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Pathognomonic features and ultrastructural of Koi Herpesvirus infected *Oreochromis niloticus*

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Abstract. *Wahidi BR, Yanuhar U, Fadjar M, Andayani A. 2019. Pathognomonic features and ultrastructural of Koi Herpesvirus infected* Oreochromis niloticus. *Biodiversitas 20: 497-503.* Koi Herpesvirus (KHV) has caused considerable losses in the cultivation of the Cyprinidae family worldwide. In addition to infecting Cyprinidae, KHV can also infect other freshwater fish. This study reports histopathology and ultrastructural analysis of KHV that infect Nile tilapia. Although there are no specific clinical symptoms, pathognomonic indicating Nile tilapia fish has been infected with KHV have been found, i.e. through the formation of intranuclear inclusion body and cell swelling which essentially experience chromatin margination. In addition, histopathology results indicate changes or damage to other tilapia tissue, i.e. necrosis, hypertrophy, edema, hemorrhage, inflammation, congestion, degeneration, and apoptosis. These results indicate that KHV has been distributed and replicated in tilapia organs. Based on the Transmission Electron Microscopy (TEM) analysis, KHV virions have been detected in the cytoplasm or outside the cells on a size of 150-170 nm.

Keywords: KHV, Pathognomonic, TEM, *Oreochromis niloticus*

INTRODUCTION

Since Koi Herpesvirus (KHV) was first discovered in Israel in 1998, KHV has spread to several countries in the world and caused so many deaths in the cultivation environment. KHV infections cause high mortality in goldfish in Indonesia. Sunarto et al. (2005) reported that at the end of December 2003, KHV infection had caused carp mass deaths up to 80-99% of the population and material losses up to IDR 15 Billion. It has been believed that KHV can infect only the specific host of Cyprinidae (Hedrick et al. 2000). This is reinforced by Perelberg et al. (2003) in their study on the susceptibility of several species of freshwater fish (*Oreochromis niloticus*, *Bidyanus bidyanus*, *Hypophthalmichthys molitrix*, *Carassius aurata*, and *Ctenopharyngodon idella*) to Herpesvirus (KHV) infection and transmission mode. It is stated that only *Carasius aurata* alone is susceptible to KHV infection, while other fish have no clinical symptoms of KHV infection and are able to survive. Dishon et al. (2005) used fish feces as one of the markers of KHV antigen presence and they did not find KHV in the feces of Nile tilapia. However, recent studies have successfully revealed that KHV can infect fish other than Cyprinidae (Sadler et al. 2008; Fabian et al. 2013).

Several studies have been undertaken to determine the susceptibility of Non Target Species (NTS) to KHV (Bretzinger et al. 1999; Perelberg et al. 2003; Fabian et al. 2013). The results of these studies are in line with the one by Hedrick et al. (2000) stating that KHV cannot infect fish other than the carp or Cyprinidae. In addition to NTS, a study has been conducted to investigate the susceptibility of hybrid fish (Hedrick et al. 2006; Bergmann et al. 2010); it shows that hybrids are susceptible to KHV infection and are potentially a carrier. Associated with the carrier potential possessed by hybrid fish, rainbow trout also has potential as a KHV carrier (Bergmann et al. 2016).

Tilapia (*Oreochromis* sp.) is one of the freshwater fish species introduced from Eastern Africa (Nile River), then brought to Europe, America, Middle East, and Asia including Indonesia. Tilapia is widely cultivated in Indonesia because it can reproduce quickly, is resistant to high density and tolerant to poor water quality. As one of the exporting countries of tilapia, it is a disadvantage if tilapia is attacked by pathogens, resulting in a decrease in customer interest. In Indonesia, over the past 4 years, tilapia have been reported to be infected with KHV—based on periodic monitoring results by Fish Quarantine-Ministry of Maritime Affairs and Fisheries. In addition, Suprapto and Kartika (2012) and Wahidi (2014), using molecular assay (Polymerase Chain Reaction), detected the KHV genome in the gills of *Oreochromis niloticus*, but the results were not supplemented by histopathology examination to see the tissue damages and KHV ultrastructure so pathognomonic and the distribution of KHV infection in each organ was unknown.

