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PCR-based quantitation of *Cryptosporidium parvum* in municipal water samples

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Abstract

A PCR method for the quantitation of *Cryptosporidium parvum* oocysts in municipal drinking water samples was investigated. Quantitative PCR uses an internal standard (IS) template with unknown target numbers to compare to standards of known concentrations in a standard curve. The IS template was amplified using the same primers used to amplify a portion of a 358 bp gene fragment that encodes a repetitive oocyst wall protein in *C. parvum*. Municipal water samples spiked with known numbers of *C. parvum* oocysts were tested by quantitative PCR using the IS and the Digene SHARP Signal™ System Assay for PCR product detection. The absorbance readings for target DNA and IS templates versus the number of molecules of the target DNA were plotted to generate standard curves for estimating oocyst numbers. The method allowed the quantitation of oocysts from log 3 to log 5 spiked into municipal water samples.

Keywords: *Cryptosporidium parvum*; Detection; Digene SHARP Signal System™; Municipal water; Oocyst

1. Introduction

Cryptosporidium parvum is a waterborne pathogen responsible for outbreaks of gastrointestinal illness worldwide. The presence of *C. parvum* in water supplies during outbreaks of cryptosporidiosis is a result of either human or animal fecal contamination (Rose et al., 1997). Because the relationship between the prevalence of the infection, incidence of disease, and oocyst numbers in water is not well understood, the significance of low numbers of oocysts in drinking water remains uncertain. Low level oocyst contamination could indicate the need for caution and control measures. As such, the United States Environmental Protection Agency has advised monthly monitoring of drinking water for *Cryptosporidium* oocysts in municipal water plants serving populations greater than 100 000 people in the United States (Morgan and Thompson, 1998).

The low infectious dose, defined as the number of

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C. parvum oocysts required to produce infection in 50% of healthy adult individuals has been reported to be 132 oocysts (DuPont et al., 1995). The finding that 30–100 oocysts are capable of causing infection (DuPont et al., 1995) prompted the need to establish a baseline in outbreak situations where oocyst numbers may exceed acceptable levels of risk (Haas et al., 1996). To implement an action level for *Cryptosporidium*, sensitive methods of quantitating oocyst numbers in drinking water are necessary.

The development of PCR methods to estimate the numbers of target molecules or organisms in biological samples may be useful for *Cryptosporidium* (e.g. Wilkinson et al., 1995; Jenkins et al., 1997; Morgan and Thompson, 1998). The development of these methods is necessary in assessing both the presence and prevalence of pathogens in municipal water sources. One approach involves the establishment of a competitive PCR system in which a single reaction contains both target DNA and control DNA. The use of controls having the same primer template sequences as the target makes it possible to determine the amount of target DNA by allowing known amounts of competitor DNA to compete with the target for primer binding, when both sets of products are compared, the amount of competitor DNA that yields equal molar amounts of products gives the initial amount of target gene (Siebert and Larrick, 1992). In this paper, we used a competitive PCR method coupled with the Digene SHARP Signal™ System Assay for the quantitation of *Cryptosporidium parvum* oocysts. Water samples spiked with known numbers of oocysts were assessed using this method and estimates obtained were compared to original oocyst numbers.

2. Materials and methods

2.1. Parasites

Cryptosporidium parvum oocysts used in all studies were obtained from K. Dianne Swabby-Cahill, Parasitology Research Labs (P.R.L.), Phoenix, Arizona, USA. The oocysts were of bovine origin and had subsequently been passaged in 8–12 week old C57BL/6 mice immunosuppressed with dexamethasone at P.R.L.

2.2. Inoculation and recovery of oocysts from municipal water samples

The technique of Aldom and Chagla (1995) was used to filter 1000 l of municipal water from Guelph, Ontario, Canada inoculated with 10^3–10^5 formalin-killed *C. parvum* oocysts. Oocysts were recovered using an 8.0-μm average pore size cellulose acetate membrane filter contained within a Millipore (Nepean, Ontario, Canada) 293-mm stainless steel filtration unit. The membrane and sample were subsequently dissolved in 100% acetone and oocysts washed successively with 95% (v/v) ethanol, 70% (v/v) ethanol, and eluting fluid [1% (w/v) sodium dodecyl sulfate (SDS), 1% (v/v) Tween 80, 1X phosphate-buffered saline (PBS), 0.001% (w/v) Sigma Antifoam, (Sigma Chemical Company, St. Louis, MO, USA)]. Oocysts were recovered after the final wash by centrifugation at $650 \times g$ for 15 min at room temperature and resuspended in ~10 ml eluting fluid and counted using the MERIFLUOR immunofluorescence assay from Meridian Diagnostics (Cincinnati, Ohio, USA).

