Global Journal Of Biodiversity Science And Management, 3(1): 62-67, 2013
ISSN 2074-0875

Cloning And Prediction Of B Cell Epitopes Of Indonesian Koi Herpesvirus Membrane Protein

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ABSTRACT

Koi herpesvirus (KHV) is a viral disease attacking common carp (*Cyprinus carpio*) and koi (*Cyprinus carpio koi*), very contagious and causing mass mortality. Until now, vaccine for KHV still not present. The purposes of this study were to clone membrane protein (MP) gene of Indonesia's KHV isolate and to predict its B-cell epitopes. Indonesian KHV was isolated from carper gills which obtained from Lake of Toba, North Sumatera. Gene of MP KHV was amplified using PCR (polymerase chain reaction) with specific primers which were designed according to GenBank Data. The product of amplification was ligated to pBluescriptII KS +/- vector and transformed into *Escherechia coli* DH5α. The presence of insert in recombinant plasmid which was isolated from transforman was confirmed by digesting with restriction enzyme, then the recombinant was sequenced. Similarity, T and B-cell epitopes prediction, were analyzed by using programs of BLAST, Genetyx-Win, and IMF Bioinformatic Tool. The result showed that gene of MP KHV was successfully cloned into *E.coli* DH5α. The length of MP KHV encoding gene is 837 bp. BLAST analysis indicated that MP KHV gene of Indonesian isolate has similarity of 99% with Israel's isolate (AB78324) and Japan's isolate (AB178537). Analysis of deduced amino acid sequence revealed that MP KHV Indonesian isolate have 17, 4, and 8 T-cell epitopes of IAd, IEd, and Rothbard/Taylor patterns, respectively. Whereas analysis of IMF Bioinformatic Tool program indicated that MP KHV has 7 B-cell epitopes. Based on these results, it can be theoretically concluded that MP KHV Indonesian isolate has some putative immunogens.

Key word: *KHV, membrane protein (MP), B and T-cell epitopes.*

Introduction

Since 1998, mass mortality of common carp and koi have observed in countries throughout the world, including the U.S.A., Germany, England, Italy, Netherland, Israel, and Indonesia. Losses tended to occur seasonally, especially when water temperatures ranged from 18°–25°C. A herpes-like virus, referred as koi herpesvirus (KHV), has been isolated and identified from common carp and koi in some cases where mass mortality have happened (Gilad *et al*., 2003).

For aquaculture industry, losses which are caused by disease and antibiotic used must be controlled (Gudding *et al*., 1999). The alternative solution is to prepare subunit vaccine where specific components of disease-causing agents are isolated and used as vaccines. In order to increase the quantity of available antigens, one of the recent efforts is to clone gen encoding specific antigen from pathogen and expressing it in bacteria or yeast as recombinant vaccine (Karunasagar *et al*., 1999). Membrane protein of koi herpesvirus can be predicted as an antigen which act as a potential vaccine from common carp and koi.

Materials and Methods

Primer construction:

Designed of forward and reverse primer were based on the sequence data of KHV from GenBank with accession number AB178537. Sequence of *Eco*RI and *Hind*III were selected due to there no restriction site of both in sequence of KHV's membrane protein gene, then they were added to foreward and reverse primer respectively.

KHV DNA isolation:

DNA of fish gills tissue infected by KHV was extracted by using NTE (NaCl, Tris-Cl, EDTA). Tissue (100 mg/ml) was homogenized in NTE buffer, then it was added proteinase-K (100 µg/ml) and SDS which final concentration was 0,5%. Afterwards, the homogenized sample was incubated at 37°C for two hours.

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Furthermore, DNA from the sample was extracted and purified with phenol-chloroform-isoamylalcohol, and than was diluted with ethanol and Na-acetat. After centrifugation (5000 rpm) for 10 minutes, the DNA obtained was diluted in steril aquabidest (Artama, 1991).

PCR assay:

Amplification of ORF3 KHV (candidate MP KHV) was carried out by PCR using kit PuRe Taq ready to Go PCR beads (Amersham Biosciences; diluted in 20 µl aquabidest) mixed with 1 µl ORF3-F KHV and 1 µl ORF3-K KHV primers as well as 3 µl KHV DNA template. The PCR protocol consists of an incubation period of 5 minutes at 95^{o}C, followed by 25 cycles where each cycle consists of denaturation process at 95^{o}C for 30 seconds, annealing for 30 seconds at 55^{o}C, and elongation period for 1 minute at 72^{o}C. After completed the cycles, there was additional extention at 72^{o}C for 5 minutes. The product was precipited by phenol-choloroform-isoamyalcohol.

