A New Duplex Reverse Transcription PCR for Simultaneous Detection of Viable *Cryptosporidium parvum* Oocysts and *Giardia duodenalis* Cysts

SEHEE NAM AND GYUCHEOL LEE[1]

Water Analysis and Research Center, K-water, Daejeon 306-711, Republic of Korea

Objective To simultaneously detect viable *Cryptosporidium parvum* oocysts and *Giardia duodenalis* cysts for the purpose of reducing time and cost spent. **Methods** A duplex reverse transcription polymerase chain reaction (RT-PCR) method was newly developed. **Results** Using duplex RT-PCR method for the *hsp*70 gene, viable (oo)cyst concentrations of 10^1 and 10^3 (oo)cysts/100 μL could be detected for *C. parvum* and *G. duodenalis*, respectively. However, after heat-shock stimulation the expression of *hsp*70 mRNAs was detectable at 10^0 and 10^1 (oo)cysts/100 μL concentrations of *C. parvum* and *G. duodenalis*, respectively. Thus, the detection sensitivity was significantly increased when the viable (oo)cysts were exposed to heat shock. **Conclusion** This study describes a new duplex RT-PCR method for *hsp*70 gene to detect the viable (oo)cysts of the *C. parvum* and *G. duodenalis* with less time consumed and at a lower cost. This newly developed duplex RT-PCR method may be used to detect these parasites not only in aquatic environments but also in clinical samples.

Key words: *Cryptosporidium parvum*; *Giardia duodenalis*; Heat-shock; RT-PCR

INTRODUCTION

Cryptosporidium parvum and *Giardia duodenalis* are respective causative agents for cryptosporidiosis and giardiasis. They are well known waterborne protozoan parasites that are transmitted via fecal-oral routes[1-2]. The (oo)cysts of these organisms have durable and thick cell walls providing resistance against various types of environmental stress. For example, resistance against the chlorination process in water treatment plants has been reported[3], and these organisms have the ability to maintain their vitality for several months.

In an outbreak caused by *C. parvum* at Milwaukee, Wisconsin, USA in 1993, over 400 000 people were infected resulting in more than 100 deaths[4]. In 1996, a massive outbreak of cryptosporidiosis due to the contamination of water occurred in Saitama Prefecture, Japan[5]. In South Korea, although no massive outbreaks caused by *C. parvum* or *G. duodenalis* have been reported, studies on the detection and surveillance of these parasites in aquatic environments have been conducted[6-7].

Since *C. parvum* and *G. duodenalis* exist in aquatic environments at very low concentrations, detection methods with very high sensitivity are required. Currently, the United States Environmental Protection Agency (US-EPA) method 1 623 is used for this purpose[8]. However, this method has several shortcomings: it is time-consuming, unable to discriminate between viable and dead (oo)cysts, and inconsistent results may be obtained by researchers with different levels of experience. To overcome these limitations, molecular based techniques, such as polymerase chain reaction (PCR) and real-time PCR, have been developed. Several reports demonstrated excellent sensitivity and specificity of these molecular based methods in analyzing environmental samples[9-13]. However, neither PCR nor reverse transcription (RT)-PCR is able to verify the viability of detected (oo)cysts. In an attempt to address this issue, Gobet *et al*. (2001) and other researchers developed *hsp*70 RT-PCR as a tool to discriminate between viable and dead oocysts of *C. parvum*[14-16] , and RT-PCR method to detect *hsp*70 mRNA of *G. duodenalis* was developed by Lee *et al*. (unpublished data). However, performing separate RT-PCR assays in order to detect each protozoan will be more time consuming and expensive. Hence, this study proposes a duplex RT-PCR method to simultaneously detect viable (oo)cysts of both *C. parvum* and *G.*

[1]Correspondence should be addressed to Gyucheol LEE. Tel: 82-42-629-2034. Fax: 82-42-629-2079. E-mail:gclee@kwater.or.kr

Biographical note of the first author: Sehee NAM, male, born in 1973, senior researcher at Water Analysis and Research Center, K-water, Republic of Korea, majoring in Parasitology.

0895-3988/2010
CN 11-2816/Q
Copyright © 2010 by China CDC

duodenalis.

