

Detecting *Fasciola hepatica* and *F. gigantica* microRNAs using loop-mediated isothermal amplification (LAMP)

Tran Hong Diem, Phung Thi Thu Huong*

Nguyen Tat Thanh Hi-Tech Institute, Nguyen Tat Thanh University

*ptthuong@ntt.edu.vn, thdiem@ntt.edu.vn

Abstract

Fascioliasis is a parasitic infection typically caused by two common parasites of class Trematodo, genus *Fasciola*, named *Fasciola hepatica* and *F. gigantica*. The widespread appearance of these species in water and food makes fascioliasis become a global zoonotic disease that affects 2.4 million people in more than 75 countries worldwide. Typically, *F. hepatica* and *F. gigantica* can be recognized with parasitological techniques to detect *Fasciola* spp. eggs, immunological techniques to detect worm-specific antibodies, or molecular techniques for instance polymerase chain reactions to detect parasitic genomic DNA. Recently, miRNAs have been recognised a key regulator and potential diagnostic biomarkers of diseases, including parasitic infection. An isothermal PCR called LAMP (loop-mediated isothermal amplification) is rapid, sensitive, and this amplification is very extensive, making it well-suited for field diagnostics. LAMP reaction for miRNA detection has been introduced and is able to detect miRNA in the range between 1.0amol and 1.0pmol, showing high selectivity to differentiate one miRNA sequence from others. Here, we introduced a modified LAMP to detect a typical miRNA of both *F. hepatica* and *F. gigantica*. Our method does not demand an initial heating step and the reactions have a high sensitivity even 1,000 times higher in comparison to that reported in previous studies. These results create a promising technique basis for some novel and simple device to diagnose fascioliasis and other parasitic diseases at point-of-care.

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1 Introduction

Fascioliasis, a parasitic infection, is one of the major neglected tropical diseases caused by flatworms *Fasciola hepatica* and *F. gigantica*, two species of trematodes that mainly affect the liver. They are also known as “the common liver fluke”[1]. Fascioliasis is waterborne and foodborne zoonotic disease in which human are incidental hosts and get infected by consuming contaminated watercress or water[1-3]. This disease is found in all five continents, in over 75 countries and infects at least 2.4 million people worldwide[4]. As the result, fascioliasis diagnostic methods have always been of interest and on improvement. Normally, the infection confirmation is abided by different ways of diagnostic techniques. The typical criteria to confidently confirm a person is infected with *Fasciola* spp. is by observing the parasite[2]. This

parasitological technique is set up to find *Fasciola* spp. eggs in feces specimens[2]. However, it can be hard to search for eggs in stool specimens from patients with light infections. Thus, the infection has to be diagnosed by alternative methods rather than by examining stool samples[2]. Specific and sensitive molecular diagnostic methods, including polymerase chain reactions (PCR), enzyme-linked immunoelectrotransfer blot (EITB), and enzyme-linked immunosorbent assay (ELISA), have been developed for fascioliasis[2,4]. However, these tests require advanced skills and equipment that is not available in resource-limited settings, especially in isolated areas where the disease is widespread.

Recently, the discovery of microRNAs (miRNAs), a short non-coding RNA molecular that has about 21-25 nucleotides of length in eukaryote cells, has expanded our understanding of the pathogens’ mechanisms[5], and has



created new changes for developing novel techniques to detect them. Clearly, miRNAs play a pivotal role in regulating pathogen gene expressions with a variety of manners[5-8]. The presence of miRNAs in serum has been proven to be an important biomarker for the diagnosis of certain diseases such as viral infections, cardiovascular and nervous system disorders, and diabetes[5]. The interest in the role of small RNAs in parasitic infections has been rapidly growing currently. Importantly, miRNAs are identified as one of the key regulators in nematode development[9]. Parasitic circulating miRNAs has been shown to be detected in the biological fluids of infected hosts, such as serum, saliva and others[10-15].

