

Development and comparison of loop mediated isothermal amplification assay and polymerase chain reaction based on major capsid protein gene for detection of CyHV-2 infection in goldfish *Carassius auratus* (L.)

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Abstract

Cyprinid herpesvirus 2 (CyHV-2) is the etiological agent of goldfish herpes viral haematopoietic necrosis (GHVHN) that cause high economic losses in goldfish aquaculture. In this study, a loop-mediated isothermal amplification (LAMP) assay and a polymerase chain reaction (PCR) targeting major capsid protein (MCP) gene of CyHV-2 were standardised and assessed for CyHV-2 detection. CyHV-2 was purified from infected fantail goldfish fin (FtGF) cells using ultracentrifugation and used as template for developing the diagnostic assays. The nucleic acids of other pathogens tested, such as Cyprinid herpesvirus-3 (CyHV-3), spring viraemia of carp virus (SVCV), viral nervous necrosis virus (VNNV), *Aeromonas hydrophila*, *A. caviae*, *A. veronii*, *Edwardsiella tarda*, *Vibrio parahaemolyticus*, *V. harveyi*, *V. anguillarum* and *Proteus hauseri*, were not amplified by both the assays. Among the two assays developed, LAMP was found to be more sensitive and efficient for detecting the plasmid construct upto 10 copies containing 942 bp fragment of CyHV-2 MCP gene, while PCR could detect only 100 copies. The LAMP assay developed is a simple, reliable and rapid method for CyHV-2 infection detection which can also be used in field conditions.



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Introduction

Cyprinid herpesvirus 2 (CyHV-2) is the causative agent for goldfish herpes viral haematopoietic necrosis (GHVHN), which is a serious disease of goldfish (*Carassius auratus*) (Goodwin *et al.*, 2006a). The disease has also been reported from Prussian/gibelio carp (*C. gibelio*), Crucian carp (*C. carassius*) with the virus detected in asymptomatic goldfish and common carp (*Cyprinus carpio*) (Wang *et al.*, 2012; Panicz *et al.*, 2019). Mass mortality also occurred in gibelio carp aquaculture in China during 2011-12 (Wang *et al.*, 2012). The first report of CyHV-2 was from the mass mortality of juvenile goldfish in Japan in 1992-93 (Jung and Miyazaki, 1995) and thereafter been detected from more than 13 countries

(Adamek *et al.*, 2018). In India, CyHV-2 disease outbreak was confirmed in goldfish in West Bengal during 2015-16 (Sahoo *et al.*, 2016). The disease outbreaks due to CyHV-2 have resulted in huge mortalities and economic losses worldwide and the implementation of control measures warrants the development of rapid and specific detection methods and improved CyHV-2 culture methods.

The diagnosis of GHVHN is generally based on clinical signs, post-mortem lesions, histopathology and the demonstration of herpesvirus particles in tissues of infected fish by electron microscopy (Jung and Miyazaki, 1995; Chang *et al.*, 1999). Different types of molecular and immunological analyses such as polymerase chain reaction

(PCR) (Jeffery *et al.*, 2007; Waltzek *et al.*, 2009; Xu *et al.*, 2013), loop-mediated isothermal amplification (LAMP) assay (He *et al.*, 2013; Liang *et al.*, 2014; Zhang *et al.*, 2014; Zhu *et al.*, 2015); real-time PCR (Goodwin *et al.*, 2006b) immuno-histochemistry assay (Kong *et al.*, 2017), indirect immunofluorescence antibody test (Nanjo *et al.*, 2016) and fluorescence *in situ* hybridisation (Ding *et al.*, 2014) have been designed for the rapid detection of CyHV-2. In addition, CyHV-2 can be isolated using cell lines from other fish species such as fathead minnow (FHM) (Jung and Miyazaki, 1995), koi fin I (KF-1) (Jeffery *et al.*, 2007) and *Cyprinus carpio koi* fin (CCKF) cell line (Sahoo *et al.*, 2016). Over the last few years, species-specific cell lines, namely GiCB (Ma *et al.*, 2015), CAF and SRTF (Ito and Maeno, 2014) have been established for continuous propagation of CyHV-2. We have also developed a species-specific cell line from caudal fin of fantail Goldfish (FtGF) for the isolation of CyHV-2 (Dharmaratnam *et al.*, 2020). Recently, three new cell lines like fantail goldfish gill (FtGG), fantail goldfish brain (FtGB) and fantail goldfish liver (FtGL) were developed from the gill, brain and liver tissues of *C. auratus* respectively and it was confirmed that the CyHV-2 titre in FtGG was higher than the other three cell lines (Swaminathan *et al.*, 2021).

