

A new immunomagnetic bead separation–surfactant extraction treatment protocol for rapid and sensitive quantitative PCR detection of *Cryptosporidium parvum* DNA

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ABSTRACT

The *Cryptosporidium* oocyst is encased in a robust wall that is extremely resistant to detrimental environmental factors such as chlorine used to disinfect potable water. Therefore, extracting oocyst DNA is not a trivial undertaking. Standard procedures used to extract DNA from oocysts, such as freeze–thaw (F/T) methods and DNA purification kits, are time-consuming and expensive and are difficult to implement in routine clinical practice. Therefore, we developed a surfactant extraction treatment (SET) that efficiently extracts DNA from the oocyst. Immunomagnetic separation (IMS) combined with quantitative real-time polymerase chain reaction (qPCR) detects pathogenic microorganisms with high sensitivity. The objective of the present study was to evaluate SET for its ability to simplify qPCR detection of 18S rDNA directly from immunomagnetic bead–oocyst conjugates. DNA extracted directly from the conjugates using SET did not affect DNA amplification in the qPCR assay. Further, the rate of DNA amplification using IMS–SET was greater than that using F/T combined with the DNA purification kit. The rate of recovery of oocysts from surface water samples spiked with oocysts did not differ significantly from previously published values. These data demonstrate that the new IMS–SET protocol using qPCR can simplify the recovery and detection of *Cryptosporidium* oocysts.

Key words | *Cryptosporidium* DNA, immunomagnetic beads, oocysts, real-time PCR, surface water, surfactant extraction treatment

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INTRODUCTION

Cryptosporidium parvum was first recognized as a human pathogen in 1976 (Nime *et al.* 1976), nearly 70 years after the first cryptosporidial species was identified in mice (Tyzzer 1907). *Cryptosporidium* infects various vertebrate hosts, including mammals, rodents, birds, reptiles, and fish; and oocysts excreted by these hosts are present in the environment (Smith & Nichols 2010). *Cryptosporidium*

parvum is a major zoonotic species that causes neonatal diarrhea in livestock, which is accompanied by significant economic losses (Smith & Nichols 2010).

Cases of cryptosporidiosis with HIV/AIDS were reported in the early 1980s. The link with AIDS was so strong that cryptosporidiosis became one of the defining features of the syndrome before the discovery of the human immunodeficiency virus (HIV) (Tzipori & Widmer 2008). *Cryptosporidium* is a significant pathogen in all populations regardless of the prevalence of HIV. It is the second most common pathogen in infants (Kotloff *et al.* 2013). In 1993, in

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the largest outbreak associated with a contaminated public water source reported to date in the United States, *Cryptosporidium* made 403,000 residents of Milwaukee, WI sick (Corso *et al.* 2003). This outbreak was caused by *Cryptosporidium* oocysts that were not removed by one of the city's water treatment plants, and the victims experienced watery diarrhea, abdominal cramps, fever, and vomiting (MacKenzie *et al.* 1994). In 2010, a waterborne cryptosporidiosis outbreak occurred in Östersund, Sweden, and affected at least 12,700 inhabitants (Widerström *et al.* 2014).

The infectious dose varies depending on the *Cryptosporidium* isolate, and as few as 10 oocysts cause disease (Okhuysen *et al.* 1999). *Cryptosporidium* represents a major challenge to the delivery of safe drinking water because the oocysts can survive and remain infectious for 16 months in disinfected-treated water supplies (Bridle *et al.* 2012). The oocysts are encased in a durable oocyst wall comprising inner and outer walls composed of a protein-lipid-carbohydrate matrix that serves as a highly effective protective barrier against deleterious environmental factors (Fayer 2008). Thus, early detection of oocysts in water sources is essential to ensure the safety of drinking water (Widerström *et al.* 2014).

Successful detection of nucleic acids from purified oocysts usually requires complex extraction and purification processes aimed at digesting their protective wall. These protocols may involve techniques such as freeze-thaw (F/T) cycles as well as treatments using enzymes and surfactants before nucleic acid amplification. These procedures take at least 1 h (Gao *et al.* 2014).

