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DETECTION AND QUANTIFICATION OF *CRYPTOSPORIDIUM* IN HCT-8 CELLS AND HUMAN FECAL SPECIMENS USING REAL-TIME POLYMERASE CHAIN REACTION

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Abstract

Cryptosporidium is a significant cause of diarrheal illness worldwide, especially among children and immunocompromised patients. Currently used diagnostic techniques are time-consuming, require skilled technicians, and are not useful for quantification of oocysts in fecal and environmental samples. In this study, we examined the use of a real-time polymerase chain reaction (PCR) assay for detecting and quantifying *Cryptosporidium parvum* in three distinct and progressively more complex matrices: phosphate-buffered saline (PBS), HCT-8 cells (human ileocecal carcinoma), and human fecal specimens. A reliable standard curve was generated using the PBS samples spiked with pure oocysts, and oocyst starting quantities were calculated for the infected HCT-8 cell and spiked fecal samples. The assay detected *Cryptosporidium* in samples infected/spiked with $\geq 10^3$ oocysts/sample and detected both *C. hominis* and *C. parvum* in clinical specimens. This assay is useful in a variety of samples in the research laboratory and will likely prove to be a useful tool in the clinical laboratory.

INTRODUCTION

Cryptosporidium is a major cause of diarrheal illness among children and patients with AIDS and has frequently been responsible for waterborne outbreaks resulting from contaminated drinking water and recreational waters.^{1,2} Current immunofluorescence and enzyme-linked immunoassays can have sensitivities and specificities of 100% for clinical diagnosis,³ but they are not sensitive enough for epidemiologic studies of asymptomatic individuals or for environmental samples. Additionally, oocysts cannot be readily quantified in environmental or fecal samples using these techniques.

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Polymerase chain reaction (PCR), however, has been used to detect *C. parvum* with extreme sensitivity under experimental conditions.⁴ As little as one purified oocyst isolated by micromanipulation in a 10- μ L PCR buffer solution was detected in 38% of 50 replicates. Real-time quantitative PCR (qPCR) has also been used to quantify *C. parvum* oocysts over six orders of magnitude in water samples with a sensitivity of five oocysts.⁵ qPCR has been used to quantify *C. parvum* oocysts in diarrheal feces from calves, achieving a result within one log of the 3.1×10^6 oocysts/mL of diarrhea calculated by immunofluorescence antibody (IFA) microscopy.⁶ A quantitative real-time reverse transcription PCR assay has also been used recently to assess drug efficacy against *C. parvum in vivo*.⁷ Compared with immunofluorescence, real-time PCR is quick and easy to perform. A sensitive and reliable qPCR assay for *Cryptosporidium* in human fecal specimens is desirable but has not been developed.

In this study, we sought to examine the limits of qPCR to quantify *C. parvum* oocysts in three distinct and progressively more complex matrices: phosphate-buffered saline (PBS), HCT-8 cells (human ileocecal carcinoma), and human fecal specimens. Thus, we evaluated qPCRs potential for quantification of *Cryptosporidium* in the research laboratory and in the clinical laboratory. We also analyzed clinical stool specimens to confirm the clinical utility of our assay and its ability to detect other *Cryptosporidium* species implicated in human disease.

MATERIALS AND METHODS

Sample preparation.

Cryptosporidium parvum oocysts from bovine sources were cleaned of fecal debris by density gradient centrifugation methods and counted with a hemacytometer as previously described.⁸ The oocysts (Iowa isolate, as well as preliminary studies with similar results, but not included in this manuscript, with the Beltsville isolate) were stored for 8–10 weeks in 2.5% PBS at 4°C until used. Serial dilutions in PBS were performed to make solutions of 5×10^2 – 5×10^7 oocysts/mL PBS. Aliquots of 200 μ L from each solution (yielding pure oocyst samples of 1×10^2 – 1×10^7 oocysts/sample) were stored at 4°C overnight.

