A new method for efficient detection of *Cryptosporidium* RNA by real-time reverse transcription-PCR with surfactants

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ABSTRACT

Cryptosporidium is one of the most common causes of waterborne diseases worldwide. Its oocysts possess a robust wall that is extremely resistant to the chlorine used for potable water disinfection. The current procedures of nucleic acid extraction and purification, such as the freeze–thaw (F/T) method and the commercial kits, are time consuming and expensive. To this end, a surfactant extraction treatment (SET) was developed as a method to extract nucleic acids from *Cryptosporidium* using only surfactants. The use of 18S rRNA improves the sensitivity of *Cryptosporidium* detection for real-time polymerase chain reaction (PCR), because 18S rRNA molecules are constitutively present in high copy numbers. Therefore, we applied SET to the detection of *Cryptosporidium* 18S rRNA using reverse transcription (RT)-PCR for the first time. RT-PCR was inhibited by 0.01% of the anionic surfactant sodium dodecyl sulfate (SDS), whereas the inhibition did not occur with 5% of the nonionic surfactants Tween 20, Triton X-100, Tween 80, and Triton X-114. However, the nonionic surfactants could not completely suppress the inhibition induced by 0.1% SDS. We successfully extracted 18S rRNA genes from oocysts by SET without the F/T method and detected them by real-time RT-PCR.

Key words │ 18S rRNA, *Cryptosporidium parvum*, nucleic acid extraction, oocyst, RT-PCR, surfactant

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INTRODUCTION

Cryptosporidium is a protozoan parasite distributed worldwide. It is excreted in the feces of infected humans or animals and is the causative agent of cryptosporidiosis, whose symptoms include watery diarrhea, vomiting, and fever. While *Cryptosporidium parvum* and *C. hominis* are the primary species known to infect humans, recent studies suggest that *C. cervine*, *C. felis*, and *C. meleagridis* may also cause diarrhea in humans (Carey *et al.* 2004). The oocysts of *Cryptosporidium* spp. are shed in the feces and may then enter sewage treatment facilities via wastewater or persist in the environment. A previous study has demonstrated that some sewage treatments are not efficient enough to eliminate all the oocysts prior to water discharge (Bonadonna *et al.* 2002), which can lead to outbreaks of cryptosporidiosis. The largest outbreak of watery diarrhea was recorded in Milwaukee, WI, USA, in 1993 and was caused by *Cryptosporidium* oocysts that were not removed by the filtration system of one of the city's water treatment plants. Over 400,000 residents in the Milwaukee area presented watery diarrhea, abdominal cramps, fever, and vomiting symptoms (Mac Kenzie *et al.* 1994). *Cryptosporidium* oocysts present a robust wall resistant to several environmental factors as well as to many of the processes and substances normally used for water disinfection. The robust nature of the oocyst wall requires more stringent treatments for disruption (Carey *et al.* 2004). Thus, early detection of oocysts in untreated water sources is essential to ensure efficient quality control for drinking water.

Cryptosporidium oocysts in water and fecal samples have been generally purified with antibody magnetic beads or

sucrose density-gradient centrifugation for *Cryptosporidium* testing (Simmons *et al.* 2001; Wohlsen *et al.* 2004). Then, the purified oocysts are identified by microscopy with fluorescent staining or genetic testing. Recently, the performance of real-time polymerase chain reaction (PCR) has been improving and has proven to be highly sensitive in detecting the presence of *Cryptosporidium* (Marangi *et al.* 2015; Operario *et al.* 2015). Successful detection of nucleic acids from purified oocysts usually requires complex extraction and purification processes aimed at digesting their protective wall. These may involve the use of methods such as freeze–thaw (F/T) cycles, enzymatic treatment, and surfactant treatment prior to nucleic acid amplification. Some of the most common methods used to extract nucleic acids from oocysts include freeze–thawing the samples in a lysis buffer or using commercially available nucleic acid extraction kits with proteinase K and a lysis buffer. The lysis buffer generally contains the anionic surfactant sodium dodecyl sulfate (SDS) (Webster *et al.* 1993; Leng *et al.* 1996; Nichols & Smith 2004; Schiffner *et al.* 2005). However, SDS is an inhibitor of PCR even at extremely low concentrations; therefore, a cleaning step is required to eliminate any trace of SDS prior to PCR amplification (Weyant *et al.* 1990). Furthermore, common procedures to extract and purify nucleic acids, such as the F/T method or those followed using commercial kits, are time consuming and expensive. Thus, there is an interest in the development of faster and more inexpensive methods to extract nucleic acids from oocysts.

