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DEVELOPMENT OF NEW PCR PRIMERS FOR DETECTION OF KOI HERPES VIRUS (KHV)

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Outbreaks of a new disease responsible for mass mortality in common carp (*Cyprinus carpio*) and koi carp (*Cyprinus carpio* koi) may have occurred as early as 1996 (Haenen *et al.*, 2004). First scientific report on the disease appeared in 1998 and the disease-causing agent was termed as koi herpes virus (KHV). Therefore, this virus was first reported as the cause of mass mortality among koi carps from Germany, Israel and USA in 1998 (Bretzinger *et al.*, 1999; Hedrick *et al.*, 2000). Since then, outbreaks of KHV disease have been regularly reported from Europe, South Africa, USA and Asia. In Asia, KHV has been reported from Israel, Indonesia, Taiwan, China (Hong Kong), Thailand, Japan and Malaysia. KHV is also called as carp interstitial nephritis and gill necrosis virus or cyprinid herpesvirus-3. The disease is temperature dependent, occurring at water temperatures between 16-25 °C. The disease causes severe financial losses and the mortality rates have consistently been more than 80% in affected populations. The genome of KHV is a double stranded DNA sequence of 295 kbp belonging to family of *Alloherpesviridae*. Entire genome sequencing of three KHV strains from Japan, USA and Israel has been completed (Aoki *et al.*, 2007). Molecular methods such as DNA hybridization, polymerase chain reaction (PCR) and enzyme linked immunosorbent assay have been used for detection of KHV in fish (Gray *et al.*, 2002; Bercovier *et al.*, 2005; Adkison *et al.*, 2005). The objective of the study was to design specific and sensitive PCR primers for detection of major capsid protein (MSP) gene of KHV in koi and common carps.

Positive DNA controls of KHV were generously gifted by Prof. Moshe Kotler, The Hebrew University Hadassah Medical School, Jerusalem, Israel. MSP gene sequences were collected from NCBI with different accession no. AP008984, DQ657948, DQ177346, EF025505, AY787402, AY939865 and AY939864. These nucleotide sequences were aligned by ClustalX 1.83 software and the conserved region of MSP gene was selected. Primers were designed with Oligo 4.0 software. The highly conserved region between nucleotide 334–351 and nucleotide 642–665 was selected for designing specific primers from the complete open reading frame of 3807 bp of major capsid protein gene and commercially synthesized. Details of primer sequences are given in Table 1. The designed primers were then simultaneously compared to the other sequences in the Genbank database to verify their identity and similarity to Koi herpes virus and with those of other related herpes viruses.

PCR amplification was performed using designed primer sets in this study. We used PCR reaction mixture (50 µl) consisted of 10 ng of KHV genomic DNA, 1.5 units of Taq DNA polymerase, 5 µl of 10X PCR amplification buffer (100 mM Tris-HCl, 15 mM $MgCl_2$, 500 mM KCl, pH 8.3), 200 µM deoxynucleotide triphosphates and 10 pmoles of each primer. Samples were subjected to 35 cycles of amplification (94°C for 1 min, 55°C for 1 min and 72 °C for 1 min on a Master cycler (Eppendorf). A final extension at 72°C for 10 min was used. Ten µl of the reaction mixture was then analyzed by submarine gel electrophoresis in 1.2% agarose. The PCR products were visualized under gel documentation system.

Amplified PCR products of 331 bp was ligated into cloning vector pTZ57R/T (Fermentas) and transformed into DH5-á cell (*E. coli* competent cells) as per the instructions of manual. White colonies were selected from Luria Bartini agar plate. Recombinant clones were confirmed by colony PCR. Positive clones of major capsid protein gene showed 331 bp product. Plasmids were purified from positive clones by using commercially available kit. Insertion of desired fragment in the purified plasmids was also confirmed by PCR amplification as described above. All purified plasmid were found positive in PCR. The positive plasmid was used as positive DNA control for testing the sensitivity of primers.

