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Research Article

Clinical and Molecular Study of Koi Herpesvirus (KHV) Emerged in *Oreochromis niloticus* from Indonesia

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Abstract

Background and Objective: Koi Herpesvirus (KHV) can infect some species of freshwater fish, other than *Cyprinidae*. As well this possibility, in which tilapia may be infected with KHV, this study aimed to detect KHV from tilapia in Gresik, East Java, Indonesia using clinical and molecular study. **Methodology:** The study was carried out by examining clinical symptoms of tilapia and PCR examination followed by a sequence analysis and then aligned with BioEdit and the construction of phylogenetic trees was determined using the Neighbors-Joining. **Results:** The clinical symptoms observed from tilapia were discoloration on the skin and white patches on the gills. A sequence analysis using TK gene and PCR duplex analysis (marker I and marker II) indicated that the KHV genotype of the tilapia was identical to the A1 variants and displayed allele I^{++} II^{+} allele of the Asian genotype. **Conclusion:** The genetic variations of KHV observed from tilapia clearly indicated that the KHV genotype infecting tilapia is an Asian genotype. However, although tilapia was infected with KHV, no specific apparent of clinical symptoms and no mortality was seen in tilapia.

Key words: KHV, *Oreochromis niloticus*, cyprinidae, clinical symptoms, white patches, PCR examination, Asian genotype, Indonesia

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Koi Herpesvirus (KHV), also known as Cyprinid herpesvirus 3 (CyHV-3) is one of the viruses that attack koi and carp[1] and infect any sizes of fish[2]. The KHV was first identified in 1998 in Israel and caused mass mortality of koi in both juvenile and adult stages[3]. In the Asian regions, KHV infection causes mortality in common carp and koi, in Japan, China, Taiwan, Indonesia, Korea, Malaysia, Singapore and Thailand[4-6]. The KHV entered Indonesia in 2002 through koi imported from China to Surabaya[6]. This virus resulted in mass of 80-95% of the population and material losses resulting from the disease reached IDR 15 billions at the end of December[6] 2003. In addition, KHV also attacked carp cultivated at Lake Kasumigaura with a total loss of 1,200 t or close to 20% of annual production in Japan[7]. Clinical symptoms of KHV are highly variable and generally non-specific. Symptoms found include fish swimming to the surface to take the air or gasping because of high respiratory frequency. In addition, fish often lose sight and swim with irregular movements[8,9], suffer from hepatic dysfunction and osmoregulation systems that are susceptible to secondary pathogen infection[2,10].

The KHV can infect freshwater fish other than *Cyprinidae*, which is believed to be the specific host for KHVD. This allows the tilapia as the KHVD host as well[11,12]. However, previous studies did not specify that tilapia could be artificially infected by KHV either through injection or oral application[2,13,14], several studies have proven that fish other than *Cyprinidae* is susceptible to KHV infection[3,10,15,16]. In addition, hybrids are also known to be susceptible to KHV[1,17]. The KHV can be subdivided into European and Asian genotype or variants and variant of KHV in Indonesia more closely to Asian genotype[18]. In addition, based on epidemiological investigations, the spread of KHV in Indonesia originated from a single introduction[19].

The data was obtained from these results i.e., clinical symptoms and genetic variation can be used as a reference if there are similar infections emerged in other freshwater fish, especially in Indonesia. The prevention and control of KHV distribution in Indonesia has been pursued in both host and non-host. The fact that tilapia may become a KHVD host (or as a carrier) and that there has not been any clarity about the clinical symptoms and genetic variation of KHV-infected tilapia has urged for efforts to KHV disease managements. Thus, this study will report the detection and characterization of KHV DNA in tilapia based on clinical symptoms and molecular assay followed by a sequence analysis.

MATERIALS AND METHODS

Sample collection: The research was conducted between March and August, 2018 in Indonesia. Samples were obtained from Gresik, East Java, where reports on mortalities of carp kept together with tilapia occurred. The carp and tilapia were cultivated for 1 month as reported by the fish owner. Water quality parameters (pH 6, 8; temperature 28°C; DO 4 mg L^{-1}; nitrate 0,2 mg L^{-1}; nitrite 0,1 mg L^{-1} and ammonia 0,5 mg L^{-1}) showed normal results. Tilapia was grouped into two in a plastic tank and packed using a special sample plastic with oxygen supply and taken to Fish Quarantine Laboratory for dissection on a sterile condition. The dissected organ was taken and kept at -70°C for DNA extraction.