Knowledge on the distribution of KHV in tilapia organ is important to get the pattern of virus infection in cells or tissues and the change of cell and tissue function. In addition, information related to changes in tissue and ultrastructure KHV on tilapia can inform the potential of Nile tilapia as a carrier of KHV. In relation to the infinite information on pathology, as well as the form and size of KHV virions infecting *Oreochromis niloticus*, the purpose

of this study is to determine the pathognomonic distribution and morphological characteristic of KHV virions found in tilapia.

MATERIALS AND METHODS

Sample collection

The tilapias used in this study were obtained from traditional fish farming in Abar-Abir Village, Bungah District, Gresik Regency, East Java. We used 23 of tilapia, whose length were 5-7 cm, taken randomly and in a living condition. They were brought to BKIPM Laboratory Surabaya II for necropsy and anatomical pathology examination. In addition, the fish were isolated for molecular assay, histopathology and TEM examinations.

DNA Extraction

Total DNA was extracted from organs using SilicaExtraction Kit (Genereach Biotechnology). 25 mg of organ was crushed and homogenized in 900 µl GT buffer, centrifuged at 12,000 rpm for 3 minutes. 600 µl of supernatant was mixed with 40 µl of silica and centrifuged at 12,000 rpm for 15 seconds. The supernatant was removed afterwards. Pellet silica was washed with 500 µl GT buffer, centrifuged at 12,000 rpm for 15 seconds and removed. 1 µl of 70% ethanol was added, centrifuged at 12,000 rpm for 15 seconds and ethanol was removed. 1 ml of ddH_2O was added, vortexed to homogenise and incubated for 10 minutes at 55 ^{0}C and continued to centrifuge at 12,000 rpm for 2 minutes. 500 µl of supernatant was taken and stored at-20 ^{o}C for the following process.

DNA Amplification

TK gene specific primers (Bercovier et al. 2005) were used for KHV DNA amplification: Forward (F: 5 '-GGG TTA CCT GTA CGA G-3) and Reverse (R: 5'-CAC CCA GTA GAT TAT GC-3'), which results in amplification products at 409 bp. Amplification was performed using Go Tag Green Master Mix (Promega) with a total volume of 25 µl. Thermalcycler Mastercycler Personal (Eppendorf) was used with temperature settings: 1 cycle at 95^{o}C for 5 minutes; 35 cycles at 95^{o}C for 30 seconds, 52^{o}C for 30 seconds and 72^{o}C for 1 minuteand 1 cycle at 72^{o}C for 10 minutes. The amplification product was then electrophoresed in 2% (w / v) agarose gel (Scie-Plus) using 1x TAE buffer (Thermo Scientific) for 1 hour with a voltage of 100V and continued with Sybr Green (Lonza) staining. DNA tape was visualized using Gel-Documentation (Uvitech)

Histopathology and Immunohistochemistry Examination

The organ sample was fixed using 10% Formal Neutral Buffer (NBF) solution. Samples were then processed by embedding and cut with a thickness of 10 µm. After that, the sections were stained with hematoxylin and eosin (HE) and observed using a microscope at 400x magnification.

Transmission Electron Microscopy (TEM)

Tilapia gill tissue with 2-millimeter thickness was isolated and fixed using 2.5% glutaraldehyde, then fixated using Karnovsky's for 4 hours at room temperature and continued for 24 hours at 4°C. It was rinsed 3 times in 0.1 M PBS solution and finally fixed with 1% osmium tetroxide. The tissue was then stained using the uranyl acetate and lead citrate. Preparations were observed with magnification up to 50,000 times.

RESULTS AND DISCUSSION

Pathological examination on the anatomy of the tilapia

Tilapia suspected to be infected with KHV in the external part, i.e. the skin and fins, did not have any signs of pathological lesions, but showed the depigmentation or discoloration of the skin, i.e. the skin had become darker, when compared with normal tilapia. While on the internal organs, i.e. the gill, there was an indication on the presence of light necrosis marked by some white parts on the ends of the gill sheets (Figure 1).