MATERIALS AND METHODS

Preparation and Identification of the (Oo)cysts

The (oo)cysts of *C. parvum* and of *G. duodenalis* were purchased from Waterborne Inc. (New Orleans, LA, USA). The (oo)cysts were inactivated by heating at 150 ℃ for 15 minutes[17]. The identification process of the (oo)cysts was established as follows. The samples containing (oo)cysts were dried on a well slide and fixed using 100% methyl alcohol. Then, an immunofluorescence assay kit (Aqua-glo G/C, Waterborne Inc.) was used to stain the nuclei of the (oo)cysts through a fluorescence dyeing process using 4′, 6-diamidino-2-phenylindole (Sigma Chemical Co., St. Louis, MO, USA). The verification of the (oo)cysts was performed using the immunofluorescence assay (exciter filter, 450-490 nm; dichroic beam-splitting mirror, 510 nm; and barrier or suppression filter, 515-520 nm), nucleus dyeing test (exciter filter, 340-380 nm; dichroic beam-splitting mirror, 400 nm; and barrier or suppression filter, 420 nm), and differential interference contrast observation using a fluorescence microscope (Eclipse E800, Nikon, Japan) with at least 400 magnifications.

RNA Extraction

For the extraction of the mRNAs of the *C. parvum* oocysts and *G. duodenalis* cysts, Dynabeads mRNA DIRECTTM kit (Dynal Biotech, Oslo, Norway) was used. Serial 1:10 dilutions of the (oo)cysts were prepared to obtain concentrations of 10^0 to 10^4 (oo)cysts/100 μL of phosphate buffered saline (PBS) solution. The number of (oo)cysts in each dilution were counted three times to ensure the accuracy of dilution steps. When necessary, a heat shock was applied in order to induce the transcription of *hsp*70 mRNA in the (oo)cysts, where samples were subjected to 42 ℃ for 20 min. After adding 200 μL of lysis/binding buffer solution (Dynal Biotech) into each (oo)cyst suspension, they were frozen in liquid nitrogen for 1 min and then thawed in a water bath at 65 ℃ for 1 min. Then, the process was repeated six times. After adding 20 μL of Dynabeads oligo $(dT)^{25}$-bead (Dynal Biotech), a reaction took place at 30 ℃ over 30 min in a mixer (Sample Mixer, MXICI model, Dynal Biotech), and magnetic particles bound with a nucleic acid were separated using a magnetic particle concentrator. After completion of these processes, samples were washed using the washing buffers A and B, which were also included in the kit. Then, mRNAs were separated from the magnetic body through a reaction at 80 ℃ for 2 min with a 50 μL of ice-cold 10 mmol/L Tris-HCl solution. After the complete removal of DNAs through the treatment of separated mRNAs with 0.01 U/μL DNase I (ABgene, Epsom, UK), it was used as a template for an RT reaction.

RT-PCR

For the RT reaction, a Sensiscript RT kit (Qiagen, Hilden, Germany) was used. The mRNA templates were mixed with the following: 2 μL of 10× RT buffer, 1 μL of reverse transcriptase, 2 μL of PCR primers (10 pmol/μL), 1 μL of 10 U RNase inhibitor, and 2 μL of dNTP mixture to obtain a final volume of 20 μL, which was adjusted using RNase-free water (Qiagen). Table 1 shows the list of each primer used in the RT-PCR and duplex RT-PCR. Following the completion of RT reaction at 37 ℃ for 60 min, a PCR process was conducted using the synthesized cDNA as a template by mixing 3 μL of the *hsp*70 cDNA, 2 μL from each of PCR primers (10 pmol/μL), 10 μL of 2× Taq DNA polymerase premixture (Taq DNA polymerase 0.1 unit/μL, dATP, dGTP, dCTP, and dTTP for each of 0.5 mmol/L and 4 mmol/L $MgCl_2$), and 3 μL of nuclease free water (Ambion, Austin, TX). The final volume was adjusted to 20 μL. Then, it was denaturalized at 94 ℃ for 1 min, extended at 72 ℃ for 1 min, and annealed at 51 ℃ for 1 min from the completion of the reaction process at 94 ℃ for 5 min. This process was repeated 40 times, and then it was reacted at 72 ℃ for 7 min. For this process, a GeneAmp PCR System 9 700 (Applied Biosystems, Foster City, CA) was used as a PCR device.