DNA extraction: Total DNA was extracted from the organs according to the procedure of the DNA Easy Blood and Tissue kit (Qiagen). As much as 25 mg of organ was crushed using pellet pestle and homogenized in 180 µL of an AL buffer using a microtube and digested in 20 µL proteinase K at 56°C for 10 min. Then 200 µL of ethanol (100%) and vortex was added to the crushed organ. Then, it moved the supernatant into a new tube, centrifuged it at 8000 rpm for 1 min, took it, then we added 500 µL of buffer AW1 and centrifuged it again at 8000 rpm for 1 min. The step continued as we took supernatant and added 500 µL buffer AW2 and centrifuged it at 14000 rpm for 3 min to dry the DNeasy membrane. The last step was added 200 µL of AE buffer directly onto the DNeasy membrane and incubate it for 1 min at room temperature before centrifuged at 8000 rpm for 1 min. The total DNA produced was dissolved in 50 µL of the AE buffer and stored at -20°C for further process.

DNA amplification and genotype analysis: The KHV DNA amplification was done using the primers specific of TK gene[20], Forward (F: 5'-GGG TTA CCT GTA CGA G-3) and Reverse (R: 5'-CAC CCA GTA GAT TAT GC-3'), which would produce amplification products at 409 bp. Amplification was performed using PCR Mix Kappa (Biosystems) with a total volume of 25 µL. Thermal cycler personal (Eppendorf) was used with a temperature setting: 1 cycle at 95°C for 5 min; 35 cycles at 95°C for 30 sec, 52°C for 30 sec and 72°C for 1 min and 1 cycle at 72°C for 10 min.

The genotype analysis was performed on tilapia, which gave positive signals of KHV. Four pairs of primers were used to determine the relationship between KHV in tilapia and other KHV variants[21,22]. The primer sets used for KHV genotype analysis is presented in Table 1.

Table 1: Primer sets and thermal cycling profiles used for KHV genotype analysis

Gene	Primer Sequences	PCR profiles	PCR product	References
TK	F: 5'-AACGCGGGCCAGCTGAACAT-3'	1 cycle: 95°C, 15 min; 49 cycle: 95°C, 30 sec;	1001 bp	Kurita *et al.*[21]
	R: 5'-TGTGTGTATCCCAATAAACG-3'	58°C, 30 sec; 72°C, 2 min; 1 cycle: 72°C, 10 min		
Marker I	F: 5'-CTACTCAGGAGCCATCATCG-3'	1 cycle: 95°C, 15 min; 49 cycle: 95°C, 30 sec;	J strain: 168 bp	Bigarre *et al.*[22]
	R: 5'-AGGACTTGGTAGGTGCCTCC-3'	60°C, 30 sec; 72°C, 2 min; 1 cycle: 72°C, 10 min	I/U Strain: 130 bp	
Marker II	F: 5'-GCTCATTTTAGCGCTTCTGTG-3'	1 cycle: 95°C, 15 min; 49 cycle: 95°C, 30 sec;	J strain: 325 bp	Bigarre *et al.*[22]
	R: 5'-CGCTGCCTACCCAATTCGCT-3'	60°C, 30 sec; 72°C, 2 min; 1 cycle: 72°C, 10 min	I/U Strain: 278 bp	

The amplification product was then electrophoresis in 2% (w/v) agarose gel (Scie-Plus) using 1x TAE buffer (1st Base) for 1 h with 100 V voltage and followed by Sybr Green (Lonza) staining. The DNA signals were visualized using Gel-Documentation (Uvitech). In addition, amplification products were also purified using DNA Clean and Concentrator (Zymo Research) to be used as template sequencing. Sequencing was done at 1st Base Laboratories-Selangor, Malaysia. Sequencing data were analyzed using sequence of BioEdit and NCBI-BLAST program. The construction of phylogenetic trees was determined using the Neighbors-Joining method[23].