2.3. Quantitative PCR

Quantitative PCR using an internal standard template involves the quantitation of samples with unknown target numbers by comparison against standards of known concentrations used to generate a standard curve. Because quantitative PCR requires comparison of PCR product concentrations between individual reactions, it is necessary to assess tube-to-tube variations in amplification and to correct for differences to obtain accurate determinations. We used an internal standard (IS) template that was amplified by the same set of primers as the *C. parvum* target DNA (Fig. 1). The IS template was introduced into all PCR mixtures to a final concentration of 5×10^{-6} amol μl^{-1}. It competed with *C. parvum* template DNA for amplification. If large amounts of *C. parvum* template were present, the amount of IS product generated would be relatively small, having been out-competed by the more abundant *Cryptosporidium* target. Conversely, if small amounts of *C. parvum* template were present, there was less competition, and this would result in greater amplification of the IS template. By examining the

A.

5′ **GCCCACCTGG ATATACACTT TC***AAGTTTCG TGAGCTGATT GCAGAGTTCT*
CCAAAATGGC TCGTGACCCT CCCCGCTATC TTGTTATACA GGGAGATGAA
AGGATGCACT TGCCTAGCCC TACAGATTCC AAGTTTTATC GCACCCTGAT
GGAGGAGGAG GACATGGAAG ACATTGTGGA TGCAGATGAG TATCTTGTCC
*C***TCCTGTTGG TACTAGAGAG GGGGA** 3′

B.

5′ **GCCCACCTGG ATATACACTT TC***TGGATCCC AATGCGAGCA AATAAAAGAA*
GCACCTCCTG TTTCAGAATG TCCACCAGGA TATAAACTTC AAGGAAATCA
ATGTACTGCA CTAAAAATGA TCGATGCTAT CTGCCCAGAT GGATTTTTAC
CAAATGGAGA CGATTGTATC CAATTTTCTC CTGCTTCAAC TGTATGTCCT
ACTGGATTCA CTCTACAAAA TCAACAGTGT GTTCAAACAA CTACCTCACC
AAAAACACCA GAATGTCCTC CAGGTTCTGC GTTGGATGGA GACTCGTGCA
*CAAGACTTGT TCCCGGGGCT CTTCAATACG TTTG***TCCTGT TGGTACTAGA**
GAGGGGGA *3′*

Fig. 1. (A) Nucleotide sequence of the internal control template (225 bp); (B) Nucleotide sequence of a portion of the gene fragment CpR1 from *C. parvum* (358 bp) (GenBank accession #M95743). The primer annealing sites (common to both) are underlined and bolded. The sequences involved in hybridization with RNA probe for the Digene SHARP Signal™ System Assay are shown in italics (WT probe = 310 bp, IS probe = 180 bp).

ratio of *Cryptosporidium* to IS PCR product generated in each reaction, tube-to-tube variation in amplification efficiency may be reduced.

2.4. Construction of purified Internal Standard and Wild-Type templates

The Clontech PCR MIMIC construction kit (Clontech Laboratories Incorporated, Palo Alto, California, USA) was used to prepare an IS template. This IS template was introduced into reaction mixes for co-amplification with *C. parvum* sample DNA. The sequence of the IS template consisted of HB-1 (5′-GCCCACCTGGATATACACTTTC-3′) and HB-2 (5′-TCCCCCTCTCTAGTACCAACAGGA-3′) *C. parvum* primer annealing sites flanking a segment of DNA unrelated to *Cryptosporidium* (Fig. 1) (see Laberge et al., 1996; Chung et al., 1998; Kostrzynska et al., 1999). Quantitation of the IS template was achieved by comparison to a ϕX174/*Hae*III digest used as a molecular weight marker (Clontech Laboratories Incorporated, Palo Alto, California, USA) electrophoresed in a 2% (w/v) agarose gel stained with ethidium bromide. When the IS template was added into PCR mixes containing *C. parvum* DNA, two products resulted from amplification using the same set of primers. The PCR product resulting from amplification of *C. parvum* DNA (i.e., Wild Type (WT) or WT PCR product) was distinguished from the IS amplicon by their different sizes (358 bp versus 225 bp, respectively).

The WT PCR product was purified through QIAquick PCR product purification columns (Qiagen Incorporated, Chatsworth, California, USA). The concentration of WT product was determined by imaging densitometry using the Gel Doc 1000 image analysis system (Bio-Rad Laboratories, Hercules, California, USA) by comparison to a ϕX174/*Hae*III digest molecular weight marker. Concentrations were confirmed by fluorimetry using a solid state model SSF-600 fluorimeter (Tyler Research Instruments, Edmonton, Alberta, Canada). A 100 amol μl^{-1} stock solution was prepared from which ten-fold serially diluted working solutions were made in 50 $\mu g\ ml^{-1}$ ultrapure glycogen (Boehringer Mannheim Canada, Laval, Quebec, Canada). The lowest WT concentration used in constructing the standard curve was 1×10^{-7} amol μl^{-1}.

