

Development and evaluation of a reverse transcription-loop-mediated isothermal amplification assay for rapid and high-sensitive detection of *Cryptosporidium* in water samples

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ABSTRACT

We describe a novel assay for simple, rapid and high-sensitive detection of *Cryptosporidium* oocysts in water samples using a reverse transcription-loop-mediated isothermal amplification (RT-LAMP). The assay is based on the detection of 18S rRNA specific for *Cryptosporidium* oocysts. The detection limit of the developed RT-LAMP assay was as low as 6×10^{-3} oocysts/test tube, which theoretically enables us to detect a *Cryptosporidium* oocyst and perform duplicated tests even if water samples contain only one oocyst. The developed RT-LAMP assay could more sensitively detect *Cryptosporidium* oocysts in real water samples than the conventional assay based on microscopic observation.

Key words | Cryptosporidiosis, *Cryptosporidium*, LAMP, oocysts, RT-LAMP

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INTRODUCTION

Members of the genus *Cryptosporidium* are protozoan parasites that can cause the gastrointestinal disease cryptosporidiosis (O'Donoghue 1995). Cryptosporidiosis remains a public health concern, as demonstrated by continued outbreaks of this disease (Nichols 2008). Waterborne cryptosporidiosis is particularly important because *Cryptosporidium* oocysts are resistant to disinfectants (such as chlorine)

commonly used for water treatment (Peeters *et al.* 1989; Carpenter *et al.* 1999). Waterborne outbreaks have been reported in not only developing countries but also developed countries. The most notorious outbreak occurred in Milwaukee, Wisconsin in 1993 where more than 400,000 suspected and 5,000 confirmed cases of clinical cryptosporidiosis (MacKenzie *et al.* 1995; Cicirello *et al.* 1997; Corso *et al.* 2003).

Although detection of *Cryptosporidium* oocysts in water samples was traditionally carried out using either direct microscopic visualization of oocysts by staining techniques and fluorescent antibodies or enzyme immunoassays or cell culture, these detection methods are labor-intensive, require a large number of oocysts for positive detection and are not suitable for high-throughput processing of samples (Ramirez & Sreevatsan 2006). Instead of these conventional methods, molecular (nucleic acid based) techniques have been developed for rapid detection of *Cryptosporidium* oocysts from water samples. Most molecular techniques are based on polymerase chain reaction (PCR) (Monis & Saint 2001; Hirata & Hashimoto 2006; Masago *et al.* 2006). PCR is sometimes combined with cell culture (cell culture-PCR) (Di Giovanni *et al.* 1999). However, these techniques require a high precision instrument (thermal cycler) to amplify target nucleic acids and elaborate methods such as gel electrophoresis for detection of amplified products. Therefore, simpler detection techniques are strongly needed for the routine detection of *Cryptosporidium* oocysts in drinking water treatment plants, bathing facilities, and wherever water quality monitoring is needed.

Loop-mediated isothermal amplification (LAMP) is a novel nucleic acid amplification technique that relies on an autocycling strand displacement DNA synthesis performed by the *Bst* DNA polymerase large fragment (Notomi *et al.* 2000). A large amount of DNA can be synthesized in a short time (15–60 min). LAMP enables nucleic acid amplification under isothermal conditions ranging from 60 to 65°C, and therefore, LAMP can be performed using a simple incubator or a water bath instead of a thermal cycler (Tani *et al.* 2007). Furthermore, gel electrophoresis is not needed because the LAMP method synthesizes a large amount of DNA so that the products can be detected by simple turbidity (Ohtsuka *et al.* 2005). Thus, LAMP is faster and simpler to perform than PCR. Recently, we have developed the LAMP assay for rapid detection of *Cryptosporidium* oocysts (Momoda *et al.* 2009). The detection limit of the assay was 0.8 oocysts/LAMP test tube, and the sensitivity of the assay was enough to detect even one oocyst. However, it is too difficult to extract and concentrate all target DNA to one test tube because the sample volume of the LAMP assay is usually only 5 µL. Therefore it is necessary to develop a more sensitive assay to obtain stable results even if only one

oocyst exists in water samples. To increase the sensitivity, we focused on rRNA in *Cryptosporidium* oocysts because the number of target (18S) rRNA is much greater than that of target rDNA. We were not sure of the amount of ribosomes in a *Cryptosporidium* oocyst, but expected a large quantity of rRNA. At least some bacterial cells have several thousand of ribosomes per cell (Fegatella *et al.* 1998), and approximately 80 percent of the total RNA in rapidly growing mammalian cells (e.g., cultured HeLa cells) is rRNA (Lodish *et al.* 2000). Moreover detection of nucleic acid sequences would theoretically be a good indicator of which cells are living, since the nucleic acid in a dead cell in a fresh water environment is likely to be degraded by endogenous and environmental nucleases within a relatively short turnover time (Paul *et al.* 1989).