A **B**

Figure 1. A. The tilapia suspected as infected with KHV show blackish skin color; B. White parts on the gill sheets.

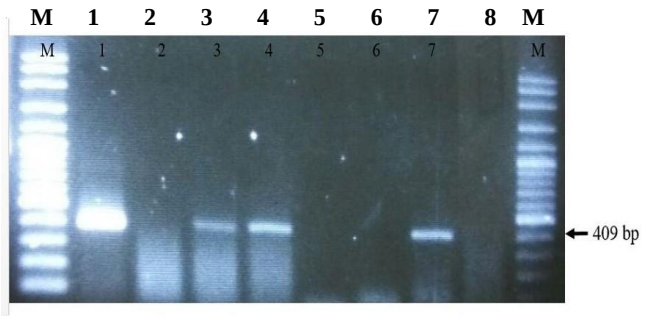

Figure 2. PCR test result on Tilapia Isolate Using TK Gene Primer; M. 100-bp DNA ladder; 1. Positive Control; 2. Negative Control; 3. Gills; 4. Kidney; 5. Liver; 6. Brain; 7. Intestine

KHV detection using molecular assay

Assessment result using the PCR method exhibited that the tilapia organs such as gills, kidneys and intestines were KHV positive. It indicated that the organs suffered KHV infection. Whereas the other organs, namely the brain and the liver, exhibited negative results. This indicates that KHV infection has been distributed to several tilapia organs in addition to the gills which are the target organs of KHV infection.

Histopathological examination

Microscopic observation results indicated that the infected tilapia showed lesions in the internal organs, i.e. gills, kidneys, brain, liver and intestine. The gill organs (Figure 3a) shows that gills undergo fusion between adjacent secondary lamellae, eosinophilic inclusion body and cell swelling (hypertrophy) resulting in chromatin margination. Histopathological features also show a proliferation of macrophages and edema. The occurrence of edema causes epithelial desquamation of the primary and secondary lamellae.

Similarly, tissue damage is also seen in the kidneys, i.e. (Figure 3b) hemorrhage, inflammation, apoptosis, necrosis, inclusion body and chromatin margination cells. The kidney also shows the occurrence of hydropic degeneration, namely the swelling of cells that cause pressure on the capillaries of the organ. Other damages to the kidneys are edema and apoptotic lesions. Histopathological observation of damage to liver (Figure 3c) of KHV-infected fish shows hemorrhage, inflammation, hydrophilic degeneration, hyaline degeneration, and necrosis, followed by chromatin margination cells, and eosinophilic inclusion bodies.

The intestine is one of the target tissues, which can be used to detect the presence of KHV because it becomes one of the target organs for KHV infection leading to pathological changes in the intestinal tissues directly or indirectly. The intestine (Figure 3d), suspected to be infected with KHV, shows hemorrhage, necrosis, and swelling of cells—in short, chromatin margination happens in the suspected virus-infected cells, and eosinophilic inclusion body has also been seen. Hyperplasia or goblet cell proliferation has also been seen in the intestinal epithelium, which is one of the initial defense response to damage in the intestine. Infection due to KHV disease in tilapia is thought to cause an increase in goblet cell proliferation as it is considered to respond to the presence of foreign substance. The results of examination in the brain show the brain experiencing hemorrhage, congestion, edema, inflammation and necrosis (Figure 3e). Brain edema generally results from inflammatory reactions in the brain.