2.5. PCR conditions

The PCR mixture contained 1X PCR buffer II (10 mM Tris–HCl pH 8.3, 50 mM KCl; Perkin-Elmer Cetus, Norwalk, Connecticut, USA); 2.5 mM $MgCl_2$;

10 μM each of forward (HB-1) and reverse (HB-2) primers; 0.2 mM each of dATP, dGTP, and dCTP; 0.4 mM dUTP; 100 μg ml^{-1} bovine serum albumin; 0.5 U uracil-*N*-glycosylase; 3.25 U AmpliTaq Gold (Perkin-Elmer Cetus); 5×10^{-6} amol μl^{-1} internal standard (IS) DNA; and 5 μl sample DNA (unknown concentration). Reactions were adjusted to a final volume of 50 μl with sterile ultrapure water. For multiple reactions, a master mix of all PCR components except sample DNA was prepared and aliquoted into individual tubes prior to sample template addition. Samples were assayed by PCR in duplicate. PCR was carried out in an automated DNA thermal cycler (model 9600, Perkin-Elmer Cetus) with 15 s denaturation at 94°C, 1 min annealing at 50°C, and 1 min extension at 72°C for 40 cycles. Prior to cycling, a 5 min incubation at 50°C was employed for uracil-*N*-glycosylase activity (PCR product carryover-prevention kit, Perkin-Elmer Cetus), followed by 10 min incubation at 95°C to inactivate the glycosylase and activate AmpliTaq Gold (Perkin-Elmer Cetus) as per the manufacturer's instructions. After 40 cycles, a 6 min final extension was performed at 72°C followed by a 4°C soak.

2.6. Construction and application of the standard curve

The generation of a standard curve using competitive PCR data has been previously described (Siebert and Larrick, 1992; Zachar et al., 1993; Wilkinson et al., 1995). Briefly, the target DNA is coamplified in the same tube as an IS sample, and the IS serves as a control for all reactions. Following PCR, the control and target products are compared, and the amount of competitor DNA that yields equal molar amounts of products gives the initial amount of target gene (Siebert and Larrick, 1992). The set of standard templates for constructing the standard curve consisted of a ten-fold serial dilution of *C. parvum* PCR product (designated WT) generated using the HB-1 and HB-2 primer set. For competitive PCR, the IS template was added to both the standard and sample reactions to a final concentration of 5×10^{-6} amol μl^{-1} (i.e. 30 molecules). The number of molecules per PCR for each water sample was calculated using the regression equation of the standard curve, plotted as log (WT/IS) vs. log (≠ molecules WT). The number of molecules per PCR divided by 4 (4 sporozoites per 1 oocyst based on the presumed single copy of the oocyst wall protein gene (Spano et al., 1997)) was used to estimate the number of oocysts in the original spiked samples (Table 1).

2.7. The Digene SHARP Signal™ System Assay

The Digene SHARP Signal™ System Assay (Digene Diagnostics Incorporated, Beltsville, Maryland, USA) for PCR product detection was used to probe the WT and IS PCR products from the standards and samples. PCR product concentrations were obtained using a V_{max} kinetic microplate reader (Molecular Devices Corporation, Sunnyvale, California, USA). Optimization of the assay for the detection of the WT and IS PCR products involved determining the linear range of the assay with respect to input PCR product concentration, and finding suitable concentrations of RNA probe for hybridization to the WT and IS PCR products.

The following modifications were made to minimize intra-assay variation between replicate wells: (1) the PCR product was diluted ten-fold in sample diluent (Digene); (2) a volume of 20 μl was added to 200 μl of sample diluent and 100 μl of denaturation solution (Digene) in a sterile 1.5-ml Eppendorf tube, after 10 min denaturation at 22°C, 100 μl of 200 pmol l^{-1} RNA probe mix (Digene) was added and the tube was vortexed; (3) a volume of 100 μl of hybridization mixture was transferred to each of three capture wells.

Hybrid capture and detection steps were carried out according to the manufacturer's instructions. Microplates were read after 2 h of incubation at 37°C, and after overnight incubation at 4°C where necessary. Optical density readings were taken at 405 nm with subtraction of reference absorbance readings taken at 650 nm.

2.8. Imaging densitometry

The PCR product concentration was estimated using imaging densitometry to analyze product bands in 2% (w/v) agarose gels stained with ethidium bromide. The Bio-Rad Gel Doc 1000 image analysis system with Molecular Analyst Software was used to

Table 1
Inter-assay variation of the Digene SHARP Signal™ System Assay

Oocysts per sample standard[a]	OD_{405} nm				Standard deviation[d]	Coefficient of variation[e]
	Trial 1[b]	Trial 2[b]	Trial 3[b]	Mean OD[c]		
3×10^5(A)	0.663	0.636	0.414	0.571	0.137	23.900
3×10^4(A)	0.630	0.463	0.402	0.498	0.118	23.700
3×10^3(A)	0.454	0.332	0.334	0.373	0.070	18.700
3×10^2(A)	0.419	0.246	0.303	0.323	0.088	27.300
3×10^1(A)	0.312	0.186	0.223	0.240	0.065	26.900
3×10^0(A)	0.052	0.029	0.040	0.040	0.012	28.500
3×10^5(B)	0.203	0.203	0.106	0.171	0.056	32.800
3×10^4(B)	0.130	0.134	0.097	0.120	0.020	16.800
3×10^3(B)	0.086	0.090	0.087	0.088	0.002	2.300
3×10^2(B)	0.073	0.056	0.074	0.068	0.010	14.900
3×10^1(B)	0.031	0.024	0.029	0.028	0.004	12.800
3×10^0(B)	0.002	0.001	0.004	0.002	0.002	65.400
						Mean CV = 24.540