In previous studies, we developed a surfactant extraction treatment (SET) as a simple alternative to extract DNA from *C. parvum* oocysts using only an anionic surfactant for PCR and loop-mediated isothermal amplification (Sekikawa & Kawasaki 2008; Sekikawa *et al.* 2011). Here, we explore the use of nonionic surfactants in suppressing the inhibition induced by SDS and the efficiency of the extraction method in amplifying DNA without a nucleic acid purification step.

The use of 18S rRNA improves the sensitivity of *Cryptosporidium* detection for real-time PCR, because 18S rRNA molecules are constitutively present in high copy numbers (Fontaine & Guillot 2003; Kishida *et al.* 2012). However, the efficacy of the SET extraction procedure in 18S rRNA detection has not been evaluated. Therefore, we examined the efficacy of SET combined with real-time reverse transcription (RT)-PCR as a fast method for extracting 18S rRNA from *C. parvum* oocysts.

METHODS

C. parvum oocysts

Purified and quantified *C. parvum* oocysts (Iowa isolate) were obtained from Waterborne Inc. (New Orleans, LA, USA). One week after administering the oocysts to C57BL/6 mice orally, the oocysts were purified from fecal samples using a sucrose gradient and Percoll density gradient with centrifugation at $1,200 \times g$ for 10 min. The interface between the sucrose and the Percoll layers was washed two times using deionized water. The oocysts were stained using a fluorescent antibody, Aqua-Glo GC Direct antibody (Waterborne Inc.), and quantified by flow cytometry with cell sorting using a Becton Dickinson Aria II instrument (Becton Dickinson, San Jose, CA, USA). Finally, the living oocysts were inactivated without affecting the oocyst wall by gamma irradiation under 10 kGy (Joung *et al.* 2011) and stored at 4 °C until further use in order to prevent a change in the number of the 18S rRNA gene copies in the oocysts. The oocysts were used for this experiment within a month after inactivation.

DNA and RNA templates

We extracted and purified nucleic acids by freeze–thawing oocysts between −80 and 37 °C in Tris-EDTA buffer five times (Promega, Madison, WI, USA) and DNeasy Blood & Tissue kits (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. Immediately after the last purification step, extracted and purified nucleic acid solutions were cooled in ice and used as DNA and RNA templates for the amplification reactions.

RT

An RNA template was subjected to RT using a PrimeScript RT Master Mix kit (TaKaRa Bio Inc., Shiga, Japan) to obtain cDNA (Kishida *et al.* 2012). The RT reaction mix included the sample in a final volume of 10 μl. The RT reaction was performed using a Thermal Cycler Dice Real Time System (TaKaRa Bio Inc.) with the following program: RT at 37 °C for 15 min and enzyme inactivation at 85 °C for 5 s. The synthesized cDNA was further used for *Taq*Man PCR.

*Taq*Man PCR

A Premix EX *Taq* kit (TaKaRa Bio Inc.) was used for PCR using a *Taq*Man probe. The primer Crypt-193f for *Cryptosporidium* 18S rRNA was used as the forward primer (5′-GGA AGG GTT GTA TTT ATT AGA TAA AGA ACC A-3′) and Crypt-374r (5′- CTC CCT CTC CGG AAT CGA A-3′) was used as the reverse primer. We used the *Taq*Man probe Crypt-276p (5′- CAT TCA AGT TTC TGA CCT ATC AGC TTT AGA CGG-3′) (Miller *et al.* 2006). *Taq*Man probe oligonucleotides were labeled with 6-FAM at the 5′ end and the quencher TAMRA at the 3′ end. The PCR assay using the *Taq*Man probe was conducted in a Thermal Cycler Dice Real Time System (TaKaRa Bio Inc.). After 30 s of hot start at 95 °C, the amplification conditions involved 50 cycles of 5 s at 95 °C and 30 s at 60 °C. Real-time *Taq*Man PCR was conducted in a Thermal Cycler Dice Real Time System (TaKaRa Bio Inc.). Each PCR assay contained 400 nM of each primer, 80 nM of the *Taq*Man probe, a Premix EX *Taq* kit (TaKaRa Bio Inc.), and 2 µl of the sample in a final volume of 25 µl. A cycle threshold analysis tool integrated in Thermal Cycler Dice Real Time System Lite software was used to compute the threshold levels.