Recently, we developed a new method – a surfactant extraction treatment (SET) that is simple to perform and inexpensive (Sekikawa & Toshiki 2015). SET uses an anionic surfactant, sodium dodecyl sulfate, or SDS, a common component of lysis buffers generally (Webster *et al.* 1993; Leng *et al.* 1996; Nichols & Smith 2004; Schiffner *et al.* 2005). The SET method can extract nucleic acids by incubating the oocyst in 0.1% SDS solution at 90 °C for 15 min, without F/T and purification kits. Because SDS inhibits polymerase chain reaction (PCR) assays even at extremely low concentrations, a purification step is then required to eliminate any trace of SDS. In the SET assay we overcame inhibition by adding 5% Tween 20 to the PCR reactions (Sekikawa & Toshiki 2015).

Immunomagnetic separation (IMS) combined with quantitative PCR (qPCR) is an effective and sensitive method for

detecting pathogenic microorganisms in environmental samples (Hallier-Soulier & Guillot 2000; Hwang *et al.* 2007; Gao *et al.* 2014). Therefore, IMS is generally used in combination with qPCR for detecting *Cryptosporidium* DNA in sources such as surface water and feces (Fontaine & Guillot 2003; Gao *et al.* 2014). We reasoned that DNA extracted directly from an immunomagnetic bead (IMB)-oocyst conjugate using 0.1% SDS could be applied prior to the qPCR assay to provide a simple method for detecting oocysts. Thus, the objective of the present study was to demonstrate the use of SET to extract the 18S rDNA gene directly from IMB conjugates for detection of *Cryptosporidium* without diminishing the sensitivity of the PCR assay.

METHODS

C. parvum oocysts

Standard suspensions containing 10^5 *Cryptosporidium parvum* oocysts (Iowa isolate, AccuSpike-IR) were obtained from Waterborne™, Inc. (New Orleans, LA, USA).

River water sample collection and water quality analysis

A surface water sample was obtained from the drinking water intake of the Okitsu River in Shizuoka City during March 2015. The surface water of the Okitsu River is a major source of drinking water for the residents of the Simizu-Ku area of Shizuoka City. Digital meters were used to measure the pH, temperature, electrical conductivity (EC), and total dissolved solids (TDS) of each water sample. The formazine standard solution, adjusted to 400 nephelometric turbidity units (NTUs) (Wako Pure Chemical Industries, Osaka, Japan), was used as a standard. Turbidity was determined at 660 nm using a spectrophotometer (UV-1600, Kyoto, Shimadzu) (Ortmanis *et al.* 1991).

DNA standard

A 192-bp fragment of standard DNA that included the partial sequence of the 18S rRNA gene of *C. parvum* (Accession No. L16996; nucleotides 187–378) was

chemically synthesized and sequenced by Eurofins Genomics (Tokyo, Japan).

SET

To extract nucleic acids, *Cryptosporidium* oocysts or IMB–oocyst conjugates were incubated at 90 °C for 15 min in a SET tube containing Tris-EDTA (TE) buffer (Promega Corporation, Madison, WI, USA) and 0.1% SDS and used as a SET product (Figure 1). A SET procedure includes addition of the nonionic surfactant Tween 20 to a PCR tube at a final concentration of 5% in order to suppress PCR inhibition induced by SDS (Sekikawa & Toshiki 2015). After extracting nucleic acids, Tween 20 was added to a PCR tube containing FastStart Essential DNA Probes Master Mix (Roche Diagnostics, Mannheim, Germany), and 2.0 µL of the SET product was vortexed and added immediately to the PCR tube.

TaqMan PCR

A FastStart Essential DNA Probes Master Mix (Roche Diagnostics) that included a TaqMan probe was used for PCR assays. *Cryptosporidium* 18S rDNA was amplified using the forward primer Crypt-193f and the reverse primer Crypt-374r (Miller *et al.* 2006). DNA amplification was monitored using the TaqMan probe Crypt-276p (Miller *et al.* 2006). The TaqMan probe was labeled with 6-FAM and the quencher TAMRA at the 5' and 3' ends, respectively. The qPCR assay was conducted using a LightCycler Nano system (Roche Diagnostics). After 10 min at 95 °C, the DNA was amplified for 50 cycles of the denaturation step at 95 °C for 10 s and the probe measurement was taken during the annealing/extension step at 60 °C for 30 s. Each

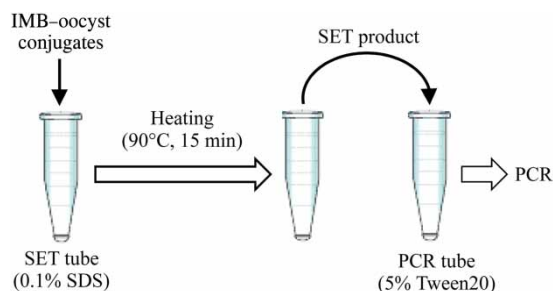


Figure 1 | SET applied to IMB–oocyst conjugates. SET, surfactant extraction treatment; IMB, immunomagnetic bead.