[a] Molecule number per PCR for a series of ten-fold serially diluted standards. (A) = Ten-fold dilution of PCR product prior to performing the SHARP assay; (B) = 100-fold dilution of PCR product prior to performing the SHARP assay.
[b] Three trials of the SHARP assay were performed over three days on the same PCR product. Fresh dilutions were prepared for each trial. Values are OD readings at 405 nm.
[c] Mean optical density reading (Abs = 405 nm) of the three trials was calculated for each standard.
[d] Standard deviation of the three trials was calculated for each standard.
[e] Coefficient of variation of the three trials was calculated for each standard.

generate volume integration reports of molecular weight size standard band intensities from which standard curves could be constructed and semi-quantitation of the WT and IS PCR products achieved.

2.9. Spiking municipal water pellets with purified oocysts

Filtered municipal water sample pellets (free of *Cryptosporidium* oocysts) were divided into 1.5 ml aliquots and pelleted by centrifugation at 16 000 × *g* for 10 min. A 1-ml aliquot of supernatant was removed and the pellet seeded with 1 ml of oocysts from a ten-fold dilution series. In this manner, filtered municipal water samples containing 10^2, 10^3, 10^4, 10^5, and 10^6 oocysts in 1.5 ml volumes were obtained (corresponding to 10^2–10^6 oocysts per 100 l). Oocyst DNA was isolated from these samples following the protocol described in Chung et al. (1998).

2.10. Denaturation and hybridization of PCR products

Attempts were made to reduce the intra-assay variability of the Digene SHARP Signal™ System Assay. Modifications to the protocol in the denaturation and hybridization steps of the assay were attempted. The PCR product was first diluted ten-fold in each sample. A volume of 20 μl was added to 200 μl of sample diluent and 100 μl of denaturation solution in a sterile 1.5-ml Eppendorf tube. After 10 min denaturation at 22°C, 100 μl of 200 pmol l^{-1} RNA probe mix was added and the tube gently vortexed. A volume of 100 μl of hybridization mixture was transferred to each of three capture wells. Hybrid capture and detection steps were carried out according to the manufacturer's instructions. Microplates were read after 2 h of incubation at 37°C, and after overnight incubation at 4°C where necessary. Optical density values were read at 405 nm with subtraction of reference values read at 650 nm.

2.11. Quantitative PCR on spiked municipal water samples using the Digene SHARP Signal ™ System Assay

DNA isolated from eight municipal water samples were diluted ten-fold and amplified by PCR in duplicate together with IS at a concentration of 5×10^{-6} amol μl^{-1}. In the same PCR experiment, ten-fold serial dilutions of the WT template were also amplified in duplicate with the IS DNA at a concentration of 5×10^{-6} amol μl^{-1}. PCR products from the duplicate reactions were pooled together and diluted ten-fold. Aliquots were hybridized to WT- and IS-specific RNA probes (produced as described in Chung et al., 1998) in separate reaction tubes. Hybridization mixtures were assayed in triplicate in SHARP assay microwells.

The mean optical density readings for triplicates of the amplified *C. parvum* PCR product and the IS PCR product were obtained for each of the eight municipal water samples. The ratio of *C. parvum* product to IS product (y) was applied to the linear regression equation of the standard curve to obtain the number of PCR input molecules of *C. parvum* template (x).

3. Results and discussion

3.1. Optimization of the quantitative PCR reaction parameters

Initial conditions for the quantitative amplification of both the WT and IS PCR products were investigated. Known amounts of the WT template were serially diluted ten-fold and co-amplified with a constant amount of IS template. The concentration of the WT template in this series ranged between 3 and 300 000 molecules per PCR. The concentration of the IS template was fixed at 30 molecules per PCR. Amplified PCR products (Fig. 2) corresponded to the predicted product sizes (WT = 358 bp, IS = 225 bp). As the amount of the WT product increased, the amount of the IS product decreased, indicating that the two products were competing in the amplification reaction (Fig. 3).

Accurate quantitation of PCR products requires the number of PCR cycles to be within the exponential phase of amplification. Under our reaction conditions, forty cycles of amplification were suitable for the quantitation of the amplified *C. parvum* PCR product. An increasing linear trend in PCR product quantity was observed between 25 and 45 cycles of amplification for 30 000 input template molecules using the SHARP assay, suggesting the reactions had not reached the non-exponential plateau phase.

3.2. Optimization of the Digene SHARP Signal ™ system assay

3.2.1. RNA probes

PCR products were analyzed using the Digene SHARP Signal™ System Assay with sequence-specific RNA probes. The most suitable RNA probe concentration for detection of the WT was between 150 and 200 pmol l^{-1}. A concentration of 200 pmol l^{-1} of IS-specific RNA probe was also suitable for detection of the IS PCR product.

