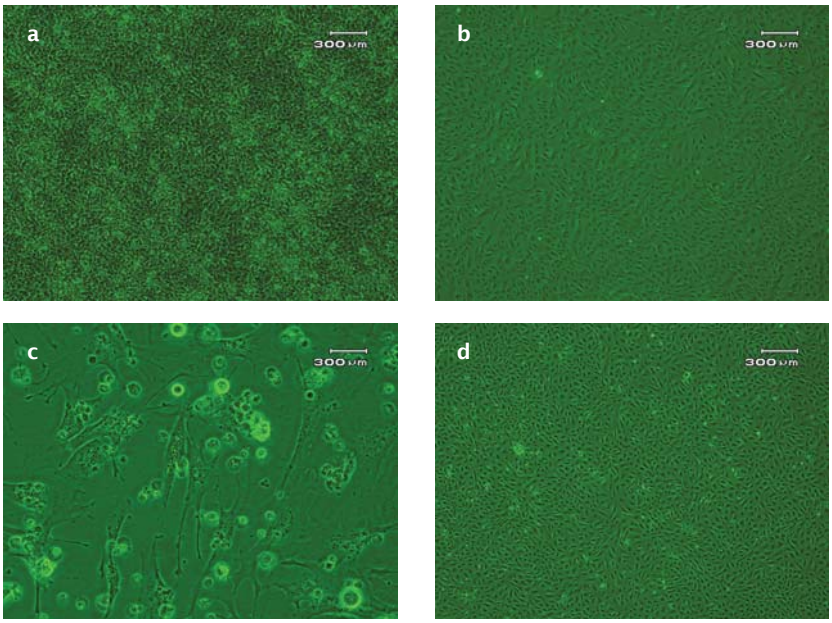

Figure 2. Photograph of experiment 1: cell cultures infected with tissue extract at day 10 post inoculation. a. SSN-1 cell, b. KF-1 cell, c. KT-2 cell, d. Un-infected KT-2 cell (control flask). Note the cytopathic effect in KT-2 infected cells (c)

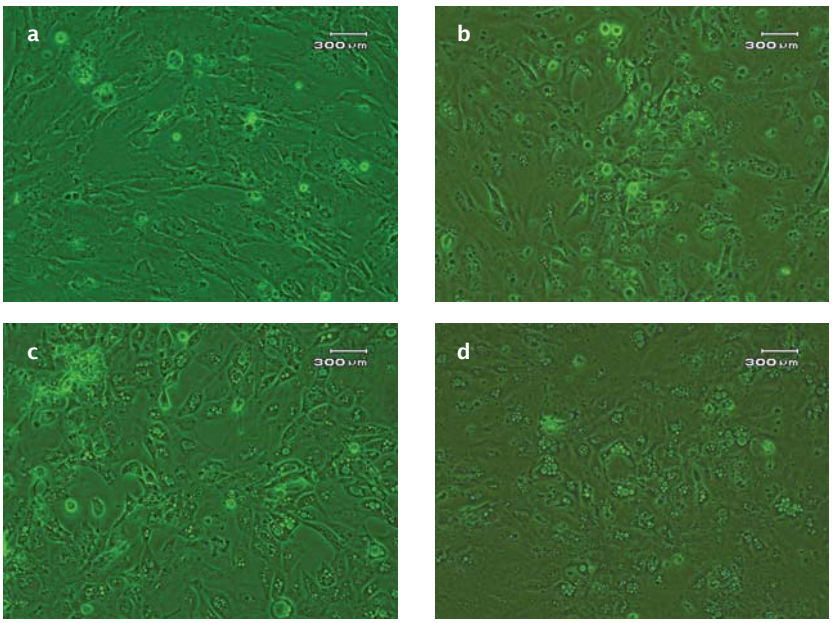

Figure 3. Photograph of KT-2 cells infected with various passages of KHV at day 7 post inoculation a. Primary virus of KHV, b. Passage 1, c. Passage 2 and d. Passage 3. All the virus preparations induced CPE characterized by an extensive vacuolization of infected cells

Table 1. PCR assay against KHV of infected KT-2 cells and its cell-free tissue culture supernatant

Virus passage	PCR assay	
	Cells	Culture fluid
Primary	+	+
Passage 1	+	+
Passage 2	+	+
Passage 3	+	+

