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EARLY DETECTION OF *TRICHINELLA SPIRALIS* DNA IN RAT FECES BASED ON TRACING PHOSPHATE IONS GENERATED DURING LOOP-MEDIATED ISOTHERMAL AMPLIFICATION

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KEY WORDS

Trichinella spiralis
LAMP
1.6-kb repetitive element
Fecal samples

ABSTRACT

Early diagnosis of trichinellosis is still difficult because of the lack of specific symptoms and limited window for serological detection. Here we established an assay based on tracing phosphate ions generated during loop-mediated isothermal amplification (LAMP) to detect *Trichinella spiralis* DNA in rat feces during its early stage of infection. By targeting a 1.6-kb repetitive element of *Tri. spiralis*, the assay was able to detect *Tri. spiralis* DNA in the feces of all infected rats as early as 1 day postinfection (dpi). The positive detection lasted to 7 dpi in the rats infected with 250 muscle larvae, and 21 dpi in the rats infected with 5,000 larvae. The assay was highly sensitive, and could detect 1.7 femtograms (fg) of *Tri. spiralis* DNA with high specificity, and with no cross reactivity with the DNA from *Anisakis pegreffii*, *Gnathostoma spinigerum*, *Angiostrongylus cantonensis*, *Enterobius vermicularis*, *Schistosoma japonicum*, and *Trypanosoma evansi*. Our present study provided a reliable technique for the early diagnosis of trichinellosis with the advantages of simplicity and speed, as well as high sensitivity and specificity.

Trichinellosis is caused by the consumption of *Trichinella*-infected raw or undercooked meat of various kinds of animals (Pozio, 2015). The disease is a public health problem, not only in human infection but also in an economic loss of porcine production and food safety (Dupouy-Camet, 2009). Parasite nematodes of the genus *Trichinella* are distributed worldwide with 9 species and 3 genotypes (Pozio and Zarlenga, 2013). Among these species, *Trichinella spiralis* is the most common species that could infect pigs, horses, rats, and many carnivores (Pozio and Murrell, 2006). Surveillance and diagnosis of *Trichinella* infection using rapid, sensitive, and accurate diagnostic tools to detect *Trichinella* in wildlife hosts are necessary for the evaluation of the prevalence and risk of transmission from wildlife to humans. The diagnosis relies largely on the serodiagnostic procedures, which are of great value but unfortunately miss the enteric phase (Gamble et al., 2004). Because of the limited detection window, conventional methods cannot detect anti-*Trichinella* IgG antibodies in the several weeks of the early stage of infection or result in a high rate of false-negative results (Bruschi et al., 1990; Liu et al., 2013). This causes a serious diagnostic problem at an early stage in the absence of corresponding epidemiological data, typical symptoms, and pathognomonic signs of the disease.

Molecular detection methods such as polymerase chain reaction (PCR) and real-time PCR are alternative techniques offering both high sensitivity and specificity, and have been used to detect *Trichinella* DNA in blood (Li et al., 2010), feces (Golab et al., 2009; Liu et al., 2017) and muscle (Guenther et al., 2008). In the past decade, the use of PCR has resulted in a significant improvement in both *Trichinella* taxonomical identification (Tantrawatpan et al., 2012) and early detection of experimentally infected animals (Tantrawatpan et al., 2013) or naturally infected wildlife (Cuttell et al., 2012). Real-time PCR not only can quantify *Trichinella* in biological samples, but also has superior sensitivity. Despite these advances, diagnosis of *Trichinella* infection remains unsatisfied because PCR-based assays have not yet attained a sufficient level of sensitivity, and are limited due to expensive equipment and long reaction times.

Loop-mediated isothermal amplification (LAMP) is one of the nucleic acid tests used in various fields, including diagnosis and identification of infectious organisms. This assay uses *Bst* polymerase, which has displacement activity and a set of 4 specially designed primers that recognize a total of 6 distinct sequences of the target DNA (Notomi et al., 2000). It has been used to detect parasitic DNA with high sensitivity and specificity, such as *Plasmodium* (Iseki et al., 2010), *Trypanosoma* (Wastling et