A molecular assay that is highly sensitive, specific, rapid, easy to use and economical is warranted for timely diagnosis of CyHV-2 in goldfish. For the present study, we have developed a sensitive polymerase chain reaction (PCR) and Loop mediated isothermal amplification (LAMP) assay based on major capsid protein (MCP) gene of CyHV-2. The sensitivity and specificity of both the assays developed were compared with each other as well as with the available PCR assays.

Materials and methods

Propagation and purification of CyHV-2

Diseased goldfish showing clinical signs of GHVHN were collected from ornamental fish farms in Kochi, Kerala, India during January 2018. Gill, spleen and kidney of the affected fish were collected and stored at -80°C. DNA was isolated from the stored samples and screened for the presence of CyHV-2 infection using PCR (Engelsma *et al.*, 2013). The PCR positive tissue samples were pooled and homogenised with sterile Leibovitz's (L-15) medium supplemented with 1000 IU ml⁻¹ penicillin, 1000 µg ml⁻¹ streptomycin and 2.5 µg ml⁻¹ amphotericin B (Invitrogen, Carlsbad, California, USA). The 0.22 µm filter (Millipore) filtered homogenised tissue samples were inoculated onto a confluent monolayer of fantail goldfish fin (FtGF) cell line (Dharmaratnam *et al.*, 2020) at 30th passage in a 25 cm² flask (Nunc) for isolation of CyHV-2. CyHV-2 virus particles were purified from infected goldfish cells by centrifugation at 35,000 *g* followed by sucrose gradient ultracentrifugation (Beckman Model L Ultracentrifuge). Presence of viral DNA was confirmed by PCR reaction using DNA polymerase gene (Engelsma *et al.*, 2013). CyHV-2 PCR positive cell culture supernatants were stored at -80°C for further use.

Primer design

The outer primers, inner primers and loop primers for LAMP assay for specific detection of CyHV-2 were designed from the major

capsid protein (MCP) gene sequence (GenBank Accession No. NC019495) using Primer Explorer V4 (<http://primerexplorer.jp/lamp>) (Table 1). In addition, a new set of primers (IF 5'CCC GTC TGA GAA AGT GCT TC3'; IR 5'AAG GCG CTT GGG AAG TAG AT3'), targeting a 942 bp fragment of MCP gene was also designed for detection of CyHV-2 using PCR. The primers were synthesised commercially (Sigma, India).

Table 1. Details of the LAMP primers for the amplification of CyHV-2

F3- CyHV2	ATCGGATGCCTGGGACAC
B3- CyHV2	GAACGGGCATGACGGAGA
FIP-CyHV2	GCGAGTAGTGACAGGCGATTTTGGCCCTCCGCTGAGAAAGT
BIP-CyHV2	CTGCTCGTCAACCAGCGGAACCTTTTTCATGCGTCTGACCACCAC
LF-CyHV2	GCGGGCAAGTGTTGAAGC
LB-CyHV2	ATAAGCAGTATTACTCTGGATGGGG

