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Research article

Establishment of loop-mediated isothermal amplification assay for detection of parasitic ciliate *Ichthyophthirius multifiliis* in cyprinid fish

Surachai Pikulkaew[1,*] and Panyisa Potibut[2]

[1]*Department of Food Animal Clinic, Faculty of Veterinary Medicine, Chiang Mai University, Chiang Mai 50100, Thailand.*
[2]*Aquaculture Service Development and Research Team, National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, Pathum Thani 12120, Thailand*

Abstract

The objective of this research was to establish loop-mediated isothermal amplifications (LAMP) that could be used to detect parasitic ciliate *Ichthyophthirius multifiliis* (*I. multifiliis*) in freshwater cyprinid fish. Primers were developed from the distinguishing fragments of 18S ribosomal RNA of *I. multifiliis* and the LAMP test was then used to evaluate and optimize various concentrations of chemicals, time and temperature. The results indicated that LAMP required 1.6 µM of FIP primers and BIP primers, 0.2 µM of F3 and B3, 2 mM of Mg^{2+}, 1 M of Betaine, and 0.6 mM of dNTP. This assay was able to detect parasite DNA within a 40 min period of incubation and at a constant optimal temperature of 64°C. The positive sample appeared as a clear ladder like pattern on gel electrophoresis, while a yellowish green color appeared with SYBR Green I under ultraviolet light with the use of a heating block. The LAMP test was determined to be more sensitive than conventional PCR in the detection of *I. multifiliis*. In conclusion, we have presented a sensitive and specific rapid detection system for *I. multifiliis* based on isothermal DNA amplification. Importantly, this system could then be employed as an alternative and effective diagnostic method in place of other molecular techniques.

Keywords: Cyprinid fish, Detection, *Ichthyophthirius multifiliis*, LAMP

Corresponding author: Surachai Pikulkaew, Department of Food Animal Clinic and Research Center of Producing and Development of Products and Innovations for Animal Health and Production, Faculty of Veterinary Medicine, Chiang Mai University, Thailand. Tel.: +66 53 948023; Fax: +66 53274710. E-mail: surapikulkaew@gmail.com.

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INTRODUCTION

Freshwater cyprinid fish are the most popular order of fish that are kept as pets worldwide. Examples of these freshwater fish include koi carp (*Cyprinius carpio* koi) and goldfish (*Carassius auratus*). However, a number of serious problems have been associated with the keeping of these fish. These problems have resulted from inappropriate management practices, while disease outbreaks that occurred as a result of the presence of various pathogens such as bacterial infections (McDermott and Palmeiro 2020; Saengsitthisak et al., 2020), viruses (Pikulkaew et al., 2009; Pikulkaew et al., 2020) and parasitic infections (Saengsitthisak et al., 2021, Sirri et al., 2020). Importantly, *Ichthyophthirius multifiliis* (*I. multifiliis*) is the known cause of freshwater white spot disease, or ichthyophthiriasis, and other serious ectoparasitic infestation/infections in freshwater cyprinid fish along with other freshwater species of fish worldwide (Dickerson and Clark, 1996). At present, treatment with chemicals is the only known method of control for this disease. However, it can be difficult to treat parasites after they have penetrated the fish's epithelium. Consequently, ichthyophthiriasis infections frequently lead to high morbidity and mortality rates on fish farms (Noga, 2010).

The life cycle of a parasite is composed of an infective theront, a feeding or parasitic trophont, and a reproductive tomont (Matthews, 2005; Noga, 2010). During the course of the infective stage of *I. multifiliis*, it typically penetrates the epidermis of the skin and gills in order to feed on the fish and move about. This results in an impairment of the physiological regulation of any affected organs. Infected fish also display a range of behavioral abnormalities including anorexia, irritation and discoloration (Xu et al., 2012). Moreover, *I. multifiliis* may act as a carrier of bacterial pathogens associated with secondary infections and it has the potential to kill large numbers of fish in a short period of time (Matthews, 2005; Noga, 2010).