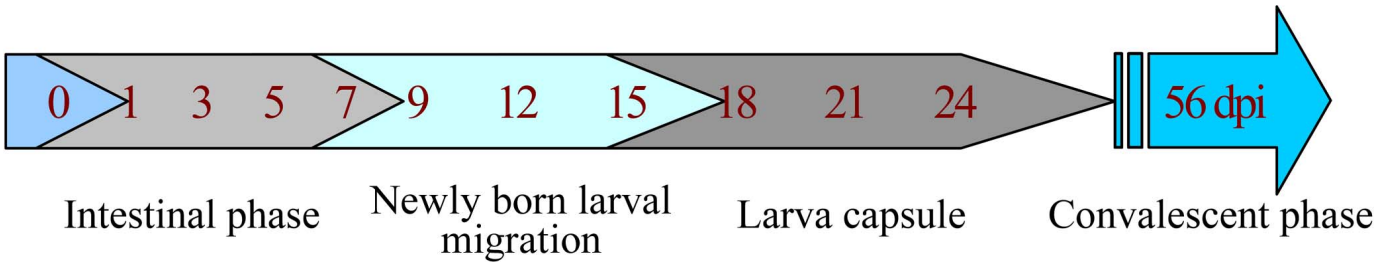

Figure 1. *Trichinella spiralis* life-cycle stages relative to experimental time points postinfection (days postinfection). Color version available online.

al., 2010), *Schistosoma japonicum* (Tong et al., 2014), *Toxoplasma gondii* (Kong et al., 2012) and *Angiostrongylus cantonensis* (Chen et al., 2011). LAMP assay has been tried to detect *Tri. spiralis* larvae in muscles by targeting a 1.6-kb repetitive element of the *Trichinella* genome (Li et al., 2012) and mitochondrial large subunit ribosomal DNA in experimentally infected animals (Lin et al., 2013). However, the effects of LAMP on such detection of the infectious dose of *Trichinella* parasites and of the duration of the postinfection period have not been ascertained. And also, there is a high risk of aerosol contamination because of the large amount of LAMP products. In the present study, we developed the sensitive and simple assay based on tracing phosphate ions (Pi) generated during LAMP and used a closed device to avoid aerosol contamination. Diagnostic validity assessment of this assay in rat feces was evaluated, with the aim of providing a potential tool for rapid detection of *Tri. spiralis* DNA in early stage of infection.

MATERIALS AND METHODS

Parasites and animals

The parasite *Tri. spiralis* (ISS413) used in the study was maintained in rats by serial passages in the laboratory at the Zhejiang Provincial Experimental Animal Center, Zhejiang Academy of Medical Sciences, Hangzhou, China. Muscle larvae (ML) were collected from the infected rat muscles by the digestion of carcasses with 1% pepsin (Activity, 3,000–3,500 [National Formulary Unit] NFU/mg, Sangon Biotech Co., Ltd., Shanghai, China) and 1% hydrochloric acid according to Forbes et al. (2003).

Infection of rats, collection of fecal samples, and DNA extraction

Twelve Sprague-Dawley rats (250 g weight) were divided into 4 groups (A–D) with 3 rats each at random, which were infected with 0, 250, 2,500, or 5,000 ML of *Tri. spiralis* by oral gavage, respectively. The parasite infection dose in rats used in this study was consistent with other published studies (Bell et al., 1987; Fenwick et al., 1990). Rats were fed with a standard granulated feed and water and each of them was kept independently in a metabolic cage. Fecal samples from each cage were collected at 1, 3, 5, 7, 9, 12, 15, 18, 21, 24, and 56 days postinfection (dpi), as shown in Figure 1. The DNA was extracted from 200 mg of fecal samples, that is, approximately 5% of the daily fecal output of each infected rat feces by using QIAamp DNA Stool Mini Kit (QIAGEN, Germantown, Maryland) according to the manufacturer's instructions. Genomic DNA of *Tri. spiralis* and other parasites were extracted using DNeasy Blood & Tissue Kit (QIAGEN) according to the manufacturer's instructions. The purified DNA was dissolved in 80 µl of double-distilled water and 2 µl of the resulting supernatant was used as the template for the subsequent detection.

Table I. Specific loop-mediated isothermal amplification (LAMP) primers used for specific detection of *Trichinella spiralis*.