Detection of CyHV-2 by MCP PCR

DNA from purified CyHV-2 was extracted using DNA isolation kit (Qiagen, India), following manufacturer's instructions. PCR was performed in a final volume of 25 µl containing 0.25 µl of *Taq* DNA polymerase (5 U µl⁻¹), 2.5 µl of 10X *Taq* buffer, 0.5 µl of dNTPs (2 mM), 1.5 µl (10 pmol) of each primer, 1 µl of total DNA and ddH₂O to make final volume to 25 µl. PCR cycling conditions included, denaturation at 95°C for 2 min; followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 15 min. The amplified PCR products were checked on 1.2% agarose gels, and cleaned PCR products were sequenced using an Applied Biosystems AB 3730 XL capillary sequencer. The raw DNA sequences were edited using BIOEDIT version 7.0.5.2 and deposited in GenBank and the sequence was then aligned with other known CyHV-2 sequences. PCR was also carried out with DNA isolated from goldfish samples suspected to be infected with CyHV-2.

Development of CyHV-2 DNA standard

The CyHV-2 MCP gene (this study), DNA polymerase gene (Jeffery *et al.*, 2007) and Helicase gene (366 bp and 357 bp fragments) (Waltzek *et al.*, 2009; Xu *et al.*, 2013) were amplified, purified and cloned into pCR2.1 Vector (Thermo Fisher Scientific, India). Plasmid vectors containing the MCP gene, DNA polymerase gene and Helicase gene of CyHV-2 were isolated using QIAprep Spin Miniprep Kit (Qiagen, India), quantified using spectrophotometer (Eppendorf) and were verified by gel electrophoresis and sequencing (SciGenome, Kerala, India). These purified plasmids were used as standards and the copy number of standard plasmid DNA of CyHV-2 was calculated by following the method described by Whelan *et al.*, (2003). After quantification, the CyHV-2 plasmid was serially 10-fold diluted from 1 X 10⁸ to 1 X 10¹ copies µl⁻¹ to prepare the standard template. The respective plasmid constructs were used to compare the sensitivity of the newly developed PCR and LAMP assays based on MCP gene with that of the other PCR methods based on DNA polymerase gene (Jeffery *et al.*, 2007) and helicase gene of CyHV-2 (Waltzek *et al.*, 2009; Xu *et al.*, 2013) and the experiment was repeated thrice.

Optimisation of LAMP for the detection of CyHV-2

The LAMP reaction was optimised at different reaction temperatures of 60, 61, 62, 63, 64 and 65°C and different MgCl_2 concentrations of 0, 2, 4, 6, 8 and 10 mM for 60 min. The reaction mixture (25 μl) contained 1.6 μM each of LAMP inner primers, 0.4 μM each of outer primers and 0.2 μM each of forward and backward loop primers, 1 mM of dNTP mix, 8 mM MgCl_2 , Bst 2 warm start DNA polymerase, 1 $\times$ buffer and 5 μl template DNA prepared from the purified CyHV-2. The products were analysed by 2% agarose gel electrophoresis. Besides, visual examination of CyHV-2 LAMP amplification was carried out by adding 5 μl SYBR Green I (Invitrogen, CA, USA) (1:100 dilution stock) to the reaction tube. The colour change in the tubes was observed with naked eye under natural light; positive samples gave a green fluorescence while negative samples gave an orange colour. DNA isolated from mock infected goldfish fin cell line was used as negative control. For the samples collected from goldfish suspected to be infected with CyHV-2, the LAMP reaction was performed at 60°C for 60 min. Amplified products were also analysed by agarose gel electrophoresis.

Determination of sensitivity and specificity of LAMP and PCR

To determine the sensitivity of the LAMP and PCR assay, the plasmid construct with MCP gene was diluted (from 1×10^8 to 1×10^1 copies μl^{-1}) and spiked with CyHV-2 negative goldfish tissue homogenate was used as template. The LAMP and PCR assays were performed according to the reaction conditions described above. To determine the specificity of the newly designed LAMP and PCR primers, assays were performed using CyHV-2 DNA, fish viral pathogens namely Cyprinid herpesvirus-3 (CyHV-3), spring viraemia of carp virus (SVCV) and viral nervous necrosis virus (VNNV) and bacterial pathogens including *Aeromonas hydrophila*, *A. caviae*, *A. veronii*, *Edwardsiella tarda*, *V. parahaemolyticus*, *V. anguillarum*, *V. harveyi* and *Proteus hauseri*. After the reaction, LAMP and PCR products were electrophoresed on a 1.5% agarose gel and visualised under a gel documentation system.

