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Cloning ORF2 Membrane Protein of Koi Herpesvirus Lake Toba, Indonesian Isolate

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Koi herpesvirus (KHV) caused significant morbidity and mortality in koi and common carp. KHV which showed strong antigenic property implied that KHV virion or proteins may be used as antigen to raise antibody or vaccine to increase the resistance. The objectives of this research were to (i) clone KHV membrane protein ORF2, (ii) analysis on immunogenicity, and (iii) genetic tracing. Based on genbank data, one pair of primers was designed to amplify KHV ORF2. The KHV ORF2 can be amplified using infected fish DNA which originally from Toba Lake, Sumatera, Indonesia. The KHV ORF2 composed of 699 nucleotides encoded for 292 amino acids. BLAST analysis showed that KHV ORF2 had 100% homology with KHV-J and KHV0301 strains from Japan; 98 and 91% homology on nucleotides and amino acids respectively with both KHV-U strain from Unites State and KHV-I strain from Israel. KHV in Indonesia was most likely to have originated from Japan via spreading directly or not directly to China or Hongkong. Based on T- and B-cell epitopes prediction, membrane protein ORF2 was proposed has a potency to be used in development vaccine and immunodetection.

Key words: genetic tracing, koi herpesvirus (KHV), membrane protein, ORF2

INTRODUCTION

Koi herpesvirus (KHV), which also known as Carp interstitial nephritis, and gill necrosis virus (CNGV), Koi herpes-like virus, and Cyprinid herpesvirus 3 (CyHV-3), caused significant morbidity (sickness or disease) and mortality in koi and common carp (*Cyprinus carpio*) (Hedrick *et al*. 2000; Pikarsky *et al*. 2004; Dishon *et al*. 2005; Waltzek *et al*. 2005). Mortality due to infection of other freshwater fish species, such as goldfish (*Carassius auratus*), grass carp (*Ctenopharyngodon idellus*), silver carp (*Hypophthalmichthys molitrix*), silver perch (*Bidyanus bidyanus*), and tilapia (*Oreochromis niloticus*), by this virus has not been observed naturally or by experimental infection (Perelberg *et al*. 2003).

Historically, the first outbreak of KHV was reported in 1998 and confirmed in 1999 in Israel. Since then, other cases have been confirmed in the United States, Europe and Asia (Hedrick *et al*. 2000). KHV in Indonesia, having started in the area of Blitar in East Java in March 2002. Since then, it has spread rapidly throughout Java Island, causing very high mortality (80-90%). From Java island the KHV spreaded into Sumatera and Borneo islands the infected fishes were lethargic, showed loss of balance and gasped for air, sloughing off the epithelium with loss of mucus and rough appearance of the skin or showed a blister-like lesion on the skin, haemorrhages of operculum, fins, tail and abdomen, and severe gill damages (Sunarto *et al*. 2005).

KHV virion is an icosahedron morphology resembling herpes viruses but its genome as 277 kb double-stranded DNA is larger than that of *Herpesviridae* family members (Ronen *et al*. 2003). The initial characterization on virion polypeptide and restriction fragment length polymorphism analysis of genomic DNA showed that KHV was different with Herpesvirus cyprini (CHV) and Channel catfish virus (CCV) (Gilad *et al*. 2002). A comparison of virion polypeptides and genomic restriction fragments of seven geographically diverse isolates of KHV indicated that with one exception they represented a homogeneous group (Gilad *et al*. 2003).

The variability in nucleotide sequence can be a very potent tool in understanding the epidemiology of disease. Nucleotide sequence analysis is potential tool to trace the origin and movement of viruses (Walker & Cowley 2000). Partial regions of *gag* and *pol* and the entire *env* Jembrana virus of have been used to trace disease from Bali, Sumatra, and South Kalimantan (Desport *et al*. 2007). Gilad *et al*. (2003) found the homogeneity among KHV isolates and proposed that to more precision in distinguishing between geographically diverse isolates, the sequencing of PCR amplicons and more variable regions of the viral genome was promised. Using whole virus, Ronen *et al*. (2003) found that KHV had strong antigenic property and high antibody titer was found in immunized fish. Those result imply that the KHV or it‘s proteins may be used for antigen to raise antibody or vaccine to increase resistance against KHV. In this study, we cloned Koi herpesvirus membrane protein gene (ORF2) from Toba isolate, analyzed the immunogenicity of the membrane protein and traced the origin of the KHV isolate.