Transmission Electron Microscopy (TEM) Examination

In addition, the virion capsid in the mature phase contains a solid material with electrons (electron-dense cores) and has a clear circular shape around the core. In the cytoplasm, the morphology of KHV virus capsid may be different in shape based on its morphology, i.e. (i) capsid containing internal circular structure, as if there is an additional circle in the capsid, (ii) capsid containing a solid nucleus with electrons, and (iii) capsid with no additional material or empty capsid (Miwa et al. 2007)

Discussion

The histopathological observations on the tilapia organ shows that all examined organs have tissue damage. Gills have the highest tissue damage—this is possible because KHV is a waterborne virus, so it does not take long for the gill to become infected. Gilad et al. (2004) state that gills take only one day to be exposed to the virus, while Yuasa et al. (2012) report that it takes 3 days for KHV to infect the gills, whereas it will be longer for the kidney and brain—approximately 7 days. The existence of inclusion body is one of the signs of virus infection in a tissue. Research on the formation of eosinophilic inclusion bodies with a slightly basophilic core of hypertrophy and chromatin in KHV-infected tissue has also been suggested by Hedrick et al. (2000), Perelberg et al. (2003), Miyazaki et al. (2008), Cheng et al. (2011), Monaghan et al. (2015), Rahmati-Holasoo et al. (2016).

The liver is one of the organs that often suffered damage caused by the presence of toxic substances and antigen. Hedrick et al. (2000), Gray et al. (2002) and Gilad et al. (2003) report on the liver becoming one of the target organs of KHV. Furthermore, Gilad et al. (2004) have found high levels of KHV DNA in the liver organ accompanied by pathological abnormalities due to KHV infection. The liver is an organ very sensitive to antigens and toxic substances; this is related to the metabolic function in the liver cells. Hedrick et al. (2000) and Perelberg et al. (2003) report that fish infected with KHV will experience liver dysfunction. In the liver of KHV-infected tilapia, some pathological changes have been seen, such as the presence of inflammation and degeneration, although only slight ones, indicate the beginning of a mild infection. Two things may cause hydrophic degeneration of the liver, lack of oxygen in the tissues and the presence of intoxication or pathogenic agents.