TABLE 1

Primers Used for *hsp*70 RT-PCR and Duplex RT-PCR

Primers		Sequences (5′-3′)	Size (bp)	Target Region	References
C. parvum	F	AAATGGTGAGCAATCCTCTG	361	*hsp*70	Rochelle *et al.*[15]
	R	CTTGCTGCTCTTACCAGTAC			
G. duedenalis	F	GTTTAAGAAGAAGCACGGGA	148	*hsp*70	Newly designed for this study
	R	GAAGTCTATGCCCTCGAAAA			

RESULTS AND DISCUSSION

C. parvum and *G. duodenalis* are transmitted via fecal-oral route as important causative agents for waterborne outbreaks[18]. Currently, in addition to the US-EPA 1 623 method, molecular based methods such as PCR are used for the detection of these protozoan parasites[8]. However, microscopic and molecular based methods are unable to discriminate between viable and dead (oo)cysts, representing a major disadvantage. In order to verify the viability of (oo)cysts, additional assessments such as experiments using animal models[19-20], *in vitro* excystation[21] and dye exclusion assay[22] are required. Albeit various techniques have been developed to detect the viable (oo)cysts[14-16,23-25], it is necessary to simultaneously detect the two pathogens rather than separate detection of each individual pathogen. Such an approach would reduce the time needed for analysis and decrease the cost. Considering this unmet need, we developed a duplex RT-PCR method which is able to detect the two pathogens in one tube.

First, it was investigated whether the monoplex *hsp*70 RT-PCR method developed by Rochelle *et al.*[15] for *C. parvum* and the monoplex *hsp*70 RT-PCR method by Lee *et al*. for *G. duodenalis* can detect the mRNAs of the *hsp*70 transcribed by their viable (oo)cysts. Ten-fold serial dilutions of the viable and dead (oo)cysts were carried out to obtain concentrations ranging between 10^0 and 10^4 (oo)cysts/100 μL of PBS solution and then some (oo)cysts were treated by heat shock at 45 ℃ for 20 minutes, whereas others were not exposed to heat. *hsp*70 mRNAs of dead (oo)cysts were not expressed in *hsp*70 RT-PCR assay (Table 2), which may be explained by the absence of vitality. On the other hand, in case of the viable oocysts of *C. parvum*, the amplicon amplified from *hsp*70 mRNA detected 10^1 cysts/100 μL when heat shock was not applied, and detection of 10^0 cyst/100 μL was possible in samples exposed to heat shock (Fig. 1a and Table 2). For the viable cysts of *G. duodenalis*, *hsp*70 mRNA could be first detected at concentrations of 10^0 cysts/100 μL and 10^3 cysts/100 μL in samples with and without heat shock application, respectively (Fig. 1b and Table 2).

TABLE 2

Comparison of Detection Sensitivity of Monoplex and Duplex RT-PCR

Protozoa	Viability	Heat shock	Methods	Number of (oo)cysts/100 μL					
				10^4	10^3	10^2	10^1	10^0	0
C. parvum	–	–	M[a]	–	–	–	–	–	–
			D[b]	–	–	–	–	–	–
		+	M	–	–	–	–	–	–
			D	–	–	–	–	–	–
	+	–	M	+	+	+	+	–	–
			D	+	+	+	+	–	–
		+	M	+	+	+	+	+	–
			D	+	+	+	+	+	–
G. duodenalis	–	–	M	–	–	–	–	–	–
			D	–	–	–	–	–	–
		+	M	–	–	–	–	–	–
			D	–	–	–	–	–	–
	+	–	M	+	+	–	–	–	–
			D	–	–	–	–	–	–
		+	M	+	+	+	+	+	–
			D	+	+	+	+	–	–

Note. M[a], monoplex RT-PCR; D[b], duplex RT-PCR.