MATERIALS AND METHODS

Primers. The genbank data on AB178537 denoted for Koi herpesvirus genes contained 6814 nucleotides and encoded five ORFs, included ORF2. The primers were designed to amplify full length ORF2 and *Bam*HI and *Eco*RI restriction

sequence was added on the 5' of forward and reverse primer respectively. The sequences primer were KHV-ORF2-F 5'-TTCAGGATCCATGTCTCCTTTGTGCGGT-3' and KHV-ORF2-R 5'-CCTGGAATTCCTATACGCGCTCATAACC-3'. For checking the present of KHV genome in the samples, specific primers for KHV designed by Gilad *et al*. (2002) F 5'-GACGACGCCGGAGACCTTGTG-3'/R 5'-CACAAGTTCAG TCTGTTCCTCAAC-3' were used for PCR detection which produces 484 bp amplicon.

Samples. The gill tissues of common carp (*Cyprinus carpio*) which originally from Toba lake fish farmer were received from Fish Health Laboratory, Research Centre of Aquaculture at Pasar Minggu Jakarta.

Genomic DNA and Plasmid Extraction. A 50-100 mg of gills were homogenized on 400 ìl of TNE buffer (1 M Tris-HCl (pH 7.5), 5 M NaCl, 0.5 mM EDTA) using pestle on 1.5 ml. Proteinase K (10 mg/ml) as 3 ìl; and SDS as final concentration 0.5% were added into mixture followed by incubation 42 °C for 2 hours. After incubation, the mixtures were extracted using phenol-chloroform-isoamylalchohol twice, and the aqueous phases were collected. The DNAs were precipitated with Na-acetat and ethanol absolute and washed with 70% ethanol. DNA pellets were suspended with TE buffer (100 mM Tris-HCl, 10 mM EDTA, pH 7.6).

The plasmids were extracted using lysis alkali minipreparation method (Sambrook & Russel 2001). A 1.5 ml of overnight bacteria cultured was spinned at 15,000 g for 3 minutes. The pellet was resuspended in 150 ìl of cold Solution I (25 mM Tris-HCl, 50 mM Glucose, 10 mM EDTA, pH 8) and incubated for 1 minute in ice. Solution II (0.2 N NaOH, 1% SDS) was added as 180 ìl, mixed gently and incubated for 1 minute in ice. The cold Solution III (3 M Na Acetat) as 120 ìl was added, mixed gently and incubated in ice for 1 minute. After incubation, the mixtures were centrifuged at 15,000 g for 5 minutes. The supernatants were collected, extracted with phenol-chloroform-isoamylalchohol. Plasmids were collected from aqueous phases by adding 2.5 volume of cold ethanol absolute and centrifuged at 15,000 g for 5 minute. The pellets were suspended with TE buffer.

DNA Amplification and Colony PCR. The amplification of ORF2 KHV was done with PCR using Ready to go PCR kit (Amersham) and DNA samples as templates with KHV-ORF2-F/ KHV-ORF2-R pair primers. PCR was done as follow 95 °C for 30 sec, 55 °C for 30 sec, 72 °C for 1 minute with 25 cycles. After amplification, the PCR products were electrophoreted on a 1.5% agarose-TAE (40 mM Tris-Acetate pH 8.3, 1 mM EDTA) gel and stained with ethidium bromide. Amplification for checking the present of KHV using Gilad's primer was done with same procedure as above. For colony PCR, the overnight old colonies were picked up using sterile toothpick and suspended into sterile water. Five ìl of bacterial suspensions were used as template, and continue following procedures as above.