Target	Primer	Sequence (5′–3′)
1.6-kb repetitive element	T.s-F3	GCCATGTCTGTAACACCAG
	T.s-B3	AGTATTACATTCCATAGAGAGGA
	T.s-FIP	CAAACCGCTCATATTCGTTAGGGTA AATACTCGTGAAGCTTTGCT
	T.s-BIP	AAGGGTTCTTCTTACCAATTTTCCG ACCATTACCAAACATGTGTG
	T.s-LF	CGCTGTGAAATGCAATG
	T.s-LB	TTGTACCTGTGTTTCTCCG

Plasmid construction and control samples

The sequence for the 1.6-kb repetitive element (GenBank: X06625.1) was amplified by PCR, using the following primers: forward: 5′-CTTGAAATTGGTGGAGCCTCT-3′; reverse: 5′-CTCGAGTCAGAGGAACTCTGGCGTCT-3′. The amplicon was cloned into a vector named as pMD19-T-RE for sequencing. The DNA extracted from the fecal samples of uninfected rats was used as negative control. Two sets of positive-control samples were prepared. The first set consisted of recombinant plasmid pMD19-T-RE dilutions ranging from 17 pg to 0.17 femtograms (fg) in DNA isolated from healthy rat feces. The other set was prepared as simulated samples. In brief, 1 g of feces from healthy rats muscle together with 30 *Tri. spiralis* larvae were homogenized with a glass tissue grinder and 200 mg of homogenate was used for DNA extraction. Simulated samples with concentrations that ranged from 30 to 0.003 larvae into 1 g of feces were prepared by dilution of the purified DNA with double-distilled water.

Detection assay based on LAMP

Based on the sequence of the 1.6-kb repetitive element (GenBank: X06625.1) of *Tri. spiralis*, a primer set for use in the LAMP was designed using Primer Explorer V4 (http://pri-merexplorer.jp/elamp4.0.0/index.html). The primer sequences are listed in Table I. The procedures for the LAMP methods were carried out according to the description by Kong et al. (2012). The closed device was used as previously described (Tong et al., 2018). In brief, the 25-µl reaction mixture in tube R1 of the closed device containing 12.5 µl of 2× reaction mix, 5 pmol of each the F3 and B3 primers, 40 pmol of each the FIP and BIP primers, 20 pmol of

