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Sequence Analysis of Thymidine-Kinase Encoding Gene of Koi Herpesvirus Infection Case in Malang and Batu City–Indonesia

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Abstract

Background: Koi Herpesvirus (KHV) infection evidently gave a serious impact to the ornamental fish industry in Indonesia. Measures have been managed to control the outbreak in local fisheries, however, the infection is somehow persistent due to presumptive broad contamination to native fishes, water body, and poor fishpond management. This study investigated the KHV infection cases occurrence in Malang and Batu city based on pathological changes found in deceased fish followed by partial thymidine kinase (TK) encoding gene analysis. **Methods:** Five and two koi fish were dissected to obtain gill tissue and preserved in non-buffered 10% formalin for histopathologic examination. Two gill tissues were performed polymerase chain reaction (PCR) and an amplicon with prominent specific band were sequenced using Sanger sequencing method. **Results:** The smaller fish presented lesser gills necrosis with multifocal whitish fibrinous exudate plaques and the larger fish tended to develop a vast area of necrosis. The amplicons partially covered about 410 base pairs (bp) of nucleotides of the total about 998 bp length nucleotides of TK encoding gene that matched to KHV 3 TK encoding gene sequences from NCBI Genbank. **Conclusions:** There was no distinct grouping of haplotypes based on KHV TK encoding gene sequences.

Keywords: Haplotype, Histopathology, Koi herpesvirus, Phylogeny, Thymidine kinase

1. Introduction

Known as high valuable ornamental and decorative fish for either outdoor or indoor ponds, koi fish has been extensively bred and contributed to Indonesia national commodity and income. During 2017 to 2018, the koi fish industry has culminated at 1.22 billion fish exported to so-called countries including the United States, United Kingdom, Australia, Japan, China, and Singapore. This indeed directly gave an impactful increase of income to Indonesia fisheries communities especially those who relied on ornamental fish's industry, reporting an increase of income from USD 21 million to USD 33 million in the last five years. Ornamental fish industry also apparently developed during the Covid-19 pandemic, giving alternative sources of revenue [1].

Though this koi fish industry seems promising for supporting national economic development, several infectious diseases including Koi Herpesvirus (KHV) infection have been reported deteriorating the fish health, giving a serious hit to koi population in the breeding ponds. These viruses evidently cause rapid death with high morbidity and mortality especially those that attack parent stocks leading to serious economic loss. Several KHV infection cases have been reported in many places in Indonesia since 2002 predominantly in Java Island including Blitar, Subang, and Cirata. In 2003, a similar case was reported in Lubuk Lingau, South Sumatra presenting a list of highly identical lesions to what were reported in Java. Considering the spread of infection, the contamination of KHV in a broader region, however, is not reported, is highly possible [2].

Several pathological lesions were reported associated with KHV infection though they were not pathognomonic merely in this infection. Fatal defects inducing systemic failure of organs were reported in kidneys and gills which alter the primary organ of fish osmoregulation. It might be associated with virus portal entry through superficial tissues including gills. Other studies suggested that fins may contribute as a primary portal of entry for KHV prior to reaching systemic organs [3]. In fact, gills were apparently the most reported organ presenting pathological lesions associated with KHV infection [4]. Analysis of pathological changes in organs of deceased fish due to KHV infection might help to provide symptomatic treatment and prophylactic therapy at the earliest time of infection.

Koi herpesvirus is a double stranded DNA virus (dsDNA) that causes infection in common carp (*Cyprinus carpio*) and koi carp (*Cyprinus carpio koi*). According to ICTV nomenclature, Koi herpesvirus is classified as *Cyvirus cyprinidallo3* belonging to the family of Alloherpesviridae, genus *Cyvirus*. It has an estimated 277 kbp genome size, greater than any herpesvirus, and encodes about 136 proteins. The primary protein of koi herpesvirus to infect the cell host is a glycoprotein. Glycoprotein mediates DNA virus injection to the nucleus for replication. The synthesis process of viral protein occurs in the nucleus. The protein will be transported to the Golgi apparatus to assemble its components and released into the environment [5]. At least, three major KHV lineages–American, Asian, and Europe– have been identified based on genotype to trace the transmission route according to their geographic origins.

