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An immunomagnetic separation–real-time PCR method for quantification of *Cryptosporidium parvum* in water samples

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Abstract

The protozoan parasite *Cryptosporidium* parvum is known to occur widely in both saw and drinking water and is the cause of waterborne outbreaks of gastroenteritis throughout the world. The routinely used method for the detection of *Cryptosporidium* oocysts in water is based on an immunofluorescence assay (IFA). It is both time-consuming and nonspecific for the human pathogenic species C. parvum. We have developed a TaqMan polymerase chain reaction (PCR) test that accurately quantifies C. parvum oocysts in treated and untreated water samples. The protocol consisted of the following successive steps: Envirochek® capsule filtration, immunomagnetic separation (IMS), thermal lysis followed by DNA purification using Nanosep® centrifugal devices and, finally, real-time PCR using fluorescent TaqMan technology. Quantification was accomplished by comparing the fluorescence signals obtained from test samples with those from standard dilutions of C. parvum oocysts. This IMS-real-time PCR assay permits rapid and reliable quantification over six orders of magnitude, with a detection limit of five oocysts for purified oocyst solutions and eight oocysts for spiked water samples. Replicate samples of spiked tap water and Seine River water samples (with approximately 78 and 775 oocysts) were tested. C. parvum oocyst recoveries, which ranged from 47.4% to 99% and from 39.1% to 68.3%, respectively, were significantly higher and less variable than those reported using the traditional US Environmental Protection Agency (USEPA) method 1622. This new molecular method offers a rapid, sensitive and specific alternative for C. parvum oocyst quantification in water.

Keywords: *Cryptosporidium parvum*; Quantitative; Real-time PCR; TaqMan; Water

1. Introduction

Cryptosporidium parvum is a coccidian protozoan parasite responsible for gastrointestinal illnesses in humans, particularly in immunocompromised individuals (Current and Garcia, 199 1; Fayer et al., 2000). A large number of waterborne outbreaks of cryptosporidiosis have been reported worldwide in the last few decades (Fricker and Crabb, 1998; Smith and Rose, 1998). The parasite can be transmitted through water in an oocyst form that is highly resistant to conventional disinfection treatments (Korich ct al., 1990). To date, 8- 10 known species have been identified in the *Cryptosporidium* genus, of which C. parvum is considered to be the main species associated with human diseases (Fayer et al., 2000; Xiao et al., 1999).

The conventional methods used to detect this parasite in water sources (Anonymous, 1999a,b) were

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developed by the US Environmental Protection Agency (USEPA) and are based on filtration through a cartridge membrane associated with immunomagnetic separation (IMS) and microscopic detection by immunofluorescence assays (IFA). These methods are limited by the difficulties in detecting oocysts by microscopy and their nonspecificity for the human pathogenic species *C. parvum*.

The development of molecular detection methods based on the polymerase chain reaction (PCR) has been reported in a growing number of studies. These methods have been shown to be more rapid, sensitive and specific for detection of the *C. parvum* parasite in environmental water samples (Chung et al., 1998; Hallier-Soulier and Guillot, 1999, 2000; Johnson et al., 1995; Kostrzynska et al., 1999; Wiedenmann et al., 1998). However, PCR-based methods are inappropriate for an accurate quantification of the target sequence because such assays rely on endpoint measurements. By contrast, a real-time PCR method using the TaqMan fluorogenic detection system focuses on the early exponential phase of the amplification reaction when the amount of amplified product is proportional to template DNA concentration. This method uses an oligonucleotide probe labeled with two fluorescent dyes (a reporter and a quencher) that hybridizes to the target DNA. The probe is included in the PCR mix in addition to the primers. This detection system permits the quantification of the target DNA sequence by continuous measurement of products throughout the reaction (Livak et al., 1995; Heid et al., 1996). Real-time PCR reactions are characterized by the point in time during cycling when the amplified PCR product is first detected rather than by the amount of PCR product accumulated. This point, called the threshold cycle (Ct), is logarithmically proportional to the initial quantity of target nucleic acid. When samples of known starting amounts are amplified simultaneously and under the same conditions as unknowns, a standard curve can be constructed and the starting amount of DNA in each unknown sample can be determined. Coupled with the ABI Prism® 7700 Sequence Detection System (Applied Biosystems, Courtaboeuf, France), quantification can be monitored in real time during the amplification process with no postamplification handling.