Common clinical detection and diagnosis of *I. multifiliis* infection include gross observation, microscopic identification, and histopathological examination (Noga, 2010); nevertheless these methods cannot detect the early stage of infection of *I. multifiliis* as molecular methods are necessary. Recently, several molecular methods have been developed to detect and quantify *I. multifiliis* such as real-time PCR (Jousson, 2005; Howell et al. 2019) and PCR (Fariya et al. 2016). However, the above techniques are time-consuming and their implementation may require expensive instruments. Loop-mediated isothermal amplification (LAMP) has been developed for the detection of various pathogens in both humans and animals. LAMP requires only simple equipment, can give an accurate result, and is not difficult to perform (Notomi et al., 2000). In this research work, we have developed a LAMP detection system for the rapid identification of the parasitic ciliate *I. multifiliis* in freshwater cyprinid fish. This diagnosis assay is simple, sensitive, and effective for the specific detection and diagnosis of ichthyophthiriasis in freshwater fish.

MATERIALS and METHODS

Animal and parasitic collection

I. multifiliis was originally isolated from freshwater cyprinid fish, including goldfish and koi carp, obtained from ornamental fish shops located in Meuang District, Chiang Mai Province. The fish were maintained in the laboratory facility of Aquatic Medicine, Faculty of Veterinary Medicine, Chiang Mai University. Parasites were collected according to the method described by Zhang et al. (2013). Briefly, collection of the trophont stage of *I. multifiliis* was achieved by means of skin and fin biopsies, while confirmation of infection was established by examination under a microscope. Trophonts can be identified by the presence of typical, often horseshoe shaped macronucleus and motile holotrich ciliates (Noga, 2010). The use of animals in this study was conducted in accordance with the experimental methods approved of by the Animal Ethics Committee, Faculty of Veterinary Medicine, Chiang Mai University (R8/2557).

DNA extraction

Accordingly, 20 trophonts of *I. multifiliis* collected from 3 individual infected fish (replications employed in all PCR and LAMP experiments) were obtained for DNA extraction using a NucleoSpin® Tissue Kit (Macherey-nagel GmbH & Co. KG, *Düren*, Germany) based on the specific protocol provided by the manufacturer. DNA concentration values were measured and the purification of each sample was determined using a spectrophotometer (Du 730, Beckman Coulter®, CA, USA). The DNA samples were kept in -20 ºC prior to being used for further experimentation.

Polymerase chain reaction

The primers used to amplify the region of the internal transcribed spacers of nuclear ribosomal of *I. multifiliis* were S01-F (5'- GTA CTT TAT TTA GGA GGA GGA CT -3') and S02-R (5'- TGT TTA ACG AGA GAA AAT CAT AAA T-3'). These primers were used to amplify a 341 bp PCR product that was specifically established for *I. multifiliis* (Chen *et al*., 2008). PCR reactions were performed at a final volume of 25 µl containing 50 ng of template DNA, 0.5 pmol of each primer, 4 mM of $MgCl_2$, 1x *Taq* buffer, 200 µM of dNTPs, and 0.5 IU of Taq DNA polymerase (Fermentas, USA). The PCR conditions were established under the following optimized conditions: 95ºC for 5 min, followed by 35 cycles of 94ºC for 30 sec, 53ºC for 30 sec and 72ºC for 60 sec, followed by a final extension step at 72ºC for 5 min.

Loop-mediated isothermal amplification

Primer design

A conserved sequence of the 18S ribosomal RNA of *I. multifiliis* (GenBank: U17354.1 >gi|2182270|gb|U17354.1|IMU17354) was selected as the target for the LAMP reaction (Jousson *et al*., 2005). LAMP primers were designed using online PrimerExplorer V4 software (available at http://primerexplorer.jp/elamp4.0.0/index.html). The LAMP primers with the best parameters were selected (primers were specified at the 5′ to 3′ end with 40 - 60% GC content and the primer melting temperature (Tm) of each primer had a maximum difference of 5°C) including the outer forward primer (F3), the outer backward primer (B3), the forward inner primer (FIP), and the backward inner primer (BIP). The primer sequences are shown in Table 1 and the positions of the LAMP primers in the target sequences are presented in Figure 1.