Cloning and Sequencing. The PCR products were purified using phenol extraction and followed by digestion with *Bam*HI and *Eco*RI. After digestion, the PCR products were agarose electrophoreted and DNA bands were recovered from agarose using glass powder method (Murwantoko 2004). Purified PCR fragments were ligated into pBSKSII (Strategene) which digested using T4 DNA ligase (Toyobo) at 16 °C for over night. Ligation mixtures were transformed into *Escherichia coli* DH5α using heat shock on 42 °C for 90 sec followed incubation on ice. The bacteria were cultured on LB agar plate containing 50 mg/l amphicilin for over night. The growth colonies were cultured in LB broth containing 50 mg/l amphicilin at 37 °C for over night. The colony PCR was performed to verify the present of recombinant plasmid.

The recombinant plasmids were purified using PEG method (Murwantoko 2004), briefly the plasmids were treated with RNAse at 37 °C for 30 minutes. The solution containing 50% PEG, 1.6 M NaCl was added on same volume and incubated on RT for 15 minutes. The plasmids were precipitated using centrifugation and washed with 70% ethanol. The purity of plasmids were evaluated using UV spectrophotometer. The high quality plasmids were sequenced using Big DyeTM terminator (PE biosystem, USA), T3 and T7 primers and performed on automatic thermal cycler Perkin-Elmer 480 and analyzed on an ABI310 DNA auto-sequencer (Perkins Elmer).

Genomic Analysis. Sequences from DNA sequencer were analyzed using Genetyx program. Sequences of clone were analyzed on their homology with other sequences on gene bank by BLAST method (Altschul *et al*. 1990). The T-cell epitope was predicted by Genetyx; and B-cell epitopes were analysis by Bcepred soft ware (Saha & Raghava 2004).

RESULTS

DNA Amplification. The Toba isolate samples are positively contain KHV genome as a 484 bp band is appeared when used as template for PCR with KHV specific primer designed by Gilad *et al*. (2002) as shown in lane 3 Figure 1a. From those samples, the membrane protein ORF2 can be amplified using KHV-ORF2-F/KHV-ORF2-R primers on the condition as explained in methods. The single band in 700 bp size was produced as we expected which shown in Figure 1a lane 1 and 2.

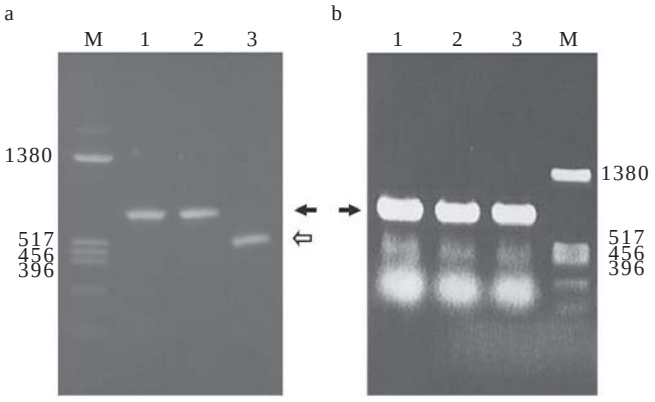

Figure 1. Agarose electrophoresis of PCR products. a. Amplification of ORF2 using KHV-ORF2-F/KHV-ORF2-R, black arrow (lane 1, 2); checking the present of KHV using Gilad's primer open arrow (lane 3). b. Evaluation of recombinant plasmid by colony PCR, all three clones (lane 1-3) contain DNA inserts as shown the present of a single bands as 700 bps in size (black arrow). M = DNA size marker BSM13/*Hinf*I with indicated size.

Cloning. Transformation of ligation mixtures of PBSKSII and PCR products into *E. coli* DH5á produced some colonies appear in LB agar containing amphicilin. Evaluation the present of recombinant using colony PCR from 3 colonies showed single band with 700 bp in size was appeared from all colonies (Figure 1b, lane 1-3). The PCR products from colony PCR are same size with PCR product of amplification of ORF2. This result showed that the ORF2 of KHV gene was successfully cloned. The plasmid of positive clones were isolated and sequenced using T3 and T7 primers. Sequences from those primers were proceed and combined to determine the full length sequence of membrane protein gene ORF2. The sequence also was analyzed on the prediction of translated amino acids using Genetyx program. The nucleotides and amino acids sequence of coat protein was presented in Figure 2. Analysis using Genetyx from the sequences showed some restriction sites were present in the sequences such as *Bgl*I (514), *Nae*I (61, 256), *Sal*I (316, 400), *Xho*I (346). The ORF size is 699 nucleotides encoded 232 amino acids. This sequence has been submitted to Genbank with accession GQ121137.