Recently, an increasing number of TaqMan quantitative PCR assays have been developed for microorganisms in the environment (Bach et al., 2002; Brunk et al., 2002; Haugland et al., 2002; McAvin et al., 2001). We have previously reported the development of a *C. parvum* TaqMan quantitative PCR assay that was validated with purified oocyst dilutions (Fontaine and Guillot, 2002). In this paper, we demonstrate how we have adapted this test to water samples by developing an efficient sample treatment step compatible with TaqMan PCR that includes an initial filtration, an IMS and a DNA purification by Nanosep® centrifugal filtration. This assay was applied to treated and untreated water samples spiked with a known number of *C. parvum* oocysts.

2. Materials and methods

2.1. Oocyst stocks

Purified bovine-derived preparations of *C. parvum* oocysts (Iowa isolate) were obtained from Waterborne (New Orleans, LA, USA). Oocysts were supplied as a purified suspension in 2.5% phosphate-buffered saline solution and stored at 4 °C. Concentrations were determined by immunofluorescence microscopic enumeration using the *Cryptosporidium/Giardia* cell IFA test (Cellabs, BMD, France) and differential interference contrast microscopy.

2.2. Water sample preparation

Tap water samples (20 and 100 l) and Seine River water samples (5 l) were filtered through Envirochek® capsules (Pall Gelman Sciences, Ann Arbor, MI, USA) at a flow rate of 2 l min^{-1} and simultaneously spiked by a steady secured injection with stock dilutions ranging from approximately 5–8 to 775 *C. parvum* oocysts. Dilutions of oocyst stocks used in the spiking experiments were microscopically counted using the *Cryptosporidium/Giardia* cell IFA test (Cellabs) according to the USEPA 1622 method (Anonymous, 1999a). A HACH 2100AN.IS turbidimeter (Prolabo, Fontenay-sous-Bois, France) was used to determine turbidity levels of the Seine River samples.

Oocysts were eluted from the capsule filter with elution buffer and by wrist motion, as specified in the USEPA method 1622 (Anonymous, 1999a). Eluates were collected in 250-ml conical-bottom centrifuge

tubes, and the oocysts were concentrated by centrifugation at 1100 × *g* and 4 °C for 20 min. The supernatants from each tube were aspirated to the 5-ml mark on the 250-ml conical tubes. Distilled water was added to the pellet-eluting solution volume so that the packed pellet volumes were 10-ml samples subjected to IMS.

2.3. Immunomagnetic separation, oocyst lysis and DNA purification

Anti-*Cryptosporidium* IMS kits (Dynal Biotech, Compiègne, France) were used for separating oocysts from other interfering particulate matter present in the sample according to the manufacturer's instructions. Additional washing steps were added. For tap water, an additional wash with 1 ml of 2.5% phosphate-buffered saline solution (PBS) was carried out. For untreated water, a preliminary wash with 5 ml of 1 × SL buffer A (prepared from 10 × SL buffer stock supplied) and two final washes with 1 ml of 2.5% PBS were added. At the end of the immunocapture, Dynabeads were resuspended in 24 μl of PCR double-distilled water in the presence of 25% (w/v) Chelex-100 (Bio-Rad, Hercules, CA, USA). Genomic DNA was released by five cycles of freezing and thawing (− 80 °C for 2 min, 95 °C for 2 min). After a centrifugation step of 3 min at 10,000 × *g*, DNA in the supernatant was recovered from the Chelex and Dynabeads by 10,000 × *g* centrifugal filtration for 5 min using the Nanosep® GHP MF centrifugal devices (Pall Gelman Sciences). The total volume of purified DNA was used as template in the real-time PCR experiments.