PCR assay (final volume, 20 µL) contained 400 nM of each primer, 80 nM TaqMan probe, the master mix (Roche Diagnostics), and 2 µL sample. Tween 20 was added when required to each PCR tube at a final concentration of 5%. A cycle threshold analysis tool integrated in the LightCycler Nano software V1.1.0 was used to compute threshold levels.

Effect of IMBs subjected to SET on qPCR assays

The effects of 2 µL of IMBs (Dynabeads anti-*Cryptosporidium* [Invitrogen Dynal AS, Oslo, Norway]) with or without SET on a qPCR assay were determined using a DNA standard (3×10^4 copies/µL). The IMBs occupied 10% of the volume of the master mix.

Each reagent was independently prepared in a PCR tube. The PCR for each experiment was run together using the LightCycler Nano system (Roche Diagnostics).

Detection of *Cryptosporidium* DNA in IMB–oocyst conjugates subjected to SET

The effect of SET, and of IMB–oocyst conjugates subjected to SET, on qPCR assays was determined using standard suspensions of 10^5 oocysts added to 10 mL distilled water in a 10-mL centrifuge tube. IMB–oocysts were prepared and isolated using 100 µL of the IMB kit described above, according to the manufacturer's instructions (Dynabeads anti-*Cryptosporidium* Catalog 2012). The IMB–oocyst conjugates were resuspended in 100 µL of TE buffer (Promega). To compare IMS recovery rates, oocysts were isolated by centrifugation. For comparison with SET, DNA was isolated from oocysts using five cycles of F/T (at –80 °C and 37 °C) (Kishida *et al.* 2012; Ichikawa-Seki *et al.* 2015) in TE buffer and then purified using a commercial DNA purification kit (DNeasy Blood & Tissue kits; QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. Immediately after the last purification step, the nucleic acid solutions were cooled in ice. The theoretical concentration of oocysts was $10^5/\mu\text{L}$ in the SET tube after SET or after the IMB–oocyst conjugate was subjected to SET. Two microliters of the SET product was vortexed and immediately added to the PCR master mix. Serial dilutions of the SET product ($1-10^4$ fold) were used to prepare the samples for PCR assays. Amplifications of free oocyst DNA and the

conjugates after SET were performed with or without 5% Tween 20 in a PCR tube. Each of the four reagents were independently prepared in a PCR tube. The PCR with the three prepared reagents was run together using the Light-Cycler Nano system (Roche Diagnostics).

qPCR detection of *Cryptosporidium* DNA in water samples spiked with oocysts that were subjected to IMB-SET

Water sample collection, *Cryptosporidium* oocyst isolation and detection were processed according to the method described by the Japan Water Works Association (JWWA) (Japan Water Works Association 2003). Oocysts were added to distilled water and river water samples to compare recovery rates. Briefly, 10 L of water samples added to a polyethylene tank were spiked with 10^5 oocysts and were filtered through a $5.0\ \mu\text{m}$ pore PTFE membrane filter (Millipore, Carrigtwohill Co., Cork, Ireland). *Cryptosporidium* oocysts collected on the filter membrane were eluted by vortexing the filter in PET eluting solution (Inoue *et al.* 2003). The eluted material was concentrated by centrifugation ($1,050\times g$, 10 min) in a 50-mL centrifuge tube. The oocysts were isolated using 100 μL of IMBs and processed according to the manufacturer's instructions. The IMB-oocyst conjugate was resuspended in 100 μL TE buffer. Serial dilutions ($1-10^4$ fold) of the SET product were used to prepare the samples for PCR. Each DNA amplification was performed with or without 5% Tween 20 in a PCR tube. The recovery from each water sample spiked with oocysts was independently performed. Each reagent was independently prepared in a PCR tube. The PCR for each experiment was run together using the Light-Cycler Nano system (Roche Diagnostics).