Before infection with *C. parvum*, 1×10^5 HCT-8 cells (passage 29) in Roswell Park Memorial Institute (RPMI) medium with L-glutamine containing 10% fetal bovine serum (FBS), 0.5% streptomycin and penicillin, and 1% sodium pyruvate were seeded into 12-well plates. The cells were incubated to 90% confluency in 5% CO₂ at 37°C for 2 days. The medium for each well was changed before infection. *C. parvum* oocysts were excysted (65% excystation rate) by incubating them for 10 minutes at room temperature in a solution of 1 part laundry bleach to 5 parts of dH₂O. The oocyst suspension was centrifuged at $2800 \times g$ for 3 minutes, all but 100 μ L of supernatant was aspirated, and 900 μ L of Hanks balanced salt solution (HBSS) with phenol red (Cambrex, Walkersville, MD) was added. This process of centrifugation, aspiration, and addition of HBSS was repeated until the HBSS with phenol red retained its original color. The HCT-8 cells were infected with 100 μ L aliquots of excysted *C. parvum* oocysts (yielding 1×10^2 – 1×10^7 excysted oocysts/well) and incubated in 5% CO₂ at 37°C, and the media were replaced 4 hours after infection. Cells were harvested after 24 hours using 500 μ L of Accutase (Innovative Cell Technologies, San Diego, CA), and the wells were washed with PBS to ensure that all cells were harvested. Cell suspensions were centrifuged at $5000 \times g$ for 5 minutes, and all but 100 μ L of supernatant was aspirated. Samples were stored at –20°C overnight.

In a separate experiment to assess the effectiveness of the washing steps for removing extracellular parasites, 1×10^5 HCT-8 cells (passage 9) were seeded into 6-well plates and incubated to 90% confluency as described above. Three wells were infected with excysted *C. parvum* oocysts to yield 1×10^5 oocysts/well as described, and three wells were infected with excysted oocysts that had been heat-killed after excystation by placing them in a 95°C heat

block for 2 hours. The cells were washed and harvested at 4, 16, or 28 hours after infection. The DNA was extracted and examined in triplicate by real-time PCR as described below.

A *C. parvum*-negative stool sample was obtained from an 11-month-old white female infant and stored at -20°C for 7 weeks. From each oocyst stock dilution, 200 μL was added to a 200 ± 10 -mg aliquot of the negative stool sample (yielding 1×10^2 – 1×10^7 oocysts/sample), and the samples were vortexed until homogenized and stored at -20°C overnight.

DNA extraction.

Oocysts were lysed in the pure oocyst and spiked stool samples by freezing in liquid nitrogen for 1 minute and thawing in boiling water for 1 minute for a total of six freeze-thaw cycles. Although we used boiling to better control temperature stability, we suspect that lower temperatures could also be used for thawing. DNA was extracted from the pure oocyst samples using a DNeasy Tissue Kit (QIAGEN, Valencia, CA), eluted in 200 μL elution buffer, and stored at -20°C .

The DNA was extracted from the spiked stool samples using a QIAamp DNA Stool Kit (QIAGEN) as follows. First 200 μL proprietary Quagen stool lysis (ASL) buffer was added to each sample, and each sample was vortexed at full speed until homogenized. An additional 1.2 mL of ASL buffer was added and vortexed. The samples were incubated at 95°C for 5 minutes. The “Protocol for Isolation of DNA from Stool for Human DNA Analysis” (QIAGEN) was followed to completion, and the DNA was eluted in 200 μL elution buffer and stored at -20°C . For the infected HCT-8 cells, the DNA was extracted using a DNeasy Tissue Kit (QIAGEN), eluted in 200 μL elution buffer, and stored at -20°C .

Real-time PCR.

The forward and reverse oligonucleotide primers used for real-time PCR were as follows: forward, 5'-CTGCGAATGGCTCATTATAACA-3'; reverse, 5'-AGGCCAATACCCTACCGTCT-3'.⁹ They were designed to detect the 18S rRNA gene of as many *Cryptosporidium* species as possible, with sequences matching *C. hominis* (AF093491), *C. parvum* (AF164102), *C. meleagridis* (AF112574), *C. canis* (AB210854), and *C. suis* (AF108861) completely and with a single base pair (bp) mismatch at forward primer position 5 of 22 for *C. felis* (AF112575) and position 18 of 22 for *C. muris* (X64343). A 5- μL aliquot of DNA was added to the 20 μL reaction mixture containing Bio-Rad iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) and 0.25 $\mu\text{mol/L}$ forward and reverse primers. After a 13.5-minute preincubation at 95°C , PCR amplification was performed for 50 cycles (15 seconds at 95°C , 15 seconds at 60°C , 20 seconds at 72°C), followed by melt curve analysis (8 minutes and 20 seconds at 75°C). The extracted DNA was analyzed in triplicate. The reaction was performed in a Bio-Rad (Hercules, CA) iCycler iQ using iCycler software (version 3.0), and the results were analyzed with a user-defined threshold of 200 PCR baseline subtracted curve-fit relative fluorescence units (CF RFU).