In this study, we developed a one-step reverse-transcription LAMP (RT-LAMP) assay for rapid and high-sensitive detection of *Cryptosporidium* oocysts, and applied the assay to the detection from real water samples collected from surface water and ground water.

METHODS

RT-LAMP assay

The RT-LAMP assay was performed in 25 µL of the total reaction mixture with a Loopamp DNA amplification kit (Eiken Chemical Co., Ltd., Tokyo, Japan) containing 5 pmol (each) of outer primers CryF3 and CryB3, 20 pmol (each) of loop primers CryLF and CryLB, 40 pmol (each) of inner primers CryFIP and CryBIP, 1.5U of reverse transcriptase (Roche) and 5 µL of extracted RNA samples. Sequences of these primers are shown in Table 1. The reaction mixture was incubated at 63°C for 60 min, and turbidity was continuously monitored using a Loopamp real-time turbidimeter (LA-320C; Eiken Chemical CO., LTD., Tokyo, Japan). Positive and negative controls for the RT-LAMP reaction were included in every reaction. The positive control was made of RNA with an artificial sequence, which enables one to distinguish the RT-LAMP products between the artificial sequence and the real *Cryptosporidium* sequence by banding patterns on the gel after electrophoresis. The artificial sequence had all primer sequences

Table 1 | Primer oligonucleotides used for RT-LAMP

Primer name	Sequence (5' → 3')	Location*	Reference
CryFIP	TACTTAACTC ATTCCAATTA GAAAACCCAG GGAGGTAGTG ACAAG	502	Momoda et al. 2009
CryBIP	ATAAACCCCT TTACAAGTAT CAATTTATAC GCTATTGGAG CTGG	502	
CryF3	GCGCAAATTA CCAATCC	413	
CryB3	ACTACGAGCT TTTTAACTGC	611	
CryLF	CCAAAAAGTC CTGTATTG	477	
CryLB	GAGGGCAAGT CTGGTG	528	

*Corresponding nucleotide position of *Cryptosporidium parvum* 18S rRNA gene (Accession No. L16996) of the 5' end.

and the *Eco* RI restriction enzyme site. After an oligo DNA synthesis of the artificial sequence with a T7 RNA polymerase binding site, the positive control was made by using the T7 RNA polymerase (Stratagene). The negative control was the pure water included in the kit.

RNA extraction

Immediately after five freeze (−80°C) and thaw (37°C) cycles, the sample solution including *Cryptosporidium* oocysts was incubated at 60°C for 30 min using a heat block with the solution for nucleic acids extraction (TE: 20 mM, NaCl: 0.1%, TrironX-100: 2 mM, DTT: 0.2 µg/mL, Proteinase K: 6 mAnson-U/mL). After that, the sample solution was sonicated for 2 min and incubated at 75°C for 10 min. Then, the solution was incubated at 95°C for 5 min to deactivate Proteinase K. Finally, the extracted RNA solution was immediately cooled down with ice.

Sensitivity test of RT-LAMP assay

Cryptosporidium parvum oocysts (H8 strain, Yagita et al. 2001) which was maintained in our laboratory by passages in infected mice were used for a sensitivity test of the RT-LAMP assay. Oocysts were purified from the feces by a combination of discontinuous density sucrose gradient centrifugation and cesium chloride gradient centrifugation and enumerated with a hemacytometer. The RNA of purified and enumerated oocysts was extracted, and used as template RNA. The template RNA was prepared as 10-fold serial dilutions to obtain final concentrations of 6×10^{-5} – 10^2 oocysts/5 µL. Then, the RT-LAMP assay was performed in duplicate for each diluted sample.

Detection of *Cryptosporidium* oocysts in real water samples by conventional microscopic observation and RT-LAMP

Twenty-two surface water samples and nine ground water samples were collected in two Japanese area (Tokyo Metropolitan, Miyagi prefecture). Twenty liters of water samples were concentrated to 5 ml by vacuum filtration with 5 µm polytetrafluoroethylene (PTFE) membrane filters (90 mm diameter, Omnipore, Millipore). Typically between 1 to 3 filters, or a maximum 10 filters were required to process the entire 20 L sample. Half the volume of concentrated sample solution, namely 2.5 ml of the concentrate which was equivalent to 10 L of water sample, was used for conventional assay based on microscopic observation, and the other half volume was used for the RT-LAMP assay.