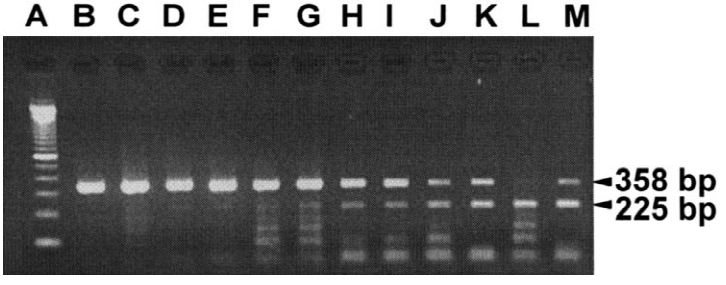

Fig. 2. Agarose gel electrophoresis of PCR products from competitive amplification of a ten-fold serial dilution of the WT standards (358 bp) with the IS (225 bp) at a constant concentration of 5×10^{6} amol μl^{-1}. Lane (A) = 100 bp molecular weight marker; Lanes (B) and (C) = 300 000 molecules of WT + 30 molecules of IS; Lanes (D) and (E) = 30 000 molecules of WT + 30 molecules of IS; Lanes (F) and (G) = 3000 molecules of WT + 30 molecules of IS; Lanes (H) and (I) = 300 molecules of WT + 30 molecules of IS; Lanes (J) and (K) = 30 molecules of WT + 30 molecules of IS; Lanes (L) and (M) = 3 molecules of WT + 30 molecules of IS.

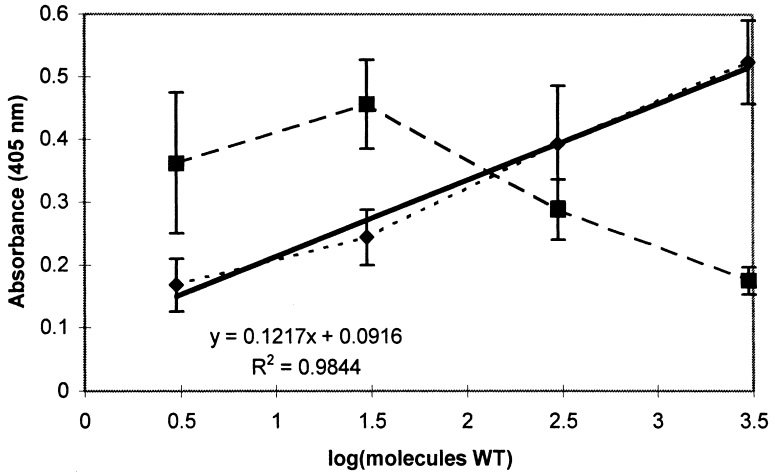

Fig. 3. Absorbance reading at 405 nm vs. number of input molecules of WT for PCR products as described in Fig. 2 detected using the Digene SHARP Signal™ System Assay. Values are mean±SD for triplicate wells of WT (♦) and IS (■). The linear regression line that fits the curve of WT standards is also shown along with its equation and correlation coefficient (R^2).

The probes for the WT and IS PCR products were tested for cross-reactivity by hybridizing samples that contained the WT product alone with the IS sequence-specific probe or the IS product with the WT sequence-specific probe. The resulting OD values from the assay were about equal to the background signal of approximately 0.100 OD units (data not shown).

The WT- and IS-specific probes hybridized to their respective PCR products generated in competitive PCR. The mean OD readings for triplicates of each WT and IS PCR product standard were obtained and plotted against the number of PCR input molecules of WT. An increasing trend for the OD readings of WT and a decreasing trend for the OD readings of IS were observed as the number of input WT molecules increased (Fig. 4). The pattern of decreasing IS amplification with increasing input WT molecules suggests that the two products were competing in this series of amplification reactions. The ratio of WT OD to IS OD was calculated for the standards and plotted against the number of PCR input molecules to obtain the standard curve for quantitation of samples (Fig. 4). A curve was generated to fit the data.

The length of incubation time of the Digene SHARP Signal™ System Assay was examined for its effect on the linearity of a standard curve generated using starting WT amounts ranging from 3 to 300 000 molecules. After incubation for 1, 2, 3, and 4 h at 37°C and overnight at 4°C, no effect was observed on the linearity of the standard curve for the mean values among triplicate wells of selected standards.

3.3. Denaturation and hybridization of PCR products

Incorporating the modifications to the protocol described above, coefficients of variation between triplicate wells were consistently $\leq 15\%$. When the protocol was followed according to the manufacturer's instructions, variation could increase, averaging approximately 20% or higher. Inter-assay variation was measured for three identical assays performed over three days. The coefficient of variation between assays was about 25% (Table 1). These results were consistent with findings reported by other users of this assay (Boivin et al., 1997).