Thymidine kinase is one of the important enzymes of koi herpesvirus that plays a critical role in replication and virulence. It is located within Open Reading Frame 55 of the koi herpesvirus genome. Thymidine kinase catalyzes the transfer of phosphate groups from ATP to thymidine, producing thymidine monophosphate (TMP). Thymidine monophosphate enters the nucleotide biosynthesis pathway, and undergoes several phosphorylation steps to become thymidine diphosphate (TDP) and eventually thymidine triphosphate (TPP). TTP is a key component of DNA synthesis, ensuring there are enough nucleotides available for koi herpesvirus replication. Thymidine kinase activity enables the virus to replicate more quickly and increase the viral load in the koi fish, leading to higher virulence. [6]

Herpesvirus infects many other animals, such as reptiles and mammals. They include in the subfamily Alphaherpesviridae—several symptom signs documented, such as stomatitis, conjunctivitis, and tumors. The analysis of herpesvirus from captive Chalonians in Europe during 2016 – 2020 [7]. With 4,797 samples, they found herpesvirus from captive chelonians 312 samples. Other herpes viruses infect cetaceans that include the subfamily Alphaherpesviridae and Gammaherpersviridae. The 966 tissues of cetaceans in Spain and 121 were tested positive [8]. It infected several tissues in cetaceans, such as the skin, urinary bladder, heart, and suprarenal glands.

Our study investigated the KHV infection cases occurrence in Malang and Batu city based on pathological changes found in deceased fish followed by partial thymidine kinase (TK) encoding gene analysis. As we could tell, there is no report of KHV detection performed in Malang and Batu City confirmed with polymerase chain reaction (PCR) and also we use thymidine kinase due to there are numerous references and gene databases, providing ample data for analysis. The genetic analysis of KHV TK sequences were aimed to predict the putative geographic origin of the KHV found in Malang and Batu City based on phylogenetic tree and haplotype network analysis.

2. Materials and Methods

2.1 Ethical Approval

This research has received ethical clearance from the Animal Care and Use Committee of Universitas Brawijaya (No. 085-KEP-UB-2023).

2.2 Study Period and Location

The study was conducted in June - July 2023 at the Laboratory of Veterinary Anatomic Pathology and Laboratory of Animal Disease Diagnostic, Faculty of Veterinary Medicine, Universitas Brawijaya.

2.3 Necropsy and Sample Collection

Five and two koi fish were submitted from local ponds in Malang and Batu City respectively in frozen condition. The fish were dissected in the Laboratory of Veterinary Anatomic Pathology, Faculty of Veterinary Medicine, Universitas Brawijaya to obtain gill tissues and other organs were inspected for any abnormality. All tissues predicted containing lesions were preserved in non-buffered 10% formalin for histopathologic examination. Those tissues were processed following histotechnique protocol in our laboratory.

2.4 Histopathology Preparation

The tissue processing was performed in routine protocol: fixation, trimming, dehydration, clearing, paraffin embedding and sectioning. Initially, the gill tissues were fixed in a 10% formalin for 24 hours. The tissues were sliced to approximately 0.5 cm thick. The tissues in the cassette were then dehydrated in series of ethanol with increasing concentration (70%, 80%, 85%, 90%, and 95%) followed by three immersions in absolute ethanol for one hour each. The clearing was done in xylenes three times for five minutes, five minutes, and 10 minutes, respectively. For paraffin embedding, the tissues were placed in melted paraffin wax three times for 60 minutes each inside an incubator set to 56°C. Embedding was completed by pouring liquid paraffin into metal molds to form tissue blocks. Finally, sectioning was performed using a microtome to cut the paraffin blocks into 5-micron thick sections, which were floated into a water bath and mounted onto glass slides.

For routine hematoxylin-eosin staining, following protocols were performed: deparaffinization with xylene three times for 20 minutes each, hydration in series of ethanol with decreasing concentrations for five minutes each, hematoxylin as primary staining for 15 minutes, decolorization with acid alcohol for four seconds, bluing in tap water for 20 minutes, counter staining with eosin for 10 minutes, dehydration with series of ethanol in increasing concentrations for five-seconds each, and finally clearing with xylene three times for 20 minutes each. At last, the slides were mounted with cover slip using Entellan® and observed under Olympus® CX-23 microscope using OptiLab® Advance Plus mounted camera. Pictures were taken in three magnifications: 40x, 100x, and 400x.