Starting quantities were calculated for the HCT-8 cell and spiked stool samples by using the pure oocyst samples as reference standards. A linear regression calculated from the pure oocyst samples was used in these calculations. Losses of yield for the HCT-8 cell and spiked stool samples were obtained by subtracting calculated starting quantities from known starting quantities and the Student *t* test performed using SPSS 12.0 software (Chicago, IL) to compare the two sample types. An analysis of variance (ANOVA) and *post hoc* analyses were also performed to assess the statistical significance of the differences between C_t values of samples with differing concentrations for both the infected HCT-8 cell and spiked stool samples.

Assessment of PCR inhibition.

To assess for the presence of residual inhibitors in our samples, DNA was first extracted from three 1×10^5 -cell aliquots of uninfected HCT-8 cells and three 200-mg aliquots of negative stool as described above. Four-week-old oocysts (Iowa isolate) were excysted using the bleach method described above, and 20- μ L aliquots from the excysted oocyst solution were added to 200- μ L aliquots of PBS, DNA extracted from the uninfected HCT-8 cells, and DNA extracted from the negative stool samples to yield concentrations of 1×10^5 excysted oocysts/sample. Real-time PCR was performed as described above on all three sets of samples in triplicate. The Student test was performed using SPSS to assess the statistical significance of the differences between C_t values of the three sets of samples.

Clinical samples.

Two sets of clinical samples were analyzed: diarrheal stools (frozen at -80°C) from 36 patients hospitalized from June to October 2004 in South Africa and DNA extracted from diarrheal stools from Brazil. The guidelines for human investigation of the University of Virginia, the Research and Ethical Committee of the University of Venda (Thohoyandou, South Africa), the Department of Health and Welfare, and the Department of Education in Polokwane (Limpopo province, South Africa), and the Federal University of Ceara (Fortaleza, Brazil) were followed, and informed consent was obtained from all patients. The DNA was extracted from 200-mg aliquots of the South African stool samples and analyzed using real-time PCR as described above, and samples testing positive for *Cryptosporidium* were confirmed by repeating the real-time PCR assay on another aliquot of the remaining stool. Positive samples were also confirmed and speciated using a previously described PCR-restriction fragment length polymorphism (RFLP) technique with few modifications.¹⁰ DNA for the PCR-RFLP was extracted from new aliquots of the original stool samples to ensure that positive real-time PCR results were not caused by contamination.

DNA had been previously extracted and stored at -20°C from 200-mg aliquots of stool samples obtained from patients from October 1989 through April 1993 in the urban shantytown of Goncalvas Dias, Fortaleza, Brazil, as a part of a larger project.¹¹ Eleven samples were selected, all of which had been previously analyzed using fluorescence microscopy (MeriFluor *Cryptosporidium/Giardia*, Meridian Bioscience, Cincinnati, OH). Five of these were *Cryptosporidium* negative, and six were positive by microscopy, with oocyst counts ranging from 10^5 to 10^7 oocysts/mL stool. These samples were selected in hopes of comparing real-time PCR quantification to oocyst counts obtained using microscopy. Real-time PCR was performed as described above, and the Student *t* test was used to assess the differences between C_t values for samples with 10^5 and 10^7 oocysts/mL stool as calculated by microscopy.