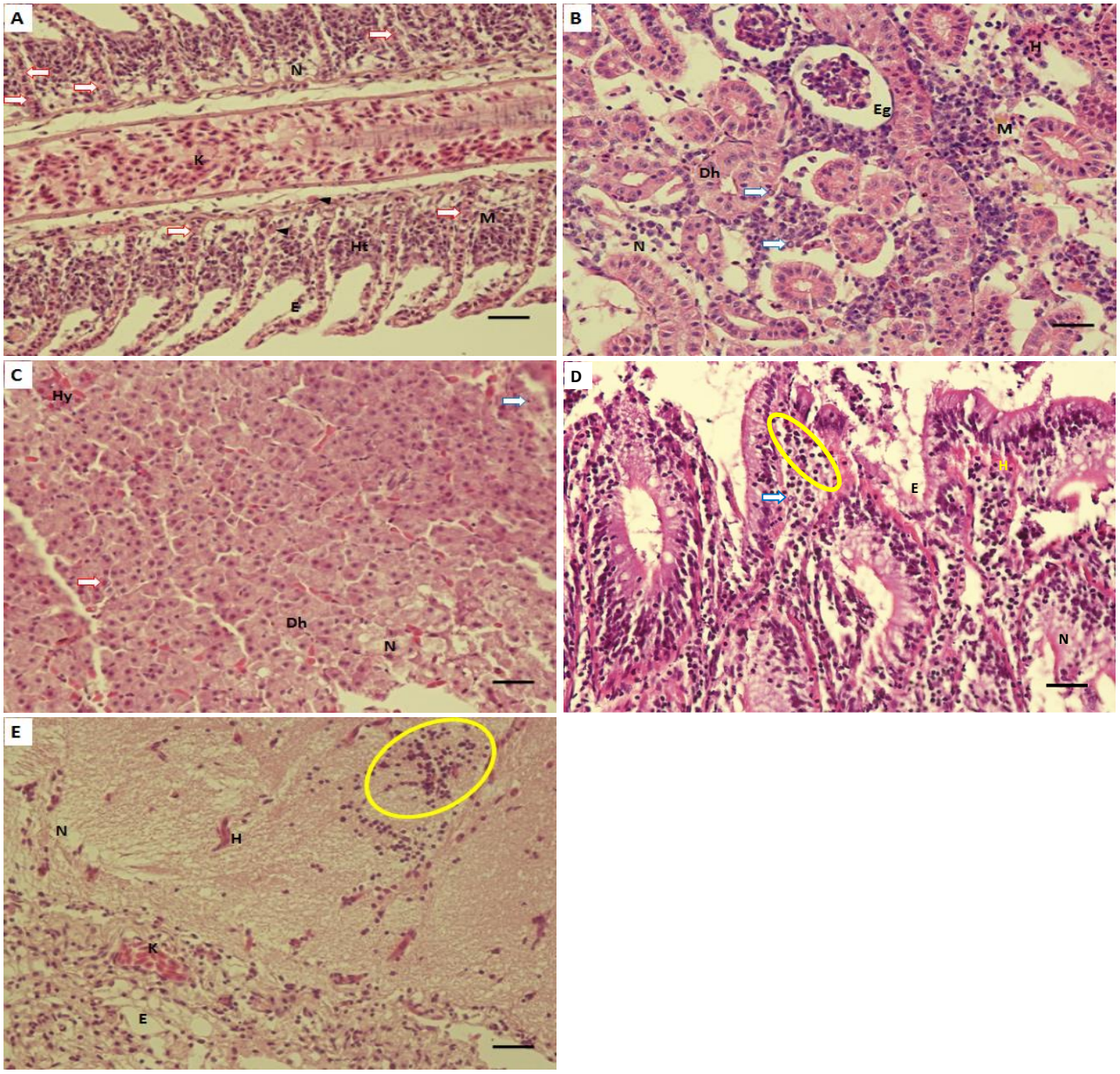

Figure 3. Histopathology of organs with H&E staining. **A**. Gills experience hypertrophy (Ht), Congestion (K), Edema (E) necrosis (N), Macrophage (M), eosinophilic inclusion body (red arrow) and cell apoptosis (black arrow). **B**. The kidney undergoes hydropic degeneration (Dh), hemorrhage (H), Glomerular Edema (Eg), Macrophage (M), necrosis (N), and chromatin margination (blue arrow) cells. **C**. The liver experiences hydrophobic degeneration (Dh), hyaline degeneration (Hy), chromatin margination (blue arrow), eosinophilic inclusion body (red arrow) and necrosis (N). **D**. The intestine experiences hemorrhage (H), and necrosis (N), glomerular edema (E), inflammation (yellow circle), followed by cell enlargement, essentially chromatin margination (blue arrow), suspected of virus-infected cells. **E**. The brain experiences hemorrhage (H), congestion (K), Edema (E), necrosis (N), and inflammation (yellow circle). Bar = 1 μm.

Similar to the liver, the kidney also suffered damage due to KHV, i.e. the formation of inclusion body. Pathological observation results of the kidneys show interstitial nephritis, the damage caused by the thickening of the epithelium in the tubules, inflammation, and necrosis. In a study conducted by Pikarsky et al. (2004), KHV infection that reaches the kidney also brings abnormalities to the interstitial nephritis. Acute damage to the kidney may result from a disturbance of the tubule epithelium by infection, the direct toxin effect, or ischemia. In the histopathological image of the KHV-infected tilapia, tubular epithelial cells also experience necrosis, and the eosinophilic inclusion body is followed by the magnification of the nuclei that experience chromatin margination. Miwa et al. (2015) state that the presence of cells whose nuclei experience chromatin margination indicates that the cell has been infected with KHV. Rahmati-Holasoo et al. (2016) also report that KHV-infected Koi fish also suffers kidney damage in the form of necrosis and an intranuclear inclusion body that resembles a ring shape with clear chromatin borders.