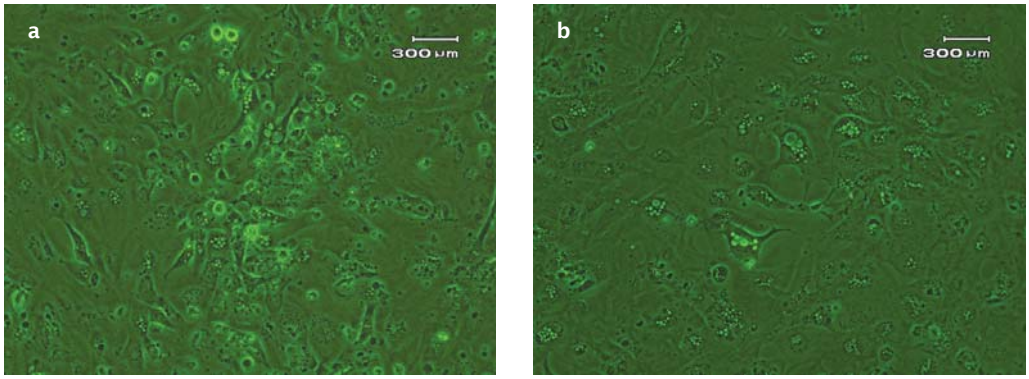

Figure 4. Photograph of experiment 2: cell cultures inoculated with virus stock of KHV. The virus only induced CPE at KT-2 cell line (a) and KF-1cell line (b)

Table 2. The susceptibility of fish cell cultures to Koi herpesvirus

Cell line	Inoculation with tissue extract (experiment 1)	Inoculation with virus stock of KHV (experiment 2)
KT-2	+	+
KF-1	-	+
SSN-1	-	-

Note: + : showing CPE; - : not showing CPE

gills of koi which were collected from several farms suffered mass mortality in West Java using KT-2 cell line (Sunarto *et al.*, 2005[b]). The development of CPE was first observed at day 6 after inoculation with tissue extract prepared from diseased fish. The type of CPE in this KT-2 cells was identical to CPE in KF-1 cells infected with KHV as described previously (Hedrick *et al.*, 2000). However, the onset of CPE in KT-2 (4-6 days) was faster than the onset of CPE in KF-1 cells (7-10 days).

The CPE at KF-1 and KT-2 cell lines was characterized by an extensive vacuolization of the cell. The vacuolated cells were swollen, rounded, and some of the cells were fused with adjacent cells. The cells were retracted from the flask and completely detached after 2 weeks post inoculation. Although KHV can grow in both KF-1 and KT-2 cells, but experiment 1 showed that KT-2 was more sensitive for virus isolation of KHV from Indonesian fish than KF-1 cell. In other word, the use of KT-2 cells in

an attempt to isolate KHV from Indonesian fish increased the isolation rate. However, further research is needed to ascertain the exact isolation rate of KHV using KT-2 cell line.

KT-2 and KF-1 cell lines were originated from tail and fin of koi (Cyprinidae), respectively. SSN-1 cell line was derived from whole fry tissue of striped snakehead (Channidae). Table 2 showed that KHV only grew and caused CPE in the cell cultures originated from cyprinid fish. The results are in line with the previous work Davidovich *et al*., 2007) that most cyprinid cell cultures are susceptible to CyHV-3 (KHV). However, other carp cell lines (Epithelioma Papulosum Cyprini, EPC) was restrictive to the virus. On the other hand, SSN-1, a non-cyprinid cell line, was resistant to KHV. It is interesting to note that the results of the experiments in cell cultures perfectly reflect the situation in fish farm in which KHV only affects koi and carp.

As there are no treatment for KHV, attempt to control KHV outbreak in farm level is focused on the prevention of getting infected with the virus by applying biosecurity concept. However these practices may only be effective for isolated farm such as indoor koi farm, but not for raceway and cage culture system in open water (Sunarto *et al.,* 2005[c]). In this case, good aquaculture practices (GAPs) such as vaccination programme would be methods of choice. The success of the isolation of KHV from Indonesian fish using KT-2 cell and the subsequent application of the cell culture for the study of biological properties of KHV *in vivo*, may lead to a better understanding of the pathogenesis of the virus both *in vivo* and *in vitro*. In long term, the data generated from these researches may provide a basic step toward the development of vaccination programme to control the outbreaks of koi herpesvirus.

CONCLUSION

Cell lines originated from cyprinid fish (KT-2 and KF-1) were susceptible to Koi herpesvirus. However, cell line derived from non-cyprinid fish (SSN-1) was resistant to KHV. KT-2 cell was more sensitive for isolation of KHV from Indonesian fish than KF-1 and SNN-1 cells.

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