3.4. Dilution of PCR products

It was determined from further optimization of the SHARP assay that a ten-fold dilution of the PCR product was necessary to avoid saturation of the assay wells with the product when a large number of input molecules were used (i.e. $>30\,000$ molecules; Fig. 5). When undiluted PCR product was used in the

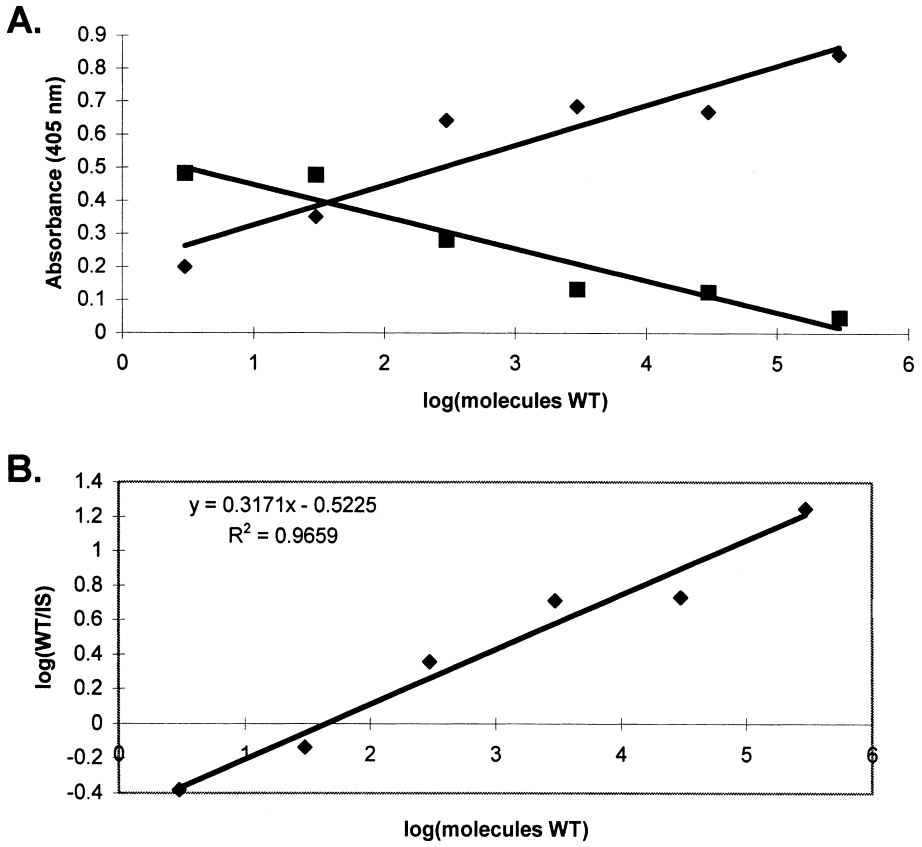

Fig. 4. (A) = Absorbance reading at 405 nm versus number of input molecules of WT for the pooled WT (♦) and the IS (■) PCR product standards diluted ten-fold and assayed by the Digene SHARP Signal™ System Assay. Values are mean±SD for triplicate wells. (B) = The ratio of WT:IS absorbance readings at 405 nm versus number of input molecules of WT for competitive PCR products of WT standards amplified together with the IS (♦). The regression line for the curve and its equation and square of the correlation coefficient (R^2) are also shown. The regression equation is in the form $y = mx + b$, where m is the slope and b is the y-intercept. The regression line was described by the equation $y = 0.3171x - 0.5225$ with an R^2 value of 0.9659, where ($0.000 \leq R^2 \leq 1.000$) and an R^2 value of 1.000 gives a straight line.

assay, the absorbance readings reached a plateau for reactions containing high numbers of input molecules.

3.5. Quantitative PCR on municipal water pellets spiked with known numbers of C. parvum oocysts

The Digene SHARP Signal™ System Assay enabled the quantitation of 10^3–10^5 oocysts in municipal water pellets which corresponded to 10^3–10^5 oocysts per 100 l. Fig. 6 shows the corresponding standard curve used to obtain the number of PCR input molecules and for subsequent determination of oocyst concentration. Ten-fold differences in oocyst number were distinguishable using the SHARP assay, and numbers of oocysts predicted by PCR were lower than the original numbers detected from spiked samples (Table 2). The samples spiked with 10^2 and 10^6 oocysts were beyond the quantitative range of this standard curve (data not shown).