2.4. TaqMan probe and primers for real-time PCR

The primers and TaqMan probe used for the real-time PCR were positioned inside a specific 452-bp *C. parvum* sequence reported by Laxer et al. (1991) and referenced by Deng et al. in GenBank AF188110. The fluorescent TaqMan probe (CCAATCACAGAATCATCAGAATCGACTGGTATC) was labeled at the 5′-end with the 6-carboxyfluorescein reporter dye and at the 3′-end with the 6-carboxy-tetramethylrhodamine quencher dye (Fontaine and Guillot, 2002). The primer pair (forward primer 5′-CGCTTCTCTAGCCTTTCATGA-3′ and reverse primer, 5′-CTTCACGTGTGTTTGCCAAT-3′) amplified a 138-bp fragment inside the 452-bp fragment. Primers and probes were obtained from a commercial laboratory (Applied Biosystems).

2.5. Real-time quantitative PCR

Amplification reactions (50 μl) contained 12.5 μl of TaqMan Universal PCR Master Mix (Applied Biosystems) composed of 5 mM $MgCl_2$, ROX (6-carboxy-X-rhodamine) as an internal fluorescence reference, 200 μM each of dATP, dCTP and dGTP, 400 μM dUTP, 1 U AmpliTaq Gold DNA polymerase for hot-start PCR and AmpErase uracil-*N*-glycosylase which degrades PCR carry-over products from previous PCR runs. $MgCl_2$ was added to a final concentration of 6 mM. Two microliters of 0.1N HCl were also added to the PCR mix. The concentrations of fluorescent probe and primers were, respectively, 200 and 300 nM. Purified DNA obtained either from water samples or oocyst dilutions was added into each assay tube. Negative controls with no oocyst or template DNA (replaced with sterile distilled water) in both the IMS and PCR steps were performed for each reaction series. MicroAmp optical caps and tubes were supplied by Applied Biosystems. After 2 min at 50 °C followed by AmpliTaq Gold activation for 10 min at 95 °C, the amplification conditions were 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Amplification and detection were performed using the ABI Prism® 7700 Sequence Detection System (Applied Biosystems). Post-PCR data analysis was performed using the Sequence Detector Software (Applied Biosystems).

The degradation of the probe by the DNA polymerase in each elongation step induces an increase in fluorescence that can be monitored during PCR amplification. The fluorescence signal is normalized by dividing the reporter dye emission (6-carboxy-fluorescein) by the emission of the passive reference (6-carboxy-X-rhodamine). The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed. The Ct (threshold cycle) parameter is defined as the fractional cycle number at which the fluorescence of the reporter generated by cleavage of the probe crosses an arbitrarily defined threshold (defined to be 0.1 in all our reactions) within the logarithmic phase. Hence, this parameter can be used to compare different amplification reactions.

Standard curves were generated by plotting Ct values as a function of the logarithm of known spiked oocyst quantities. To construct the standard curve, tenfold serial dilutions of purified *C. parvum* oocysts ranging from 1–5 to 7.7×10^5 were amplified in triplicates by real-time PCR. Oocysts were lysed by thermal shocks in the presence of 25% (w/v) Chelex-100 followed by DNA purification by filtration through 0.45 μm Nanosep® GHP MF centrifugal devices. The amplification efficiency (E) was estimated by the formula $E = 10^{-1/s} - 1$, where s is the slope of the standard curve. From such standard Ct plots, it was possible to quantify samples with unknown amounts of oocysts.