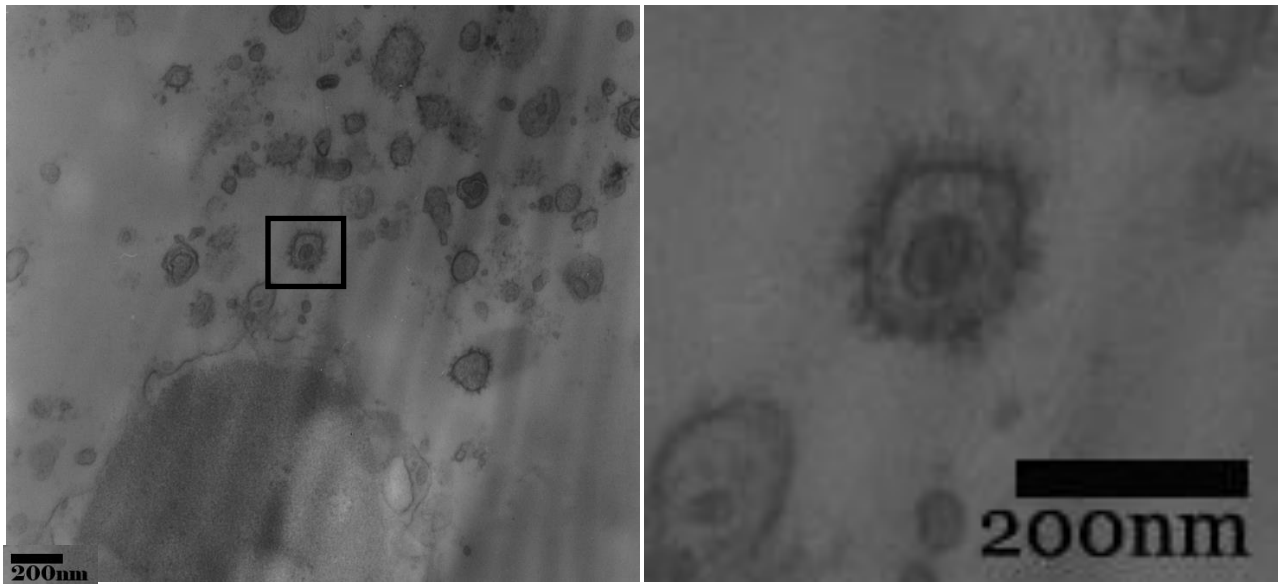

Figure 4. The TEM results indicate the presence of viruses that are morphologically consistent in size with viruses from the *Herpesviridae* family. Viral particles found in gill organs are suspected to be still in the mature phase and are in the cytoplasm with a diameter of 150-170 nm.

The presence of hemorrhage in the intestine indicates the discharge of blood from the blood vessels, either into or out of body tissues. Hemorrhage occurring in the intestine can be caused by the entry of foreign materials or substances along with food that can cause lesions in the intestine. In addition to hemorrhage, the gut also undergoes necrosis which results in the loss of villi from the basal lamina. Necrosis affects the loss of function in necrotic areas. The entry of antigens into the gut will first be driven by goblet cells. Goblet cells are present among the absorptive cells of the small intestine villi containing glycoprotein acid, which serves to lubricate the intestinal wall and serve as an important defense medium against infection. This is in accordance with research by Fournier et al. (2012) which states that the KHV virus has been found after the fish eats the material containing the virus. Hedrick et al. (2000), Gray et al. (2002), and Gilad et al. (2004) suggest the intestine as one of the target organs of KHV infection (CyHV-3). In addition to Gray et al. (2002), Gilad et al. (2004) also state that specific DNA viruses have been detected at a high amount in KHV-infected carp gut.

Damage occurs in the brain including hemorrhage, inflammation or degeneration, and necrosis (each seen on a broad field of view). Results of observation in the brains of KHV-infected Nile tilapia showed mild inflammation, with visible infiltration of inflammatory cells. In addition to inflammation, other damage was found in the form of hemorrhage in the brains of Nile tilapia. Hemorrhage can cause brain neurons to experience ischemia due to lack of oxygen supply. The damaged neuron cells have an impact on the motion of the fish as a result of which swimming regularly is rather hard. Tilapia infected with KHV in this study did not show irregular swimming behavior, so the possibility was that the neuron cells in Nile tilapia have not been damaged. The absence of KHV virion in the brain, according to Miwa et al. (2015), has been due to the long time for the viral infection to reach the brain, especially to penetrate the brain's defense system (blood-brain barrier). Another possibility is that the virus enters the Central Nervous System (CNS) through the olfactory system, and the process of viral infection goes a little slow along the nervous system as it simultaneously circulates the blood system by the blood-brain barrier.