RESULTS

Clinical symptoms: In the external part of the tilapia, there were no parasites and pathological lesions but it showed the discoloration of the skin (darker skin) when compared with the normal tilapia. While on the internal organs, gill showed the presence of white parts on the ends of the gill (Fig. 1).

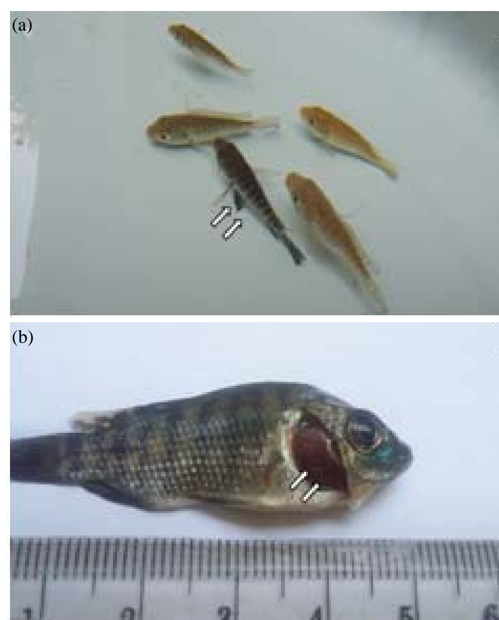

Fig. 1(a-b): (a) Blackish color of tilapia and (b) White parts on the gill

KHV detection: The KHV testing was performed on tilapia that showed clinical symptoms such as white parts on the gills and the blackish skin color. The positive signals indicated KHV infection in tilapia by using TK genefor both of 2 tilapia groups (Fig. 2).

As mentioned earlier, 11 published genomic sequences show high sequence analysis[24-26], i.e., >99%. In addition, to determine the genotype of tilapia[21], primers from Bigarre *et al.*[22] were also used, based on differences in three variable domains within two genetic marker. Marker I (the region between ORF 29 and ORF 30) and Marker II (the regionnear to the start of ORF 133) (Fig. 3).

Figure 3a showed both of 2 tilapia groups amplified using enlarged TK gene at 1.001 bp, while Fig. 3 Bamplifiedin the marker I region displayed allele I^{++} with the full length 168 bp and marker II region showed allele II^{+} with fulllength 352 bp. These suggested as allele I^{++} II^{+}, corresponding to Asian genotype.

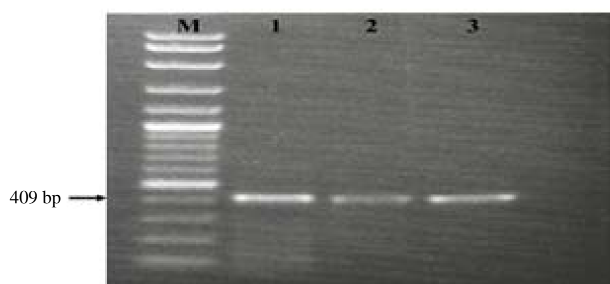

Fig. 2: TK gene PCR assay of tilapia

M: Marker 100 bp, 1: Positive control KHV, 2: Tilapia group I, 3: Tilapia group II

Results of sequencing analysis and alignment: Multiple sequence alignment based on enlarged TK gene in Fig. 4 showed that the tilapia had a high similarity with variant A1 of J strain (Asian genotype). Consistent with a previous study, all TK gene sequences clustered into two major genotype: European genotype and Asian genotype[21].

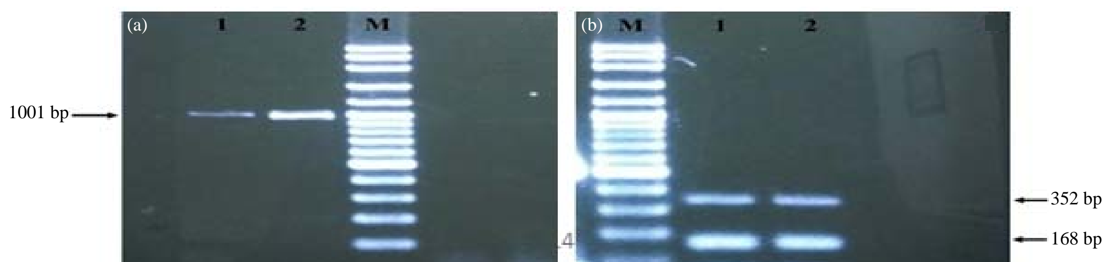

Fig. 3(a-b): (a) Enlarged TK Gene PCR assay of tilapia. Lane 1: Tilapia group I, 2: Tilapia group II, 3: 100 bp DNA ladder and (b) PCR assay targeting KHV marker I and marker II. Lane 1: 100 bp DNA ladder, 2: Tilapia group I, 3: Tilapia group II