3. Results

3.1. Determination of the standard curve

Fig. 1A and B shows the amplification plots of each *C. parvum* oocyst dilution associated with the

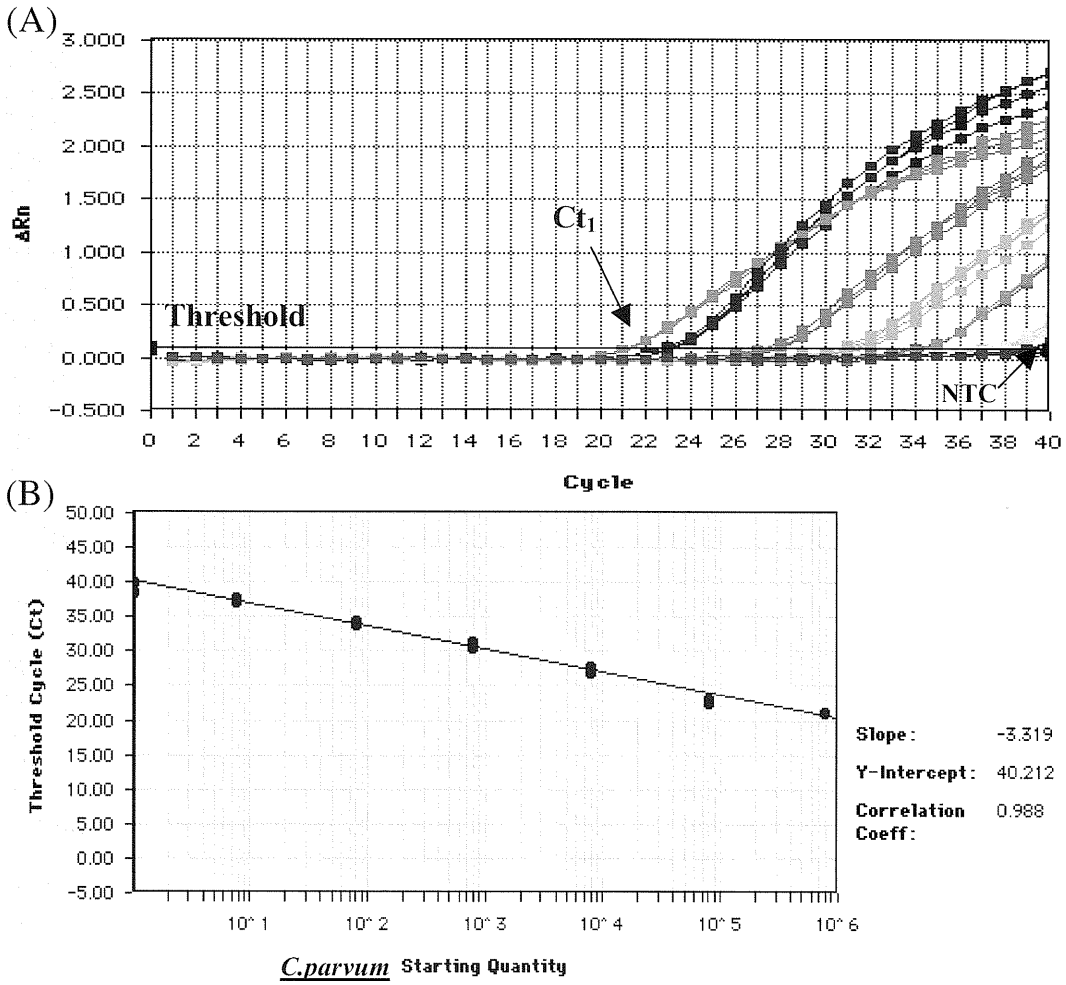

Fig. 1. Generation of standard curve. (A) Increase of fluorescence intensity with cycle number for serially diluted *C. parvum* oocysts. The relative fluorescence is the increase in reporter dye intensity relative to the passive internal reference dye. Quantities of oocysts range from 1 to 7.7×10^5. The preset threshold fluorescence value, or fluorescence value at which the threshold cycle was determined, was 0.1. Ct: threshold cycle or cycle number at which the threshold fluorescence was reached. NTC: no template control. (B) Standard curve generated from the data plotted in panel (A). Data were from 1–5 to 7.7×10^5 oocysts ($n = 3$; $r^2 = 0.988$).

standard curve obtained from these data. A linear response was observed over the six orders of magnitude and no inhibitor effect was observed. When no oocyst was added to the assay (NTC, no template control), no Ct value was achieved. The slope of the curve was −1.441 with a squared correlation coefficient (r^2) of 0.988. From the slope, the PCR amplification efficiency was estimated to be 0.999. The detection limit of the TaqMan PCR assay was approximately four to five oocysts.

