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ULTRASTRUCTURAL FEATURE OF KOI HERPESVIRUS (KHV) INFECTED CULTURED KOI FIN (KF-1) CELLS

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ABSTRACT

Koi herpesvirus (KHV), may cause significant morbidity and mortality in common carp (*Cyprinus carpio*). In the present study, an electron microscopic (EM) was performed on KHV-infected cultured koi fin (KF-1) to document the ultrastructure of the lesions. Viral particles were firstly evident in the nucleus. These viral particles observed as immature capsids and nucleocapsids. Many non-enveloped nucleocapsids have moved from the nucleus into the cell cytoplasm. The formation of subviral particles and virions, which comprised, in turn, an electron dense core, capsids with a hexagonal outline, the tegument was evident in the cytoplasm. And then, the virions with the enveloped tegument budded through the intracytoplasmic membrane. Based on EM results, the definitive pathological change was similar as those in the Family Herpesviridae.

KEYWORDS: KHV, KF-1, nucleocapsid, virion, Herpesviridae

INTRODUCTION

Koi herpesvirus (KHV) is currently classified as a DNA-virus belonging to the virus family *Herpesviridae* (i.e., a herpesvirus), whose infection was designated as Koi Herpesvirus Disease (KHVD). KHVD occurred in common carp at all ages causing mass mortalities of up to 80-95%. KHV was first isolated in 1998 from koi carp (*Cyprinus carpio koi*) and common carp (*Cyprinus carpio*) cultured in fish farms that experienced mass mortalities in the USA and Israel (Hedrick *et al*., 2000). Since then, a similar virus was also isolated after the massive mortality of carp in European countries (Haenen *et al*., 2004; Neukirch and Kunz, 2001; Schlotfeldt, 2004) and Asian countries such as Japan, Taiwan, Korea and Indonesia (Sano *et al*., 2004; Tu *et al*., 2004; Oh *et al*., 2001; Rukyani, 2002; Sunarto, 2004), revealing that this disease is rapidly spreading among world-wide carp-trading countries.

Clinical signs of KHV are often non-specific and mortality may occur rapidly. Affected fish become disoriented and swim erratically prior to death, which can occur within 24–48 hours after the onset of clinical signs (Gray *et al*., 2002; Hartman *et al*., 2004). Principle histopathological features were characterized by KHV-infection in the respiratory epithelial cells of gill lamellae: necrosis of the gills, proliferation of interlamellar epithelial cells resulting in fusion of gill lamellae and subsequent clubbing of gill filaments. Myocardial cells and hematopoietic cells were also infected with KHV by systemic infection. Under electron microscopy, assembly of im-

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mature capsids and nucleocapsids was evident in the nucleus, and the formation of subviral particles and virions was evident in the cytoplasm (Kholidin, 2007; Miyazaki, 2007; Miyazaki *et al*., 2008).

The virus was isolated from numerous tissues of fish with signs of the disease, including the gill, kidney, spleen, liver, and intestine and then cultured in some cell lines as KF-1 (koi fin), FHM (fathead minnow), EPC (*epithelioma papulosum cyprini*), brain (CCB) tissues of carp and cell line from gill (CCG) (Neukirch *et al*., 1999; Hedrick *et al*., 2000; Oh *et al*., 2001; Hutoran *et al*., 2005). Optimal growth of koi herpesvirus occurred at temperatures from 15 to 25°C. Virus detected at 4°C and 10°C and at 7 and 13 days was just above the detection limit (42 $TCID_{50}$ KHV/mL) of the assay. There was no evidence of virus growth at 30°C or 37°C (Hedrick *et al*., 2000; Gilad *et al*., 2002). However, there are a few studies on electron microscopic features of cultured cells infected with KHV especially in Indonesia. In the present study we investigated the electron microscopic features of KHV-infected KF-1 cells. We revealed the basic features of KHV-infected KF-1 cells, and morphology and morphogenesis of KHV in KF-1 cells.

MATERIALS AND METHODS

Cell culture

KF-1 cells were used in this study. KF-1 cells were grown in 25 cm^2 cell culture flasks to confluence in Eagle's minimum essential medium (EMEM: Nissui, Japan) supplemented with fetal bovine serum (FBS: Sigma, USA), L-glutamine, and buffered with NaHCO to pH 7.2-7.4. During the growth, KF-1 cells were incubated at 25°C, after inoculation with the virus at 20°C, respectively.

Virus stock

Virus inoculum was prepared from harvested KHV-infected KF-1 cells. The viral titer of this inoculum was calculated using $TCID_{50}$ method which resulted in $10^{6.3}$ $TCID_{50}$/mL. The virus inoculum was stored at -80°C until further use.