RESULTS

All pure oocyst samples containing 5×10^2 – 5×10^7 purified oocysts/mL (1×10^2 – 1×10^7 oocysts/sample) were detected, and their cycle threshold (C_t) values were regularly spaced (Figure 1A). When the PCR reaction was performed on the same DNA in triplicate three times, the mean C_t value difference between samples with one log difference in starting oocyst concentration was 3.7 ± 0.8 ($N = 60$). Additionally, melting curve analysis revealed a single peak at 80°C at all pure oocyst concentrations. All HCT-8 cell samples infected with $\geq 10^3$ oocysts were detected (one replicate of the sample infected with 10^2 oocysts was detected; Figure 1B). The C_t values were regularly spaced for samples a–d, but the replicates of samples e–f were inconsistent. Stool samples spiked with $\geq 10^3$ oocysts were also detected (only one replicate of the sample infected with 10^2 oocysts was detected), but their C_t values were not as regularly spaced as those in the pure oocyst samples (Figure 1C). A standard curve for this data is shown in Figure 2. The differences in C_t values between samples with different starting

oocyst concentrations were statistically significant by ANOVA ($F = 359.7$, $P < 0.001$ for infected HCT-8 cell samples and $F = 1608.1$, $P < 0.001$ for the spiked stool samples), and post hoc analysis showed statistically significant differences ($P < 0.001$) between all groups for each sample type. Because only one replicate of the samples infected with 10^2 oocysts was detected for both samples types, they were excluded from statistical analyses.

The experiments designed to assess the effectiveness of the washing step for removing extracellular parasite showed no significant amplification in HCT-8 cells infected with heat-killed oocysts. The mean C_t values for cells infected with live oocysts were 25.8 ± 0.2 , 22.1 ± 0.2 , and 20.4 ± 0.0 for cells harvested at 4, 16, and 28 hours after infection, respectively.

If the pure oocyst samples are used as reference standards and the starting oocyst quantities of the infected HCT-8 cells and spiked stool samples are back calculated, the loss of sensitivity was 1.4 ± 0.3 logs (25-fold) for the HCT-8 cells and 2.2 ± 0.5 logs (158-fold) for the spiked stool samples. There was a statistically significant difference ($P < 0.001$) between the losses of sensitivity for the two sample types. The experiments performed to assess the influence of PCR inhibitors on these losses revealed a statistically significant increase in C_t value in the stool samples compared with the PBS samples ($P = 0.017$) and the HCT-8 samples ($P = 0.018$), respectively. There was no difference between C_t values for the PBS and HCT-8 samples ($P = 0.193$). The mean C_t values for the PBS, HCT-8 cell, and stool samples were 31.6 ± 0.2 , 30.8 ± 0.8 , and 33.1 ± 0.64 , respectively.

When the assay was applied to clinical samples, 6 of the 36 South African samples were positive by real-time PCR. All six samples also tested positive by nested PCR, and restriction profiles indicated that five of these were *C. hominis* and one was *C. parvum* (Figure 3). Quantification was not possible with the Brazilian samples, but all replicates of the negative samples were negative by real-time PCR and all of the positive samples were positive by real-time PCR, yielding a sensitivity of 100% and specificity of 100% as compared with immunofluorescence microscopy. There was variability in C_t values between replicates for the positive samples, but samples with at least two positive replicates were considered positive (all C_t values were < 40). The mean C_t values for samples with 10^5 and 10^7 oocysts/mL by microscopy were 35.0 ± 0.8 and 35.3 ± 2.2 , respectively, and these two values did not show a statistically significant difference ($P = 0.832$).

DISCUSSION

The pure oocyst samples were detected with the greatest sensitivity (Figure 1A). A standard curve with strong positive correlation was generated for these samples, confirming that a reliable standard curve can be generated from pure oocyst samples. The qPCR data for the infected HCT-8 cells and spiked stool samples were not as regularly spaced, nor were samples detected with the sensitivity of the pure oocyst samples. HCT-8 cell samples infected with $\geq 10^3$ oocysts/sample showed statistically significant differences between samples infected with different amounts of oocysts, but inconsistencies within sample replicates infected with $\leq 10^4$ oocysts/sample made reliable differentiation difficult (Figure 1B). Spiked stool samples with different oocyst concentrations were easily distinguished from each other (Figure 1C). As in the HCT-8 cells, however, it is more difficult to distinguish samples with different concentrations at lower initial oocyst concentrations. Only one of the three replicates of the sample spiked with 10^2 oocysts, for example, was detected.