To assess the reproducibility and the reliability of this TaqMan PCR assay, tenfold serial dilutions of *C. parvum* oocysts were amplified by real-time PCR on three separate occasions under identical conditions. Each experiment was performed in triplicate with oocyst dilutions stored at 4 °C over 3-month periods. For each concentration and within each experiment, the mean Ct values were associated with a coefficient of variation (i.e., variations among the triplicates). These coefficients of variation obtained ranged from 0.14 to a maximum of 2.6 for concentrations below 10 oocysts (data not shown). Accordingly, we obtained, by the three repetitions in separate occasions of the amplification, means of coefficients of variation (i.e., variations among the three sets) which ranged from 0.7 to 2. Moreover, similar slope variations were observed ($s = -1.443$, −1.441 and −1.526) with the same square regression coefficients of 0.99.

3.2. Combination of IMS and TaqMan PCR

The impact of IMS, a technique proven to be an efficient pre-PCR step, was evaluated on the real-time PCR assay. Serial oocyst dilutions were subjected to IMS then lysed by thermal shocks in the presence of 25% (w/v) Chelex before the real-time PCR step. The presence of opaque beads and interfering debris disturbed fluorescent PCR signals and resulted in a very low correlation between oocyst quantities and Ct values (data not shown). By adding a DNA purification step with Nanosep® GHP MF centrifugal devices (Pall Gelman Sciences) after the thermal lysis, we obtained a good correlation between oocyst numbers and Ct values. The slopes obtained from the standard curves were $s = -1.447$ and −1.442 ($r^2 > 0.99$) with calculated PCR efficiencies of 0.99 and 1 (data not shown.).

3.3. Analysis of spiked water samples

Tap water samples (20 and 100 l) and surface water samples (5 l of Seine water) were spiked with three oocyst quantities (5–8, 78 and 775) and concentrated through Envirochek® 1 μm pore-size capsule filters then treated by IMS as described in Materials and methods. DNA from captured parasites was released by thermal shocks and purified through 0.45-μm Nanosep® GHP MF centrifugal devices. After amplification using TaqMan PCR, a typical amplification plot versus Ct was obtained for each sample. No Ct value was reached in the unspiked tap and surface water samples meaning that the tap and surface water samples analyzed were free of *C. parvum* oocysts (level below the detection limit). To determine the *C. parvum* oocyst quantity in each water sample, the fluorescent signals detected from three or five repli-

Table 1
C. parvum oocyst quantification of spiked tap and surface water samples

Mean oocyst spiked quantity	Sample	Volume filtered (l)	No. of samples tested	Mean threshold cycle (Ct)	Mean no. of oocysts quantified	Oocyst recovery (%)
5	Tap water	20	3	37.98 ± 1.02	3.8 ± 2.1	75.8 ± 42.3
8.3	Tap water	20	3	37.68 ± 1.15	4.8 ± 2.8	58.2 ± 33.4
		100	3	37.39 ± 1.57	6.5 ± 4.4	78.2 ± 61.8
	Seine water	5	3	37.7 ± 0.58	4.1 ± 1.6	49.6 ± 19.3
78.6	Tap water	20	5	33.87 ± 0.44	54.8 ± 17.5	69.7 ± 22.3
		100	5	33.54 ± 0.26	66.4 ± 7.6	84.5 ± 9.7
	Seine water	5	5	34.33 ± 0.27	36.4 ± 5.8	46.4 ± 7.3
775	Tap water	20	5	30.41 ± 0.27	551.1 ± 108.7	71.1 ± 14.0
		100	3	30.47 ± 0.36	608.5 ± 158.1	78.5 ± 20.4
	Seine water	5	5	30.78 ± 0.30	446.5 ± 83.1	57.6 ± 10.7