RESULTS AND DISCUSSION

Standard curve

Cryptosporidium parvum oocysts were used to generate a standard curve. Figure 2 shows the amplification of dilutions of the DNA extracted from the oocysts using SET. Ct values were calculated for 2 to 2×10^4 oocysts per PCR assay. Each

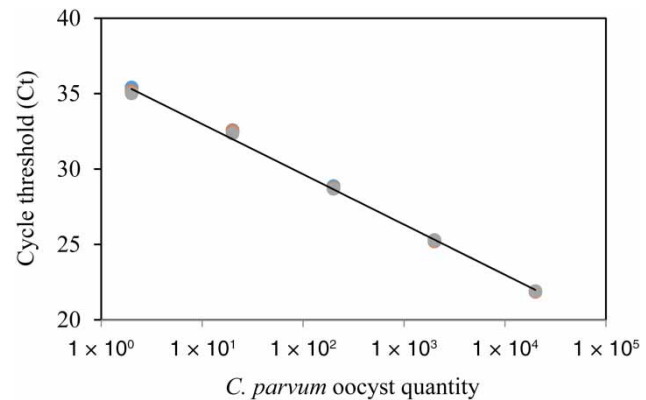


Figure 2 | Standard curve generated from 2 to 2×10^4 oocysts of *C. parvum* per PCR assay ($n = 3$, $r^2 = 0.9979$). DNA was extracted from the oocysts using SET.

point on the line represents the mean of three independent PCR amplifications. The slope = -1.447 , and the correlation coefficient (r^2) = 0.9979.

Effect of IMBs subjected to SET on qPCR assays

The results of qPCR assays using the DNA standard with or without IMBs are shown in Table 1. In the absence of Tween 20, the Ct values of the untreated IMBs and the positive control were 23.09 and 23.14, respectively, indicating that a 10% suspension of IMBs did not inhibit the qPCR assay. DNA isolated from the IMBs after SET without Tween 20 was not amplified (Table 1). In our previous study, the final concentration of SDS in the PCR mix was 0.01%,

Table 1 | qPCR assays of DNA extracted from IMBs subjected to SET

| Sample for PCR | Conc. of SDS in the PCR mix | Ct value (mean \pm SE) | |
|---------------------------------|-----------------------------|----------------------------------|------------------|
| | | Conc. of Tween 20 in the PCR mix | |
| | | 0% | 5% |
| IMBs and standard DNA | 0.0% | 23.09 \pm 0.07 | 23.42 \pm 0.01 |
| IMBs after SET and standard DNA | 0.01% | ND | 23.26 \pm 0.05 |
| Standard DNA (positive control) | 0.0% | 23.14 \pm 0.02 | 23.28 \pm 0.07 |

qPCR, quantitative real-time PCR; IMB, immunomagnetic bead; SET, surfactant extraction treatment; SE, standard error; ND, none detected. $n = 3$. Standard DNA (3×10^4 copies per reaction) was used for qPCR. The DNA standard included a partial sequence of the 18S rRNA gene of *C. parvum*.

which inhibits PCR (Sekikawa & Toshiki 2015). Further, we reported that inhibition of PCR by 0.01% SDS is reduced by adding 5% Tween 20 (Sekikawa & Toshiki 2015). Therefore, each PCR assay was performed in the presence of Tween 20 to examine the effect on DNA amplification of the IMBs after they were subjected to SET. Here, we show that Tween 20 abolished the inhibitory effect of the IMBs after SET (Table 1). The results of the Ct values in the presence of 5% Tween 20 indicate that the activity of *Taq* polymerase was not affected by SET.

Effect of IMB–oocyst conjugates subjected to SET on the qPCR assay

The Ct values obtained using oocysts and the conjugates are shown in Table 2. DNA was not detected following extraction from the oocysts or from the conjugates subjected to SET. SDS inhibited the PCR assay because the concentration of SDS in the PCR tube was 0.01%. However, it was difficult to judge whether the SET extracted DNA from the conjugates. To test whether Tween 20 suppressed the inhibition of the PCR assay by SDS, we determined the effect of combining the conjugates after SET and Tween 20. It was found that in the presence of Tween 20, the DNA extracted from the conjugates using SET was amplified and that the mean Ct value of the conjugates (25.32) was not significantly different compared with that

of the oocysts recovered using centrifugation (25.27) (Table 2). These results indicate that DNA can be extracted from conjugates and amplified after SET.

DNA detection from river water samples spiked with oocysts that were subjected to IMS–SET

We tried detecting *Cryptosporidium* DNA in the water sample obtained from the Okitsu River using filtration, IMS, F/T and the DNA purification kit (DNeasy Blood & Tissue kits; QIAGEN). PCR inhibitors were removed from the river water sample by using the DNA purification kit. As a result, *Cryptosporidium* DNA was not detected on the qPCR assay. Therefore, the Okitsu River sample was used for the preparation of a river water sample spiked with oocysts.