Virus culture

The monolayers of KF-1 cells were inoculated with KHV inoculum at 0.1 mL (MOI: 1) for 1 hour at 20°C, and then 5 mL of fresh EMEM-2 was added. Infected cells were incubated at 20°C. Cytopathic effect (CPE) was daily observed under a light microscope, and the infected cells were harvested after 14 days inoculation.

Electron microscopy

For transmission electron microscopy, a 3.0 mL of harvested medium containing KHV-infected KF-1 cells were centrifuged at 1,200 x *g* for 10 minutes, and then the precipitates were washed two times using PBS (-). The obtained pellets were fixed with Karnovsky's solution, postfixed in 1% OsO_4 and then processed as follows: dehydrated in cool ethanol (50% to100%), soaked in QY-1 and epon-812. The pellet was transferred into capsule plate containing epon-812 and hardened in oven at 60°C for 36 hours. Capsule-samples were processed for electron microscopy as previously described by Mahardika *et al.* (2008).

RESULT AND DISCUSSION

Under inverted microscopy, the KHV virus induced CPE as syncytial formation, cell fusion and intense cytoplasmic-vacuolation in KF-1 cells within 5 days after inoculation. The cells-vacuolation was evident at 7-10 days and progressed to invade all cells after 14 days (Figure 1A-D).

Similar to KHV infected KF-1 cells, different cultured cells have been reported to be sensitive to KHV infection. In FHM cell line, CPE was observed at 3 to 5 days after inoculation. A complete cell lyses could be observed within 15 days (Oh *et al*., 2001). In CCB cells, the development of CPE became obvious 5 days after inoculation. CPE was characterized by giant syncytial formation. The syncytia spread in the cell culture during the following 4 to 5 days. In CCG cells, CPE developed since day 6 post infection. In EPC cells first syncytia could be detected from day 11 after inoculation (Neukirch *et al*., 1999). In KF cells, the appearance of CPE was observed at 5 to 6 days post inoculation (Hutoran *et al*., 2005).

Electron microscopic (EM) examination of KHV-infected KF-1 cells revealed the cells with the formation of vacuola within an intracytoplasmic cell. Nuclear changes including severe hypertrophy and a diffuse appearance to the chromatin. Intranuclear virus particles were observed mainly in hypertrophied cells. The nuclear particles were circular or polygo-

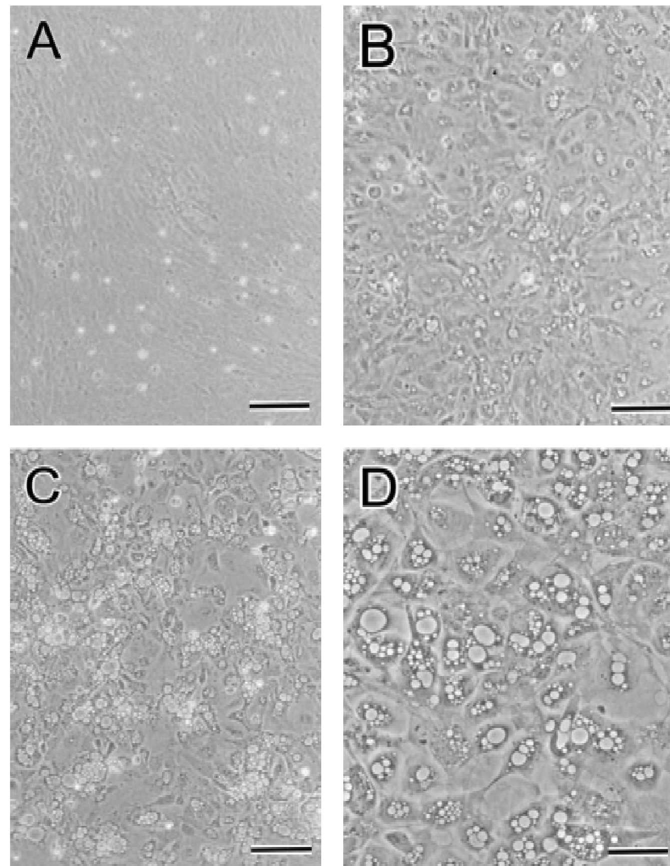

Figure 1. (A) Cytopathic effect (CPE) of KHV-infected KF-1 cells at 5 d p.i. shows some cell fusion and cytoplasmic-vacuolation (Scale bar = 15 mm). (B) CPE of KHV-infected KF-1 cells at 10 d p.i. shows many cells-vacuolation. (C) CPE of KHV-infected KF-1 cells at 14 d p.i. shows many varietes of cells-vacuolation (Scale bar = 25 mm). (D) High magnification of fig. 1C displays some vacualation within one cell cytoplasm (Scale bar = 50 mm)

