

Development of a TaqMan quantitative PCR assay specific for *Cryptosporidium parvum*

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Abstract

A rapid detection method that is both quantitative and specific for the water-borne human parasite *Cryptosporidium parvum* is reported. Real-time polymerase chain reaction (PCR) combined with fluorescent TaqMan technology was used to develop this sensitive and accurate assay. The selected primer–probe set identified a 138-bp section specific to a *C. parvum* genomic DNA sequence. The method was optimized on a cloned section of the target DNA sequence, then evaluated on *C. parvum* oocyst dilutions. Quantification was accomplished by comparing the fluorescence signals obtained from test samples of *C. parvum* oocysts with those obtained from standard dilutions of *C. parvum* oocysts. This real-time PCR assay allowed reliable quantification of *C. parvum* oocysts over six orders of magnitude with a baseline sensitivity of six oocysts in 2 h. © 2002 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: *Cryptosporidium parvum*; Quantitative; Real-time polymerase chain reaction; TaqMan

1. Introduction

Cryptosporidium parvum is a water-borne coccidian protozoan parasite responsible for gastrointestinal illnesses in humans, particularly in immunocompromised individuals. To date, of the eight species which have been identified in the *Cryptosporidium* genus, *C. parvum* is considered the main species associated with human diseases [1]. The current 1622 USEPA method [2] based on immunofluorescence assays (IFA) for the detection of *Cryptosporidium* is time-consuming and does not distinguish *C. parvum* specifically. In order to circumvent these limitations, *C. parvum* polymerase chain reaction (PCR)-based detection methods have been reported as alternative methods for rapid detection of this parasite [3,4]. We have already developed a rapid molecular test to detect the presence/absence of *C. parvum* in water samples [5,6]. However, these PCR methods could not quantitate the number of

oocysts present in the samples. In fact, although PCR-based assays have the advantage of rapid identification of pathogenic microorganisms, such assays rely on end-point measurements and are not appropriate for an accurate quantification of the target sequence. In contrast, quantification of DNA by real-time PCR is focused on the early exponential phase of the amplification reaction, when the amount of amplified products is proportional to the template DNA concentration. Real-time PCR uses the TaqMan fluorogenic detection system for a continuous measurement of products throughout the reaction. This PCR assay includes, in addition to the primers, an oligonucleotide probe labelled with two fluorescent dyes (a reporter and a quencher) that hybridizes to the target DNA [7]. Using the ABI Prism® 7700 Sequence Detection System (Applied Biosystems, Courtaboeuf, France), quantification occurs in real time during the amplification process with no post-amplification handling. This method eliminates potential sources of carry-over contamination and reduces handling time. Recently, an increasing number of studies based on real-time PCR have been described to quantify microorganisms in environmental samples [8–11]. In this study, we developed a rapid quantitative test based on real-time PCR for the automated quantification of *C. parvum* parasites.

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2. Materials and methods

2.1. Plasmid standard

The TaqMan probe and primers were based on the DNA sequence defined by Laxer et al. [3] and referenced by Deng et al. in GenBank AF188110. This 452-base sequence obtained by PCR with the primers P1 and P2 (P1: 5'-CCGAGTTTGTATCCAAAAAGTTACGAA-3'; P2: 5'-TAGCTCCTCATATGCCTTATTGAGTA-3') was cloned into the pCR[®]2.1-TOPO plasmid using the manufacturer's protocol. The resulting ligation product, Plax, was used to transform *Escherichia coli* TOPO10 competent cells (Topo TA Cloning[®] Kit, Invitrogen, The Netherlands). Purification of plasmid DNA was carried out using a plasmid extraction and purification kit (GFX[™] Micro Plasmid Prep kit, Pharmacia Biotech, France). The plasmid DNA concentration was estimated by measuring the optical density at 260 nm.