2.5 Genome Extraction

Six gill tissues were kept in a small bottle containing 70% ethanol representing specimens from Jakarta, Malang, and Batu City. The ethanol was evaporated by letting the tube open at room temperature for about 48 hours prior to DNA extraction performed. The DNA extractions were performed using the Wizard® Genomic DNA Purification Kit (Promega, USA) at Animal Disease Diagnostic (ADD) Laboratory, Faculty of Veterinary Medicine, Universitas Brawijaya following the manufactured protocol. The extracted DNA was then directly run for PCR without quantification or purification.

2.6 Amplification and Sequencing

We used a positive control for the diagnostic panel for KHV from Jakarta isolate that was previously confirmed through sequencing. The DNA isolates were put in PCR tubes and added PCR mix 5 µL (ThermoFischer) each tube for amplification. The 2.5 µL ddH2O were added in the tube and then DNA isolation samples 3 µL each tube. The two primers added on the PCR tubes were TK forward 1 µL (5'- GGG TTA CCT GTA CGA G – 3') and TK reverse 1 µL (5'- CAC CCA GTA GAT TAT GC -3') [9]. The suspensions were homogenized using a vortex mixer for 15 – 20 seconds. The amplifications were set up after a series of annealing temperature optimization in

following conditions: initial denaturation 95°C for three minutes and then 35 cycles of denaturation 95°C for 30 seconds, annealing 46°C for 30 seconds, elongation 72°C for 60 seconds and post elongation 72°C for five minutes. Amplicons were run in electrophoresis apparatus to visualize the amplicon bands. Amplicons with prominent specific bands were sequenced using Sanger sequencing method with Applied Biosystems® 3730XL with initial purification through gel extraction procedure.

2.7 Phylogenetic and Haplotype Analysis

Multiple sequence alignments were performed in MEGA 10.1.8 using ClustalW. The phylogenetic tree was constructed using maximum likelihood as statistical method with bootstrap method (1000 times replication) for test of phylogeny and Tamura 3-parameter (T92) for substitution model. Haplotype analysis was conducted using DnaSP v6.12.03 and NETWORK 10.2.0.0 to create haplotype network. For the median joining network analysis, we included TK sequences from NCBI Genbank with full coverage of our sequences consisting sequences from United Stated (NC_009127.1, HM347096.1, DQ657948.1, EU932923.1, MT914509.1, MK987087.1, MK987090.1, MK733801.1, MK733802.1), United Kingdom (HM347097.1, AB375389.1), Indonesia (HM347098.1, HM347099.1, HM347101.1, HM347102.1, HM347103.1, HM347104.1, HM347106.1, HM347107.1, HM347108.1, HM347109.1, HM347110.1, HM347111.1, HM347112.1, HM347113.1, KX544843.1, KX544844.1, KX544845.1, KX544846.1, KX544847.11, KX544848.1, HM347098, HM347110, HM347112), Iran (KX609546.1, KP280047, KT290517, KT364393, KX609547), Mexico (KY703866.1, KY703857.1, KY703858.1, KY703863.1, KY703867.1), China (JQ247182.1, JN180630.1, MG755640.1, KY703859.1, KY703860.1, JQ247183.1, KJ627438.1, KX777255.1, MK260013.1), Iraq (MW928743.1, MK817063.1), India (MZ475945.1), Israel (AJ535112.1, AB375386.1), Netherlands (AB375387.1, AB375388.1), Japan (AB458384.1), Poland (KX544842.1), Belgium (MG925485.1, MG925486.11, MG925487.1, MG925488.1, MG925489.1, MG925490.1, MG925491.1), Croatia (MN913971.1) and unknown origin (KP343683.1, KP343684.1, DQ177346.1, AP008984.1, AB375385.1, AB375390.1, AB375391.1). We also included a sequence from KHV 1 (DQ177346.1) as outgroup comparison.

2.8 Genetic Distance Analysis

We estimated the genetic distance among sequences from the NCBI Genbank database consisting of KHV 1, 2, and 3 to our specimens. We collected representative sequences from several distant regions in the world categorized as KHV 1, 2, and 3. The KHV 1 group sequences consist of KT587200.1 (Brazil), NC_019491.1 (Japan), KU672601.1 (USA), and KU672599.1 (UK). The KHV 2 group sequences consist of MN201961.1 (China), NC_019495.1 (China), and JQ815364.1 (Japan). The KHV 3 group sequences consist of MN913972.1 (Croatia), ON340600.1 (Czech), MK987097.1 (USA), KT364393.1 (Iran), MG755640.1 (China) and HM347101.1 (Jakarta). The distance estimation was computed in MEGA 11 using bootstrap method 1000 times as variance estimation method and Tamura-3 parameter as substitution model.