In contrast to previous findings stating that tilapia cannot be infected with or resistant to KHV utilizing PCR method (Perelberg et al. 2003; Dishon et al. 2005; Yuasa et al. 2009), the result from observation using molecular assay on the organs indicates that tilapia was infected by KHV. It was also confirmed by Bergmann et al. (2016) that in addition to Cyprinidae, NTS fish can also be infected with KHV. Although PCR method has a high sensitivity and specificity, but the results are less reliable because often the virus is in a carrier condition (Azila et al. 2012) and the primary PCR limit detection used in this study is only able to detect 10 fg (fentogram) of KHV DNA or 30 virion particles (Bercovier et al. 2005). KHV detection is recommended to be performed simultaneously between molecular and serological methods to support each other's specificity (Dishon et al. 2005; Eide et al. 2011; Bergmann et al. 2016; Li et al. 2017).

KHV morphological observation in this study only uses the gill samples with consideration that the gill is the main place of KHV replication. According to Hedrick et al. (2000) and Miyazaki et al. (2008), the gill, in particular the epithelial cells, is the target of KHV infection and facilitate

observation of KHV virions using TEM. Observation of viral particles showed KHV virions in a circular shape of 150-170 nm in size; the results of these observations correspond to the observations made by Miwa et al. (2007) that KHV virus particles are in circular or spherical shape and are 170-100 nm. Based on the examination, the virus in the Nile tilapia that has such morphology goes into *Herphesviridae* family (Miwa et al. 2007; Miyazaki et al. 2008). The size of the virion will change to 170-230 nm when the virion obtains the envelope upon exiting the cell (Hedrick et al. 2005), as it is known that KHV is an enveloped virion enclosing the capsid nucleus contained therein. The capsid in the mature phase, as shown in Figure 4, is a continuation phase after the un-enveloped of the virions; then, the electron-dense core will enlarge and angle to a corner, which finally during attachment to the cell nucleus membrane (inner nuclear membrane), the material in capsid will be released and replication occurs. The KHV capsid attachment process on the cell nuclear membranes is very rarely observable using TEM; this is possible because the phase is so rapid that it is difficult to observe (Miwa et al. 2007). This is also in line with Gilad et al. (2004), stating that it only takes 1 day for KHV to infect and replicate in the host's body.

Based on the pathognomonic distribution of KHV reinforced with molecular assay, although the tilapia did not show any specific clinical symptoms and did not even die, the molecular assay yield was positive on the gill, kidney, liver, and intestine. This indicates not only that KHV has been distributed and replicated in the tilapia organ, but also that there has been an interaction between the virus and the receptor on the tilapia seed organ. The interaction between the virus and the receptor is an early stage of the infection process, which will evolve toward pathogenesis and force cell function to form a stronger immune system so as to prevent the spread of infection within or between organs. Although it does not cause infection, the interaction between the virus and the receptor will still emit a transduction signal that affects the progression of the disease itself (Schneider-Schaulies, 2000). In addition, Traylen et al. (2011) state that after the virus enters the body, it replicates through the lysogenic cycle, in which within this cycle occurs the replication of the viral genome, yet the host cell is not destroyed but is inserted by nucleic acids from the virus, resulting in provirus. At this stage, the DNA of the virus merges with the cell's DNA and does not cause damage to the cell, so it does not cause the clinical symptoms of viral infection (latent). Viruses that have low levels of virulence will try to maintain low density, thus providing less threat to hosts. It is also possible that viruses in the host's body utilize the protein to survive, as the host's defense system works well and the virulence is low due to adaptation to the new host environment, then the presence of virus in the host body does not cause too many problems. The latent condition of this virus will return to active state when triggered by a combination of cellular stimulation and usually the level of virulence infection will be more severe.

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