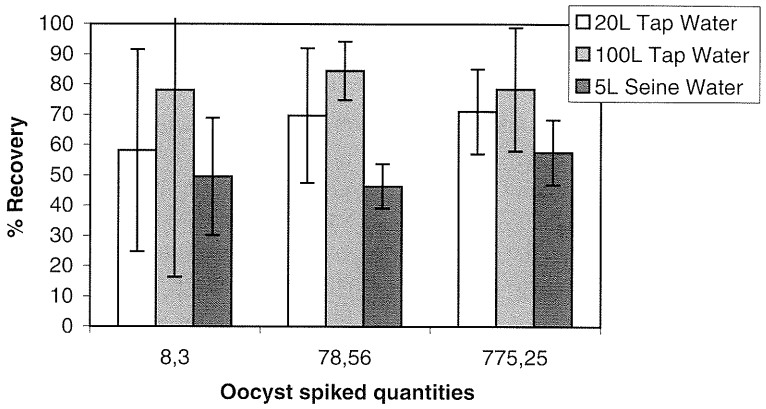

Fig. 2. Comparison of *C. parvum* oocyst recovery efficiencies with different initial quantities of spiked oocysts for different water samples.

cates in the linear range of the assay were compared to a standard curve generated with *C. parvum* oocysts during the same experiment. Oocyst recoveries (amount and percentage recoveries) from tap and surface water samples with four different concentrations of spiked *C. parvum* oocysts are summarized in Table 1 according to the volumes filtered.

The limit of the entire detection procedure was determined with low levels of *C. parvum* oocysts spiked in triplicates. Five and eight oocysts, respectively, spiked in 20 and 100 l of tap water and eight oocysts in 5 l of Seine water were detected in all cases (data not shown).

For tap water samples, the mean recovery was $69.7 \pm 22.3\%$ and $71.1 \pm 14.0\%$ (20 l samples) and $84.5 \pm 9.7\%$ and $78.5 \pm 20.4\%$ (100 l samples) for spiked oocyst quantities of 78 and 775, respectively. For Seine water samples (5 l samples) with turbidity levels ranging from 4 to 7 nephelometric turbidity units (NTU), we obtained lower mean recoveries of $46.4 \pm 7.3\%$ and $57.6 \pm 10.7\%$ for 78 and 775 spiked oocysts, respectively. These oocysts recovery rates can be visualized on Fig. 2.

4. Discussion

To date, no rapid, specific and sensitive molecular method is available to quantify the waterborne parasite *C. parvum*. Rapid quantitative tests for *Cryptosporidium* oocyst pollution are necessary for making effective management decisions and corrective actions in water treatment plants. The real-time PCR technique using fluorescent TaqMan technology was recently proven to be a good method for target sequence quantitation. Although it requires an expensive instrumentation, TaqMan PCR does offer advantages over other approaches to quantitative PCR. It is more rapid, accurate and reliable than a larger range of methods that rely on endpoint PCR measurements, such as the competitive PCR previously reported for *Cryptosporidium* (Chung et al., 1999; Udeh et al., 2000). Using the ABI Prism® 7700 Sequence Detection System (PE Applied Biosystems), quantification occurs in real time during the amplification process with no post-amplification handling, which eliminates potential sources of carry-over contamination and reduces handling time. Moreover, this method allows the simultaneous and automated analysis of 96 samples.