Evaluation of the LAMP assay and PCR using clinical samples

A total of 50 diseased goldfish samples (collected from different places of India as part of routine disease surveillance during 2018-19) were tested for CyHV-2. Samples from healthy goldfish (without CyHV-2 clinical signs) were included as known negative controls in the assays. All goldfish samples were tested using the PCR and LAMP assay developed.

Results

Isolation and propagation of cyprinid herpesvirus 2 (CyHV-2)

Following inoculation of filtered tissue homogenate in FtGF cells, cytopathic effects (CPE) started at 2 days post-infection (dpi) and typical CPE including cytoplasmic vacuolisation was observed in FtGF cells at 3 dpi and cell fusion with cell shrinkage and rounding was observed at 4 dpi and the cell monolayer was destroyed by 9 dpi (Fig. 1b). No changes were observed in morphology of mock infected FtGF cells (Fig. 1a).

Detection of CyHV-2 by LAMP and PCR

The Primer Explorer V4 was used for designing the LAMP primers and the locations are indicated in Fig. 3a. The LAMP product amplification was obtained at a temperature of 60°C for 1 h and at 8 mM MgCl_2 concentration. The MCP gene plasmid construct of LAMP reaction could detect upto 10 copies. The LAMP assay results were observed in gel electrophoresis as ladder like pattern and as fluorescent green by naked eye (Fig. 3c). In our study, the newly designed MCP primer set could amplify 942 bp of CyHV-2 MCP partial sequence and the gene sequences of CyHV-2 MCP gene isolated from goldfish were deposited in GenBank (Accession Nos. KU527546 and KU527547). A 100% sequence similarity was observed with CyHV-2 isolate SYC1 strain (KM200722) and

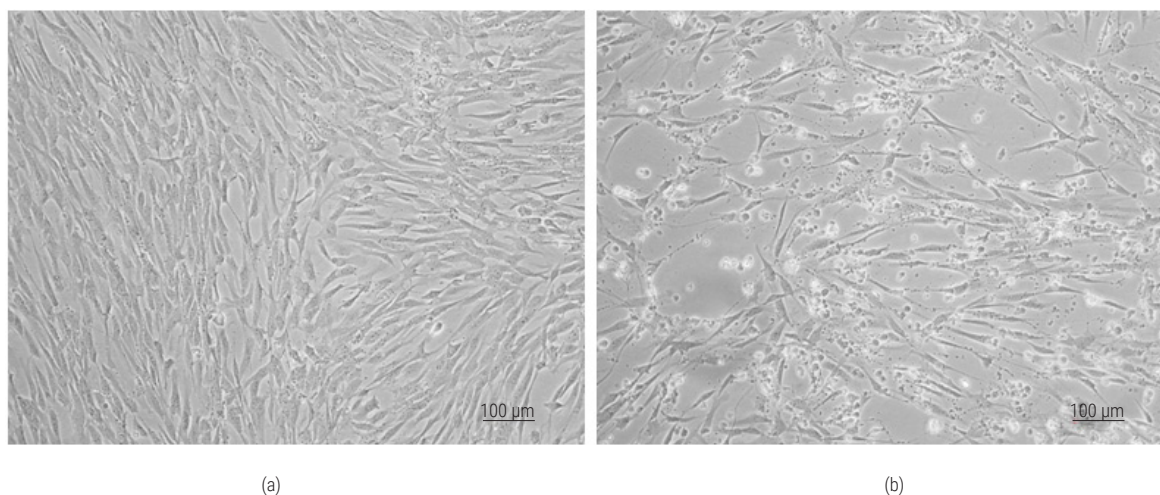


Fig. 1. Isolation of CyHV-2 in species-specific FtGF cell line. (a) Control fantail goldfish fin (FtGF) cell line, (b) Cytopathic effect after 2dpi in FtGF cell line