nal in shape. Some nuclear particles appeared empty or incompletely packaged DNA in the inner volume and were interpreted as being capsids. Capsids were 115-120 nm in diameter. Others contained an electron-dense toroidal or brick-shaped core, or a concentric ring structure, and were assumed to be nucleocapsids. Nucleocapsids were icosahedral or spherical in shape with 125-130 nm in diameter (Fig. 2A & B). Capsids and nucleocapsids were scattered throughout the nucleus of infected cells. Many non-enveloped nucleocapsids have moved into the cytoplasm, around which fine granules were increased. The nucleocapsids have been enveloped with the inner nuclear membrane when it has budding from a damage part of the inner nuclear membrane (Miyazaki, 2007). In the cytoplasm, all of mature nucleocapsids were non enveloped or naked, which indicated that they have budded without being enveloped from the outer nuclear membrane (Fig. 2C & D; Fig. 3A & C). The enveloped nucleocapsid is de-enveloped in the perinuclear membrane (Miyazaki, 2007). Moreover, mature nucleocapsids formed the tegument with a definite surface membrane to be subviral particles in the cytoplasm (Kholidin, 2008). Within small vesicles, virions with the enveloped tegument budded through the intracytoplasmic membrane. These virions were spherical in shape and 231-233 nm in diameter (Insert Fig. 3C). Thus, KHV mainly complete

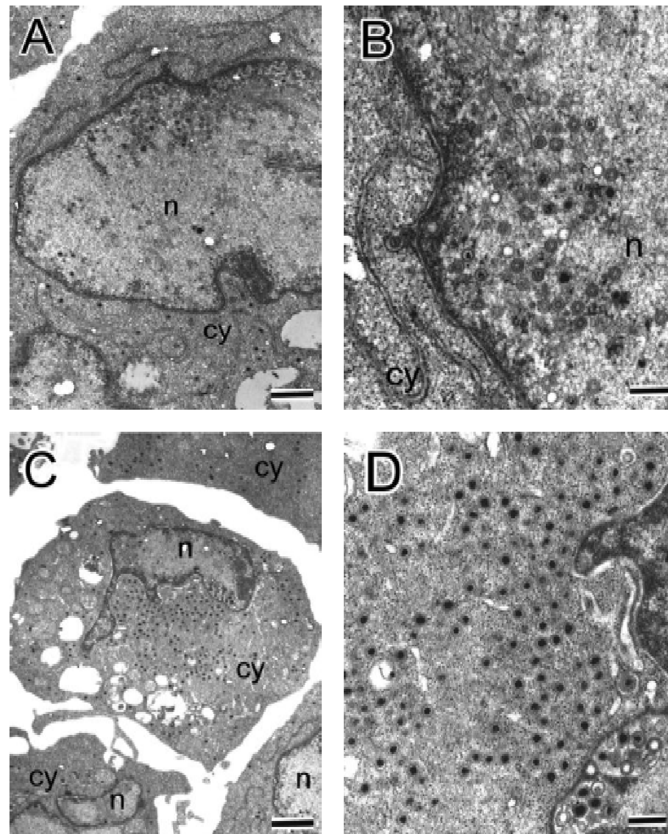

Figure 2. (A-D) Electron micrographs of cells derived from KHV-infected KF-1 cells at 14 d p.i. (A) Infected cell with an enlarged nucleus within which viral particles were assembled (Scale bar = 1000 nm). (B) High power view of cell nuclues in fig. 2A, capsids and nucleocapsids were scattered throughout the nucleus. Nucleocapsids were icosahedral or spherical in shape (Scale bar = 500 nm). (C) Infected cell shows assembly of mature nucleocapsids within the cytoplasm (Scale bar = 1200 nm). (D) High power view of infected cell in fig. 2C, many mature nucleocapsids assembly around which fine granules are increased (Scale bar = 500 nm)

maturation in the vesicle (Miyazaki, 2007; Kholidin, 20087). Some KHV-infected cells were necrotized with nuclear fragmentation and damaged organelles, whose cytoplasm still contain a small number of virus particles (Fig. 3B). And then, the mature virions were released following the cells lyses.

Ronen *et al*. (2003) reported that the mature virions contain a loosely applied envelope, giving the virion an overall diameter of 170–230 nm. The virions bear thread-like structure (tegument) on the core surface resembling those of herpesvirus. Major component of the envelope is a lipoprotein double layer. Morphology and size is similar to the viruses in the family of Herpesviridae. However the large size of KHV genome, which is estimated at 277 kbp, exceeds that of 250 kbp known for members of the family Herpesviridae.