3. Results

3.1 Gross and Histopathological Findings

The most prominent and distinguished lesion in all collected specimens was located in gills where necrotic areas cover almost all lamellae racks with severe branchitis and occasional haemorrhage (Figure 1). Grossly, there were marked multifocal extensive pale white discolorations in the primary gill lamellae with thickening lamella tips presuming lamella epithelial hyperplasia. Complete whitening of gills might be associated with prolonged icy preservation, putrefaction, or decreased blood perfusion to the gills due to hypostasis. We found that the smaller and younger fish presented lesser gills necrosis with multifocal whitish fibrinous exudate plaques. In contrast, the larger fish tend to develop a vast area of necrosis before being found deceased. There is no specific anatomic alteration deemed as pathological change in other organs. Figure 1A and 1B showed multifocal plaques of exudates in the surface of secondary lamellae of younger fish which were lesser than what were encountered in adult one. Figure 1C and 1D presented a more extensive discoloration of

Figure 1. Gross changes of koi fish gills infected with KHV presented extensive white to yellow discoloration with no marked demarcation (asterisks). Note that various degrees of suspected gill necrosis were present in pre-adult fish from Malang City (A-B) and adult fish from Jakarta (C) and Batu City (D).

gill lamellae in adult fish which might indicate a broader tissue necrosis. The difference in the area of necrosis depends on the external factor, one of which is age-related differences in the immune system development. Younger fish tend to have an immature immune system compared to adult fish. This could affect how koi fish responds to viral infection. Younger fish might exhibit a weaker immune response, while adult fish might mount a stronger immune response, leading to more extensive necrosis.

Histopathology examination revealed severe damage of secondary lamellae of gills architecture (Fig. 2). Extensive haemorrhages were present along the primary lamellas indicated by elongated clusters of nucleated erythrocytes. Either primary or secondary lamella architecture apparently underwent necrosis with abundant inflammatory cell infiltration. Rupture and fusion of secondary lamellae were distinct especially in area adjacent to necrotic tissues.

3.2 Molecular Detection and Genetic Analysis of TK Encoding Gene

The amplified amplicons using suggested primers resulted in products sitting near to the 400 bp marker as depicted in Figure 3. Lane 1 and 2 were amplicons from KHV extracted Jakarta, lane 3 to 5 were from Malang, and lane 6 was from Batu City. Multiple bands were observed in lane 1, 4, and five while smears occurred in all amplicons except lane 6 which template was extracted in separate time. A single band in each lane confirmed as a fragment of TK encoding gene based on Sanger sequencing was present in various intensity indicating the amount of viral DNA extracted and load of virion in tissue collected might vary. Interestingly, amplicons in lane 1 and 4 showed multiple bands almost in exact size compared to markers which apparently was not present in other lane.