The extreme stability of the secret miRNAs is believed to be due to their release within micro-vesicles or exosomes or by forming complex with special protein[13]. Studies on *Heligmosomoides polygyrus*'s excreted materials have proved that certain miRNAs excreted by parasites are covered in the extracellular vesicles[17]. Moreover, those parasitic miRNAs in the exosomes are also transported to host cells[17]. Exosome-like vesicles containing miRNAs are reported to be released from the infective L3 stage of the human filarial parasite *Brugia malayi*[18]. Importantly, release of exosomes derived from *F. hepatica* has also been demonstrated[19]. Despite the fact that there was no mutuality between the microfilariae number and miRNA quantity[20], the gathered information significantly demonstrates that the particular parasitic miRNAs present in the host circulatory system advantageously appear as non-invasive markers for the detection of specific infections. Furthermore, the detailed profiles of miRNAs expression of parasitic helminthes have recently been created, including fluke, nematodes, and tapeworms such as *F. gigantica* and *F. hepatica*[21, 22]. The research reports the comparison of miRNA expression profiles of *F. gigantica* and *F. hepatica* and shows that there are 11 miRNAs shared by the two kinds of worm, including 8 conserved and 3 novel miRNAs[22]. All the conserved miRNAs are the same as those from *Schistosoma japonicum* in the miRBase database. Besides, 8 and 5 miRNAs were identified as *F. gigantica*- and *F. hepatica*-specific, respectively[22].

detecting miRNAs is challenging because they are short and highly homologous[23]. Different methods for detection of miRNAs have been developed including northern[24], reverse transcription PCR (RT-PCR)[12], microarrays and others; however, each method has its particular restrictions. Currently, different detection methods have been produced, such as isothermal exponential amplification-based methods, cleavage-based methods, rolling cycle amplification-based methods, AuNPs-based methods, quantum dot-based methods, capillary-electrophoresis-based assay[25]. A shared idea

between these recently created methods is the combination of multistep signal enhancement and sensitive signal detection to accomplish great cognitive efficiency. A loop-mediated isothermal amplification (LAMP) to detect specific miRNA has recently been introduced[25] (Fig. 1). LAMP can be accomplished with only one kind of DNA polymerase without requirement of any modified or labeled DNA probes to markedly decrease the cost and make the experimental procedure simpler. A conceivable disadvantage of the LAMP is the need of a template DNA, forward inner primer (FIP), backward inner primer (BIP), and backward outer primer B3[26]. However, LAMP reactions merely need little amount of primers and template, making this assay still cost-effective. Moreover, LAMP was demonstrated to be able to detect the target miRNA amounts in the range from 1.0amol to 1.0pmol, and shows marked selectivity to distinctly distinguish one-base difference among miRNA sequences [26]. However, LAMP reactions merely need little amount of primers and template, making this assay still cost-effective. Moreover, LAMP was demonstrated to be able to detect the target miRNA amounts in the wide range of 1.0amol to 1.0pmol, and displayed marked selectivity to distinctly distinguish one-base difference among miRNA sequences [26]. In this study, we have developed a modified LAMP method to sensitively and accurately detect the miRNA species-specific for *Fasciola* spp. By using this technique we achieved to detect specific miRNA of *F. hepatica* and *F. gigantica* at the amount of 1zmol in short time and simpler process at a constant temperature.

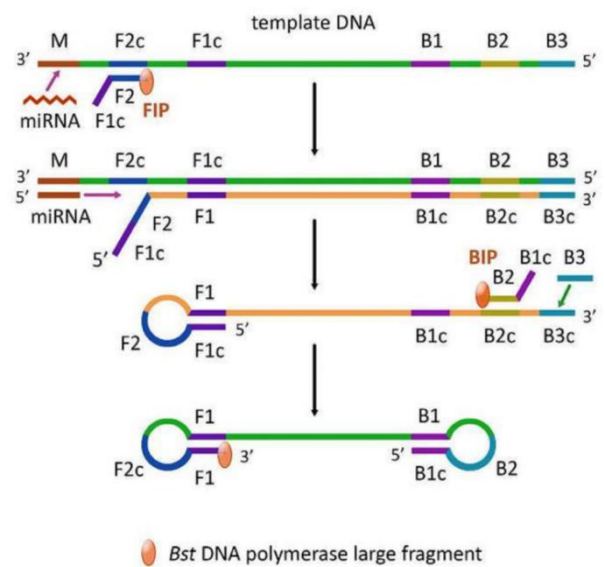


Figure 1 LAMP reaction initiated by the target miRNA (adopted from[26]). All the sequences of the DNA template, FIP primer, BIP primer, B3 primer and parasite miRNA are listed in Table 1.

2 Materials and Methods

2.1 Nucleotides, enzymes, and chemicals

The oligonucleotides used to perform LAMP reactions were synthesized commercially from IDT (Skokie, Illinois, USA). Isothermal Master Mix was purchased from OptiGene (Horsham, West Sussex, UK). Bovine serum was obtained from Sigma-Aldrich (St. Louis, Missouri, USA). Nucleic acid gel stain GelRed was provided by Biotium (Fremont, CA, USA).