Histopathological features of KHV infected fish (*in vivo*) have already been shown by Hedrick *et al*. (2000). According to Hendrick *et al*. (2000), KHVD was characterized by affection of gills: necrosis of respiratory epithelia of gill lamellae, fusion of gill lamellae, hyperplasia of interlamellar epithelial cells resulting in clubbing of gill filaments, and

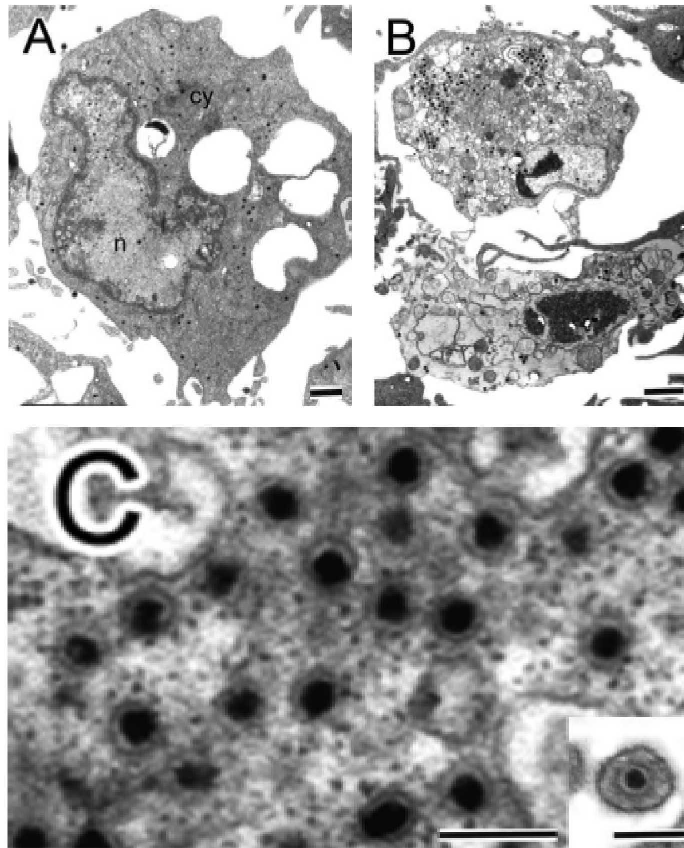

Figure 3. (A-C) Electron micrographs of cells derived from KHV-infected KF-1 cells at 14 d p.i. (A) Infected cell shows some mature nucleocapsid scattered within the cytoplasm as well as assembly of capsids and nucleocapsids within the nucleus (Scale bar = 800 nm). (B) Infected cell (upper) contained many mature nucleocapsids, degenerated mitochondria and the formation of vacuoles within the cytoplasm. Another cell became necrotic and organelles have fragmented within the transparent cytoplasm without formation of nucleocapsids (Scale bar = 1000 nm). (C) Detail of assembly of mature nucleocapsids within the cytoplasm around which comprised with granules and microfilaments (Scale bar = 300 nm). Insert, within small vesicles, virions with the enveloped tegument budded through the intracytoplasmic membrane (Scale bar = 250 nm)

subsequent systemic infection as in the liver, renal tubular epithelia, hematopoietic tissue and intestine. These histological findings were the same as those found in common carp suffering from natural or experimental KHV disease in Indonesia (Kholidin, 2007). On the other hand, in the case of Israel, although the etiological virus was the same, a different disease name was proposed: Carp interstitial nephritis and gill necrosis (CNG) based on the pathological changes occurred in the kidney, that the causative virus markedly infected inflammatory cells surrounding the nephrons (Perelberg *et al*., 2003; Pikarsky *et al*., 2004). Moreover, Kholidin (2007) reported that the KHV invaded gill epithelial cells, replicated within them and subsequently entered the blood stream of lamellar capillaries, resulting in systemic infection. Severe damage of the respiratory epithelia appeared to cause hypoxia that was evidenced by gasping behavior of diseased fish.

In summary, morphogenesis and morphology of KHV (*in vitro*) under EM as follows assembly of immature capsids and nucleocapsids was evident in the nucleus. The formation of subviral particles and virions, which comprised, in turn, an electron dense core, capsids with a hexagonal outline, the tegument was evident in the cytoplasm. These findings were similar to those in common carp infected with KHV (*in vivo*) (Miyazaki, 2007; Kholidin, 2007). These morphology and morphogenesis were the characteristics of viruses in the Family Herpesviridae.

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