CyHV-2 isolate STJ1 strain (JQ815364) by GenBank BLAST search. The newly designed MCP primer amplified 100 copies of the CyHV-2 MCP plasmid construct (Fig. 2a). Further, the primers of DNA polymerase gene (362 bp) and helicase gene (366 bp and 357 bp) of plasmid construct could detect 100, 1000 and 100 copy numbers respectively in our study (Fig. 2b, c, d). The newly designed MCP PCR and LAMP primers were found to be specific for CyHV-2 and they did not amplify the nucleic acids of CyHV-3, SVCV, VNNV, *A. hydrophila*, *A. caviae*, *A. veronii*, *V. parahaemolyticus*, *V. anguillarum*, *V. harveyi* and *P. hauseri* (data not shown).

A total of 50 goldfish samples suspected for CyHV-2 were screened by the LAMP method and MCP PCR. In total, 45 (90%) and 38 (76%) samples were CyHV-2 positive by LAMP and MCP PCR, respectively. These results indicate a higher detection capacity of the LAMP assay compared to that of MCP PCR.

Discussion

CyHV-2 infection results in high mortality and poses global threat to the goldfish aquaculture industry with potential for enormous economic losses. There is a need to develop strategies to minimise

the heavy losses to aquaculture industry due to this viral disease. Thus, it is necessary to develop an accurate, rapid and simple method for the detection of this virus. The preferred detection methods for most of fish viral diseases recommended by World Organisation for Animal Health (OIE) are isolation of the virus and detection of virus using molecular methods. Currently, molecular methods are effectively and frequently used for diagnosis of this disease. Different molecular methods such as real-time PCR and PCR have been developed for the specific and sensitive detection of CyHV-2 (Goodwin *et al.*, 2006a; b; Waltzek *et al.*, 2009).

A very quick and accurate diagnostic technique would aid in the early identification of CyHV-2 in goldfish populations and help to impede its spread. In our study, the detection limit of the LAMP assay was 10 copies of the recombinant plasmid construct with the 942 bp MCP gene of CyHV-2. The sensitivity of the developed LAMP assay is equivalent to that reported previously in LAMP assay based on intercapsomeric triplex protein gene of CyHV-2 (Liang *et al.*, 2014), whereas, it was 10 times higher than the sensitivity reported for LAMP assay based on helicase gene (Zhu *et al.*, 2015). Furthermore, LAMP assay of CyHV-2 (at 64°C for 60 min) based on helicase gene could result in amplification of up to 10^{-3} dilution of CyHV-2 DNA isolated from positive clinical samples and