Table 1 Primers sets for the LAMP reactions of this study.

Primer	Sequence (5' → 3')	Primer length
F3	AAC AG CA CAC CAG AAG TG	18
B3	GGT TAA GGT CTC GTT CGT TAA	21
FIP	AAT CCC TTC TCT GTC TTG CGC TTT TAC CTG CGG CTT AAT TTG AC	44
BIP	TGA GAG CTC TTT CTT GAT TCT TTG GTT TTG AAT TAA CCA GAC AAA TCA CTC C	52

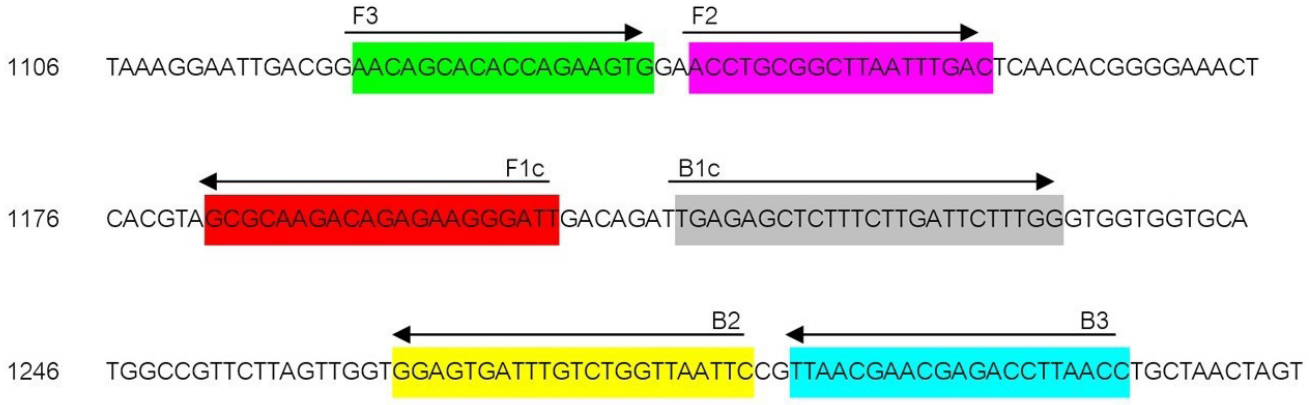

Figure 1 Partial coding sequences of 18S ribosomal RNA genes used to design the primers. LAMP primers are indicated by arrows.

Optimization of LAMP reaction

A mixture was performed to optimize the LAMP reaction including the primers of FIP and BIP (concentrations were varied at 0.8, 1.2 and 1.6 µM), 10 µmole F3 and B3 outer primer, Betaine (concentrations varied at 0.2, 0.6 and 1 mM), dNTPs (concentrations varied at 0.2, 0.4 and 0.6 mM), 4.8 IU *Bst* DNA polymerase large fragment (Lucigen Corporation, WI, USA), 1× DNA polymerase buffer B (20 mM Tris-HCl pH 8.8, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton X-100), and 50 ng of *I. multifiliis* DNA to which ultrapure water was added to a final volume of 25 µl.

The LAMP reaction was evaluated with gradient constant temperatures of 56, 58, 60, 62, and 64° C to establish the optimal temperature. The optimal temperature was then found to vary at 10, 20, 30, 40, 50, and 60 min for the incubation period. At the end of the incubation period, the LAMP reactions were terminated at 80° C for 2 min.