2.2. Source of *Cryptosporidium* oocysts and DNA

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 using the *Cryptosporidium*/*Giardia* cell IFA test (Cellabs, BMD, France) and differential interference contrast microscopy. *C. muris* (RN66 isolate) were also obtained from Waterborne. The DNA from the following *Cryptosporidium* species: *C. baileyi*, *C. andersoni*, *C. felis*, *C. wrairi*, *C. serpentis* and *C. meleagridis*, were obtained from CDC (L. Xiao, Centers for Disease Control and Prevention, Atlanta, GA, USA).

2.3. DNA extraction

Oocyst genomic DNA was released in the presence of 25% (w/v) Chelex-100 (Bio-Rad, Hercules, CA, USA) by five cycles of freezing and thawing (−80°C for 2 min, 95°C for 2 min). The lysates were centrifuged for 3 min at 10000×g and the DNA in the supernatant was used as template in the quantitative 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. [3] and referenced by Deng et al. in GenBank AF188110. These sequences were designed using Primer Express[™] software (Version 1.0, Applied Biosystems) and OLIGO version 6 (National Biosciences, Plymouth, MN, USA). The fluorescent TaqMan probe (CCAATCACAGAATCATCAGAATCGACTGGTATC) was labelled at the 5'-end with the 6-car-

boxyfluorescein reporter dye and at the 3'-end with the 6-carboxy-tetramethylrhodamine quencher dye. The set of primers selected (forward primer 5'-CGCTTCTTAGCCTTTCATGA-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

Real-time PCR uses the fluorescent TaqMan technology and its capacity to report fluorescence in real time. The TaqMan reaction requires a hybridization probe labelled with two different fluorescent dyes. 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. For real-time PCR assay, 50 µl PCR mixture contained 12.5 µl of TaqMan Universal PCR Master Mix (Applied Biosystems) composed of 5 mM MgCl₂, ROX as an internal fluorescence reference, 200 µM each 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. MgCl₂ was added to a final concentration of 6 mM. HCl 0.1 N (2 µl) was also added to the PCR mix. The concentrations of fluorescent probe and primers were respectively 200 nM and 300 nM. Plasmid standard (5 µl) or 10 µl of oocyst lysate solution was added into each assay tube. Negative controls with no template DNA were performed for each reaction series. MicroAmp optical caps and tubes were used (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).

3. Results and discussion

To date, there is no rapid specific and sensitive method available for quantifying the water-borne parasite *C. parvum*. Only a few studies regarding molecular methods for the quantification of the parasite *C. parvum* have been reported [12–14]. By comparison, a large number of PCR-based methods have been described for the detection of *Cryptosporidium* oocysts in environmental water samples [5,15,16] but they failed to accurately quantify oocyst numbers in the samples analyzed. Recently, real-time PCR using a TaqMan probe and the ability to monitor the progress in real time of the amplification reaction has been described [17]. Real-time PCR reactions are charac-