Oocysts were recovered from samples of distilled water or river water using IMS, and DNA was extracted from IMB–oocyst conjugates using SET. The limit of detection of the qPCR assay was two oocysts/PCR reaction mixture (Table 3). The Ct values without Tween 20 at the dilution ratio of 1 were not detected because the concentrations of SDS in the PCR mix were 0.01%. The PCR assay is not inhibited by 0.001% SDS (Sekikawa & Toshiki 2015). Therefore, the amplification of DNA in 10 L distilled water with Tween 20 using a 10^{-1} dilution was not detected because the concentration of SDS was 0.001%.

In general, oocyst recoveries depend on the compositions of water samples because river water contains inhibitory turbid materials (Fontaine & Gulliot 2003). Here, when we determined the ability of IMS–SET without Tween 20 to detect *Cryptosporidium* DNA in the river water sample diluted to 10^{-1} concentration (0.001% SDS), DNA amplification was not detected (Table 3). There are various substances and particles in the river water sample. Some substances and particles attach to the surface of IMB–oocyst conjugates. The SET product contains 0.1% SDS, free oocyst DNA and the conjugates with the substances and particles. Serial dilutions of the SET product ($1-10^4$ fold) were used to prepare the samples for PCR assays. Therefore, there is a possibility that the substances or the particles affected DNA amplification. DNA in the river water sample diluted to 10^{-2} concentration was able to be amplified as well as DNA in the distilled water sample because PCR inhibitors

Table 2 | Effect of IMB–oocyst conjugates combined with SET on the qPCR assay

| Sample for DNA extraction | DNA extraction method | Ct value (mean ± SE) | |
|---------------------------|---------------------------|----------------------------------|--------------|
| | | Conc. of Tween 20 in the PCR mix | |
| | | 0% | 5% |
| IMB–oocyst conjugates | SET | ND ^a | 25.32 ± 0.03 |
| Oocysts | SET | ND ^a | 25.27 ± 0.06 |
| Oocysts | F/T, DNA purification kit | 25.64 ± 0.02 | 25.70 ± 0.03 |

IMB, immunomagnetic bead; SET, surfactant extraction treatment; qPCR, quantitative real-time PCR; SE, standard error; ND, none detected.

^a0.01% SDS. $n = 4$. Oocysts were recovered from 10 mL of distilled water (DW) spiked with 10^5 oocysts using immunomagnetic separation (IMS) or centrifugation. Oocyst DNA was extracted using SET or F/T combined with a DNA purification kit.

Table 3 | qPCR detection of oocyst DNA in a 10 L water sample spiked with oocysts that were collected using IMS-SET

| Dilution of sample for PCR | Theoretical number of oocysts in the PCR mix | Ct value (mean ± SE) | | | |
|----------------------------|--|---|--------------|--|--------------|
| | | Distilled water (DW) sample Conc. of Tween 20 in the PCR mix | | River water sample Conc. of Tween 20 in the PCR mix | |
| | | 0% | 5% | 0% | 5% |
| 1 | 2000 | ND ^a | 25.64 ± 0.17 | ND ^a | 26.04 ± 0.20 |
| 10 ⁻¹ | 200 | 29.28 ± 0.17 | 29.01 ± 0.15 | ND | 29.39 ± 0.20 |
| 10 ⁻² | 20 | 32.46 ± 0.14 | 32.30 ± 0.16 | 32.80 ± 0.17 | 32.70 ± 0.18 |
| 10 ⁻³ | 2 | 36.32 ± 0.22 | 35.79 ± 0.25 | 36.31 ± 0.27 | 36.19 ± 0.23 |
| 10 ⁻⁴ | 0.2 | ND | ND | ND | ND |

qPCR, quantitative real-time PCR; SE, standard error; IMS, immunomagnetic separation; SET, surfactant extraction treatment; ND, none detected.

^a0.01% SDS. $n = 3$. IMS was used to recover oocysts from 10 L of DW or river water spiked with 10⁵ oocysts.

in the sample were diluted until their effect disappeared. The inhibition induced by the substances and particles was prevented by adding Tween 20, and further studies are required to identify these inhibitors.