Homology analysis of the ORF2 cloned using BLAST method (www.ncbi.nih.gov./blast) showed only five data with high homology of ORF2 Indonesian isolate (Table 1). The E-values with those five data were 0.0. The 100% homology was found with accession number AP008984 and AB178537 derived from KHV-J and KHV0301 strains which originated from Japan. The 98 and 91% homology on nucleotide and amino acid respectively were found with accession number DQ657948, DQ177346, AB178324 which derived from KHV-U strain from Unites State; KHV-I strain and standard strain from Dr. R. Hedrick originated from Israel. The differences between Indonesian KHV-Toba to KHV-U and KHV-I strains is an insertion of 12 nucleotide or 4 amino acids. KHV Indonesia strain which closely related to Japan strain than Israeli or US strain imply that out break disease in Indonesia is more possible some how through Japan, compare that directly from Israel, as the first confirmed country outbreak of KHV.

The antigenicity of protein was determined by epitopes. T-cell and B-cell epitopes of membrane protein ORF2 KHV was analyzed as shown in Table 2. The number of predicted epitope using IAd and Rothbard/Taylor patterns were 6 and 10 epitopes respectively. B-cell epitopes were predicted in 4 positions.

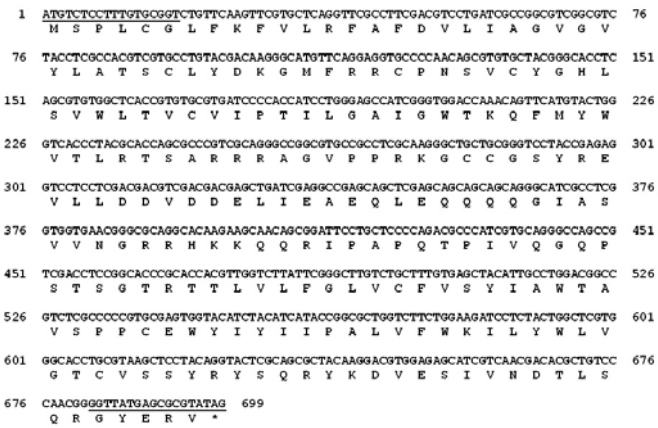

Figure 2. Sequences and translation of membrane protein of koi herpervirus (KHV) ORF2 gene (Genbank accession GQ121137). Sequences with underline indicate of primer sequences used in this study.

Table 1. The homology of Koi herpesvirus ORF2 sequences as result of BLAST analysis

Accession	Description	Query coverage (%)	E-value	Max ident (%)
AP008984.1	Koi herpesvirus DNA, complete genome, strain KHV-J	100	0.0	100
AB178537.2	Koi herpesvirus ORF1, ORF2, ORF3, ORF4, ORF5 genes for hypothetical protein, membrane protein, membrane protein, major envelope protein, hypothetical protein, complete cds, strain:KHV0301	100	0.0	100
DQ657948.1	Koi herpesvirus strain KHV-U, complete genome	100	0.0	98
DQ177346.1	Koi herpesvirus strain KHV-I, complete genome	100	0.0	98
AB178324.2	Koi herpesvirus genes for membrane protein, major envelope protein, hypothetical protein, complete cds	100	0.0	98

Tabel 2. Prediction of T-cell and B-cell epitopes of putative amino acid ORF2 of koi herpesvirus