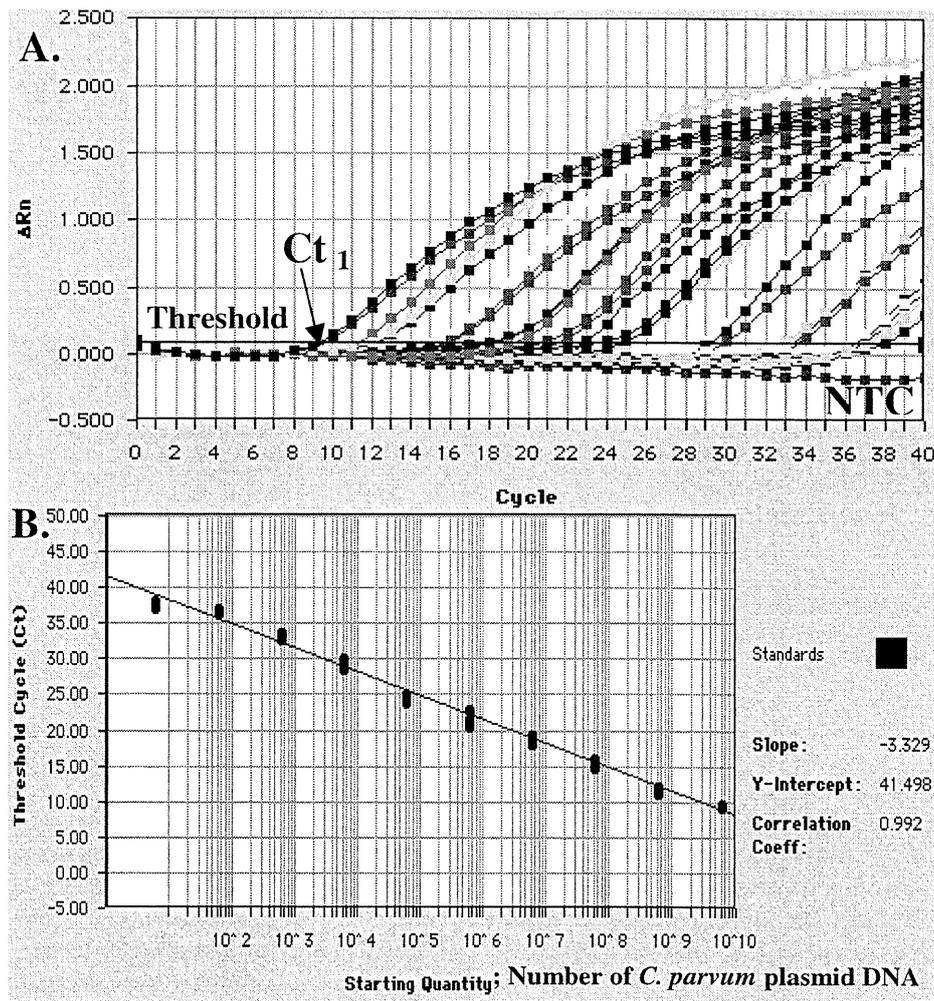


Fig. 1. Generation of standard curve. A: Increase of fluorescence intensity with cycle number for serially diluted *C. parvum* recombinant plasmid (Plax). The relative fluorescence is the increase in reporter dye intensity relative to the passive internal reference dye. Quantities of plasmid units are from 6 to 6×10^9 . The threshold fluorescence, or level at which the threshold detection cycle was determined, has a value of 0.1 . B: Standard curve generated from the data plotted in A ($n = 3$; correlation coefficient = 0.992).

terized by the point in time during cycling (Ct or threshold cycle) when amplification of the PCR product is first detected rather than the amount of PCR product accumulated. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed. This Ct parameter is defined as the number of cycles at which the fluorescence of the reporter generated by cleavage of the probe (ΔRn) is greater than a fixed threshold above the baseline. Quantification of test samples is performed by determining the Ct value and the use of a standard curve to determine the starting copy number. This amount of target sequence is automatically calculated by the software and, contrary to a standard PCR, no post-amplification step is required.

In this study we have developed a TaqMan PCR assay for the rapid quantification of *C. parvum* oocysts. The design of the primer pair and TaqMan probe was per-

formed using software to target a genomic sequence reported by Laxer et al. [3].

3.1. Optimization of the TaqMan PCR test

The optimization of primer and $MgCl_2$ concentrations was performed with 30 ng of recombinant standard plasmid DNA. $MgCl_2$ concentrations were tested from 0 to 7 mM. Results showed that a minimum concentration of 4 mM was necessary to generate PCR products but a variation of concentration from 4 to 7 mM did not change the Ct value (data not shown). As too little or too much free magnesium ion can reduce the amplification efficiency or result in amplification of non-target sequences, an optimal $MgCl_2$ concentration of 5 mM had to be chosen. By testing primer concentrations of 50 , 200 , 300 and 600 nM, the Ct value was found to be constant over this range. A