Confirmation of LAMP products

To test the corrected target, the specific restriction sites of the LAMP products were selected with the use of a NEB cutter V2.0 (http://nc2.neb.com/NEBcutter2/). The reaction was then achieved by selection of the restriction enzyme *Alu*I targeting 5′-AG/CT-3′ at the restriction site. LAMP products were digested using restriction enzymes *Alu*I, *Ban*I, and *Apa*I (New England Biolabs® Inc., UK). The restriction enzymes were kept at 37°C for 3 hrs. followed by electrophoresis in 1.5 % agarose gel.

Sensitivity and specificity test of LAMP and PCR

In order to evaluate the sensitivity of LAMP, reactions were performed under the optimal conditions described in the above studies. LAMP results were compared to conventional PCR results. For the detection limits of the study, genomic DNA of *I. multifiliis* were diluted in 10-fold serial dilutions (ranging from 50 ng to 50 ag) and used as a template at 2μL under the same conditions.

To evaluate the degree of specificity, four different external parasites that are commonly found to have infected cyprinid fish were used, namely *Argulus* sp., *Dactylogyrus* spp. *Gyrodactyrus* spp, and *Trichodina* sp.

Detection of PCR and LAMP products

PCR products and LAMP products were evaluated using gel electrophoresis on 1.5% (w/v) of agarose gel and stained with 0.05 μl mL^{-1} of RedSafe™ (iNtRON Biotechnology Inc., Korea) in 1x TAE buffer (pH 8.0). Furthermore, 100 bp marker ladder was used as the DNA molecular weight standard control. A ladder-like pattern would be shown to indicate a positive sample in the LAMP reaction.

Detection and visualization of I. multifiliis DNA with heating blocks

Various DNA concentrations obtained from *I. multifiliis* were used to establish field applicability. LAMP reactions were incubated in a simple machine or a heating block under appropriate conditions of the LAMP assay. Visual assessment of the LAMP reaction was achieved by adding 0.5 μL of the diluted SYBR® Green I (Invitrogen®, CA, USA) under UV transillumination.

RESULTS

Optimization of LAMP conditions

To establish the optimized conditions of LAMP, optimal concentrations of FIP, BIP, dNTP, and Betaine, along with the optimal temperature of the LAMP reaction, were used to produce the desired results in 1.5% agarose gel. The optimal amount of LAMP products was found markedly at 1.6 μM of FIP and BIP primers (Figure 2A), at 0.6 mM of dNTP (Figure 2B), and at 1 M of the Betaine concentration (Figure 2C). The optimum temperature of this assay was 64°C (Figure 2D).