Standard curve

A 192-bp fragment of standard DNA was chemically synthesized and sequenced by Eurofins Genomics (Tokyo, Japan). The synthesized standard DNA included the partial sequence of the 18S rRNA gene of *C. parvum* (Accession No. L16996; nucleotide position: 187–378). Serial dilutions of the standard DNA (range: $5–5 \times 10^6$ copies per reaction) were used to prepare the standard curve. All experiments were repeated twice in individual runs.

SET and real-time RT-PCR assay

Three hundred oocysts were incubated in 750 µl of Tris-EDTA buffer (Promega) with 0.1% SDS at 90 °C for 15 min to extract all nucleic acid content and used as the SET product. Figure 1(a) shows the combined SET and real-time RT-PCR method to detect 18S rRNA. The final concentration of oocysts and SDS in the SET product were 0.4 oocysts/µl and 0.1%, respectively. Subsequently, 1.0, 2.5, or 5.0 µl of the SET product was subjected to RT

using a PrimeScript RT Master Mix kit (TaKaRa Bio Inc.) to obtain cDNA. The RT reaction was performed in a final volume of 10 µl with or without 5% Tween 20 to evaluate the effect of suppression of the inhibition induced by SDS using a Thermal Cycler Dice Real Time System (TaKaRa Bio Inc.) with the following program: RT at 37 °C for 15 min and inactivation of the enzyme at 85 °C for 5 s. All the experiments were repeated twice in individual runs.

Real-time RT-PCR with or without surfactants

DNA or RNA templates from oocysts were amplified using real-time RT-PCR with or without surfactants. We compared the outcome of real-time RT-PCR in the presence of the anionic surfactant SDS (Promega) and/or the nonionic surfactants polyoxyethylene (20) sorbitan monolaurate (Tween 20) (Promega), polyoxyethylene (20) sorbitan monooleate (Tween 80) (Wako, Tokyo, Japan), octylphenol (ethyleneglycol)$_{9,6}$ ether (Triton X-100) (Wako), and octylphenol(ethyleneglycol)$_{7,5}$ ether (Triton X-114) (Wako). The structure and properties of the surfactants are shown in Table 1. Figure 1(b) shows the real-time RT-PCR method to detect 18S rRNA in the RNA template with surfactants. We first tested the effect of adding a surfactant to the RT or PCR reaction using real-time RT-PCR. In addition, we tested the effect of adding a mixture of SDS and a nonionic surfactant to the RT or PCR reaction using real-time RT-PCR. All experiments were repeated twice in individual runs.