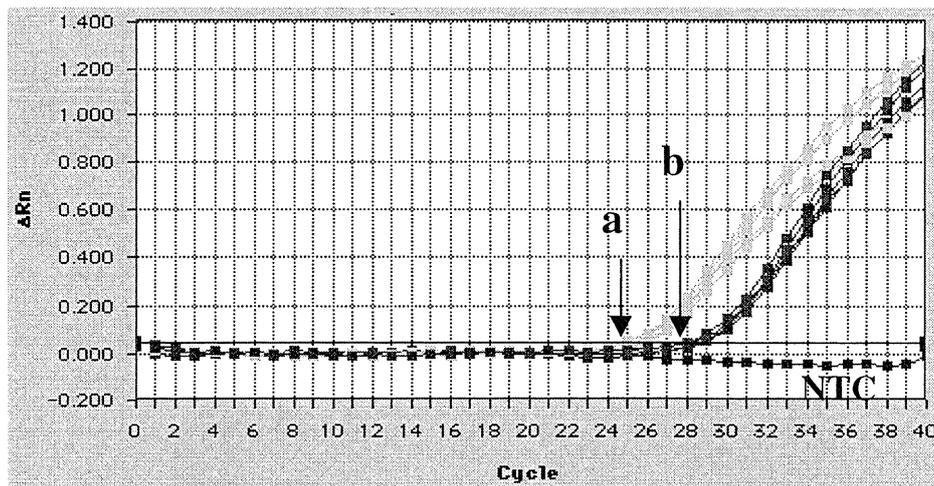


Fig. 2. Effect of Chelex-100 (concentration of 25% w/v) added prior to lysis by thermal shocks on the amplification of *C. parvum* oocysts. Curve a: oocysts (10^5) amplified after Chelex-100 treatment; curve b: oocysts (10^5) amplified without Chelex-100 treatment; NTC: no-template control; $n=5$.

concentration of 200 nM for each primer was selected to ensure that the amount of primer was not limiting to the reaction.

3.2. Specificity

This TaqMan PCR assay detected the *C. parvum* human and bovine genotypes, the most common causes of human cryptosporidiosis. The *C. meleagridis* genotype, a genetically close species, was also detected (data not shown). This species has been shown to be associated with some cases of human cryptosporidiosis [18]. The TaqMan test did not cross-react with *C. baileyi*, *C. andersoni*, *C. felis*, *C. wrairi*, *C. serpentis* and *C. muris*.

3.3. Sensitivity

Sensitivity and linearity of the real-time PCR assay was

evaluated by performing amplifications on serial 10-fold dilutions of *C. parvum* plasmid DNA using the optimal primer and $MgCl_2$ concentrations. The plasmid concentration was estimated by measuring optical density at 260 nm. Based on the total length of the recombinant plasmid containing the 452-bp insert (4360 bp), the equivalence of the amount of plasmid to copy number was calculated using the molar conversion for nucleic acids ($1 \mu g = 0.35 \text{ pmol} = 2.1 \times 10^{11}$ molecules). The standard plasmid solution was calculated to be $60 \text{ ng } \mu l^{-1}$ (i.e. 12.6×10^9 molecules μl^{-1}) and was diluted by serial 10-fold dilutions. Amplification by real-time PCR was carried out in the presence of 6×10^9 to 6 Plax plasmid DNA copies in the PCR mix and three replicates for each dilution. Fig. 1A shows the increase in fluorescence intensity with increasing number of cycles (Ct) and Fig. 1B shows the resulting standard curve. The Ct values increased from 10 to 37. Quantification was linear and reproducible over the nine