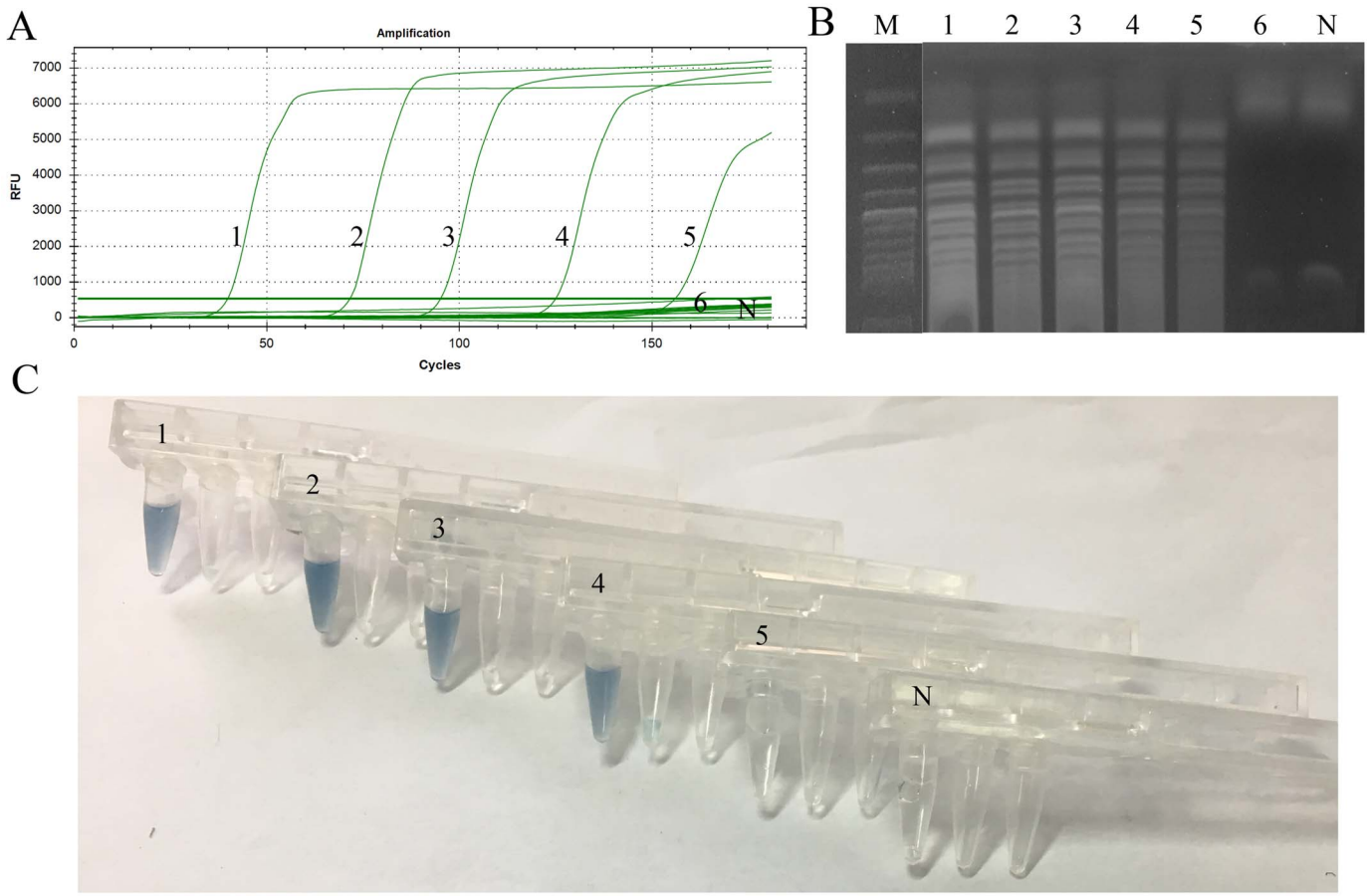

Figure 2. Sensitivity test of loop-mediated isothermal amplification (LAMP) for the detection of *Trichinella spiralis*. (**A**) Detection limit of the real-time LAMP. Lanes 1–7 represent 17 picogram (pg), 1.7 pg, 170 femtogram (fg), 17, 1.7, and 0.17 fg, and negative control. (**B**) Detection limit of *Tri. spiralis* by tracing Pi generated during LAMP. Lanes 1–6 represent 1.7, 170, 17, 1.7, and 0.17 fg and negative control. Color version available online.

each the LP and LF primers, 1 µl of PPase (New England Biolabs, Beijing, China), 1 µl of Bst 2.0 WarmStart®DNA polymerase (New England Biolabs) and 2 µl of DNA template. Meanwhile, 4 µl of acidic molybdate and potassium antimonyl tartrate solution (21 mM ammonium molybdate, 2 mM potassium antimonyl tartrate, 5 M sulfuric acid) with 106 µl ddH_2O were added in tube R2, 2 µl of 10% ascorbic acid with 68 µl ddH_2O were added in tube R3. The closed device was then incubated in a normal water bath at 65 C for 30 min. At the end of LAMP reaction, solutions in tube R2 and R3 were mixed with LAMP products in tube R1 by inclining the closed device. Positive reactions would turn into dark molybdenum blue, and negative reactions remain colorless. For agarose gel electrophoresis analysis, all positive LAMP reactions produced a typical ladder of multiple bands on the 1.5% agarose gel stained with GelRed™ (Biotium Inc., Hayward, California). The same primers were used in real-time LAMP, which was carried out in a 25-µl reaction mixture contained 12.5 µl of 2× Reaction Mix, 5 pmol each of the F3 and B3 primers, 40 pmol each of the FIP and BIP primers, 20 pmol each of the LP and LF primers, 1 µl of 25 µf SYTO 13 green fluorescent nucleic acid stain (FD; Thermo Fisher Scientific, Waltham, Massachusetts), 1 µl of Bst 2.0 WarmStart®DNA polymerase (New England Biolabs, Beijing, China), and 2 µl of DNA template. The LAMP amplification was performed on a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, California). The procedure started with 65 C for 1.5 hr (data collection), followed by 95 C for 5 min.

RESULTS

Sensitivity test of the LAMP method

The positive-control plasmid pMD19-T-RE diluted in DNA from healthy mouse feces were used to test the sensitivity of the LAMP method. As shown in Figure 2A, the detection limit was found to be 1.7 fg of the plasmid by real-time LAMP. Furthermore, LAMP reactions were produced a typical ladder of multiple bands on the 1.5% agarose gel stained with GelRed™ (Biotium Inc.; Fig. 2B), which indicated the production of stem-loop DNA with inverted repeats of the target sequence.

In addition, the serially diluted templates were used to determine the detection limit of LAMP based on tracing Pi. The sensitivity of the detection presented a same detection limit with real time LAMP as shown in Figure 2C.