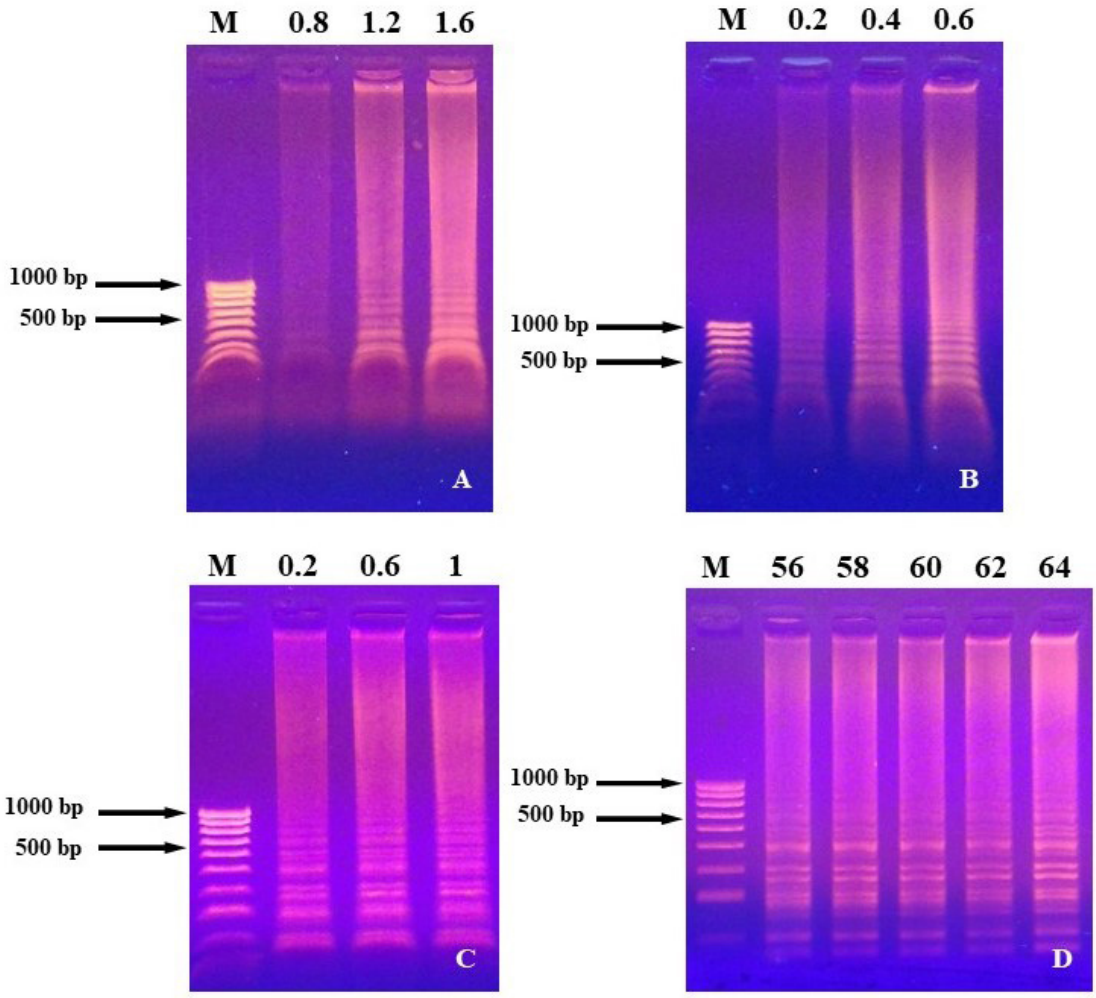

Figure 2 Optimization of LAMP assay with various concentrations of chemicals. (A): Effect of FIP and BIP primers; (B): Effect of dNTP; (C): Effect of betaine; and (D): Effect of various temperatures on LAMP reactions. Lane M: DNA marker and Lane N: ultrapure water.

Confirmation of LAMP products

To confirm the specifications of the reaction, the LAMP product was analyzed by AluI restriction enzyme digestion and the results indicated predicted sizes of 95 bp on 1.5% of agarose gel, otherwise the *Ban*I and *Apa*I restriction enzyme could not digest the LAMP product and resulted in a ladder-like form on gel electrophoresis (Figure 3).

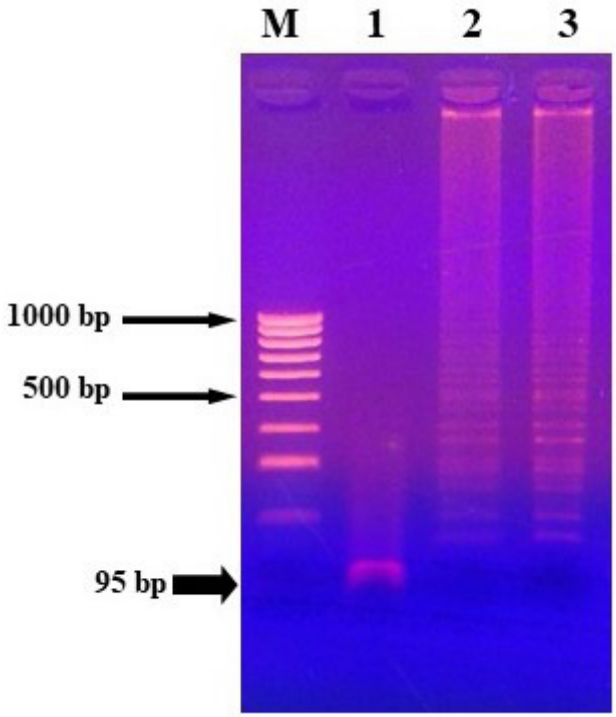

Figure 3 LAMP products digested by restriction enzyme. Lane 1-3: LAMP products were cut by *Alu*I, *Ban*I and *Apa*I, respectively.