3.6. Quantitative PCR on municipal water samples

The number of oocysts in the municipal water samples, as predicted using PCR and the Digene SHARP Signal™ System Assay, were within a one-log range of the values determined using IFA detection (Table 3), with most of the values pre-

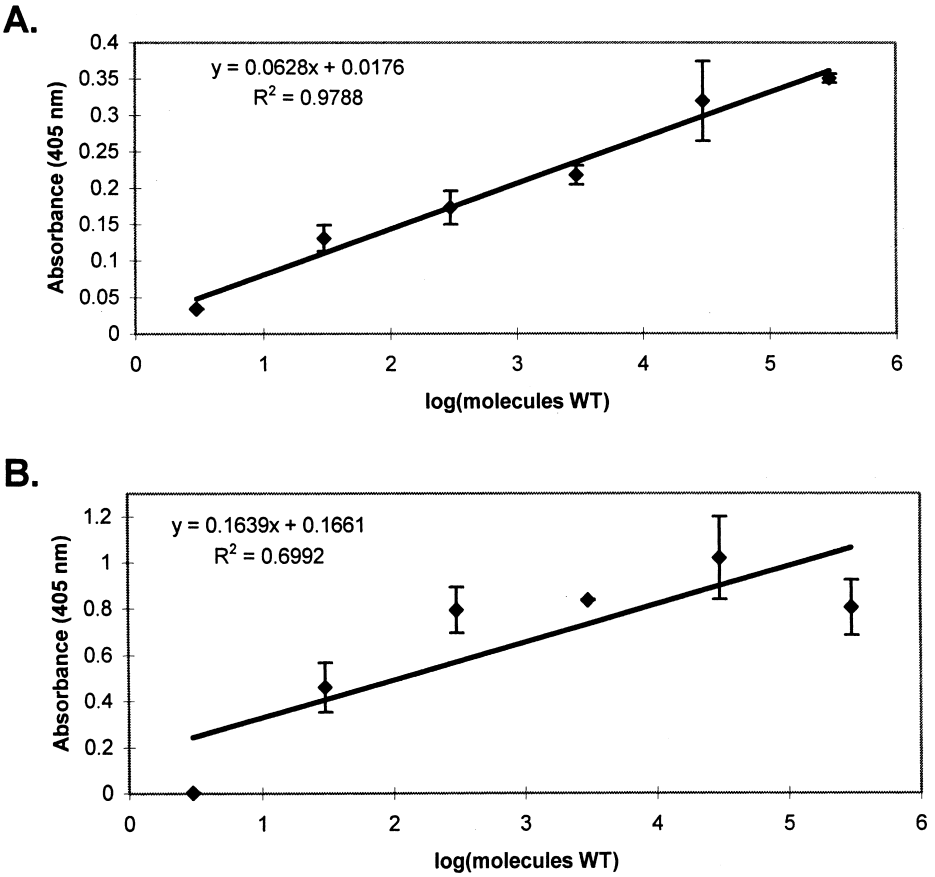

Fig. 5. (A) = Absorbance reading at 405 nm vs. number of input molecules of WT for undiluted PCR products generated from a ten-fold serial dilution of input WT molecules (range: 3–300 000 molecules) assayed by the Digene SHARP Signal™ System Assay. Values are mean ± SD for triplicate determination (♦). The linear regression line that fits the curve and its corresponding regression equation are also shown. (B) = Absorbance reading at 405 nm vs. number of input molecules of WT for ten-fold diluted PCR products generated from a ten-fold serial dilution of input WT molecules (range: 3–300 000 molecules) assayed by the Digene SHARP Signal™ System Assay.

dicted by PCR being somewhat higher than the numbers detected by IFA (four higher, three lower, one equal). Fig. 4 shows the corresponding standard curve used to obtain the number of PCR input molecules, from which the oocyst concentrations were determined.

3.7. General discussion

Quantitation in municipal water samples spiked with known numbers of oocysts was achievable within one-log range. The numbers of oocysts in spiked samples as predicted by PCR were similar to those detected by IFA. Both methods detected oocyst numbers that were within a one-log range of the original spiking levels.

Quantitative PCR was dependent on the success of the amplification and detection steps involved in the process. PCR amplification of the WT standards together with the IS template was reproducible provided that strict control of reagent quality and template storage conditions were maintained. The linearity of the quantitative PCR standard curve was affected by the individual competitive PCR runs for each WT standard. In standards containing low input molecule numbers (i.e. < 30 molecules WT), amplification was less reliable, with an increased frequency of failed reactions. The efficiency of com-