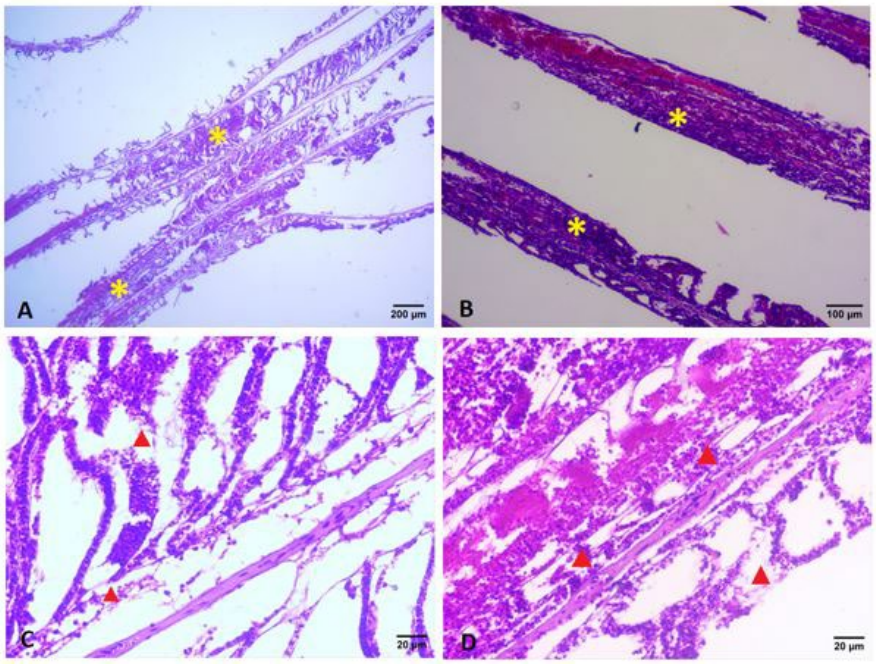

Figure 2. Necrotizing and hemorrhagic branchitis with secondary lamellar fusion (yellow asterisks) were found in all fish gills with white to yellow discoloration (Figure 2A, 40x magnification; Figure 2B 100x magnification). Extensive necrosis of primary and secondary lamellae (red arrow heads) messed the gill architecture to be tissue debris (Figure 2C-D, 400x magnification).

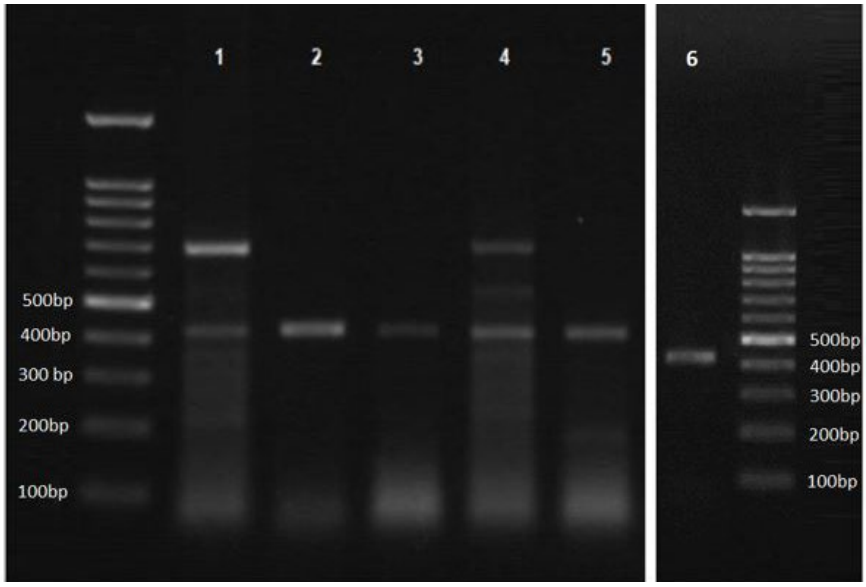

Figure 3. Presentation of TK encoding gene amplicons with 100 bp marker as reference in electrophoresis gel. Respectively from left to right lane were specimens from Jakarta (lane 1,2), Malang (lane 3,4,5) and Batu (lane 7). The desired TK encoding gene amplicons were located near to the 400 bp marker band.

We aligned the representative sequences of KHVs infecting koi fish in Jakarta, Malang, and Batu City with thirty sequences of TK encoding genes archived in NCBI Genbank. Our amplicons partially covered about 410 base pairs (bp) of nucleotides of a total about 998 bp length nucleotides of TK encoding gene that matched to KHV 3 TK encoding gene sequences from NCBI Genbank. These amplicons cover the nucleotide region from 358 to 458 of complete TK encoding genes. Our metaanalysis based on phylogenetic analysis and genetic distance among sequences

concluded that only slight genetic distance (0.00-0.04) observed among them indicating a high conserved gene even from about a decade ago archived sequences. The phylogram of our amplicons compared to NCBI Genbank TK encoding gene sequences is presented in Figure 4. Our analysis in haplotype diversity based on TK encoding gene sequence presented a major cluster grouping Indonesia, United State, Poland, Netherland, Iran, Israel, Japan, United Kingdom, Belgium, some of Mexico and China sequences into a single haplotype (Figure 5). Several sequences from Mexico, China, and Iran were grouped to separate smaller haplotype groups. The genetic distance estimation among KHV 1, 2, 3 and our specimens also presented a distinct distance especially between KHV 3 group and other groups (Table 1). The genetic distance between KHV 3 group (where our specimens were included) and KHV 2 group ranges from 0.65 to 0.68 and