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Alignment of TK gene:

NBJT_1                771 TGCGCCCATC GTGTCAGCCG CTCCACCTCG TTCTTGTAAC AT--CTATCC TGTGATGGTG TGTGTGGAAC CAATAAAATA ATGTGCGACT TGAATATGGT 870
J strain              771 TGCGCCCATC GTGTCAGCCG CTCCACCTCG TTCTTGTAAC AT--CTATCC TGTGATGGTG TGTGTGGAAC CAATAAAATA ATGTGCGACT TGAATATGGT 870
I strain              771 TGCGCCCGTC GTGTCAGCCG CTCCACCTCG TTCTTGTAAC ATATCTATCC TGTGATGGTG TGTGTGGAAC CAATAAAAT- -TGTGCGACT TGAATATGGT 870
U strain              771 TGCGCCCGTC GTGTCAGCCG CTCCACCTCG TTCTTGTAAC ATATCTATCC TGTGATGGTG TGTGTGGAAC CAATAAAAT- -TGTGCGACT TGAATATGGT 870
A1 (AB375390)         771 TGCGCCCATC GTGTCAGCCG CTCCACCTCG TTCTTGTAAC AT--CTATCC TGTGATGGTG TGTGTGGAAC CAATAAAATA ATGTGCGACT TGAATATGGT 870
A2 (AB375391)         771 TGCGCCCATC GTGTCAGCCG CTCCACCTCG TTCTTGTAAC AT--CTATCC TGTGATGGTG TGTGTGGAAC CAATAAAATA ATGTGCGACT TGAATATGGT 870
E1 E2 E3 (AB375385)   771 TGCGCCCGTC GTGTCAGCCG CTCCACCTCG TTCTTGTAAC ATATCTATCC TGTGATGGTG TGTGTGGAAC CAATAAAAT- -TGTGCGACT TGAATATGGT 870
E4 (AB375387)         771 TGCGCCCGTC GTGTCAGCCG CTCCACCTCG TTCTTGTAAC ATATCTATCC TGTGATGGTG TGTGTGGAAC CAATAAAATA ATGTGCGACT TGAATATGGT 870
E5 (AB375388)         771 TGCGCCCGTC GTGTCAGCCG CTCCACCTCG TTCTTGTAAC ATATCTATCC TGTGATGGTG TGTGTGGAAC CAATAAAAT- -TGTGCGACT TGAATATGGT 870
E6 (AB375386)         771 TGCGCCCGTC GTGTCAGCCG CTCCACCTCG TTCTTGTAAC ATATCTATCC TGTGATGGTG TGTGTGGAAC CAATAAAAT- -TGTGCGACT TGAATATGGT 870
E7 (AB375389)         771 TGCGCCCGTC GTGTCAGCCG CTCCACCTCG TTCTTGTAAC ATATCTATCC TGTGATGGTG TGTGTGGAAC CAATAAAAT- -TGTGCGACT TGAATATGGT 870