Specificity test of the LAMP method

Genomic DNA samples of *Tri. spiralis*, *Anisakis pegreffii*, *Gnathostoma spinigerum*, *Angiostrongylus cantonensis*, *Enterobius vermicularis*, *S. japonicum*, and *Trypanosoma evansi* were used to

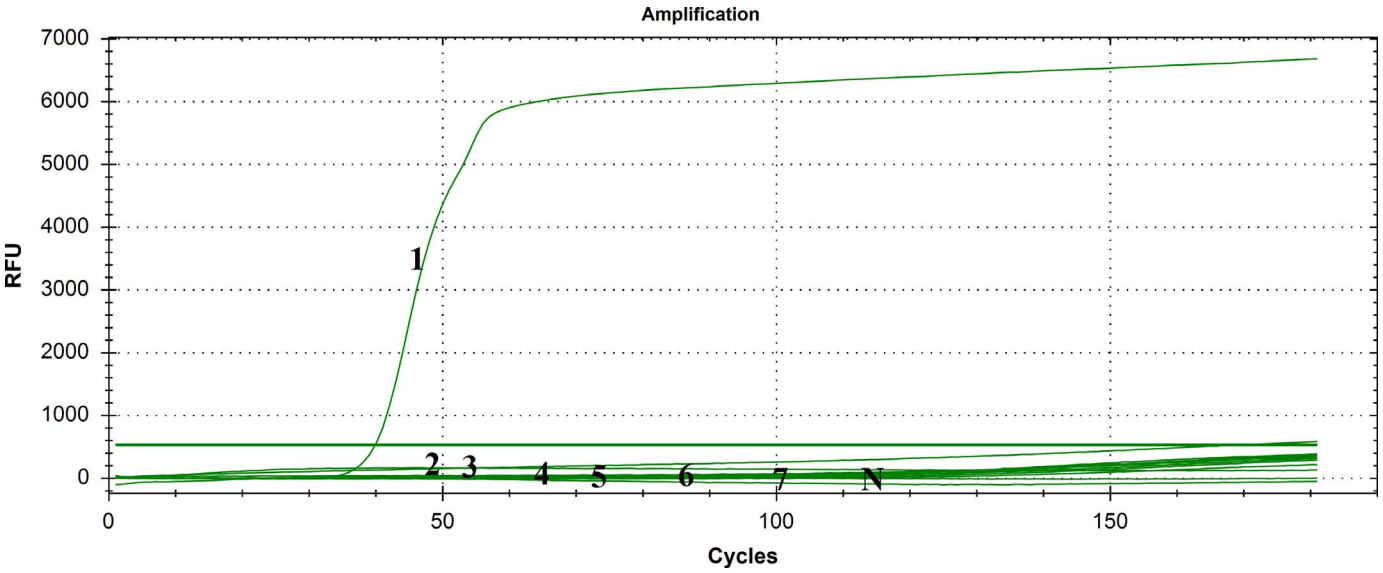

Figure 3. Specificity test of LAMP for the detection of *Trichinella spiralis*. Lanes 1–7: genomic DNA of *Trichinella spiralis*, *Anisakis pegreffii*, *Gnathostoma spinigerum*, *Angiostrongylus cantonensis*, *Enterobius vermicularis*, *Schistosoma japonicum*, and *Trypanosoma evansi*, respectively; lane 8: negative control. Color version available online.

test the specificity of the LAMP assay. Amplification signal was detected with the template of *Tri. spiralis* while the rest of templates showed no signal (Fig. 3).

LAMP detection of fecal samples from rats

Trichinella spiralis DNA was detected from 1 to 28 dpi in fecal samples collected from each of the 4 groups. The average percentage of positive LAMP results for the 21 days were found to be 48.1%, 70.4%, and 85.2% for rats infected with 250, 2,500, or 5,000 ML of *Tri. spiralis*, respectively (Table II). In individual rat feces, DNA were detected on 1–7, 1–18, and 1–21 dpi for 250-, 2,500-, and 5,000-ML doses, respectively.

Table II. Detection of *Trichinella spiralis* DNA in fecal samples of rats by loop-mediated isothermal amplification (LAMP). Groups A–D of 3 rats each were infected with 0, 250, 2,500, or 5,000 muscle larvae (ML) of *Tri. spiralis* by oral gavage, respectively.