The conventional assay was performed by Japanese standard method for detection of *Cryptosporidium* in water supply systems (Ministry of Health, Labour & Welfare 2007). After purification through immunomagnetic separation (IMS) (Dynabeads GC Combo, Invitrogen), *Cryptosporidium* oocysts were separated from magnetic beads using hydrochloric acid, and fixed on a membrane filter. The fixed sample was stained with EasyStain antibody stain (BTF), and observed using an epifluorescent and differential interference contrast microscope.

RNA extraction was performed while *Cryptosporidium* oocysts were captured by magnetic beads. After the washing step in a 1.5 ml tube, the *Cryptosporidium* and beads complex were subjected for RNA extraction in a 20 µl solution. Then, the RT-LAMP assay was performed for the extracted RNA samples. To confirm the results, 1 µL

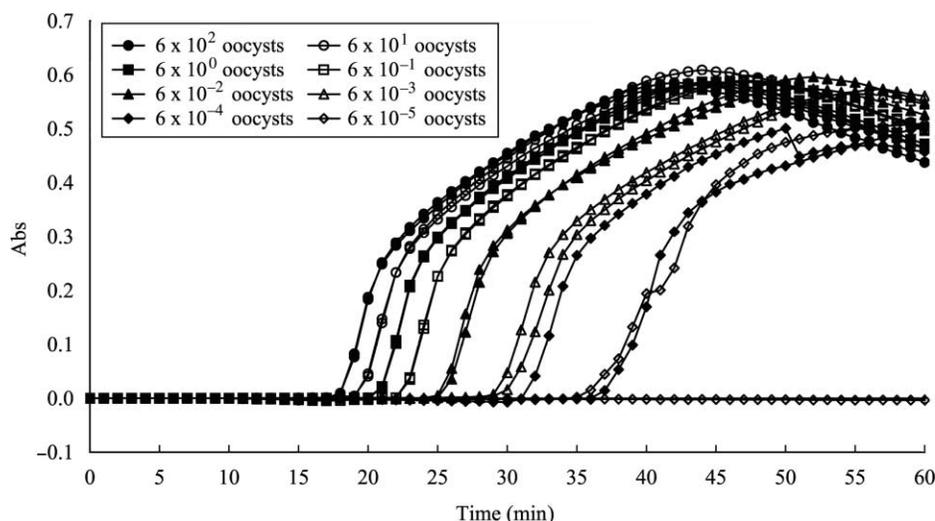


Figure 1 | Detection limit of the RT-LAMP assay for the detection of *Cryptosporidium* oocysts using 10-fold serial dilution of template RNA extracted from *Cryptosporidium parvum* oocysts.

aliquots of RT-LAMP products of positive samples were electrophoresed on a 2% agarose gel in Tris-borate-EDTA buffer (TBE buffer), followed by staining with ethidium bromide and visualization on a UV transilluminator.

RESULTS AND DISCUSSION

Sensitivity of RT-LAMP assay for the detection of *Cryptosporidium* oocysts

Figure 1 shows sensitivity test results for the developed RT-LAMP assay using 10-fold serial dilution of template RNA extracted from *Cryptosporidium parvum* oocysts. Threshold time (T_t), which is defined as the time at which the differential calculation value, exceeds 0.07, increased with the decrease in the template RNA concentration. The T_t value of each diluted sample is listed in Table 2. T_t values were unstable when oocysts concentration was 6×10^{-4} oocysts/LAMP test tube or less. When the concentration was 6×10^{-5} , one turbidity curve did not increase. On the other hand, T_t values were stable in duplicated tests when the concentration was 6×10^{-3} oocysts/LAMP test tube or more. Therefore, we judged the detection limit that shows reproducible results to be 6×10^{-3} oocysts/LAMP test tube. This value is much less than the detection limit by normal LAMP assay. In our previous study, the detection limit of the LAMP assay was investigated by almost the

same method, and the limit was shown as 0.8 oocysts/LAMP test tube. Thus, we succeeded in improving in sensitivity for the detection of *Cryptosporidium* oocysts.