2.2 The LAMP reaction

The LAMP reaction consisted of FIP, BIP, and B3 primers that were designed like previous[26]. The template was also inherited from the previous study[26] with a sequence modification which was complementary to the selected parasite miRNA. The RNA oligo which mimics the parasite miRNA was selected from the previous finding[22]. The oligonucleotides used to perform LAMP reactions are listed in Table 1. LAMP were performed in a reaction mixture (15µl) containing the indicated amount of miRNA and template, 6pmol of FIP and BIP, 0.5fmol of B3[26] and 9µl

of Isothermal Master Mix. Reactions were incubated at 60°C for 90 minutes (min). The LAMP products were then subjected to 1.5% agarose gel electrophoresis, and visualized by staining with GelRed and photographed under UV light.

3 Results

3.1 Selection of the species-specific miRNA of *Fasciola* spp. and designation of the LAMP reaction components

Based on the study establishing the miRNA expression profiles of *F. gigantica* and *F. hepatica* using an combined sequencing with bioinformatics approach and quantitative real-time PCR[22], the sequence of one *Fasciola* spp.-novel miRNA sharing between two kinds of worms was selected to serve as the biomarker for *Fasciola* spp. detection employing LAMP (Table 1). Also, we followed the LAMP components that were designed previously to conduct the LAMP reactions initiated by miRNAs[26].

Table 1 Oligonucleotides designed for LAMP reactions

No.	Nucleotide sequences (length in nt)	Name
1	5'-ACAACGTCGTGACTGGGAAAACCCCTTTTGTGCGGGCCTCTTCGCTATTAC-3' (51)	FIP
2	5'-CGACTCTAGAGGATCCCCGGGTACTTTTGTGTGGAATTGTGAGCGGAT-3' (52)	BIP
3	5'-ACTTTATGCTTCCGGCTCGTA-3' (21)	B3
4	5'-CTTTATGCTTCCGGCTCGTATGTTGTGGAATTGTGAGCGGATAACAATTCACACAG GAAACGTACCCGGGATCCTCTAGAGTCGACCTGCAGGCATGCAAGCTTACAACGTCGT GACTGGGAAAACCCCTGCGCTTACCCAACTTAATCGGTAATAGCGAAGAGGCCCGCACAA ACAGCTGTACAGTGCTTCCA-3' (199)	Template
5	5'-UGGAAGCACUGACAGCUGUUUU-3' (23)	miRNA-novel-shared-03

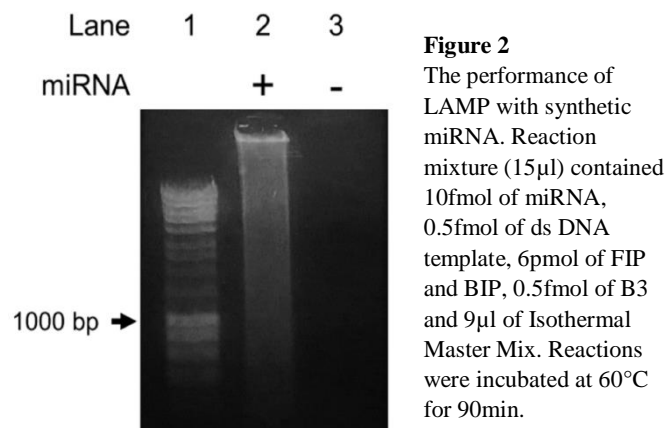
3.2 Performance of LAMP with synthetic miRNA

In this study, we used the synthetic RNA oligo to serve as miRNA specific for *Fasciola* spp. (Table 1). LAMP master mix was commercially provided by OptiGene (Horsham, West Sussex, UK). The LAMP reaction included 0.5 fmol of double-stranded (ds) DNA template, 6.0pmol of FIP and BIP primers, and 0.5fmol of B3 primer[26]. The amount of

synthetic miRNA used was 10fmol. Reactions were performed at 60°C for 90min. The results show that only in the presence of miRNA, LAMP product of different size segments formed a long smear when analyzed on gel electrophoresis (Fig. 2 lane 1). As expected, when miRNA was absent, the product cannot be observed (Fig. 2. lane 2). These data prove that positive signal of the LAMP reaction specifically corresponds to the presence of miRNA in the sample.