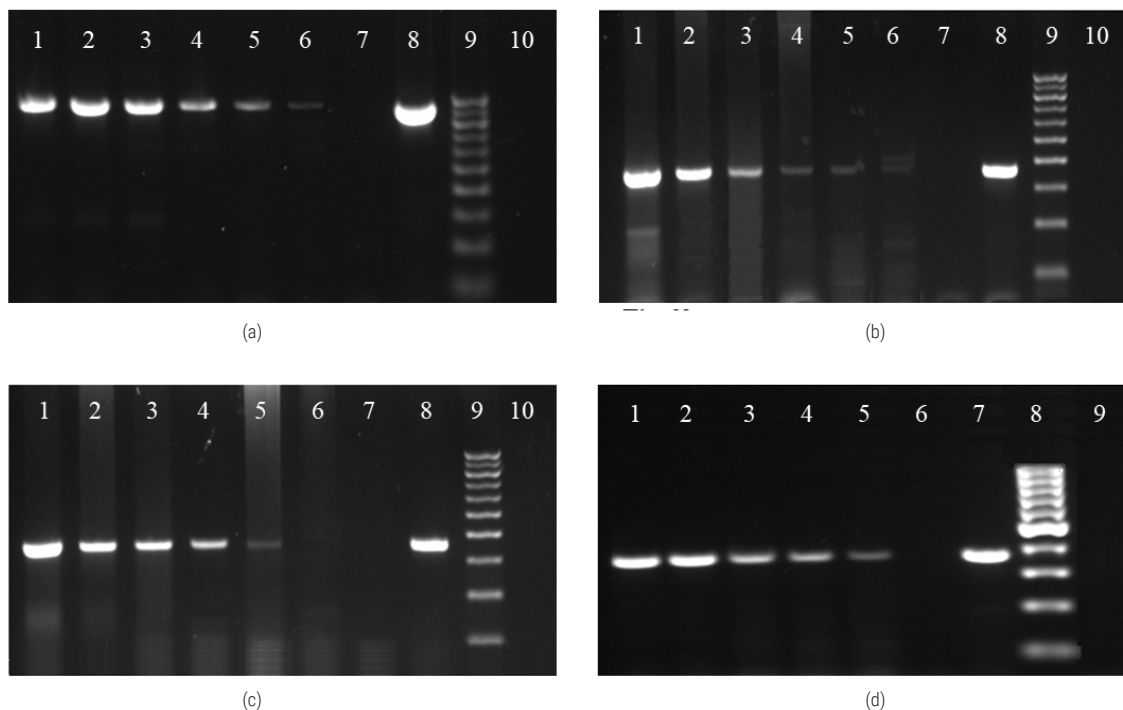


Fig. 2. Sensitivity of the different PCR primers in detecting CyHV-2. (a) Newly designed PCR primer to detect the partial fragment of major capsid protein (942 bp) of CyHV-2 DNA from infected goldfish. Lane 1 - 10^7 dilution of CyHV-2 MCP gene plasmid construct μl^{-1} ; Lane 2 - 10^6 dilution μl^{-1} ; Lane 3 - 10^5 dilution μl^{-1} ; Lane 4 - 10^4 dilution μl^{-1} ; Lane 5 - 10^3 dilution μl^{-1} ; Lane 6 - 10^2 dilution μl^{-1} ; Lane 7 - 10^1 dilution μl^{-1} ; Lane 8 - Positive control; Lane 9 - DirectLoad™ PCR 100 bp low ladder (Sigma), Lane 10: Negative control; (b) PCR primer to detect the partial fragment of DNA polymerase gene (362 bp) of CyHV-2 DNA from infected goldfish. Lane 1 - 10^7 dilution of CyHV-2 DNA polymerase gene plasmid construct μl^{-1} ; Lane 2 - 10^6 dilution μl^{-1} ; Lane 3 - 10^5 dilution μl^{-1} ; Lane 4 - 10^4 dilution μl^{-1} ; Lane 5 - 10^3 dilution μl^{-1} ; Lane 6 - 10^2 dilution μl^{-1} ; Lane 7 - 10^1 dilution μl^{-1} ; Lane 8 - Positive control; Lane 9 - DirectLoad™ PCR 100 bp low ladder (Sigma), Lane 10: Negative control; (c) Helicase gene PCR primer to detect the partial fragment of major capsid protein (366 bp) of CyHV-2 DNA from infected goldfish. Lane 1 - 10^7 dilution of CyHV-2 Helicase gene plasmid construct μl^{-1} ; Lane 2 - 10^6 dilution μl^{-1} ; Lane 3 - 10^5 dilution μl^{-1} ; Lane 4 - 10^4 dilution μl^{-1} ; Lane 5 - 10^3 dilution μl^{-1} ; Lane 6 - 10^2 dilution μl^{-1} ; Lane 7 - 10^1 dilution μl^{-1} ; Lane 8 - Positive control; Lane 9 - DirectLoad™ PCR 100 bp low ladder (Sigma), Lane 10: Negative control; (d) Helicase gene PCR primer to detect the partial fragment of major capsid protein (357 bp) of CyHV-2 DNA from infected goldfish. Lane 1 - 10^6 dilution of CyHV-2 Helicase gene plasmid construct μl^{-1} ; Lane 2 - 10^5 dilution μl^{-1} ; Lane 3 - 10^4 dilution μl^{-1} ; Lane 4 - 10^3 dilution μl^{-1} ; Lane 5 - 10^2 dilution μl^{-1} ; Lane 6 - 10^1 dilution μl^{-1} ; Lane 7 - Positive control; Lane 8 - DirectLoad™ PCR 100 bp low ladder (Sigma), Lane 9: Negative control