Sensitivity and specificity of LAMP and standard PCR

To establish sensitivity or detection limits, the results of the LAMP assay were achieved at 5 pg of *I. multifiliis* DNA (Figure 4), whereas the results of the conventional PCR were achieved at 50 pg of the template DNA concentration.

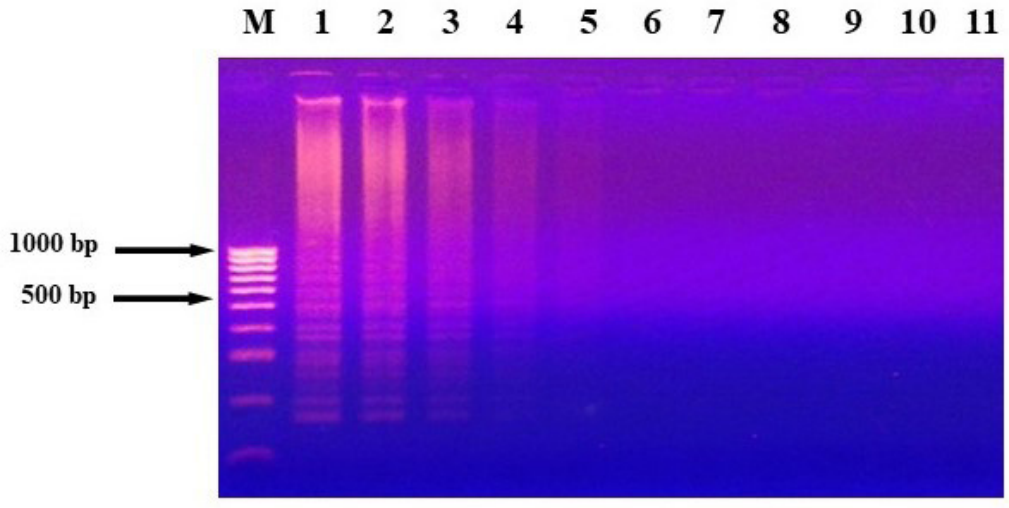

Figure 4 Detection limits of the LAMP reaction used to detect *I. multifiliis* DNA. Lane M: DNA marker; Lane 1: 50 ng; Lane 2: 5 ng; lane 3: 0.5 ng; Lane 4: 50 pg; Lane 5: 5 pg; Lane 6: 0.5 pg; Lane 7: 50 fg; Lane 8: 5 fg; Lane 9: 0.5 fg; Lane 10: 50 ag; and Lane 11: Negative control.

To evaluate the degree of specificity, the LAMP and PCR method were used to correctly identify *I. multifiliis* DNA, otherwise no amplification products were identified from any other ectoparasites of fish and the negative control. The results are shown in Figures 5A and 5B.