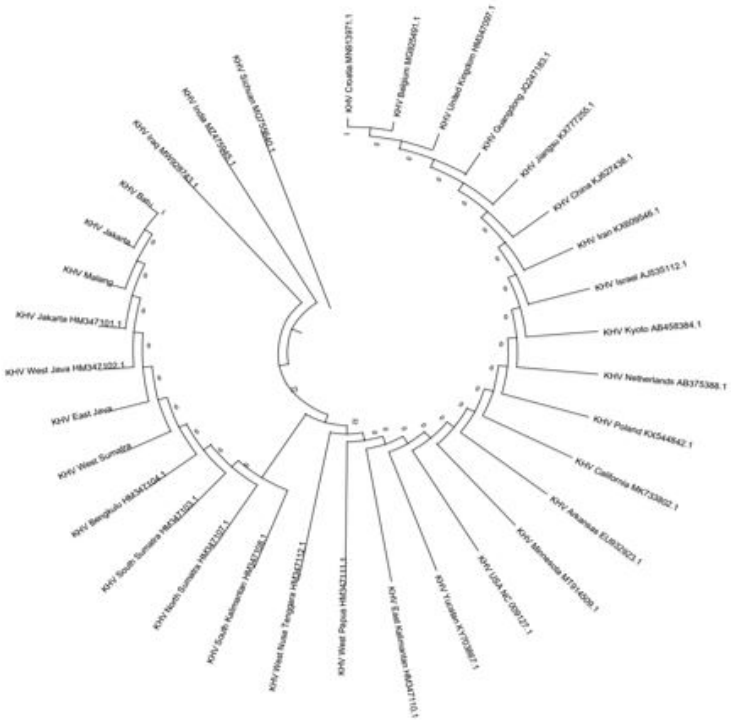

Figure 4. Phylogram of partial sequences of TK encoding gene of KHV 3 compiled from this study and archival sequences in NCBI Genbank.

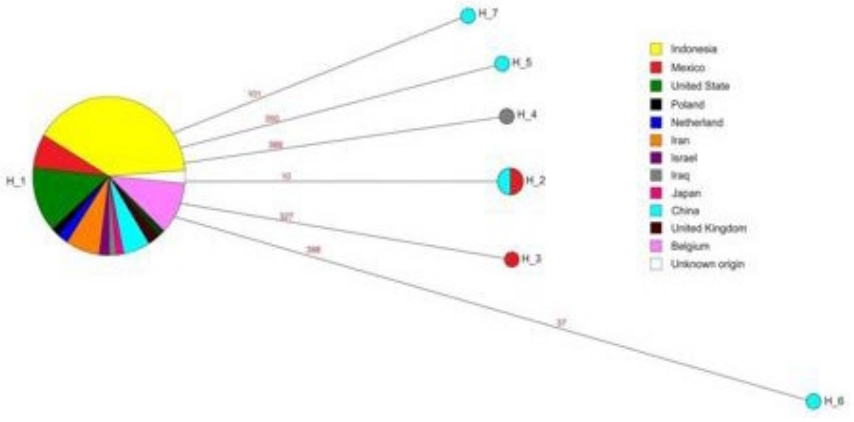

Figure 5. Haplotype Analysis of TK1 Encoding Gene

apparently were more closely related genetically to KHV 1 (0.58-0.59). The KHV 1 and 2 showed more distant relatedness rather than to KHV 3 (0.71-0.73).

Marked pathological changes were not present in other internal organs in our cases, though theoretically, the viruses must be able to travel to all organs [12]. This might be due to the lower load of KHV in other organs which was not capable of creating grossly noticeable lesions. The breeding ground in gill tissues might also induce anoxic death before other organs developed lesions. Pivotal organ that we prioritized to be inspected was kidneys since these organs provide osmoregulation ability for fish to maintain their fluid homeostasis within [13]. Freshwater fishes tend to uptake more water from the environment due to higher cytoplasmic fluid osmotic pressure than freshwater around, allowing the solvent passively diffuses to the interior part of fishes' body cavity. On a regular basis, kidneys, gills, and integuments maintain the water transport to tackle this osmotic dynamic of fluid between inner and outer body environment [14]. In case of compromised renal function, a failure of regulating the water volume in the fish body might happen followed by passive diffusion of solvent from the outer environment to fishes' body cavity. This flooding solvent creates distension inside the body cavity and shapes the fish body to be swelling tear drop-like presentation called dropsy syndrome [15]. Though this symptom might not be specific to KHV clinical signs, any renal damage inducing fluid build-up in the body cavity will end in this presentation. There was no evidence of body distension or fluid build-up in the body fish cavity in our cases, indicating that perhaps the fish died not due to systemic organ failure but rather to acute anoxic condition due to gill tissue necrosis.