DNA recombinant construction:

The product of PCR was digested by *Eco*RI and *Hind*III, and then ligated with pBSKSII (+/-) vector which previously digested by the same enzymes as PCR product. The ligation composition consists of 1 µl PCR product, 1 µl pBSKSII (+/-), 1 µl ligation buffer, 0,5 µl T4 ligase (Toyobo), 1 µl ATP, 5,5 µl ddH_2O. Ligation process took place at 16^{o}C for overnight.

Transformation:

5 µl of DNA ligated product was introduced to 100 µl competent *E.coli* (DH5α) and incubated within ice for 30 minutes, then in a short time, it was removed into waterbath at 42^{o}C for 2 minutes. After that, it was incubated again in ice for 2 minutes. Subsequently, 0,9 ml of LB broth was added to the transformation product, and the cells was grown at 37^{o}C with shaking (225 rpm) for 1 hour. Next, 100 µl transformant cells was plated on LB agar containing 50 µg/ml amphicillin and incubated at 37^{o}C overnight. The transformant cells which grown in the LB agar was subsequently lysed using alkaline of miniprep method (Yuwono, 2003).

Insert confirmation:

The presence of insert in vector pBSKSII (+/-) was evaluated by digesting the plasmid with *Hind*III and *Eco*RI respectively. The *Hind*III and *Eco*RI digesting process were carried out in a 15 µl reaction volume containing 10 µl recombinant plasmid, 1,5 µl M buffer, 1 µl *Hind*III, and 2,5 µl ddH_2O and then incubated in waterbath at 37^{o}C for 2 hours, after that, the process was continued with added 1,5 µl H buffer, 1,5 µl *Eco*RI, and 2 µl ddH_2O into the product produced before and incubated again in waterbath at 37^{o}C for 2 hours. The result was observed through electroforesis using 1% agarose.

Sequencing:

Plasmid sample which containing protein membrane gene of KHV was sent to Research and Development Centre Department of P.T. Charoen Pokphan, tbk in Jakarta in order to analyse its sequence using ABI Prism 3100-avant Genetic analyser with T3 and T7 primers.

Similarity, T-cell and B-cell epitopes prediction of KHV

Detection of similarity, T-cell and B-cell epitopes prediction of Indonesian KHV were using BLAST, Genetyx-win, and IMF Bioinformatic Tool programs.

Results:

Amplification of ORF3 KHV:

ORF3 KHV was successfully to be amplified by using PCR as figure 1.

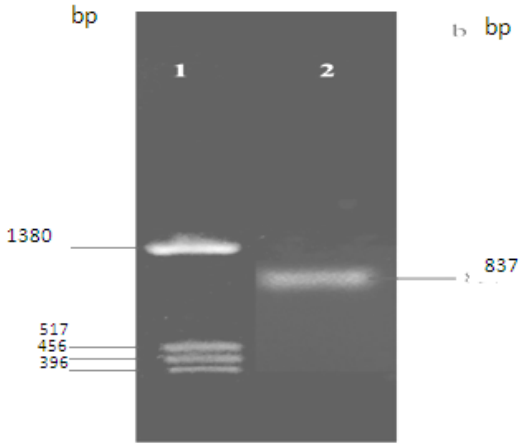

Fig. 1: Result of ORF3 KHV amplification.
1. BSM13/*Hinf*I marker.
2. ORF3 KHV

DNA recombinant and its confirmation:

Recombinant DNA as result of cloning process of DNA target which has been ligased with pBSKSII (+/-) and its confirmation can be showed on figure 2.