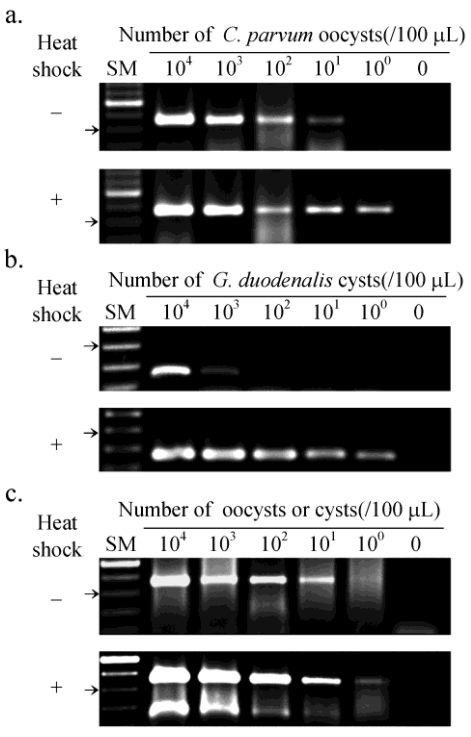

FIG. 1. Detection of viable *C. parvum* and *G. duodenalis* (oo)cysts by using monoplex or duplex RT-PCR with increasing sensitivity by heat shock. Oocysts and cysts were diluted at 1:10 in 100 μL phosphate buffered saline. Some samples received heat shock treatment whereas others were not exposed to heat. mRNA was extracted and RT-PCR and duplex RT-PCR were conducted. a. Monoplex RT-PCR for C. *parvum*; b. monoplex RT-PCR for *G. duodenalis*; c. duplex RT-PCR for *C. parvum* and *G. duodenalis*. SM, 100 bp size marker; an arrowed marker, 300 base pairs.

In order to reduce time and cost spent for analysis, a new duplex RT-PCR method was developed and tested. The *hsp*70 RT-PCR conditions for *G. duodenalis* and their primers were designed and optimized to be identical with those for *C. parvum*. Viable and dead (oo)cysts were serially diluted at 1:10 in 100 μL of PBS to obtain concentrations between 10^0 to 10^4 (oo)cycts. The amplification of *hsp*70 mRNA was not evident in dead (oo)cysts (whether heat shocked or not) (Table 2). However, for viable oocysts, the amplicon of *hsp*70 was first evident at 10^1 (oo)cysts/100 μL concentration. On the other hand, there was no amplification of *hsp*70 mRNA for the viable cysts of *G. duodenalis* by duplex RT-PCR, although they could be detected at concentrations of 10^3 cysts/100 μL by monoplex RT-PCR (Table 2). In order to increase the sensitivity, the (oo)cysts were heat-shocked at 45 ℃ for 20 min. Heat shock resulted in better sensitivity by improving the detection thresholds of *C. parvum* oocysts and *G. duodenailis* cysts to 10^0 oocyst/100 μL and 10^1 cysts/100 μL, respectively (Fig. 1c and Table 2), suggesting that viable (oo)cysts of *C. parvum* and *G. duodenalis* can be simultaneously detected at these concentrations. In conclusion, this study developed a new duplex RT-PCR method which is able to detect the viable (oo)cysts of *C. parvum* and *G. duodenalis* simultaneously , which provides rapid results at a low cost. This new duplex RT-PCR method may be used to detect these parasites not only in aquatic environments but also in clinical samples. In addition, further studies are needed to elevate the sensitivity of the duplex RT-PCR for detecting *G. duodenalis* cysts and develop the real-time duplex RT-PCR for the quantitative detection of the (oo)cysts.

ACKNOWLEDGEMENTS

We thank Sangtae LEE and Jungim LEE in K-Water for their skillful help and valuable advices.

REFERENCES

1. Adam R D (2001). Biology of *Giardia lamblia*. *Clin Microbiol Rev* **14**(3), 447-475.
2. Berkman D S, Lescano A G, Gilman R H, *et al*. (2002). Effects of stunting, diarrhoeal disease, and parasitic infection during infancy on cognition in late childhood: a follow-up study. Lancet **359**(9306), 564-571.
3. Leahy J G, Rubin A J, Sproul O J (1987). Inactivation of *Giardia muris* cysts by free chlorine. *Appl Environ Microbiol* **53**(7), 1448-1453.
4. Mac Kenzie W R, Hoxie N J, Proctor M E, *et al*. (1994). A massive outbreak in Milwaukee of cryptosporidium infection transmitted through the public water supply. *N Engl J Med* **331**(3), 161-167.
5. Yamazaki T, Sasaki N, Takahashi S, *et al*. (1997). Clinical features of Japanese children infected with *Cryptosporidium parvum* during a massive outbreak caused by contaminated water supply. *Kansenshogaku Zasshi* **71**(10), 1031-1036. (In Japanese)
6. Kim Y H, Lee S H, Lee C H (2006) Characteristics of occurrence of *Cryptosporidium* and *Giardia* in the Nakdong river. *J Kor Soc Environ Engineers* **28**(8), 843-851. (In Korean)
7. Lee M Y, Kim D Y, Cho E J, *et al*. (2000). Detection of *Giardia* and *Cryptosporidium* in water supplies in Seoul using Method 1623. *J Kor Soc Water Quality* **16**(5), 595-608. (In Korean)
8. U.S. Environmental Protection Agency (2001). US EPA method 1623: Cryptosporidium and Giardia in water by filtration/IMS/FA. EPA 815-R-05-002. Office of Water, U.S. Environmental Protection Agency, Washington, D.C.
9. Caccio S M (2003). Molecular techniques to detect and identify protozoan parasites in the environment. *Acta Microbiol Pol* **52**(Suppl.), 23-34.
10. Caccio S M (2004). New methods for the diagnosis of *Cryptosporidium* and *Giardia*. *Parassitologia* **46**(1-2), 151-155.
11. Johnson D W, Pieniazek N J, Griffin D W, *et al*. (1995). Development of a PCR protocol for sensitive detection of *Cryptosporidium* oocysts in water samples. *Appl Environ Microbiol* **61**(11), 3849-3855.