Table 1. Genetic distance estimation of KHV 1, 2, 3 from NCBI Genbank database and our specimens

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	KHV_Jakarta_HM347101.1															
2	KHV_Malang	0.00														
3	KHV_Batu	0.00	0.00													
4	KHV_Jakarta	0.00	0.00	0.00												
5	KHV_3_China_MG755640.1	0.00	0.00	0.00	0.00											
6	KHV_3_Iran_KT364393.1	0.00	0.00	0.00	0.00	0.00										
7	KHV_3_USA_MK987097.1	0.00	0.00	0.00	0.00	0.00	0.00									
8	KHV_3_Czech_ON340600.1	0.00	0.00	0.00	0.00	0.00	0.00	0.00								
9	KHV_3_Croatia_MN913972.1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00							
10	KHV_2_Japan_JQ815364.1	0.67	0.67	0.67	0.67	0.66	0.67	0.67	0.67	0.67						
11	KHV_2_China_NC_019495.1	0.67	0.67	0.67	0.67	0.66	0.67	0.67	0.67	0.67	0.00					
12	KHV_2_China_MN201961.1	0.65	0.65	0.65	0.65	0.64	0.65	0.65	0.65	0.65	0.00	0.00				
13	KHV_1_UK_KU672599.1	0.58	0.58	0.58	0.58	0.58	0.58	0.58	0.58	0.58	0.73	0.73	0.73			
14	KHV_1_USA_KU672601.1	0.59	0.59	0.59	0.59	0.59	0.59	0.59	0.59	0.59	0.71	0.71	0.71	0.02		
15	KHV_1_Japan_NC_019491.1	0.58	0.58	0.58	0.58	0.58	0.58	0.58	0.58	0.58	0.73	0.73	0.73	0.00	0.02	
16	KHV_1_Brazil_KT587200.1	0.59	0.59	0.59	0.59	0.59	0.59	0.59	0.59	0.59	0.71	0.71	0.71	0.03	0.01	0.03

4. Discussions

Our pathological examination presented identical findings to all reported KHV cases and supported that the viruses might harbour through gill tissues as initial breeding ground. Gill secondary lamellae epithelial cells apparently play a role as harbouring port of KHV to enter the systemic circulation. Assuming a similar mode of replication to other endotheliotrophic herpesviruses [10], KHV might find the shortest route to access endothelial cells to start initial replication at the site of infection. The overload volume of replicated virion parts and assembly might distend the endothelial cells causing cell lysis while virion release occurred and triggered haemorrhages to what was typically found in acute KHV-infected fishes. Soon the ischemic condition developed, most of gill tissues were degenerated and underwent necrosis leaving white anaemic dead tissues appearance as present in most gill necrosis events. The discoloration area might vary based on load virus or chronicity of the infection, but our findings showed that the adult fish tend to present wider extensive and multilamellar gill necrosis compared to the juveniles. Several anthropogenic interventions such as freezing might affect the coloration of gill tissues due to icing [11], however, typical discoloration of gill necrosis remained distinguishable.

The TK encoding gene of KHV 3 sequences evidently provide a conserved region with low potential of mutation rate and nucleotide motive diversity. Amplification of TK encoding genes from our specimens showed high similarity to KHV 3 based on obtained partial sequence alignment to the NCBI Genbank database. This might indicate that there is no meaningful point mutation happening in at least the last decade since the oldest TK encoding gene of KHV 3 reference published in the Genbank database among regions worldwide. It is supported by low genetic distance among sequences, however, full-length TK encoding gene sequence alignment of the latest cases might provide a more comprehensive dynamic of TK sequence nucleotides. This feature also makes TK encoding gene fitting as a marker for KHV detection including for discrimination of KHV 1, 2, and 3. We found that there is no distinct grouping of haplotype based on KHV TK encoding gene sequences of KHV 3, supported by the phylogram that placed our sequences in a single monophyletic cluster with KHV isolates from Indonesia. Six haplotypes were separate from the large cluster one including TK sequences from twelve countries; however, considering the genetic distance and nucleotide diversity, this might not significantly affect the viral properties or impact to the hosts [16].