RESULTS AND DISCUSSION

Standard curve determination

Figure 2 shows the standard linear response obtained on the basis of the amplification of standard DNA dilutions. C_t values were not calculated for 5×10^{-1} copies of standard DNA per PCR assay because DNA amplification was not detected. Therefore, the standard curve covered $5–5 \times 10^6$ copies of standard DNA per PCR assay. Following standard DNA amplification, we calculated the C_t values. Each point on the line represents the mean of two separate PCR amplifications. The slope of the curve was -1.457, with a squared correlation coefficient (r^2) of 0.9995.
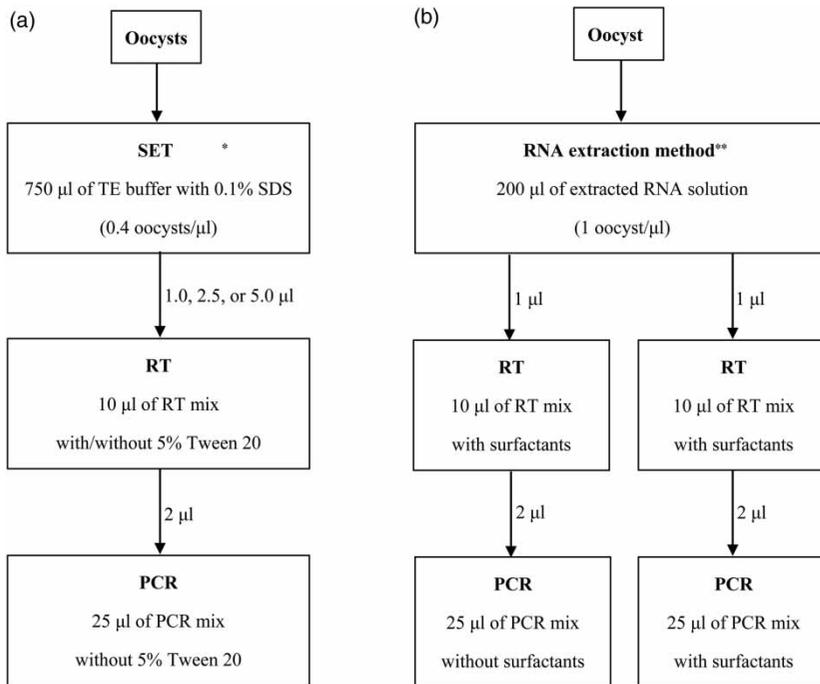
Figure 1 │ Schematic representation of the process for detecting 18S rRNA genes from *C. parvum* oocysts using real-time RT-PCR (a) with SET or (b) with DNeasy Blood & Tissue kits after the F/T method. The final solution volumes of SET, an RNA extraction method, RT, and PCR were 750 μl, 200 μl, 10 μl, and 25 μl, respectively. *We prepared a SET product from 300 oocysts. **We extracted and purified nucleic acids from 200 oocysts using DNeasy Blood & Tissue kits after the F/T method and used them as an RNA template.

Table 1 │ Structures and properties of the surfactants used in this study

| Surfactant	Structure	T	M_r	CMC (mM)	HLB
Tween 20		N	1,228	0.059	16.7
Tween 80		N	1,310	0.01	15.0
Triton X-100		N	628	0.31	13.5
Triton X-114		N	536	0.17	12.4
SDS		A	288	8.1	40.0

Note: Data are from Sivars & Tjerneld (2000).

T: type; N: nonionic; A: anionic; M_r: relative molecular mass of the monomer; CMC: critical micelle concentration; HLB: hydrophilic–lipophilic balance.

Amplification of DNA and RNA templates

DNA templates of dilution ratios of 0.01–1 and RNA templates of dilution ratios of 0.0001–1 were amplified by real-time RT-PCR. The C_t values obtained in both cases are shown in Table 2. Each *C. parvum* oocyst contains four sporozoites; therefore, there are theoretically four copies of the 18S rDNA gene in each oocyst. DNA templates of dilution
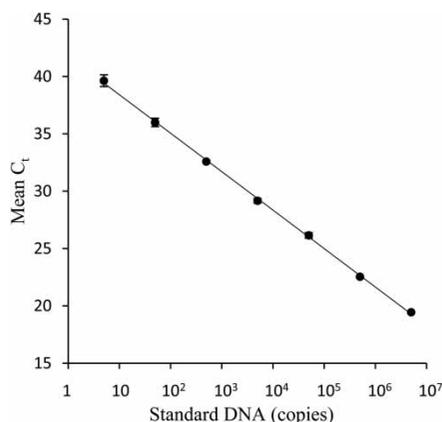
Figure 2 │ Standard curve using 5–5×10^6 copies of standard DNA in real-time PCR with a *Taq*Man probe. Each point on the line represents the mean of two separate PCR amplifications ($n = 2$). The error bars represent the standard deviation (SD).

Table 2 │ Results of real-time PCR and real-time RT-PCR using DNA and RNA templates from *C. parvum* oocysts

	C_t value (Mean $\pm$ SD)
Real-time PCR	
DNA template dilution ratio[a]	
1	32.8 ± 0.0
0.1	36.7 ± 0.3
0.01	ND
Real-time RT-PCR	
RNA template dilution ratio[b]	
1	25.8 ± 0.2
0.1	29.1 ± 0.1
0.01	32.3 ± 0.0
0.001	36.8 ± 0.9
0.0001	ND

Note: We extracted and purified nucleic acids from quantified oocysts using DNeasy Blood & Tissue kits after the F/T method and used them as DNA and RNA templates. Serial dilutions of DNA and RNA templates were subjected to PCR and RT-PCR.
ND: None detected.
[a]Two microlitres of a DNA template corresponding to 50 oocysts/µl (100 oocysts/PCR reaction) were subjected to PCR in the case of the dilution ratio of 1.
[b]One microlitre of an RNA template corresponding to 10 oocysts/µl (10 oocysts/RT reaction) was subjected to RT-PCR in the case of the dilution ratio of 1.
$n = 2$.

ratio of 0.01 corresponding to 1 oocyst/PCR reaction were not detected, although those of 0.1 corresponding to 10 oocysts/PCR reaction were detected by real-time PCR.