3.3 The LAMP reactions with double-stranded and single-stranded DNA templates

Although the LAMP reactions to detect the presence of specific miRNA was performed efficiently as reported previously, the use of ds DNA template required the need of a first heating step for a period of two to four minutes at 96 – 98°C to split the two circuits of DNA. LAMP utilizes only one enzyme *Bst* DNA polymerase which also possesses RNA polymerase (using a DNA template) and strands displacement activities. Hence, it is expected that



without the pre-heating step, the LAMP reactions should still occur. However, we found that using ds DNA template without heating first, the reaction cannot succeed (data not shown). Accordingly, this step leads to the conduct of experiments more complex and may be a constraint to future technical development at field study. With the aim of producing a simpler reaction preparation process, we utilized a single-stranded (ss) DNA template instead of the ds one. To prove that this modification does not affect the LAMP efficiency, the LAMP reactions were performed with two forms of DNA template and revealed that the efficiency of reactions is similar between two types of DNA template used (Fig. 3). Importantly, by using ss DNA template, the LAMP reactions could occur without the requirement of heat-up step which can interfere with the activity of other components in the reactions due to high temperature. Taken together, we demonstrated that the modification of using ss DNA instead of ds DNA template in the LAMP reactions led to the similar results with a marked advantage of the removal of the pre-heating step, enabling reaction preparation less complicated and quicker.

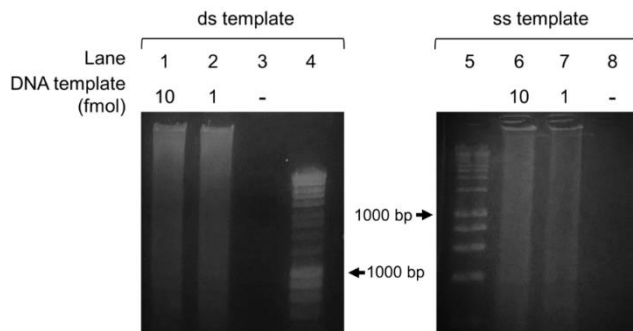


Figure 3 The LAMP reactions with different concentrations of ds and ss DNA templates. The reaction mixtures contain the indicated amount of ds and ss DNA template, 10fmol of miRNA, 6pmol of FIP and BIP, 0.5fmol of B3 and 9µl of Isothermal Master Mix.

3.4 Sensitivity of the LAMP reactions using single-stranded DNA template

The LAMP sensitivity is one of the most important factors which decide the success of the method and its possible applicability in field study. As mentioned above, LAMP utilizing ds DNA template was shown to be capable of identifying the target miRNA in the ultrasensitive range of 1amol to 1pmol[26]. In our hand, the results were revealed the same where ds-DNA-template LAMP reactions were succeeded at the lowest amount of 1amol of synthetic miRNA (Fig. 4, lanes 2 to 5). Markedly, the modified LAMP reactions with ss DNA template perform efficiently at the significant lower amount of miRNA, up to 1zmol (Fig. 4, lanes 7 to 10). These data strongly prove the superiority of ss DNA template given in our design in

comparison to the previous one[26] regarding the complexity of reaction preparation, time, and sensitivity.

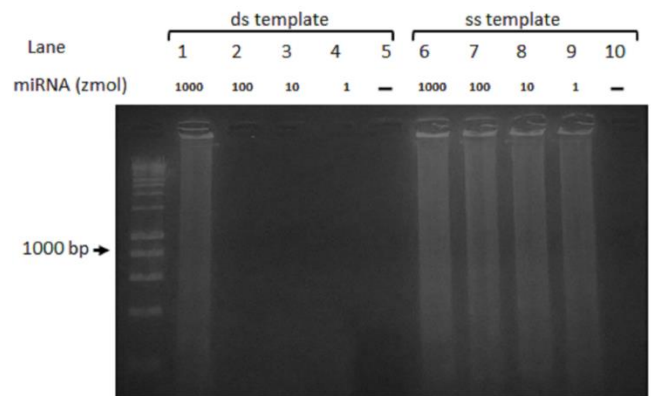


Figure 4 The sensitivity of LAMP reaction to miRNA amount. Reaction mixture contained the indicated amount of miRNA, 1fmol of ss DNA template, 6pmol of FIP and BIP, 0.5fmol of B3 and 9µl of Isothermal Master Mix.