	Days postinfection												
Group	0	1	3	5	7	9	12	15	18	21	24	28	56
A													
1	−	−	−	−	−	−	−	−	−	−	−	−	−
2	−	−	−	−	−	−	−	−	−	−	−	−	−
3	−	−	−	−	−	−	−	−	−	−	−	−	−
B													
4	−	+	+	+	+	−	−	−	−	−	−	−	−
5	−	+	+	+	+	+	+	−	−	−	−	−	−
6	−	+	+	+	+	−	−	−	−	−	−	−	−
C													
7	−	+	+	+	+	+	+	+	+	−	−	−	−
8	−	+	+	+	+	+	−	−	+	−	−	−	−
9	−	+	+	+	+	−	+	+	−	−	−	−	−
D													
10	−	+	+	+	+	+	+	+	+	+	−	−	−
11	−	+	+	+	+	+	+	−	+	−	−	−	−
12	−	+	+	+	+	+	+	+	+	−	−	−	−

DISCUSSION

In the current study, the LAMP assay targeting the sequence for the 1.6-kb repetitive element of *Tri. spiralis* was successfully established. The choice of gene sequence is critical when establishing a diagnostic method for molecular tests. After comparing 4 sets of LAMP primers targeting different gene sequences, we found the listed set presents the highest amplification efficiency which could recognize a conserved region of the 1.6-kb repetitive element of *Tri. spiralis*. In addition, other assays targeting the 1.6-kb repetitive element that were established to detect *Tri. spiralis* DNA confirm that this gene is acceptable for diagnosis (Li et al., 2012; Liu et al., 2017).

In order to determine the sensitivity of the LAMP method, the positive-control plasmid pMD19-T-RE was diluted in DNA from healthy mouse feces. Our results present that this LAMP assay was able to detect 1.7 fg plasmid DNA (Fig. 2A), and the sensitivity of LAMP based on tracing Pi was also found to be 1.7 fg, as shown in Fig. 2C. When the simulated samples were used for sensitivity test, the detection limit was found to be 0.03 larvae/g of feces. Negative results were obtained for DNA isolated from feces of the uninfected control rats. The specificity of the LAMP assay was tested by using the DNA samples of other parasites. No amplification was observed in the DNA samples of *Anisakis pegreffii*, *Gnathostoma spinigerum*, *Angiostrongylus cantonensis*, *Enterobius vermicularis*, *S. japonicum*, and *Trypanosoma evansi* (Fig. 3), which proved that the LAMP primers are highly specific for the detection of *Tri. spiralis*.

The LAMP assay was primarily applied to detect fecal samples collected from *Tri. spiralis*–infected rats. The parasite's DNA

were detected in all samples collected at intestinal phage from days 1 to 7 postinfection by LAMP, which suggested that this assay was effective for early-stage diagnosis. As shown in Table II, the percentage of LAMP positive results and the duration of the postinfection period was dose dependent. The negative control of uninfected rats (Group A) did not show any amplification of DNA fragments by LAMP, whereas in parasite-infected individual rat feces, DNA were detected during days 1–7, 1–18, and 1–21 postinfection for 250-, 2,500-, and 5,000-ML doses, respectively. According to Wassom et al. (1984), the *Trichinella* expulsion from the small intestine of mouse is completed prior to day 21 postinfection. Vallance et al. (1999) found a sharp decrease of worm load in the small bowel of mice between the 12th and 16th days and then a smaller one on the 17th to 21st days. The appearance of *Trichinella* DNA in the feces in this study was correspond with the worm burden in the small bowel of mice on different days after infection.

Another advantage of the assay is that the requirements for LAMP are relatively simple, and that it does not require high technical skills or sophisticated equipment. However, there is a high risk of aerosol contamination because of the large amount of LAMP products. Thus, in this study the closed-device format allows direct detection of LAMP products based on tracing Pi without opening tubes was used and largely reduced the possibility of aerosol contamination.

To our knowledge, this is the first attempt to detect *Tri. spiralis* DNA in rat feces during the early stage of infection. We were able to demonstrate the successful amplification of *Tri. spiralis* DNA at intestinal phase within 1 hr at 65 C using the LAMP assay. On the basis of these results, the LAMP assay can be considered one of the most accurate molecular assays because it is a specific, sensitive, and rapid diagnostic tool for the early detection of *Trichinella* in fecal samples.

In conclusion, we report the following findings: (1) We developed an assay based on tracing phosphate ions generated during LAMP targeting the1.6-kb repetitive element of *Tri. spiralis*. (2) The detection limit of the LAMP assay is 1.7 fg of *Tri. spiralis* DNA for control positive plasmid samples and 0.03 larvae/g of fecal for simulated samples. (3) The assay does not involve any cross-reactivity with the DNA of other parasites. (4) This is the first report regarding the application of the LAMP assay for early detection of *Tri. spiralis* larvae in fecal samples from experimentally infected rats. Because of its rapidity, sensitivity, and noninvasiveness for common use, we suggest that the assay could be used as an early diagnostic tool for trichinellosis.

ACKNOWLEDGMENTS

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