The presence of a conserved encoding gene might indicate the existence of a conserved protein as a target for a detection system or an antigen to generate antibodies or a vaccine. However, specificity and sensitivity of the developed KHV test or experimental vaccine employing the TK encoding gene are not largely explored. Several experimental vaccines such as live attenuated vaccines, inactivated vaccines, and live recombinant vaccines have been developed though there is only one commercial live attenuated KHV vaccine produced by a company in Israel that is applied to common carp. However, the vaccine efficacy was reduced in small carp less than 50 g due to virulence factor [17].

Other studies find effective and efficient vaccines for KHV with deletion in some open reading frames or employing recombinant proteins encapsulated in various materials. A Study by Klafack *et al*. (2022) [16], used a wild-type koi herpesvirus with alginate-encapsulated and the deletion of ORF150. The KHV is capable of interfering with the signal cascade in innate immunity and the vaccine boosted the gene expression of NF-κB as a signal for releasing pro-inflammatory cytokines such as IL-1 and IL-6. Furthermore, it could call out the immune system like macrophages from the blood vessel (monocyte form) and evolve in the tissue to eliminate the virus. A study by Huang *et al*. (2021) [18], used ORF81 of KHV delivered in a chitosan-alginate capsule. This experimental vaccine substantially induced IgM production with KHV-neutralizing activity and provided 85% protection. A study by Hu *et al*. (2020) [19], used ORF149 of KHV packed in a carbon nanotube medium that gave a protective effect of 81.95% against KHV challenge.

Genomic recombination is not yet reported among herpesvirus strains, however it is not improbable considering the nature of viral genome dynamics. Currently, the recombination rate of herpesvirus strains in the family Alloherpesviridae is low. We did not find any articles to support the recombination in strains belonging to the family Alloherpesviridae. However, recombination has occurred in the family Alphaherpesviridae. There is evidence of recombination in Equine Herpesvirus 1 (EHV-1) and EHV-4 [20]. Both herpes viruses are genetically similar, but they have different pathogenesis. A study by Vaz *et al*. (2016) [19], found that recombination events were observed in EHV-4 and only one event was observed in Equine EHV-1. The recombination rate of herpesvirus strains is low because the genome of herpesvirus is DNA. It has low nucleotide substitution and is stable in normal conditions.

The success of vaccination is indicated by the ability to induce humoral immunity. Humoral immunity prevents infection by blocking the virus from entering the host cells and neutralizing the epitope of the virus [21]. The TK encoding gene, a part of KHV genome, has been used to determine KHV infection and has become a highly sensitive diagnostic tool for KHV. In this research, we found that there are similarities among KHV genome sequences from Indonesia, Iran, and China using the gene encoding thymidine kinase. Thus, we assume that using the TK encoding gene could be a good candidate for vaccine development. Thymidine kinase protein can stimulate both cell-mediated immunity through T lymphocytes, mediated by dendritic cells, and humoral immunity through B lymphocytes mediated by internalization of thymidine kinase protein. Cell-

mediated immunity has two main functions, eliminating infected cells by cytotoxic T lymphocytes (CD8+) and promoting the formation of long-live antibodies within the assistance of helper T lymphocytes (CD4+). Humoral immunity is responsible for producing long-live antibodies in collaboration with helper T lymphocytes. However, it needs further investigation to illuminate the protective capability of the vaccine against KHV.

5. Conclusions

We reported that there was no distinct grouping of haplotypes based on KHV TK encoding gene sequences. The TK encoding gene sequences evidently provide conserved regions with a low mutation rate and nucleotide motif diversity. The presence of a conserved encoding gene theoretically fits as a target for a detection system or as an antigen to generate antibodies or vaccines. This study strengthens that TK encoding gene is plausible as a candidate for marker either in multistrain KHV detection and or vaccine development.

Availability of Data and Materials

The data are already included as part of the submitted manuscript.

Author Contribution

Conceptualization, A.B.H.; Methodology and Investigation, A.B.H., G.W.A., N.A.C., H.P.U., D.F.R., and D.R.Z.N.; Writing – Original Draft, A.B.H. and G.W.A.; Writing – Review & Editing, A.B.H. and G.W.A.

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Conflict of Interest

The authors declare no conflict of interest.

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