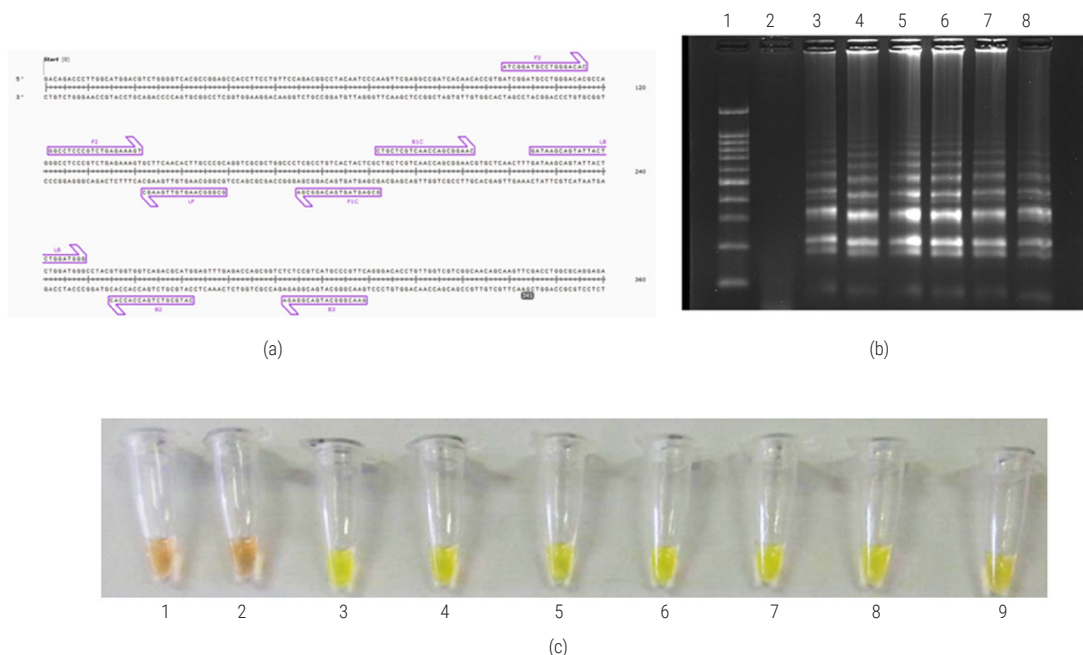


Fig. 3. Loop mediated isothermal amplification of CyHV-2. (a) Primer binding site of LAMP primer; (b) Agarose gel electrophoresis of LAMP product showing ladder like pattern; Lane 1 - DNA 100 bp ladder; Lane 2 - Known CyHV-2 negative; Lane 3 - 5 ng μl^{-1} ; Lane 4 - 2.5 ng μl^{-1} ; Lane 5 - 1.25 ng μl^{-1} ; Lane 6 - 0.625 ng μl^{-1} ; Lane 7 - 0.3 ng μl^{-1} ; Lane 8 - 0.15 ng μl^{-1} ; (c) Sensitivity of newly designed LAMP primer in the detection of CyHV-2. Tube 1 and 2 - Known CyHV-2 negative DNA; Tube 3 - 10 ng μl^{-1} ; Tube 4 - 5 ng μl^{-1} ; Tube 5 - 2.5 ng μl^{-1} ; Tube 6 - 1.25 ng μl^{-1} ; Tube 7 - 0.625 ng μl^{-1} ; Tube 8 - 0.3 ng μl^{-1} and Tube 9 - 0.15 ng μl^{-1}