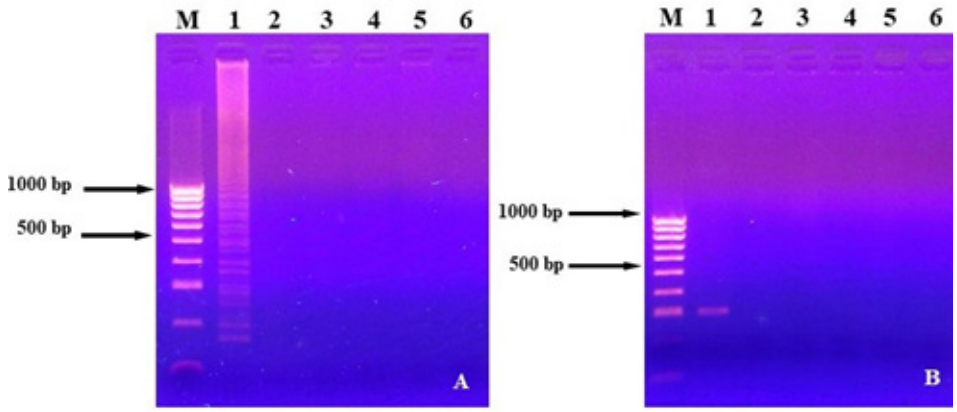

Figure 5 Specificity of the LAMP assay (A) and PCR method (B). Lane M: DNA marker; lane 1: *I. multifiliis* DNA; lane 2: *Argulus* DNA; lane 3: *Dactylogyrus* DNA; lane 4: *Gyrodactyrus* DNA; lane 5: *Trichodina* DNA and lane 6: negative sample.

Detection and visualization of *I. multifiliis* DNA with heating blocks

For the purposes of visualization, SYBR-Green I was added to the LAMP amplification tubes for fluorescence visualization detection. A yellowish green color with SYBR Green I was indicative of as a positive reaction when compared with the negative sample (Figure 6).

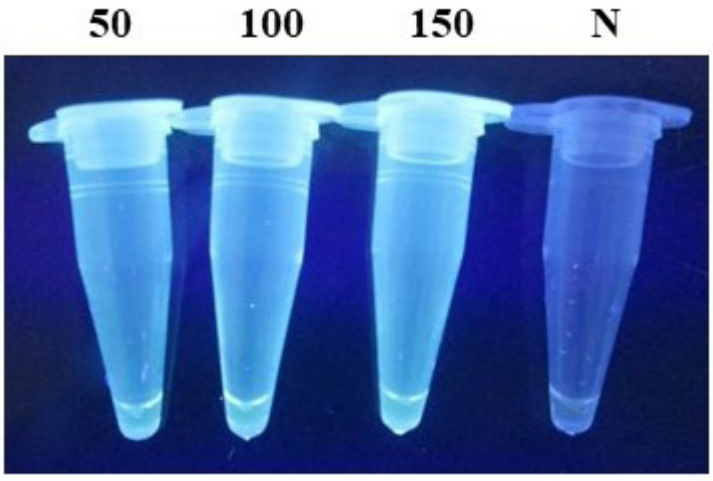

Figure 6 Detection of LAMP products by SYBR green I dye under UV light at various concentrations of DNA; 50, 100, and 150 ng. N: negative.

DISCUSSION

Ichthyophthirius multifiliis is one of the most important protozoan parasites of freshwater fish due to its high degree of virulence and that fact that it is capable of killing a large number of hosts (Dickerson, 2006; Noga, 2010). An important clinical sign of *I. multifiliis* infection is the presence of small white spots or salt grains on the skin or gills (Matthews, 2005). At the early stage of infection, the white spots may not be as obvious on fish to the naked eye; however, if the infected fish are not treated, they may become severely infected (Dickerson, 2006). Moreover, although there are limitations to gross observations with other ectoparasitic infestations, such as *Amyloodinium* sp., observations may be used to identify similar clinical signs of *I. multifiliis* infection (Noga, 2010). Therefore, if farmers or fish keepers can detect this parasite before clinical signs appear, disease outbreaks can be prevented. In effective veterinary practice, the routine diagnosis of *I. multifiliis* infection is established by the presence of mature trophonts with pleomorphic shape and horseshoe-shaped macronucleus on the skin or gills of infected fish: thus, subclinical infections often go undetected.

At present, certain molecular based approaches have been developed for *I. multifiliis* detection such as real-time PCR (Jousson, 2005) and PCR (Fariya et al. 2016). In addition, PCR was developed for effective detection along with small subunit ribosomal DNA (ssrDNA) of *I. multifiliis* in environmental samples (Howell et al., 2019). Nevertheless, the above-mentioned molecular techniques are time consuming, difficult to handle, and may require the use of expensive instruments. These factors perhaps have limited their application for diagnostics in field studies. The LAMP method was developed by Notomi et al (2000). This technique is a molecular based form of diagnosis that employs amplified DNA by using the *Bst* DNA polymerase under constant temperature (Picón-Camacho et al. 2013; Kang et al., 2015). To our knowledge, no previous reports have been published on the use of LAMP to detect *I. multifiliis* in fish.