12.Nam S H, Lee J I, Lee S T, *et al*. (2006). Detection of viable *Cryptosporidium parvum* by real-time RT-PCR. *J Kor Soc Environ Anal* **10**(3), 134-140. (In Korean)
13.Rochelle P A, De Leon R, Stewart M H, *et al*. (1997). Comparison of primers and optimization of PCR conditions for detection of *Cryptosporidium parvum* and *Giardia lamblia* in water. *Appl Environ Microbiol* **63**(1), 106-114.
14.Gobet P, Toze S (2001). Relevance of *Cryptosporidium parvum* hsp70 mRNA amplification as a tool to discriminate between viable and dead oocysts. *J Parasitol* **87**(1), 226-229.
15.Rochelle P A, Ferguson D M, Handojo T J, *et al*. (1997). An assay combining cell culture with reverse transcriptase PCR to detect and determine the infectivity of waterborne *Cryptosporidium parvum*. *Appl Environ Microbiol* **63**(5), 2029-2037.
16.Stinear T, Matusan A, Hines K, *et al*. (1996). Detection of a single viable *Cryptosporidium parvum* oocyst in environmental water concentrates by reverse transcription-PCR. *Appl Environ Microbiol* **62**(9), 3385-3390.
17.Thiriat L, Sidaner F, Schwartzbrod J (1998). Determination of *Giardia* cyst viability in environmental and faecal samples by immunofluorescence, fluorogenic dye staining and differential interference contrast microscopy. *Lett Appl Microbiol* **26**(4), 237-242.
18.Bryan R T, Pinner R W, Berkelman R L (1994). Emerging infectious diseases in the United States, improved surveillance, a requisite for prevention. *Ann N Y Acad Sci* **740**, 346-361.
19.Connelly S J, Wolyniak E A, Dieter K L, *et al*. (2007). Impact of zooplankton grazing on the excystation, viability, and infectivity of the protozoan pathogens *Cryptosporidium parvum* and *Giardia lamblia*. *Appl Environ Microbiol* **73**(22), 7277-7282.
20.Labatiuk C W, Schaefer F W 3rd, Finch G R, *et al*. (1991). Comparison of animal infectivity, excystation, and fluorogenic dye as measures of *Giardia muris* cyst inactivation by ozone. *Appl Environ Microbiol* **57**(11), 3187-3192.
21.Rice E W, Schaefer F W 3rd (1981). Improved *in vitro* excystation procedure for *Giardia lamb*lia cysts. *J Clin Microbiol* **14**(6), 709-710.
22.Schupp D G, Erlandsen S L (1987). A new method to determine *Giardia* cyst viability: correlation of fluorescein diacetate and propidium iodide staining with animal infectivity. *Appl Environ Microbiol* **53**(4), 704-707.
23.Jenkins M, Trout J M, Higgins J, *et al*. (2003). Comparison of tests for viable and infectious *Cryptosporidium parvum* oocysts. *Parasitol Res* **89**(1), 1-5.
24.Kaucner C, Stinear T (1998). Sensitive and rapid detection of viable *Giardia* cysts and *Cryptosporidium parvum* oocysts in large-volume water samples with wound fiberglass cartridge filters and reverse transcription-PCR. *Appl Environ Microbiol* **64**(5), 1743-1749.
25.Widmer G, Orbacz E A, Tzipori S (1999). Beta-tubulin mRNA as a marker of *Cryptosporidium parvum* oocyst viability. *Appl Environ Microbiol* **65**(4), 1584-1588.

(*Received July* 22, 2009 *Accepted March* 7, 2010)