was comparable to nested PCR (Zhang *et al.*, 2014). Whereas it has been reported that LAMP assay based on terminase gene of CyHV-2 could detect the virus as low as $1.09 \times 10^{-4} \mu\text{g} \mu\text{l}^{-1}$ (CyHV-2 DNA extracted from diseased goldfish), which was 10 times lower than the real-time PCR detection limit and 10^4 times lower than the detection limit of PCR (He *et al.*, 2013). The LAMP assay is reported to be of greater sensitivity in detection of pathogen mainly because of the high amplification efficiency and high SYBR Green I binding affinity to DNA with minimal time duration (Singer *et al.*, 1999). Furthermore, the use of fluorescent dye in the master mix itself reduces the chances of cross-contamination due to less requirement of repeated opening of the lid of the tubes and it also provides easy method for detecting LAMP amplification products of pathogen by naked eye (Liang *et al.*, 2014; Zhu *et al.*, 2015). SYBR Green I is a less hazardous substance and 50-100 times greater sensitive than ethidium bromide in the detection of nucleic acid and importantly, the reaction products containing SYBR green can be disposed of using water, 70% ethanol and 1 ml of 5.25% sodium hypochlorite (normal bleach) (Singer *et al.*, 1999). Moreover, in case of accidental contact with skin or eye, simple rinsing with water is effective. SYBR Green I was used by various researchers (Liang *et al.*, 2014; Zhu *et al.*, 2015) for the naked eye visualisation of LAMP products in the detection of CyHV-2.

In this investigation, a 10-fold serially diluted plasmid construct containing the CyHV-2 MCP gene fragment allowed the recently established MCP PCR to amplify 100 copies. The sensitivity limit of the newly designed PCR primer was equal to the sensitivity of PCR based on DNA polymerase gene (Jeffery *et al.*, 2007) as well as Helicase gene (Waltzek *et al.*, 2009), whereas the sensitivity was higher than that of the Helicase gene primers

(Xu *et al.*, 2013). Therefore, the newly designed MCP primers in this study provide alternate primers for detecting CyHV-2 infection.

In the present study, the new LAMP assay could detect 10 copies of plasmid construct with 942 fragments of MCP gene of CyHV-2, whereas the new MCP PCR could detect only 100 copies. Therefore, the LAMP assay developed is more sensitive in detecting CyHV-2 compared to the new PCR assay. Further, both the methods did not detect nucleic acid template from 11 aquatic animal pathogens including viruses and bacteria, suggesting that both the assays are specific to CyHV-2, in accordance with the findings of the previous reports (He *et al.*, 2013; Liang *et al.*, 2014; Zhang *et al.*, 2014; Zhu *et al.*, 2015). In our study, the new LAMP was more sensitive in detecting CyHV-2 compared to the new PCR assay.

Importantly, more goldfish were found to be positive by LAMP assay in comparison to new PCR, indicating that LAMP assay is more sensitive than PCR. The requirement of sophisticated equipment like thermal cycler and real time PCR restricts the use of PCR and real-time PCR in the detection of any pathogen in field conditions, while LAMP reaction working in an isothermal condition is comparatively easier to use in the field level. Therefore, this method is more suitable for quick detection of CyHV-2 infection in ornamental fish farms. In conclusion, the LAMP developed has prospects for rapid diagnosis of CyHV-2 in farm and would help in GHVHN surveillance.

The global trade of goldfish and koi carp could intensify the spread of cyprinid herpes viruses. The lack of rapid detection techniques could lead to potential disease risks and outbreaks in goldfish populations. The sensitive and rapid detection of CyHV-2 in the field and implementation of disease control strategies can be achieved using the newly developed LAMP assay targeting the MCP gene.

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