In the current report, LAMP techniques targeting the 18S ribosomal RNA gene have been established for rapid detection. These techniques are considered relatively convenient and reliable in the detection of *I. multifiliis* infection in freshwater fish. The critical steps of the LAMP techniques include optimal temperature, amplification time, and various concentrations of reagents (Tomita et al., 2008). Our study demonstrated that LAMP required 1.6 µM of FIP and BIP primers, 0.2 µM of F3 and B3, 2 mM of $Mg2^{+}$, 1 M of Betaine, 0.6 mM of dNTP and an optimal temperature of 64°C after a 40 min period of incubation. The LAMP primers of our study were found to be very specific to *I. multifiliis* and no amplifications were found with other common ectoparasites of freshwater fish. The development of the LAMP assay to detect a pathogenic dinoflagellate parasite, or *Amyloodinium ocellatum* (*A. ocellatum*), in marine fish also verified the specificity of *A. ocellatum* DNA when compared to closely related-dinoflagellate species (Picón-Camacho et al., 2013). In addition, the results of restriction enzyme digestion with *Alu*I digestion has further confirmed that the target gene was effectively amplified. All these results suggest that this LAMP assay was strictly specific for *I. multifiliis*.

Furthermore, the present study has demonstrated that the sensitivity of LAMP was less effective than conventional PCR in the detection of DNA. This outcome corresponds to the findings of a previous report involving LAMP as compared to the standard PCR (Tao et al. 2011; Picón-Camacho et al., 2013; Chupia et al., 2016). In addition, some studies have shown that the sensitivity and specificity of LAMP was practically in agreement with real-time PCR assays (Dokphut et al., 2021). The low detection level of LAMP assay suggests that this technique could successfully detect subclinical infections that are present not only on fish but also from their environment. For the purposes of visualization, we used SYBR green I, a fluorescent dye, which binds to the minor grooves of double strand DNA and there is then no need for detection LAMP products via gel electrophoresis. However, a limitation of LAMP is its strong reliance on indirect detection methods, such as turbidity or non-specific dyes, which can often produce false positive reactions due to cross contamination (Gadkar et al., 2018; Savan et al., 2005). LAMP assay can be performed with simple incubation instruments, such as a water bath or heating block, for isothermal heating (Tomita et al., 2008; Chen et al., 2016) Furthermore, our LAMP assay can be effectively performed on a heating block; thus, the cost per reaction of LAMP would be lower than the cost of other molecular techniques. Finally, we have presented a rapid detection system for *I. multifiliis* based on isothermal DNA amplification. This system could be employed as an alternative diagnostic tool instead of other molecular methods in the detection of *I. multifiliis* infection.

CONCLUSION

In conclusion, we have successfully established a LAMP technique to identify the parasitic ciliate *I. multifiliis* in freshwater cyprinid fish. LAMP displays a better degree of sensitivity with a lower limit of detection than standard PCR. This can be a desirable choice in providing traditional clinical diagnosis, such as microscopic examinations, and does not require expensive equipment in comparison with the other PCR based molecular approaches. Furthermore, we propose that this system can be applied as a powerful tool to monitor or achieve early diagnosis of *I. multifiliis* infection in freshwater cyprinid fish, as well as in other freshwater fish species

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AUTHORS' CONTRIBUTIONS

Conceptualization, S.P.; methodology, formal analysis, investigation and writing- original draft preparation, S.P. and P.P.; writing -review and editing, project administration and funding acquisition, S.P. All authors have read and approved of the final version of the manuscript before submission to the journal.

CONFLICT OF INTEREST

The authors declare that no conflict of interest.

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