The number of copies of the 18S rRNA gene exceeded 2×10^3 per oocyst, because DNA templates of dilution ratio of 0.001 corresponding to 2×10^{-3} oocysts/PCR reaction were detected by real-time RT-PCR. However, Kishida *et al.* (2012) have reported that a standard *Cryptosporidium* oocyst contains

approximately 32,700 copies of the 18S rRNA gene, showing a considerable difference from the observations of the present study. In the present study, we quantified the number of oocysts using flow cytometry with cell sorting before performing PCR; however, Kishida *et al.* (2012) quantified the number of oocysts using conventional microscopic observation, which could have led to the inconsistencies between their findings and the present findings. Furthermore, there are no reports concerning changes in the individual difference in the number of 18S rRNA gene copies per oocyst. Therefore, further studies are needed to clarify the natural range of 18S rRNA gene copies per oocyst, which may vary depending on environmental factors such as temperature and CO_2 concentration.

Inhibition of DNA/RNA amplification by surfactants as assessed using real-time RT-PCR

We used real-time RT-PCR to test the level of inhibition of the RT or PCR reaction in the presence of SDS, Tween 20, Tween 80, Triton X-100, or Triton X-114. Table 3 shows the results of the induced inhibition assay. When 0.1% SDS was added to the RT mix, the final concentration in the PCR assay was 0.008%, which could lead to the inhibition of PCR. Therefore, the RT mix was diluted 10 times with TE buffer before PCR to reduce the concentration of SDS below 0.001%. As Table 3 shows, real-time RT-PCR using an RT mix with 0.01% SDS was inhibited and delayed compared with the reaction using RNA templates of dilution ratio of 0.1 corresponding to 2×10^{-1} oocysts/PCR reaction (Table 2). Furthermore, DNA amplification using an RT mix with 0.1% SDS and a PCR mix with 0.01% SDS was not detected.

Suppression of the inhibition of RT-PCR induced by SDS

To test whether nonionic surfactants can suppress the inhibition of RT and PCR induced by SDS, we examined the effect of combining SDS and nonionic surfactants in the same real-time RT-PCR assay. Table 4 shows the effect of adding nonionic surfactants to RT and PCR assays. DNA amplification was delayed when 0.01% SDS was present in the RT mix, and it was completely inhibited at 0.01% SDS in the PCR mix (Table 3). However, the inhibition induced by 0.01% SDS was suppressed by adding nonionic surfactants to the RT or PCR mix prior to real-time RT-PCR.

Table 3 | Results of the inhibition of RT or PCR induced by surfactants

	C_t value (Mean ± SD)						
	Conc. of surfactants						
	SDS						
	0.001%	0.01%	0.1%	Tween 20 5%	Tween 80 5%	Triton X-100 5%	Triton X-114 5%
RT[a]	30.0 ± 0.01	38.0 ± 0.3	ND[c]	29.8 ± 0.0	29.4 ± 0.4	29.7 ± 0.1	30.2 ± 0.1
PCR[b]	29.2 ± 0.01	ND	ND	29.1 ± 0.5	29.2 ± 0.6	29.7 ± 0.1	29.4 ± 0.0

Note: We extracted and purified nucleic acids from 200 oocysts using DNeasy Blood & Tissue kits after the F/T method and used them as an RNA template (final volume: 200 μl). One microlitre of the RNA template corresponding to 1 oocyst/μl (1 oocyst/RT reaction) was used for RT-PCR.

ND: None detected.

[a]RT-PCR was performed using the RT mix with the surfactant and the PCR mix without the surfactant.

[b]RT-PCR was performed using the RT mix without the surfactant and the PCR mix with the surfactant.