For determining the sensitivity of PCR primer sets, cloned plasmid DNA (100 ng/µl) of KHV was diluted from 10^{-1} to 10^{-5} in ten-fold dilutions and amplification by PCR was done using the diluted DNA template with conditions as described above.

A total of 62 samples of koi and common carps obtained were from different districts of Uttar Pradesh, West Bengal and Kerala. Mainly, gills, kidney and intestinal tissues samples of each fish were collected in 95% ethanol and genomic DNA from samples was isolated using commercially available DNA extraction kits (Qiagen). These DNA samples were subjected to PCR for amplification of MSP gene of KHV.

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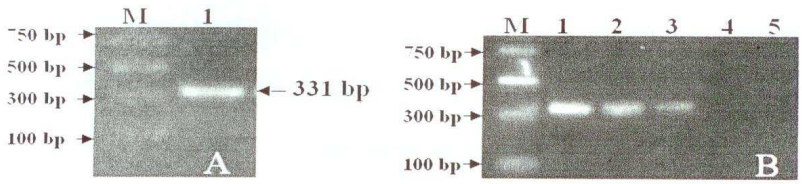

Fig. 1. Detection of major capsid protein (MCP) gene of Koi herpes virus by new MCP primers and testing its sensitivity
A: Lane M : 100 bp DNA ladder (Fermentas); Lane 1 : 331 bp MCP gene fragment of KHV
B : Lane M : 100 bp DNA ladder (Fermentas); Lane 1 : pTZ57R/T containing 331 bp MCP gene insert (100 ng);
Lane 2 : 10 ng; Lane 3 : 1 ng; Lane 4 : 0.1 ng; Lane 5 : 0.01 ng

A set of new primers was designed for specific amplification MSP gene of KHV. The primers amplifying the MSP gene were found to be specific to KHV in the BLAST analysis. A single 331 bp PCR product was obtained with the nucleic acid of the KHV positive control and cloned plasmids (Fig.1 A). The sensitivity limit of our primers for detection of major capsid protein gene of KHV was 1 ng (Fig.1 B). This shows that these primers could also be useful for screening of the fish samples for diagnosis of KHV. As any other fish virus, diagnosis of KHV in fish can be achieved by virus isolation; however, very few cell lines are susceptible to this virus. This is a great limitation for virus isolation and confirmation of the etiological agent. Also, isolation of virus in cell cultures is considered to be less sensitive the PCR-based methods to detect KHV, and is therefore not considered to be a reliable diagnostic method for KHV (Haenen *et al.,* 2004). In view of this, a number of PCR based assays have been developed for detection of KHV in clinically affected fish. Gilad *et al.* (2002) have developed a PCR based on the amplification of thymidine kinase gene for detection of KHV. This could detect 10 fg of KHV DNA and is considered most sensitive method for detection of KHV. Similarly, PCR method developed by Ishioka *et al.* (2005), based on the DNA polymerase gene has the capability to detect 100 fg of KHV DNA. The loop-mediated isothermal amplification method has also been developed for detection of KHV. This method is equally sensitive as PCR method but is more rapid than the PCR (Gunimaladevi *et al.,* 2004).

We have tested a total of 62 samples of koi and common carps were for detection of MSP gene by PCR at an optimal concentration of 1.5 mM $MgCl_2$ and primer annealing at 55 °C. All the tested samples from koi and common carps were found to be negative for presence of KHV. To confirm our results, the samples were retested with primers developed by Gilad *et al.* (2002), which again tested negative for presence of KHV. The present study will eventually help in developing diagnostic capability for detection of KHV in our country, in the event of any unexpected disease outbreak caused by this virus.

Table 1. Primers used for the detection of MSP gene of KHV

Primer code	Position	Sequence	Length
MCPF	334–351	5-CAGACCAAGAACTACGTAGG-3	20
MCPR	642–665	5-GCCTGCGCGTGAGAGTGG-3	18

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