Because all three sets of samples were spiked or infected with the same quantities of oocysts, the standard curves shown in Figure 2 allow us to compare the loss of oocysts and/or DNA in each sample set. The difference between known and calculated starting oocyst concentrations was greatest for the spiked stool samples. Two of the most likely causes of this loss of yield

are the presence of PCR inhibitors in the stool and loss of DNA during the removal of stool particulate in the DNA extraction procedure. The results of the experiments to examine PCR inhibition confirm the presence of residual PCR inhibitors in the stool samples, even after using Qiagen's InhibitEX tablets. However, the difference between the stool samples and the other sample types was less than a log (10-fold), indicating that, in addition to residual PCR inhibitors, other factors also play a role in the loss of yield. The HCT-8 cells and PBS samples showed no statistical difference and thus have similar or negligible levels of residual PCR inhibitors.

There was also loss of yield in the infected HCT-8 cells, although it was less significant than the loss in the spiked stool samples. These losses could be caused by limitations of the DNA extraction protocol, but it is also likely that oocysts and freed sporozoites that had not entered cells were lost in the washing steps after infection. We did not expect all of the oocysts to infect the HCT-8 cells because our excystation rate was < 100% and because some oocysts were inevitably non-viable at the time of infection, so we would expect to lose oocysts in the washing steps. Additionally, replication of DNA within individual HCT-8 cells would compensate for some loss of yield, which emphasizes the use of this assay for assessing infection in laboratory cell lines. A limitation of this method is that some non-viable oocysts would inevitably remain even after washing, but our experiment to assess the effectiveness of the washing step indicate that these effects are negligible. When cells were infected with heat-killed oocysts, no *Cryptosporidium* DNA was detected by real-time PCR. This indicates that non-viable oocysts were successfully removed from the wells during the washing steps. It is also possible that oocysts that were not successfully excysted before inoculation into cell culture remained attached to the HCT-8 cells but were not detected.

We can predict the sensitivity of our assay for the infected HCT-8 cells and spiked stool samples using the losses of yield described above. This prediction is fairly accurate. We calculated a 1.4 ± 0.3 log loss (25-fold) for the HCT-8 cells and 2.2 ± 0.5 log (128-fold) loss for the spiked stool samples, and we were able to detect samples initially infected with $\geq 1,000$ oocysts reliably.

The correlation between real-time PCR and PCR-RFLP for the South African stools gives support to the positive real-time PCR results. Additionally, the results of the restriction digest show that the primers used in the real-time PCR assay successfully detect both *C. parvum* and *hominis* (Figure 3), and we suspect, based on the similarity of their sequences, that they would also detect other species important in human disease, including *meleagridis*, *canis*, *felis*, *muris*, and *suis*. The results of the Brazilian stool samples analyzed by fluorescence microscopy and real-time PCR also showed the utility of this assay for analyzing clinical specimens. Unfortunately, the Brazilian stool DNA used in the assay had been stored through multiple freeze-thaw cycles for 12–16 years, thus making accurate quantification by PCR especially difficult.

The primers used in this assay amplify a short gene fragment (~230 bp, depending on species) and should allow for detection of the *Cryptosporidium* species most frequently implicated in human cryptosporidial disease. Species identification can easily be obtained because the PCR product contains a VSP1 digest site, allowing for RFLP. Additionally, this assay does not require nested amplification. Not only does this reduce costs by halving the amount of Taq polymerase required, but it also reduces contamination risks. The ability to use SYBR Green in this assay instead of more expensive TaqMan or Scorpion probes is also of considerable benefit.

These results indicate that reliable reference standards can be generated using pure oocysts in PBS with the primers and PCR protocol described. Additionally, we found that qPCR can be

used to detect *Cryptosporidium* in HCT-8 cells or stool samples infected/spiked with $\geq 10^3$ oocysts/sample, a range well below typical shedding in natural infections. Starting oocyst quantities can be calculated using pure oocysts in PBS as reference standards, and more reliable values can be calculated for higher starting oocyst concentrations. Analyses of clinical diarrheal stool specimens show that this assay has good sensitivity and specificity versus immunofluorescence microscopy and can detect *C. parvum* and *hominis* successfully. We believe that other species could be detected as well. This qPCR assay can be used to detect and/or quantitate *Cryptosporidium* in HCT-8 cells, spiked stool specimens, and clinical stool specimens, and thus has great use in both the research and clinical laboratories.