Only few studies have described a TaqMan quantitative PCR specific to *C. parvum* (Di Giovanni et al., 1999; Higgins et al., 2001; MacDonald et al., 2002). We have previously developed a TaqMan PCR test optimized for *C. parvum* purified oocysts. This test has allowed reliable quantification of *C. parvum* oocysts over six orders of magnitude after modifications for direct analysis of water samples (Fontaine and Guillot, 2002). In this study, we have developed a sample treatment protocol compatible with TaqMan PCR that effectively extracted and lysed oocysts before DNA purification. After thermal lysis, the DNA was purified using Nanosep® centrifugal devices. With this DNA purification step, the TaqMan PCR test applied on purified *C. parvum* oocyst

samples was linear for samples containing from 5–8 to more than 10^6 oocysts. The detection limit of five to eight oocysts was similar to the sensitivity of our previously reported TaqMan PCR test (Fontaine and Guillot, 2002). The slope of the standard curves generated from purified oocysts shows that amplification efficiency (E) was slightly less than 1. This slope ($s = -1.441$) was closer to the one obtained with purified *C. parvum* recombinant plasmid dilutions ($s = -1.446$) that was previously reported (Fontaine and Guillot, 2002). The linearity of the standard curves, the fact that PCR efficiencies in different experiments were constant (0.99–1) and the low coefficients of variation (<2.4), show that the test is highly reproducible and well suited for quantitative measurements (Livak et al., 1995; Heid et al., 1996). With this standard curve, it is possible to determine the oocyst content in test samples amplified simultaneously under conditions identical to those of the reference samples.

The use of Nanosep® centrifugal devices permits the retention of cellular particles, Chelex beads and magnetic beads after the thermal shock. The IMS purification was demonstrated to be an efficient pre-PCR step for separation and isolation of oocysts from water concentrates with simultaneous elimination of PCR inhibitors (Hallier-Soulier and Guillot, 2000; Lowery et al., 2001; Rochelle et al., 1999). However, with TaqMan PCR, an additional step of DNA purification was required after IMS to eliminate remaining debris and beads. In fact, the linear relationship between the initial oocyst quantity and the Ct values was only obtained when we used Nanosep® purification. This step makes it possible to avoid fluorescent signal disturbance generated by bead opacity. For these reasons, the Nanosep® DNA purification was applied both to the reference samples used to generate the standard curve and to test water samples.

The oocyst recovery rates from spiked tap and surface water samples demonstrated in this study were higher than those reported in an evaluation of USEPA method 1622 (Simmons et al., 2001a,b). In this evaluation, oocyst recoveries from reagent water averaged 47% (± 19%) and from stream water 12% (± 6%). The fact that our recovery was higher from larger volume samples (84.5 ± 9.7% and 78.5 ± 20.4% for 100 l) than from smaller volume samples (69.7 ± 22.3% and 71.1 ± 14.0% for 20 l) may have been due to the size of the centrifugal pellets. They were more visible in the former case and hence offered a lower risk of loss of material. In terms of sensitivity, this IMS–TaqMan PCR is comparable to the previously reported IMS–PCR technique (Hallier-Soulier and Guillot, 1999) since five and eight oocysts were detected in 20 and 100 l of tap water samples, respectively. This low detection limit corresponds to the sensitivity level of the entire detection procedure (filtration, IMS, DNA purification and TaqMan PCR) and confirms that loss of oocysts in each procedure step is low. With 5 l of Seine water samples, lower oocyst recoveries (averages between 46.4 ± 7.3% and 57.6 ± 10.7%) but the same sensitivity (eight oocysts) were obtained. The presence of large feed particles in raw water interferes with the ability of IMS Dynabeads to recover oocysts and inhibiting substances (particles, humus, etc.) are known to decrease PCR efficiency (Johnson et al., 1995; Lowery et al., 2000).

In conclusion, this real-time quantitative PCR assay allows reliable quantitation of *C. parvum* oocysts from treated and untreated water samples over several orders of magnitude within 3 h (without on-site filtration). This method will be useful for better health risk assessment during routine controls of drinking water quality, evaluation of treatment efficiency as well as identification of risk resources.

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