[c]The RT mix diluted ten times with TE buffer was amplified using PCR.

$n = 2$.

Table 4 | Results of suppression of the inhibition of RT or PCR induced by SDS by adding a nonionic surfactant

| | C_t value (Mean ± SD) | | | |
| | Conc. of nonionic surfactants | | | |
	Tween 20 5%	Tween 80 5%	Triton X-100 5%	Triton X-114 5%
RT[a]				
SDS				
0.01%	29.6 ± 0.1	29.9 ± 0.3	30.3 ± 0.1	29.8 ± 0.3
0.1%	31.1 ± 0.0	32.3 ± 0.2	31.6 ± 0.9	35.4 ± 0.3
PCR[b]				
SDS				
0.01%	29.0 ± 0.1	29.6 ± 0.2	29.6 ± 0.2	29.4 ± 0.5
0.1%	28.7 ± 0.1	29.4 ± 0.8	29.6 ± 0.2	ND

Note: We extracted and purified nucleic acids from 200 oocysts using DNeasy Blood & Tissue kits after the F/T method and used them as an RNA template (final volume: 200 μl). One microlitre of the RNA template corresponding to 1 oocyst/μl (1 oocyst/RT reaction) was used for RT-PCR.

ND: None detected.

[a]RT-PCR was performed using the RT mix with the surfactants and the PCR mix without the surfactants.

[b]RT-PCR was performed using the RT mix without the surfactants and the PCR mix with the surfactants.

$n = 2$.

Eriksson *et al.* (2002) reported that if a nonionic surfactant is mixed with a small amount of charged SDS, mixed micelles of the nonionic surfactant and SDS are formed. Thus, suppression of SDS-induced inhibition could be related to the formation of micelles in the RT or PCR mix.

Regarding the RT reaction, inhibition by 0.01% SDS was completely suppressed by adding any of the four nonionic surfactants. However, DNA amplification was slightly delayed in the presence of 0.1% SDS or any of the four nonionic

surfactants. The four surfactants could not completely suppress the inhibition of RT induced by 0.1% SDS. Comparing the C_t values of the RT reactions in the presence of 0.1% SDS (Table 4), the effect of suppression of the inhibition of RT decreases in the following order: Tween 20 > Triton X-100 > Tween 80 > Triton X-114. The suppression by Triton X-114 was particularly weak. As a result, an SDS content of 0.01% or less is recommended for the SET protocol, because it is difficult to completely suppress the inhibition induced by 0.1% SDS or more.

Regarding PCR, the inhibition induced by 0.01% SDS was completely suppressed by the four nonionic surfactants; however, DNA amplification was not detected for the reactions including 0.1% SDS and Triton X-114. The effect of suppression by Triton X-114 in the case of PCR was particularly weak, similar to the results obtained for the RT reactions. These results indicated that the effect of suppression by adding the nonionic surfactants differed between RT and PCR reactions.

When small amounts of SDS are mixed with a nonionic surfactant, SDS is almost totally incorporated into nonionic micelles, reducing the concentration of active SDS (Eriksson *et al.* 2002). When the concentration of SDS in the RT mix was 0.1%, none of the four nonionic surfactants could decrease the concentration of free SDS below 0.001% because of the high amount of charged SDS in the mix.

The C_t value (38.0) in the case of an RT reaction including 0.01% SDS (Table 3) appeared to be increased compared with the C_t value (29.1) in the absence of surfactants (positive control) (Table 2). The C_t value (29.6) of DNA amplification using the RT mix containing 0.01% SDS and 5% Tween 20 (Table 4) was almost the same as the C_t value (29.1) of the

positive control. These results showed that Tween 20 is effective in suppressing the inhibition of RT-PCR induced by SDS.