The result of the recovery rates of *Cryptosporidium* oocysts from water samples spiked with 10⁵ oocysts is shown in Figure 3. The recovery rates of IMS-SET from 10 mL-distilled water (DW), centrifuge-SET from 10 mL-DW, centrifuge-F/T-DNA purification from 10 mL-DW, IMS-SET from 10 L-DW and IMS-SET from 10 L-river water were 100.0 ± 2.0% (positive control), 103.5 ± 4.0%, 77.0 ± 9.0%, 80.0 ± 9.0% and 60.8 ± 7.8%, respectively. Of the three 10 mL-DW samples, the recovery rate of only the centrifuge-F/T-DNA purification sample was decreased. These results indicate that the process of F/T or DNA purification

was not able to recover the *Cryptosporidium* DNA from the oocyst as well as SET.

Substances in the river water sample were not analyzed in the present study. However, the presence of large feed particles in raw water interferes with the ability of IMS to recover oocysts, and certain substances and particles inhibit PCR assays (Fontaine & Guillot 2003). The use of a current method with IMS makes it difficult to efficiently recover oocysts from the surface waters of rivers and lakes. For example, using IMS and staining with fluorescein isothiocyanate, 44 ± 10% of oocysts are recovered from a 15 L surface water sample (Blankaart catchment site, Belgium) spiked with 100 oocysts (Ehsan *et al.* 2015). The mean recovery rate using IMS, DNA purification, and qPCR from 5 L surface water samples (turbidity, 4–7 NTU) of the Seine

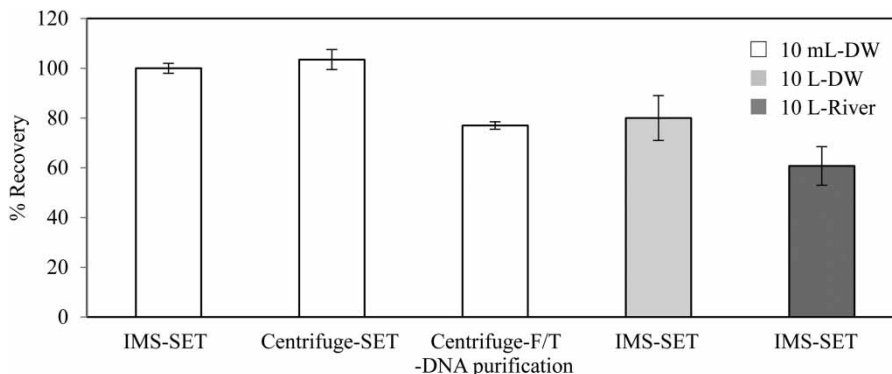


Figure 3 | The recovery rate of oocysts from water samples spiked with *C. parvum* oocysts. IMS, immunomagnetic separation. SET, surfactant extraction treatment. DW, distilled water. F/T, freeze-thaw. $n = 3$. Error bars express standard errors of the mean. IMS or centrifugation was used to collect oocysts from DW or river water samples spiked with 10⁵ oocysts. Oocyst DNA was extracted using SET or F/T-DNA purification. Each water sample without dilution was added to a PCR tube. The concentration of Tween 20 in a PCR tube was 5%. The recovery rates of oocyst from samples were calculated using a standard curve.

River that were spiked with 775 oocysts is $57.6 \pm 10.7\%$ (Fontaine & Guillot 2003). The values for the turbidity, pH, EC, and TDS of the Okitsu River sample used here were 1.2 NTU, 7.0, 144.0 μS , and 98.4 mg/L, respectively. The recovery rate from the Okitsu River sample did not differ significantly from previously published values although the quality of river water was different. These data demonstrate that qPCR assays with Tween 20 can be used for detecting oocyst DNA recovered from river water using IMS combined with SET. Consequently, the new protocol was able to simplify the operation of *Cryptosporidium* detection. However, we couldn't explain how the substances and particles in the river water sample affect the recovery rate in the present study. We need further study to determine the cause of the low recovery rate of oocysts.

CONCLUSION

- (1) A new protocol using IMS–SET can reduce the time spent on DNA extraction and purification procedures because SET takes only 15 min to extract DNA from the conjugates without the need for time-consuming processes.
- (2) The new protocol was developed to simplify the recovery and detection of *Cryptosporidium* oocysts, which can facilitate risk assessment of the quality of drinking water.
- (3) The oocyst is enclosed by a robust wall that is more resistant than the cell walls of common bacteria. Therefore, the SET protocol for extracting DNA from IMBs can be applied to numerous microorganisms present in water supplies.

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