NBJT_1                871 TGTACGGGTT TTTTT--AAC AAAAACTAAA CTACCGAAAC ACGAAACACT TGCTCTGAGC GACTTTGCGT CCAATACTTT --AAAAAAAA --GGAGATAT 970
J strain              871 TGTACGGGTT TTTTT--AAC AAAAACTAAA CTACCGAAAC ACGAAACACT TGCTCTGAGC GACTTTGCGT CCAATACTTT --AAAAAAAA --GGAGATAT 970
I strain              871 TGTACGGGTT TTTTT--AAC AAAAACTAAA CTACCGAAAC ACGAAACACT TGCTCTGAGC GACTTTGCGT CCAATACTTT TTAAAAAACA --GGAGATAT 970
U strain              871 TGTACGGGTT TTTTTTTAAC AAAAACTAAA CTACCGAAAC ACGAAACACT TGCTCTGAGC GACTTTGCGT CCAATACTTT --AAAAAAAA CAGGAGATAT 970
A1 (AB375390)         871 TGTACGGGTT TTTTT--AAC AAAAACTAAA CTACCGAAAC ACGAAACACT TGCTCTGAGC GACTTTGCGT CCAATACTTT --AAAAAAAA --GGAGATAT 970
A2 (AB375391)         871 TGTACGGGTT TTTTTT-AAC AAAAACTAAA CTACCGAAAC ACGAAACACT TGCTCTGAGC GACTTTGCGT CCAATACTTT --AAAAAAAA --GGAGATAT 970
E1 E2 E3 (AB375385)   871 TGTACGGGTT TTTTTTTAAC AAAAACTAAA CTACCGAAAC ACGAAACACT TGCTCTGAGC GACTTTGCGT CCAATACTTT --AAAAAAAA CAGGAGATAT 970
E4 (AB375387)         871 TGTACGGGTT TTTTTTTAAC AAAAACTAAA CTACCGAAAC ACGAAACACT TGCTCTGAGC GACTTTGCGT CCAATACTTT --AAAAAAAA CAGGAGATAT 970
E5 (AB375388)         871 TGTACGGGTT TTTTTTTAAC AAAAACTAAA CTACCGAAAC ACGAAACACT TGCTCTGAGC GACTTTGCGT CCAATA---- ---------- --GGA----- 970
E6 (AB375386)         871 TGTACGGGTT TTTTTTTAAC AAAAACTAAA CTACCGAAAC ACGAAACACT TGCTCTGAGC GACTTTGCGT CCAATACTTT --AAAAAAAA CAGGAGATAT 970
E7 (AB375389)         871 TGTACGGGTT TTTTTT-AAC AAAAACTAAA CTACCGAAAC ACGAAACACT TGCTCTGAGC GACTTTGCGT CCAATACTTT --AAAAAAAA CAGGAGATAT 970

Alignment of Marker I:

NBJT_1      21 GCAACCTCAA CCCCGGCAGC CTCAACCCCG GCAGCCTCAA CTTCACCTTC AGAATCTTCA ACGGTATGGA TGATAGAGTC GGAGTCGTCC ACGATGGTGA CACCCGAGAC 130
J Strain    21 GCAACCTCAA CCCCGGCAGC CTCAACCCCG GCAGCCTCAA CTTCACCTTC AGAATCTTCA ACGGTATGGA TGATAGAGTC GGAGTCGTCC ACGATGGTGA CACCCGAGAC 130
I Strain    21 GCAA------ ---------C CTCAACCCCG GCAGCCTCAA CTTCACCTTC AGAATCTTCA ACGGTATGGA TGATAGAGTC G--------- ---------- ----CGAGAC 130
U Strain    21 GCAA------ ---------C CTCAACCCCG GCAGCCTCAA CTTCACCTTC AGAATCTTCA ACGGTATGGA TGATAGAGTC G--------- ---------- ----CGAGAC 130

Alignment of Marker II:

NBJT_1      51 TAAATGAACA GAAAGAAAGC TCAGAAGTGA GATGGGCCAG AAGCACGACA AGTACCAGGG CGCCGATCTC GAGGTCGATG AACAGAAAGA AAGCTCAGAA GTGAGATGGG 160
J Strain    51 TAAATGAACA GAAAGAAAGC TCAGAAGTGA GATGGGCCAG AAGCACGACA AGTACCAGGG CGCCGATCTC GAGGTCGATG AACAGAAAGA AAGCTCAGAA GTGAGATGGG 160
I Strain    51 CAA------- ---------- ---------- ---------- ---------- ---------- ---------- -------ATG AACAGAAAGA AAGCTCAGAA GTGAGATGGG 160
U Strain    51 CAA------- ---------- ---------- ---------- ---------- ---------- ---------- -------ATG AACAGAAAGA AAGCTCAGAA GTGAGATGGG 160