T-cell epitope prediction				B-cell epitope prediction	
IAd pattern		Rothbard/taylor pattern		Amino acid position	Sequence
Amino acid position	Sequence	Amino acid position	Sequence		
23-28	VGVYLA	6-9	GLFK	36-42	GMFRRCP
25-30	VYLATS	9-13	KFVLR	75-84	VTLRTSARR
50-55	LSVWLT	13-17	RFAFD	125-135	SVVNGRRHKKQ
76-81	VTLRTS	36-39	GMFR	144-156	PIVQGQPSTSGTR
152-157	TSGTRT	64-67	GAIG		
205-210	SSYRYS	100-104	EVLLD		
		109-112	ELIE		
		122-125	GIAS		
		163-166	GLVC		
		194-197	KILY		

DISCUSSION

This study is the first time analysis on genomic analysis by cloning and sequence analysis of membrane protein gene (ORF2) of KHV in Indonesia. The publication on KHV in Indonesia is still limited. The economic impact, pathogenicity, epidemiology of KHV have been reported by Sunarto *et al.* (2005). The information of Indonesian KHV on genomic analysis was not published yet.

The KHV has been found associated with mass mortality events on most continents, including countries throughout Europe, the United States, Japan, Indonesia, South Africa, Thailand, Taiwan, China, and Malaysia. Outbreaks continue occur among cultured populations of koi in most of these areas and, alarmingly, epidemics have been observed among wild common carp populations in the United Kingdom, Japan, and most recently, several states in the United States (Aoki *et al*. 2007). Koi herpesvirus (KHV) just was first identified in 1998 as the cause of mass mortality among juvenile and adult koi and among common carp cultured in Israel (Hedrick *et al*. 2000). Since this KHV is relatively new identified virus, the genomic data is limited. Searching through NCBI entrez nucleotide of koi herpesvirus only resulted around 103 entries. Among them only three original entries show complete genome sequences for KHV strains J, U, and I with accession numbers of GenBank AP008984, DQ657948, and DQ177346, respectively. The membrane protein ORF2 of KHV gene was less analyzed, so BLAST analysis of this sequence only resulted showing high homology with 5 entries, three from complete genomes of KHV as in above, AB178537 and AB178324 (Table 1).

The comparative information gained from the Japan (strain J), the United States (strain U), and Israel (strain I) provides insights into epidemiologic features of an emerging disease threatening koi and carp worldwide. The genomes are highly similar to each other at the sequence level, with U and I more closely related to each other than either is to J. These relationships imply a history in which an ancestral KHV strain gave rise initially to two branches: the J lineage that led eventually to J and the U/I lineage, which subsequently split into the branches leading to U and I. The high degree similarity between U and I can be explained that the first cases of the disease in the eastern United States (strain U) occurred in 1998 following a koi show in New York that involved fish from Israel which may introduce strain I. This conclusion is also consistent with the pattern of differences due to insertions and deletions (Aoki *et al*. 2007). KHV Indonesian strain showed 100% homology with Japan (Strain J) or strain Ibaraki (KHV0301) and 98% homology with strain U and strain I. There are 12 nucleotides different between Indonesian strain and strain U and strain I. This result implies that KHV in Indonesia was most likely to have originated from Japan (Table 1). The first mass mortality of cultured koi (*Cyprinus carpio*) in Indonesia was recorded in March 2002 in Blitar, East Java. It occurred after heavy rains among new fishes introduced from Surabaya, East Java. The fishes were imported from China through Hongkong in December 2001 and January 2002 (Sunarto *et al.* 2005). It means some how the KHV disease was spread from Japan directly or not directly to China or Hongkong.

Using whole virus, Ronen *et al.* (2003) found that KHV had strong antigenic property, and high antibody titer was found in immunized fish. The prediction on T-cell epitope of ORF2 showed 6 and 10 epitopes based on IAd Pattern and Rothbard/Taylor Pattern respectively and B-cell epitope showed 4 locations (Table 2). This result gave possibility that membrane protein could be recognized by lymphocyte T and B; and produced immune responses. If membrane protein ORF2 can be produced, its may be used as a vaccine. The antibody can be produced by immunizing membrane protein into mouse or rabbit, so that immunodetection can be developed.

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