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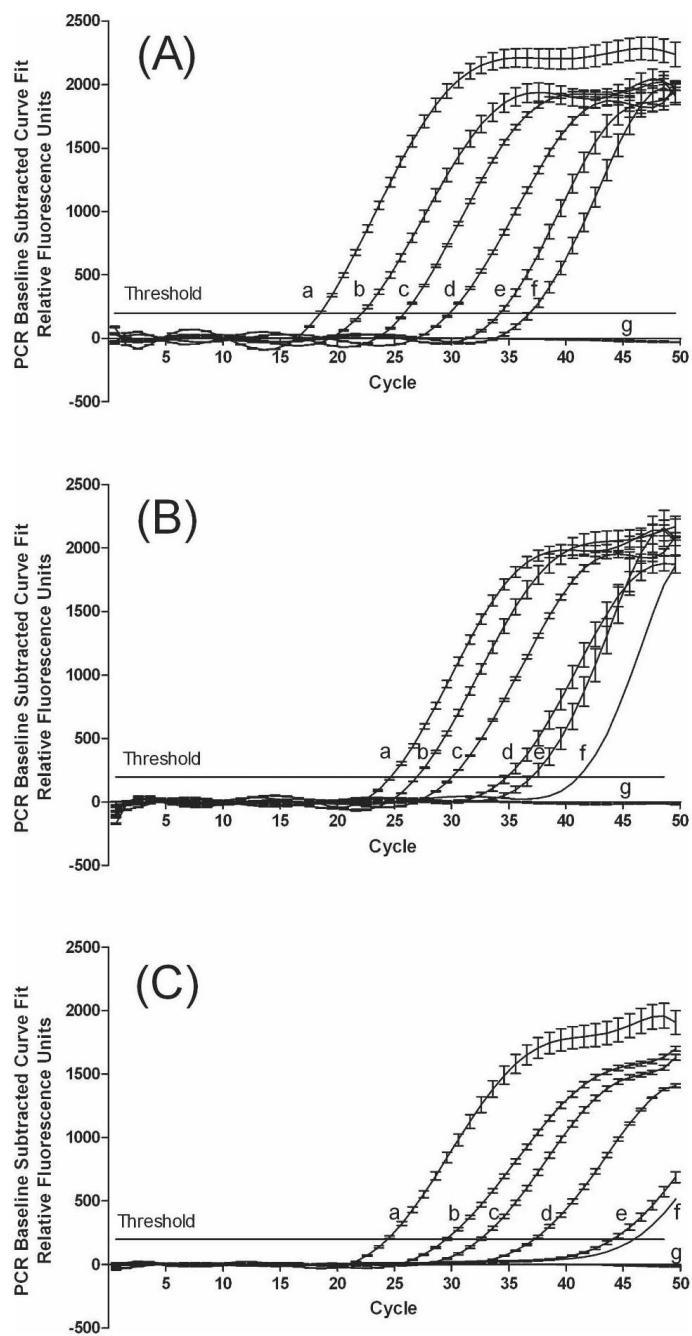


FIGURE 1.

Real-time PCR results from representative experiments using (A) 200- μ L aliquots of pure *C. parvum* oocyst samples, (B) HCT-8 cells infected with pure oocysts (each sample contained 1×10^5 HCT-8 cells initially), and (C) 200-mg stool samples spiked with pure oocysts. Samples were spiked or infected with *C. parvum* oocysts to yield samples with the following oocyst concentrations: (a) 1×10^7 , (b) 1×10^6 , (c) 1×10^5 , (d) 1×10^4 , (e) 1×10^3 , (f) 1×10^2 , and (g) 0 oocysts/sample.