NBJT_1     161 ACCAGAAGCA CGACAAGTAC CAGGGCGCCG ATCTCGAGAT CAACGACCTC GAGATCAACG TACCTATCAG CTTCATGACT CCCGAACAGA TTGCGGCGGC TGTGGGTAGA 270
J Strain   161 -CCAGAAGCA CGACAAGTAC CAGGGCGCCG ATCTCGAGAT CAACGACCTC GAGATCAACG TACCTATCAG CTTCATGACT CCCGAACAGA TTGCGGCGGC TGTGGGTAGA 270
I Strain   161 -CCAGAAGCA CGACAAGTAC CAGGGCGCCG ATCTCGAGAT CAACGACCTC GAGATCAACG TACCTATCAG CTTCATGACT CCCGAACAGA TTGCGGCGGC TGTGGGTAGA 270
U Strain   161 -CCAGAAGCA CGACAAGTAC CAGGGCGCCG ATCTCGAGAT CAACGACCTC GAGATCAACG TACCTATCAG CTTCATGACT CCCGAACAGA TTGCGGCGGC TGTGGGTAGA 270
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Fig. 4: Based on the alignment of the enlarged TK gene, the tilapia (NBJT_1) is homologous with A1 variant (J strain). Alignment of marker I and marker II of tilapia (NBJT_1) were classified as variant A1 of J strain and displayed the I^{++} II^{+}allele. The insertion of a nucleotide base A positioned at 161 bp at Marker II

Compared with three KHV strain, demonstrate that tilapia were identical as variant A1 of J strain. It can be seen that in Marker I, the tilapia has a 168 bp nucleotide length and can be denoted as I^{++}. For Marker II, the tilapia has a length of 353 bp, which can be denoted as II^{+}, identical to the J strain. Although it looks identical to J strain, in marker II, there is an insertion of base A (Adenin) at 161 nt in the middle part of the second motif.

The phylogenetic tree construction was done using a neighbor joining method in MEGA 7 software, after alignment using BioEdit software. The construction of the phylogenetic tree (Fig. 5) showed that the tilapia (code NBJT_1) were in one cluster with the J-strain isolate and the A1-A2 variant, which is a cluster of the Asian genotype. These results similar with previous findings, all TK gene of KHV isolates from Asia have a high homology with each other and there is only 1 nucleotide difference with polymorphisms at 885 nt, although the KHV-infected tilapia in this study showed no such differences[21,27].

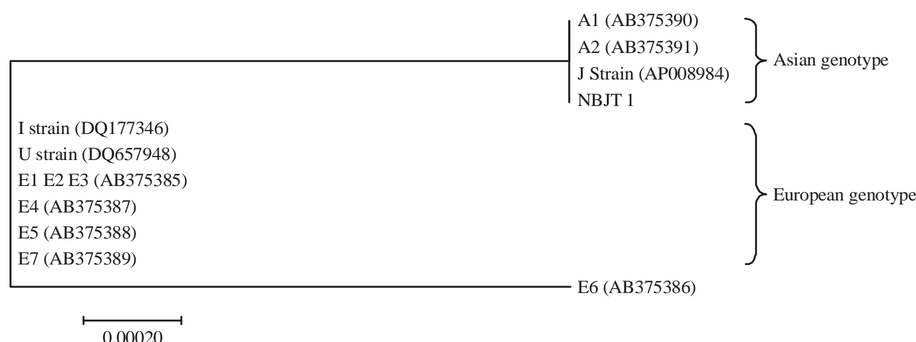

Fig. 5: Phylogenetic tree of KHV-infected tilapia (NBJT_1) with 7 comparative sequences (sequence analysis of TK Gene)

DISCUSSION

This study described the detection of KHV that emerged in tilapia from Indonesia. The results of the present clinical and molecular examination confirmed that KHV is the causative agent. Although tilapia was infected with KHV, it did not show clinical symptoms and no mortality was seen in tilapia. These finding suggested that KHV can infect tilapia but could not cause death. Bergmann *et al.*[1] also suggested, no clinical symptoms in goldfish (*Carassius auratus*) infected with KHV. The clinical symptom in the tilapia was only white parts on the gillsindicating the necrosisand skin discoloration. Skin discoloration in tilapia may be due to the proliferation of Melano-Macrophage Centers (MMC) that indicated a defense reaction in fish skin caused by environmental stress conditions. Changes that occur in the environment cause an increase in damaged red blood cells, which ultimately results in changes in pigment[28]. Changes in tilapia skin color, which becomes darker, is also possible because of an infection of the skin, which results in infiltration of inflammatory cells that extend to the epidermal layer of the body. These finding also suggested that KHV enter through the gills andmultiply. From the gills, the virus is rapidly transferred to the other tissues[9].