Evaluation of RT-LAMP assay for the detection of *Cryptosporidium* oocysts in water samples

Table 3 shows the detection results of *Cryptosporidium* oocysts in real water samples. Two out of 31 water samples were positive in conventional microscopic observation, and these two samples were positive in the RT-LAMP assay, too. Hence, false-negative results were not observed in the RT-LAMP assay. However, 5 more samples were positive in

Table 2 | T_t values of each diluted sample in the RT-LAMP assay

RNA concentration* (oocysts/LAMP test tube)	T_t value (min)	Standard deviations
6×10^2	19.8, 19.8	0.00
6×10^1	21.3, 21.2	0.07
6×10^0	22.5, 22.6	0.07
6×10^{-1}	24.3, 24.3	0.00
6×10^{-2}	27.4, 27.1	0.21
6×10^{-3}	31.3, 32.3	0.71
6×10^{-4}	39.4, 33.2	4.38
6×10^{-5}	39.0, N.D.†	–

*RNA concentration is converted to oocysts concentration in a LAMP test tube.

†Not detected.

Table 3 | Detection results of *Cryptosporidium* oocysts in real water samples by conventional microscopic observation and RT-LAMP

Sample No.	Sample type	Microscopic observation ¹ *	RT-LAMP ²
1	Surface water	–	+
2	Surface water	–	–
3	Shallow well water	–	–
4	Shallow well water	–	–
5	Surface water	–	–
6	Surface water	–	–
7	Surface water	–	–
8	Surface water	–	–
9	Surface water	–	–
10	Spring water	–	–
11	Spring water	–	–
12	Surface water	–	–
13	Spring water	–	–
14	Spring water	–	–
15	Surface water	–	–
16	Surface water	–	–
17	Surface water	–	–
18	Surface water	–	–
19	Surface water	–	–
20	Surface water	–	–
21	Surface water	–	+
22	Surface water	–	+
23	Surface water	+	+
24	Surface water	–	+
25	Surface water	–	+
26	Surface water	+	+
27	Surface water	–	–
28	Shallow well water	–	–
29	Shallow well water	–	–
30	Surface water	–	–
31	River-bed water	–	–

* +, Positive; –, Negative.

the RT-LAMP assay. Firstly, false-positive results by contamination of positive control in the RT-LAMP assay were considered as the reason. However, it was found by electrophoresis that the contamination did not occur, and RT-LAMP products of these five samples were amplified nucleic acid of *Cryptosporidium* oocysts as shown in Figure 2. Secondly, decreased sensitivity can be

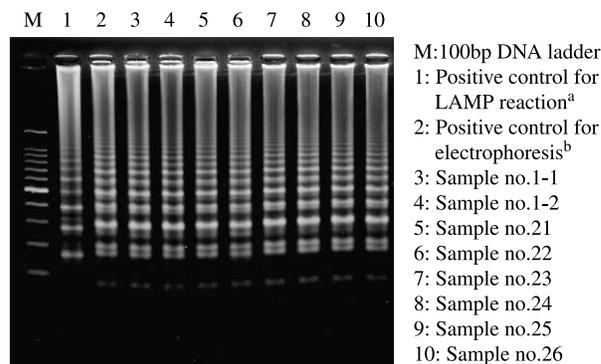


Figure 2 | Analysis of RT-LAMP products of positive samples by agarose gel electrophoresis. ^aPositive control for the RT-LAMP assay made of RNA with an artificial sequence. Electrophoretic pattern of this positive control is different from real *Cryptosporidium* oocysts. If the electrophoretic pattern of samples is the same as lane 1, it is an evidence of contamination by the positive control. ^bPositive control for electrophoresis which derives from nucleic acid of real *Cryptosporidium* oocysts. This positive control is not used in LAMP reactions.

caused by failure to separate all oocysts from magnetic beads, insufficient staining and incomplete detection by microscopic examination. Because of these reason, *Cryptosporidium* oocysts would be more sensitively detected in the RT-LAMP assay than the conventional microscopic observation.

CONCLUSIONS

We successfully developed a one-step RT-LAMP assay for the rapid and highly sensitive detection of *Cryptosporidium* oocysts. The detection limit is as low as 6×10^{-3} oocysts/test tube. Even if water samples contain only one *Cryptosporidium* oocyst, the oocyst can be detected using the developed RT-LAMP assay. Additionally, duplicated tests are possible in this assay. The RT-LAMP assay sensitively detects *Cryptosporidium* oocyst in real water samples. Use of the RT-LAMP assay instead of conventional assay based on microscopic observation will greatly decrease the labor needed for the detection of *Cryptosporidium* oocyst.

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