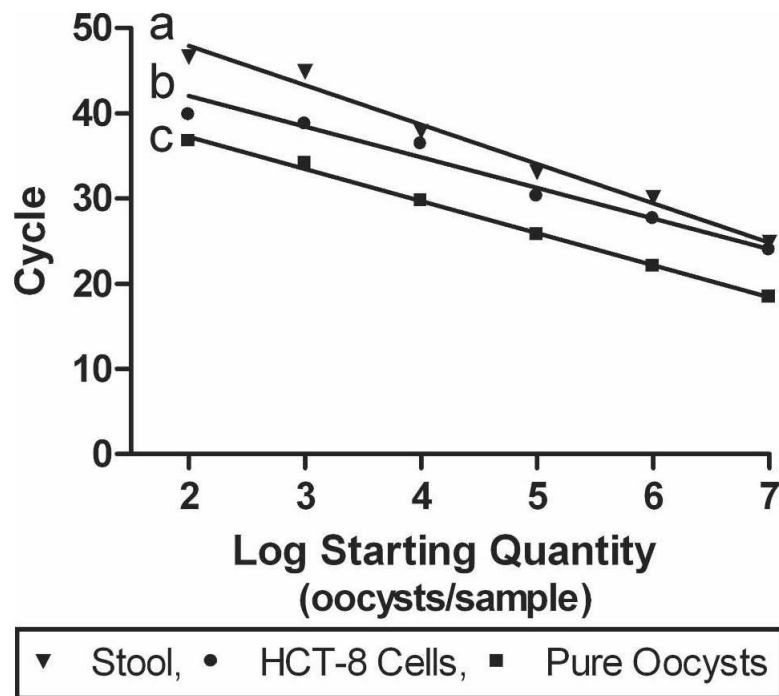


FIGURE 2.

A comparison of standard curves for pure oocyst samples, infected HCT-8 cells, and spiked stool samples. Samples were initially spiked or infected with pure oocyst solutions, yielding samples with concentrations ranging from 1×10^2 to 1×10^7 oocysts/sample. The following linear regressions (with R^2 values) were calculated: (a) $y = -4.6381x + 57.222$ ($R^2 = 0.9806$), (b) $y = -3.602x + 49.189$ ($R^2 = 0.9623$), and (c) $y = -3.771x + 44.747$ ($R^2 = 0.9957$).

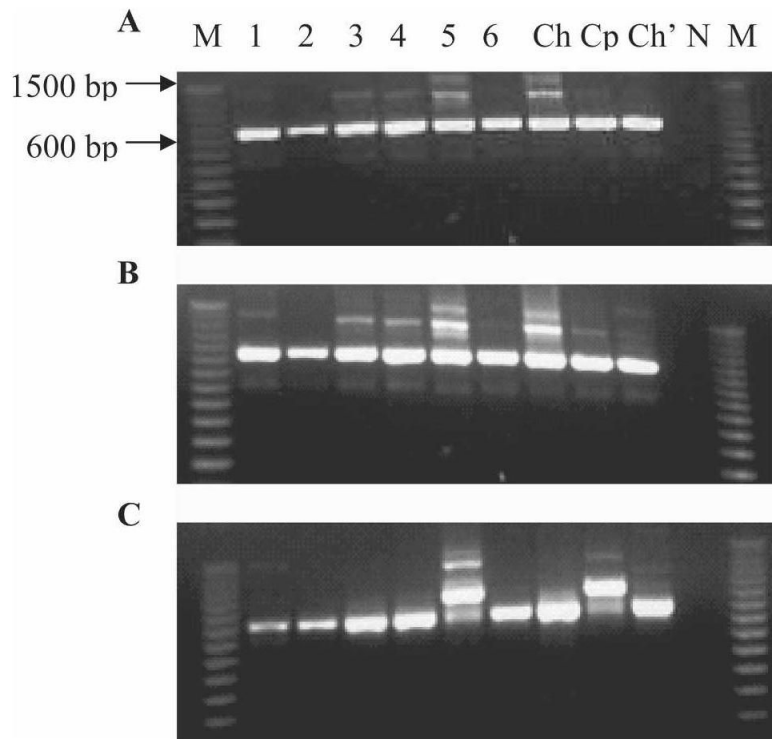


FIGURE 3.

Molecular diagnosis of *Cryptosporidium* from stool samples obtained from South African patients by a nested PCRRFLP procedure based on SSU rRNA gene sequences. **A**, Specific detection of *Cryptosporidium* spp. by nested PCR. Lane M, molecular weight markers; lanes 1–6, stool samples positive for *Cryptosporidium* after the nested PCR. **B**, Speciation of *Cryptosporidium* by *Dra*II digestion of the nested PCR products. None of the sample was digested, indicating that all were either *C. parvum* or *C. hominis*. **C**, Speciation of *Cryptosporidium* by *Vsp*I digestion of the nested PCR products, showing that sample 5 is *C. parvum*, whereas the other samples were *C. hominis*. Ch is the *C. hominis*–positive control (Ch = 50 ng, Ch' = 5 ng of genomic DNA), whereas Cp is the *C. parvum*–positive control. N is the negative control.