Test to detect 18S rRNA gene from *C. parvum* oocysts using SET and real-time RT-PCR

Nucleic acids from oocysts were extracted using SET and incubated with or without 5% Tween 20; the RT product obtained was subsequently amplified using PCR (Figure 1(a)). Table 5 shows the results of adding Tween 20 to the RT mix before real-time RT-PCR. One microlitre, 2.5 μl, or 5.0 μl of the SET product was added to each RT mix in a final reaction volume of 10 μl. Thus, the concentrations of the SET products in the RT mixes were 10%, 25%, and 50%, respectively. The maximum volume of the SET product added to the RT mix was 5.0 μl to ensure that the maximum concentration of the sample did not exceed 50% as recommended by the manufacturer. As a result, DNA amplification using the RT products as templates was not detected or was very delayed in the absence of Tween 20. When the RT product included 10% of the SET product and 0.01% SDS in the absence of Tween 20, DNA amplification was not detected. This lack of detection could relate to the low number of oocysts included in the RT reaction (0.08 oocysts) and the inhibition of the RT mix by 0.01% SDS.

When 5% Tween 20 was added to the RT mix, the speed of DNA amplification directly correlated with the concentration of the SET product in the RT mix. The speed of DNA amplification using the 25% SET products in the RT mix (corresponding

to 1 oocyst/RT reaction) was faster than that using the RNA template. These results suggested that 5% Tween 20 could suppress the inhibition induced by 0.05% SDS (Table 5). Therefore, the best concentration of the SET product in the RT mix to improve detection sensitivity is 50%. The C_t values using an RNA template and using a SET product, both corresponding to 1 oocyst/RT reaction, were 29.1 and 27.0 respectively. These data prove that SET increases the probability of extracting the 18S rRNA gene compared to F/T and presents the advantage of not requiring a nucleic acid purification step.

Although an autoclave (121 °C, 15 min) degraded *Staphylococcus aureus* and *Escherichia coli* O157:H7 rRNA enough so that signals were lost during Northern hybridization, and the rRNA could not be used as a template in subsequent RT-PCR (McKillip *et al.* 1998), Uyttendaele *et al.* (1997) found that the 16S rRNA of *Campylobacter jejuni* was stable for 5 hours following treatment at 100 °C. Regarding *Cryptosporidium*, Fontaine & Guillot (2003) reported that the 18S rRNA started decaying after treatment of *C. parvum* oocysts at 95 °C for 20 min, and the decay was still detectable 4 hours after treatment. In contrast, DNA was more heat resistant. Thus, it can be considered that SET hardly decomposes the 18S rRNA gene, because the temperature and incubation times required are lower and shorter, respectively, than those reported by Fontaine & Guillot (2003). However, F/T is known to damage nucleic acids, particularly RNA. These results demonstrate that SET can be successfully used to extract RNA more efficiently than the F/T method.

CONCLUSION

Here we assessed the effectiveness of a new method, SET, for extracting 18S rRNA from purified oocysts. The inhibition of RT-PCR induced by 0.01% SDS could be suppressed by adding one of the four nonionic surfactants tested in this study. Comparing the reactions including 0.1% SDS, a nonionic surfactant, and either RT or PCR products as the template, the C_t values observed for Tween 20, Triton X-100, and Tween 80 did not differ greatly. However, Triton X-114 was particularly weak in suppressing the inhibition induced by SDS. Among the four nonionic surfactants, Tween 20 appeared as the best suppressor of the inhibition induced by SDS. These data show that SET can be used to generate a template for RT-PCR without the need for general nucleic acid extraction methods. Consequently, our results demonstrate

Table 5 │ Results of the effect of adding Tween 20 to an RT mix on SET and real-time RT-PCR assay

Conc. of the SET product in the RT mix (%)	Conc. of SDS in the RT mix (%)	C_t value (Mean ± SD) Conc. of Tween 20 in the RT mix	
		0%	5%
10	0.01	ND	28.6 ± 0.3
25	0.025	38.5 ± 0.9	27.0 ± 0.2
50	0.05	38.1 ± 0.2	26.6 ± 0.1

Note: We prepared a SET product (final volume: 750 μl) from 300 oocysts. One microlitre, 2.5 μl, or 5.0 μl of the SET product corresponding to 0.4 oocysts/μl was used for RT-PCR with or without 5% Tween 20. Each RT mix (final volume: 10 μl) contained 10%, 25%, or 50% of the SET product corresponding to 0.4, 1, or 2 oocysts/RT reaction, respectively. ND: None detected.
$n = 2$.

that 18S rRNA genes can be efficiently extracted from the oocysts by SET. Although there is the risk of an erroneous determination of *Cryptosporidium* oocysts in environmental water samples using only microscopy, the risk would be reduced by using SET and RT-PCR together with microscopy.

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