4 Discussion

Today, one of the most important missions in managing and monitoring of neglected tropical diseases is to produce highly sensitive and proper diagnostic methods which can replace the laborious and undependable procedures. Specific and sensitive techniques to detect the early stages of *Fasciola* spp. infections can preclude irreversible pathological reactions, helping monitor and likely directing the basis for treatment failures. Fasciolosis is often popular in low-resource regions without proper laboratory equipment, thus, low-cost methods for practical diagnostics that do not need centralized laboratories are significantly required. Accordingly, researching new biomarkers for fast and accurately detecting the pathogen is highly demanded, and that can create new simpler and more appropriate techniques to diagnose diseases.

The LAMP method was used to detect miRNA in previous studied[26]; however, the results show some technical limitations. One of the biggest restraints is the need for an initial heating step at high temperatures to separate the two circuits of the ds DNA template. The heat-up step can affect the enzyme activities as well as the other reaction components. Also, this step makes reactions preparation and control became more difficult. In our study, we changed the ds DNA to ss DNA template and hence, the reactions can occur without the initial heat-up step. The ability to allow LAMP reactions to be assembled at room temperature and initiated at only one constant temperature can offer an excellent advantage in resource-limited settings. Furthermore, our LAMP reactions also provide the high level of sensitivity required for diagnosis. When investigating a limited range of detectable miRNA levels,

we succeeded in detecting as low as 1zmol of the targeted miRNA. Compared with the previous report in which the minimum miRNA level detected was 1amol[26], our results demonstrated that the sensitivity of modified LAMP was 1,000 times higher. This remarkable improvement in

sensitivity significantly increases the probability and applicability of this method in real life.

Conflict of Interest

The authors declare that there is no conflict of interest.

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Phát hiện microRNA của sán lá gan bằng phương pháp khuếch đại đẳng nhiệt trung gian vòng lặp (LAMP)

Trần Hồng Diễm, Phùng Thị Thu Hương*

Viện Kỹ thuật Công nghệ cao Nguyễn Tất Thành, Đại học Nguyễn Tất Thành

*ptthuong@ntt.edu.vn, thdiem@ntt.edu.vn

Tóm tắt Sán lá gan lớn là một bệnh phổ biến gây ra bởi hai loại ký sinh trùng thuộc lớp Trematodo, chi *Fasciola*, là *Fasciola hepatica* và *F. gigantica*. Sự có mặt rộng rãi của hai loài này trong nước và thực phẩm làm cho bệnh sán lá gan lớn trở thành một trong những bệnh truyền nhiễm từ động vật sang người phổ biến nhất, ảnh hưởng đến 2,4 triệu người thuộc hơn 75 quốc gia trên thế giới. Thông thường, *Fasciola hepatica* và *F. gigantica* có thể được phát hiện bằng các phương pháp miễn dịch (phát hiện kháng thể đặc hiệu của ký sinh trùng), kỹ thuật ký sinh trùng để phát hiện trứng sán, hay sử dụng kỹ thuật sinh học phân tử để phát hiện DNA đặc trưng của ký sinh. Trong thời gian gần đây, miRNA đã được nghiên cứu công nhận là dấu chứng chẩn đoán sinh học nhiều tiềm năng đối với các bệnh, trong đó có các bệnh gây ra bởi ký sinh trùng. Mặt khác, phương pháp khuếch đại đẳng nhiệt trung gian vòng lặp – LAMP là một phương pháp khuếch đại nhanh nhạy ở một nhiệt độ cố định. Với giới hạn phát hiện lớn, LAMP trở thành phương pháp phù hợp cho chuẩn đoán nhanh tại vùng nhiễm. Kỹ thuật LAMP đã được chứng minh có khả năng phát hiện miRNA mục tiêu trong phạm vi từ 1amol đến 1pmol. Nghiên cứu này giới thiệu kỹ thuật LAMP được sửa đổi để phát hiện một loại miRNA đặc trưng cho cả hai loài *Fasciola hepatica* và *F. gigantica*. Phương pháp này không yêu cầu bước gia nhiệt ban đầu và có độ nhạy gấp 1.000 lần so với các nghiên cứu trước đó. Kết quả này tạo nên tiền đề quan trọng cho việc phát triển các thiết bị kỹ thuật mới, đơn giản, nhanh, tại chỗ cho chuẩn đoán bệnh sán lá gan cũng như các bệnh nhiễm ký sinh trùng khác.

Từ khóa sán lá gan lớn, LAMP, miRNA