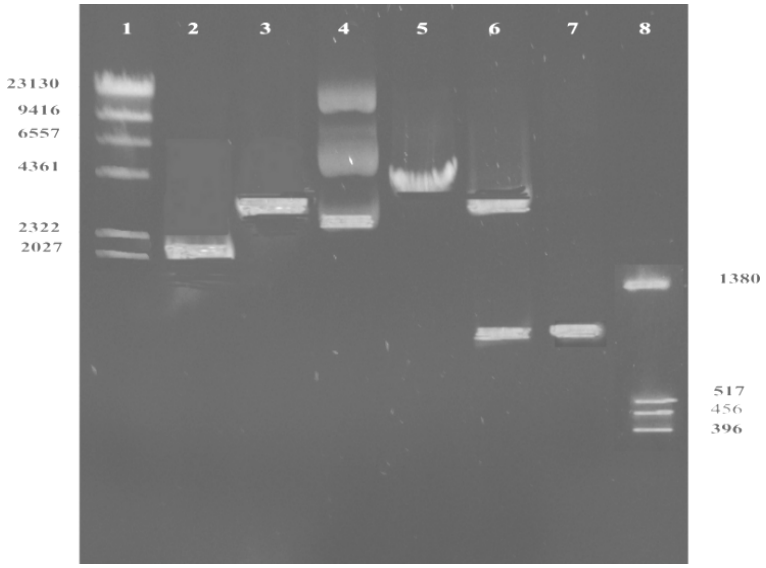

Fig. 2: Recombinant DNA and its conformation. 1. λDNA/*Hind*III marker. 2. Whole pBSKSII (+/-). 3. pBSKSII (+/-) digested by *Eco*RI. 4. Recombinant DNA. 5. Recombinant DNA digested by *Hind*III. 6. Recombinant DNA digested by *Hind*III and *Eco*RI. 7. PCR product of ORF3 KHV. 8. BSM13/*Hinf*I marker.

DNA sequence:

Sequencing of ORF3 KHV using T7 and T3 primers has respectively produced 719 and 706 bases. After aligning of both by anti-parallel method, it produced 837 bp of ORF3 KHV sequence. The ORF3 KHV sequence is showed on figure 3.

Fig. 3: Product of ORF3 KHV sequencing.

Similarity:

Homology of Indonesian ORF3 KHV gene with another KHV's genes in GenBank data was analyzed by BLAST. The result is in figure 4.

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AP008984.1	Koi herpesvirus DNA, complete genome, strain: TUMST1	1541	1541	100%	0.0	99%
DQ657948.1	Koi herpesvirus strain KHV-U, complete genome	1507	1507	100%	0.0	99%
DQ177346.1	Koi herpesvirus strain KHV-I, complete genome	1507	1507	100%	0.0	99%
AB178324.2	Koi herpesvirus genes for membrane protein, major envelope protein, hypothetical protein, complete cds	1507	1507	100%	0.0	99%
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	1314	1314	85%	0.0	99%
AY568951.1	Koi herpesvirus clone BamHI6, genomic sequence	678	678	43%	0.0	100%
DQ128164.1	Koi herpesvirus isolate 323909 genomic sequence	592	592	38%	1e-165	100%

Fig. 4: Homology of Indonesian ORF3 KHV with another genes of KHV in the GenBank data was analyzed by BLAST.

T-cell epitope prediction:

Indonesian MP KHV has 17, 4, and 8 T-cell epitopes of IAd, IEd, and Rothboard/Taylor patterns as showed in figure 5.

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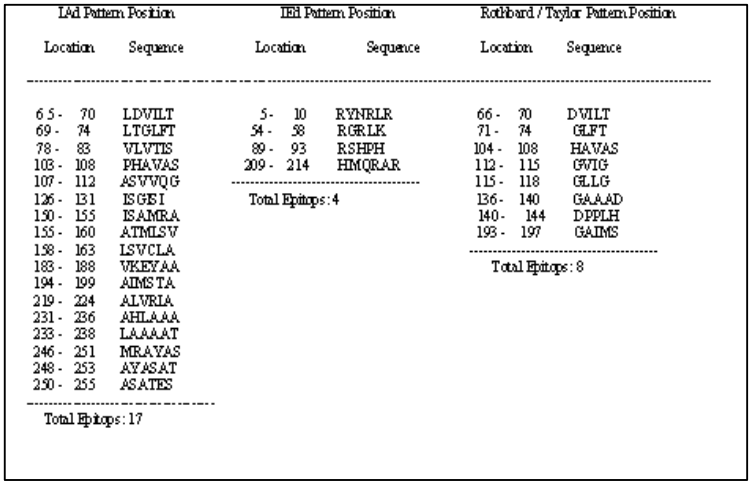

LAd Pattern Position		IEd Pattern Position		Rothbard / Taylor Pattern Position	
Location	Sequence	Location	Sequence	Location	Sequence
65 - 70	LDVILT	5 - 10	RYNRLR	66 - 70	DVILT
69 - 74	LTGLFT	54 - 58	RGRLK	71 - 74	GLFT
78 - 83	VLVTIS	89 - 93	RSHPH	104 - 108	HAVAS
103 - 108	PHAVAS	209 - 214	HMQRAR	112 - 115	GVIG
107 - 112	ASVVQG	Total Epitops: 4		115 - 118	GLLG
126 - 131	ISGISI			136 - 140	GAAAD
150 - 155	ISAMRA			140 - 144	DPPLH
155 - 160	ATMLSV			193 - 197	GAIMS
158 - 163	LSVCLA			Total Epitops: 8	
183 - 188	VKEYAA				
194 - 199	AIMSTA				
219 - 224	ALVRIA				
231 - 236	AHLAAA				
233 - 238	LAAAAT				
246 - 251	MRAYAS				
248 - 253	AYASAT				
250 - 255	ASATES				
Total Epitops: 17					

Fig. 5: T-cell epitopes prediction of Indonesian MP KHV was analyzed by Genetyx-win program.