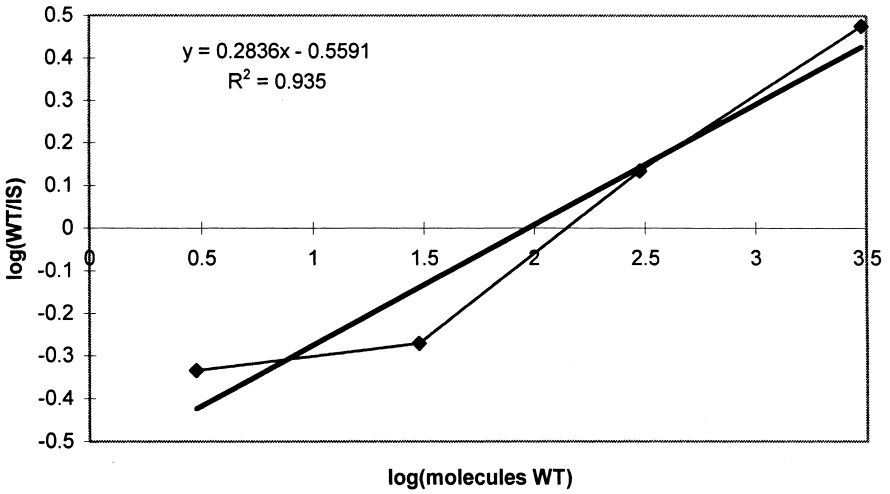

Fig. 6. Ratio of WT:IS absorbance readings at 405 nm vs. number of input molecules of WT for competitive PCR products of WT standards amplified together with IS (♦). The regression line for the curve and its equation and correlation coefficient (R^2) are also shown. The regression equation is in the form $y = mx + b$, where m is the slope and b is the y-intercept. The equation was used to calculate the number of input molecules for samples containing *C. parvum* DNA amplified together with the IS and assayed using the Digene SHARP Signal™ System Assay.

petitive PCR in municipal water samples spiked with known numbers of oocysts may have been affected by interfering substances (e.g. metals, humic material, clay particles, nucleases) in the water samples (Bej, 1995). The consistency of the method is also affected by the loss of DNA on spin columns, the effectiveness of the oocyst lysis steps, and the efficiency of centrifugation. These factors may account for predicted numbers of oocysts using WT:IS product ratios that were consistently lower than the original spiking levels.

To achieve a level of quantitation in the order of

Table 2
Quantitative PCR data using standard curve of wild-type (WT):internal standard (IS) PCR product ratios for a municipal water sample spiked with purified oocysts

log (oocysts)[a]	Mean WT (Abs = 405 nm)[b]	Mean IS (Abs = 405 nm)	Log (WT/IS)[c]	Log (oocysts in pellet)[d]
2	0.006±0.010	0.386±0.097	−1.808	0.000
3	0.257±0.027	0.191±0.006	0.129	2.602
4	0.353±0.063	0.157±0.042	0.352	3.389
5	0.492±0.131	0.113±0.006	0.639	4.400

[a] Aliquots of a municipal water pellet were spiked with 10^2, 10^3, 10^4, and 10^5 oocysts, respectively.
[b] Readings from the SHARP assay are mean±SD for $n = 3$.
[c] The ratio of WT(Abs):IS(Abs) was used to calculate the number of input molecules per PCR using the regression equation of the standard curve.
[d] Log number oocysts in each aliquot of municipal water pellet incorporating recovery efficiencies as established by multiplying the value obtained by 0.15 to account for a 1.5 ml aliquot taken from the original 10 ml pellet.

Table 3
Quantitative PCR data for selected municipal water samples

Sample desigation[a]	Estimated inoculum log (oocysts)[b]	IFA detection (total oocysts)[c]	Log (WT/IS)[d]	Log (oocysts predicted using PCR)[e]
F11	5.00	4.84	0.038	5.59
F20	4.70	4.43	−0.298	4.43
F38	4.70	4.67	−0.741	3.18
F43	4.70	4.34	−0.142	4.99
F46	5.00	4.77	−0.105	5.15
F48	5.00	4.66	−0.326	4.45
F52	5.00	4.55	−0.294	4.54
F62	4.60	4.18	−0.176	4.90

[a] Selected municipal water samples from the spike-recovery studies of 72 samples from Ontario.
[b] Log number of oocysts estimated in inoculum by haemacytometer counts.
[c] Log oocyst number recovered as determined by immunofluorescence assay.
[d] The ratio of WT(Abs):IS(Abs) was used to calculate the number of input molecules per PCR using the regression equation of the standard curve in Fig. 4.
[e] Log number of oocysts in each aliquot of municipal water pellet as determined by PCR. The formula is $x = 6.667n$, where x is the number of oocysts in the municipal water sample and n is the number of input molecules per PCR.

one *C. parvum* oocyst per PCR assay, 40 amplification cycles were required. Accurate quantitation of low target numbers can be achieved at 40 cycles since PCR product accumulation has been shown to remain in the exponential phase for starting template quantities as high as 30 000 molecules, which was equivalent to approximately 10^6 oocysts per 100 l.

A 1–4 h incubation at 37°C or overnight incubation at 4°C of the SHARP assay reactions did not affect linearity of the standard curve. However, saturation was eventually reached for the colorimetric reaction. Reactions allowed to develop overnight at 37°C were beyond the linear range of the plate reader to give reliable readings. Readings taken after 2 h incubation were suitable to cover the range of PCR product quantity in the standards.

In a study on the utility of the SHARP assay in detecting human immunodeficiency virus (HIV) RNA, Lin et al. (1996) reported between-run coefficients of variation to be between 9.1 and 24.7% for HIV-1 RNA and 10.9 and 15.1% for proviral DNA. These authors cited RNA sample preparation, the use of accurate standards, and the degree of amplification compatible with the detection method as important factors in the accurate measurement of HIV-1 levels. It is also necessary to consider the nature of the RNA target. For example, eukaryotic mRNA such as hsp70 transcripts undergo a 1000–10 000-fold increase under stress conditions (Morgan and Thompson, 1998), making their quantitation impossible.

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