This present study has found that KHV in the tilapia belongs to the variant A1 of J strain, this resultis similar with previous finding that KHV variants in Indonesia were more closely related to Asian genotype[18]. This is consistent with previous studies suggesting that all isolates from the Asian region have high homology with only one nucleotide polymorphism that distinguishes A1 and A2 variant based on TK gene analysis[21]. Based on duplex analysis, it was found that KHV genotype of tilapia identical and displayed the I^{++} II^{+} allele. Although at Marker II there is an insertion of base A (Adenine) at 161 nt, this is more related to slippage of the DNA polymerase. These results are similar to the study conducted by Sunarto *et al.*[18], which also found the insertion of base G (Guanine) at 74 nt for KHV originating from Indonesia. In addition, marker I and II can be used to distinguish variants in genetic KHV polymorphisms but it is still unable to inform us about the virulence of KHV or other viruses. This can be proven in such case-although the A1 and A2 variant contains single nucleotide polymorphism, it cannot determine which variants are more virulent. The homogeneity of KHV isolates from Asia showed that KHV invades to this region and adapts to the cooler European region[29]. This affects the proximity of KHV isolates in the Asian region and gives rise to fewer variations-unlike the KHV isolates derived from European region.

The KHV genotype of tilapia in this study is consistent with the assumption that KHV from Asia are related to each other[18,27]. However, not all KHV isolates from the Asian regions are included in the Asian genotype. Dong *et al.*[30] isolated KHV from koi, which was designated as KHV-GZ11 and suggested that the KHV outbreak in China in 2011 is caused by KHV belonging to the European lineage; based on the phylogenetic position, KHV GZ-11 is an intermediate variant, which is between the Asian and European cluster[27]. In addition, European genotype is also found in South Korea[27,31]. The European genotype found in the Asian regions is believed to be an intermediate variant between Asian and the European genotype. The KHV isolates originating from Indonesia are also believed to be intermediates[18]. The existence of this intermediate variant is important because it informs us the origin of this KHV. There is no linkage in terms of the spread between Asian and European genotype; this may occur due to koi trading activities from Asia to Europe, which indirectly also causes the spread of the KHV variant in Europe[21]. To clarify whether the Indonesian KHV from tilapia in this study is an intermediate variant, a complete genome sequence is required as conducted by Li *et al.*[25].

The distribution pattern of KHV variants in some countries is not known clearly; this may be due to international trade

involving koi as KHV hosts. Although the KHV variants showed clear geographical boundaries based on molecular markers, the distribution pattern remains unclear. Ongoing detection and monitoring is urgently needed to determine the current condition associated with the presence of KHV in the tilapia and the possibility of KHV to infect other freshwater fish outside of the specific host, as we know that the evolutionary of KHV to infect the other fish is not known yet. Implementation of strict biosecurity and quarantine measures is also indispensable especially to prevent the spread of this disease to all province of Indonesia.

CONCLUSION

This study confirmed the presence of KHV in the tilapia. The fact that KHV can infect the tilapia-as a carrier-without clinical symptoms will make difficult to identify. The KHV genotype of tilapia in this study is consistent with the assumption that KHV isolates from Asia are related to each other. It also recommend avoiding carp cultivation together with other freshwater fish.

SIGNIFICANCE STATEMENTS

This study discovered the clinical symptoms and genetic variation of KHV infected tilapia that can be beneficial for both researchers and policy makers. This study will help the researchers to uncover the critical areas of tilapia infected with KHV that many prior researchers were not able to explore. Thus, a new theory on possibility of other species to KHV may be found.

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