B-cell epitope prediction:

B-cell epitopes prediction of Indonesian MP KHV by using IMF Bioinformatic Tool was producing 7 epitopes as showed on figure 6.

n	Start Position	Sequence	End Position
1	63	PGLDVILTG	71
2	74	TFGWVLVTISVFVLDRSHP	92
3	102	NPHAVASVVQGVIGL	116
4	138	AADPPLHCFD	147
5	156	TMLSVCLANLATYLFKLTVFFQFVVASVKEYAALHS	191
6	213	ARWVALALVRIALFPMALAHLAAAAT	238
7	259	QRSFCFRVYSSTPSCA	274

Fig. 6: B-cell epitopes prediction of Indonesian MP KHV by using IMF Bioinformatic Tool program.

Discussion:

Gene cloning using PCR method (direct cloning) can produce recombinant DNA which bring specific target gene. Figure 1 pointed out that ORF3 KHV was successfully amplified using PCR and producing 837 bp band. Column 2 in figure 1 indicated that ORF3-F KHV and ORF3-R KHV primers was specific to target gene, it was proved by existing of single band in that column and expected as ORF3 KHV.

In figure 2 it can be seen that the recombinant plasmid which has digested by *Hind*III and *Eco*RI was producing two bands (column 6). The first band size is the same as pBSKSII (+/-) vector, and the other is the same as ORF3 KHV (column 7). So that, it can be concluded that the recombinant plasmid consists of pBSKSII (+/-) vector and ORF3 KHV.

Sequencing of nucleotides of the insert (ORF3 KHV) was carried out by using T3 and T7 primers. The primers was flanking multiple cloning site (MCS) where the insert was placed. Transcript direction of T3 is contrary to T7 direction, therefore they can amplify the insert in MCS. Sequencing from T3 direction was producing 706 bases, and from T7 direction was 719 bases. Aligning the sequences to each other produced 837 bases of ORF3 KHV. The ORF3 KHV sequence is showed on figure 3.

Analysis similarity indicated that homology of ORF3 KHV with Israel's KHV MP gene (AB178324) and Japan's KHV MP gene (AB178537) were 99% as pointed out in figure 4. These phenomena have shown that almost no difference between Indonesian ORF3 KHV, Israel's KHV MP, and Japan's KHV MP. It indicated that KHV has spreaded around the world. There are three mechanisms that have contributed to the rapid global

spread of KHV, i.e. intensive fish culture, koi shows and regional domestic and international trading (Gilad *et al*., 2003).

The result of T-cell and B-cell epitopes prediction analysis showed that Indonesian MP KHV has 17 T-cell epitopes of IAd pattern, 4 T-cell of IEd pattern, and 7 B-cell epitopes. IAd and IEd molecules constitutes MHC class II molecules, and because of the previously demonstrated strong correlation between MHC binding and immunogenicity, they are useful in selecting potentially immunogenic peptide regions within proteins (Sette *et al*., 1989). The production of antibody requires the presence of both B-cell and T-cell epitopes in the vaccine. T-cell have receptors on their surface (T-cell receptors), which are antibody like proteins that specifically bind to a part of an antigen which is presented by MHC. There are various subclasses of T cells that perform very different functions, and those that collaborate with B cells in the antibody response are called T helper cells or T_H cells. For an effective antibody response, the antigen molecules must bind to the receptor on the surface of T_H cells (Glazer *et al*., 1995). T-cell epitopes are presented in the context of MHC proteins. The confirmation of a T-cell epitope embedded in an MHC protein is critical for TCR (T-cell receptor) recognition (Kober *et al*., 2006).

Conclusion:

This research was successfully to clone Indonesian KHV MP gene which has 837 bp. The Indonesian KHV MP gene has 99% similarity with Israel's KHV MP gene (AB178324) and Japan's KHV MP gene (AB178537). This similarity indicated that KHV has spreaded around the world due to international trading mechanism. Based on analysis of DNA sequence using IMF Bioinformatic tool program, the Indonesian KHV MP has 7 B-cell epitopes, hence, it's can be predicted as immunogenic antigens.

Acknowledgements

The author is greatly indebted to the supervisors, Dr. Ir. Ngadiman, M.Si. and Dr. Ir. Murwantoko, M.Si., for their enthusiastic supporting and supervision.

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