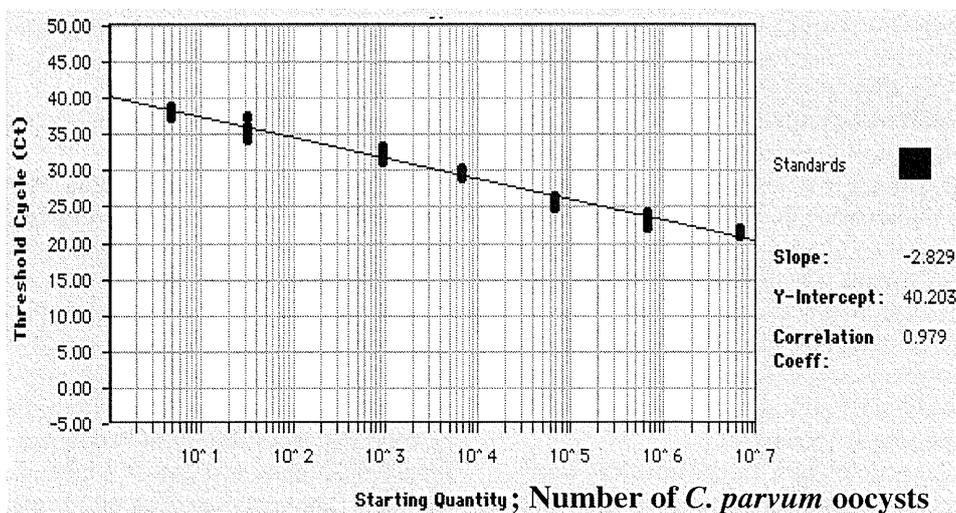


Fig. 3. Amplification of serially diluted *C. parvum* oocysts with Chelex-100 (25% w/v) and lysed by thermal shock. Generation of standard curve. Dots represent the values of five PCR amplifications for each dilution ($n=5$).

orders of magnitude. Negative controls run in parallel without any template DNA were negative. Since in theory the *C. parvum* genome contains only one copy of the target sequence reported by Laxer [3], the detection limit of six plasmid DNA copies corresponds to six *C. parvum* oocysts.

3.4. Quantification in purified oocyst solutions

The real-time PCR test performance was evaluated on purified oocyst dilutions. Addition of Chelex-100 was required prior to the lysis step by thermal shocks. By protecting the extracted DNA from degradation at high temperatures, Chelex-100 allowed a higher yield of amplification products. Fig. 2 shows the positive effect of the addition of 25% (w/v) Chelex-100 which lowers Ct values from 28 to 25 for the same oocyst amount (10^5). However, raising the Chelex-100 concentration from 15 to 75% (w/v) did not increase the PCR yield (data not shown).

To evaluate the sensitivity of the test, serial dilutions of *C. parvum* oocysts were analyzed with five replicates for each dilution. The data obtained were used to draw a standard curve relating Ct values to the number of *C. parvum* oocysts (Fig. 3). A linear response was observed over six orders of magnitude ranging from 5 to 6.6×10^6 oocysts. No inhibitor effect was observed. The detection limit was five oocysts in the PCR mix, comparable to that of conventional PCR [5]. Moreover, when no oocyst was added to the assays, no Ct value was observed. This standard curve allows the extrapolation of oocyst quantities in test samples after amplification under identical conditions. A slight difference was observed between the slopes of standard curves generated with Plax plasmid DNA (slope = 3.329) and purified oocyst solutions (slope = 2.829). This could be explained by the oocysts' tendency to agglutinate to each other at high concentrations. Thus, the use of a standard curve based on data from the amplification of *C. parvum* oocyst dilutions should give more reliable results to quantify test samples. This real-time PCR assay offers the possibility of rapidly (2 h) quantifying *C. parvum* oocysts over a large concentration range (6 log units) with sensitivity and specificity features equivalent to those of conventional PCR. Moreover, this method allows the simultaneous analysis of 96 samples and reduces the contamination risks because there is no post-PCR handling. We are currently developing a sample treatment step to eliminate TaqMan PCR inhibitors. It will permit this test to be used on water samples while maintaining a high sensitivity.

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