Development and test for long-term stability of a synthetic standard for a quantitative *Cryptosporidium parvum* LightCycler™ PCR assay

Renata Filkorn-Kaiser, Konrad Botzenhart and Albrecht Wiedenmann

ABSTRACT

A recently described quantitative rapid cycle real time PCR (LightCycler™) assay detects *Cryptosporidium parvum* after *in vitro* excystation, which is a surrogate marker for the viability of the organisms. In the original assay the quantification standard is a dilution series of *C. parvum* oocysts with a microscopically determined excystation rate. The need to keep suspensions of viable oocysts in stock and to continuously monitor their excystation rate, however, renders the assay impracticable for routine application. A synthetic standard was developed to replace the *in vivo* standard and was calibrated using oocysts with known excystation rates. The standard consists of a 486 bp DNA segment ranging from 229 bp upstream to 79 bp downstream of the actual PCR target site. Aliquots of the standard were frozen and stored at −20 °C and at −70 °C or lyophilised and stored at room temperature in the dark. For a period of one year samples preserved with each of the three methods were restored every four or five weeks. They were amplified in the LightCycler™ and the crossing points (CP) were monitored. No significant trend in the raw CP values could be observed for any of the three storage methods. However, when the methods were compared to each other by calculating the CP ratios (−20 °C/−70 °C; −20 °C/lyophilised; −70 °C/lyophilised) at the 10 monitoring dates, the CP ratios −20 °C/−70 °C and −20 °C/lyophilised showed a highly significant positive trend ($p < 0.0001$) while the CP ratio −70 °C/lyophilised did not differ from the null hypothesis ($p = 0.53$). It can be concluded that the latter two preservation methods are both appropriate, while storage at −20 °C is less advisable. Calculations based on the molecular weight of the standard and on the assumption of an average yield of three sporozoites per oocyst led to the conclusion that the target sequence is probably located on a double copy gene.

Key words | *Cryptosporidium parvum*, real time PCR, stability, storage, synthetic standard, water

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INTRODUCTION

Cryptosporidium parvum is one of the most important waterborne pathogens. Human infections and epidemics have been linked to the consumption of contaminated drinking water, to swimming pool water and to recreational water (CDC 2000).

Standard operating procedures for the microscopic enumeration of oocysts have been set up by offices such as the US Environmental Protection Agency and the British Drinking Water Inspectorate (DWI 1999; USEPA 1999). Microscopic enumeration, however, is time-consuming and requires experienced personnel. Moreover it does not allow conclusive statements on the viability of the oocysts. Numerous PCR protocols and combinations of such protocols with viability or cell culture infectivity assays have been published in an attempt to overcome these difficulties (Filkorn *et al.* 1994; Wagner-Wiening & Kimmig 1995; Rochelle *et al.* 1996; Deng *et al.* 1997; Wiedenmann *et al.* 1998). A recent protocol consists of a reproducible and straightforward *in vitro* excystation procedure performed in commercially available micro-centrifuge filter tubes, the

extraction of DNA only from excysted sporozoites and the subsequent quantitative detection of a specific gene sequence by rapid cycle real time PCR with hybridisation probes (Krüger *et al.* 2001). The PCR itself requires less than one hour reaction time and no additional sample handling is required for the quantification step. A precondition for quantification, however, is the preparation of a dilution series of viable oocysts with known concentrations and a microscopically verified excystation rate. This dilution series undergoes the same *in vitro* excystation, DNA extraction and amplification procedures as the sample oocysts. The LightCycler instrument uses the results from this dilution series to calculate a calibration curve and the sample results are quantified by comparison to that curve. The results must be corrected by multiplication with the determined excystation rate. Since the excystation ability of the oocysts changes over the course of time, and because the organisms have only a limited viability, relatively fresh oocysts (ideally not older than three months) must be kept in stock continuously and their excystation rate has to be monitored at short intervals. These requirements have rendered the whole method impracticable for routine application in most laboratories.

Consequently a synthetic standard was developed to replace the *in vivo* standard. The synthetic standard was calibrated by comparison to fresh oocysts and the stability of the standard was monitored for a period of one year under three different storage conditions. Thus, the results presented in this paper are an approach for standardising a rapid and quantitative PCR method for addressing the viability of oocysts by *in vitro* excystation. Standardisation is an essential precondition for the routine application of a method in different laboratories, the comparison of its sensitivity and specificity with other standardised methods and tests for the usefulness and limitations, e.g. with various matrices, environmentally aged oocysts or oocysts which have been subjected to disinfection procedures or treatment with antiprotozoal drugs.

MATERIAL AND METHODS

Organisms

Cryptosporidium parvum oocysts, live in water, were purchased from Waterborne Inc., USA, or Moredun Scientific Ltd., Penicuik, Scotland, UK and stored at $5 \pm 2\,^{\circ}C$. To determine the number of oocysts, 10 μL aliquots were fixed on Dynal SpotOn(TSO) microscope slides and stained with fluorescein labelled monoclonal antibodies (Cellabs) using standard procedures (DWI 1999).

Determination of the excystation rate

To determine the excystation rate oocysts were incubated in 100 μL acidified 0.09% NaCl solution, pH 2.6, for 30 minutes at 37 °C. 50 μL 3x excystation medium was added and the suspension was incubated again at 37 °C for one hour. The 3x excystation medium was prepared by rehydration of a lyophilised concentrate in 0.5 mL of 0.05 M Tris, pH 7.8, just before use. The concentrate was prepared by lyophilisation of 1.5 mL aliquots of 1.5% (w/v) tauroglycholic acid sodium salt (Merck) and 0.5% (w/v) bovine trypsin (2.2 U/mg, pH 7.6, 25 °C, Biochrom KG, Berlin, Germany). 10 μL aliquots of the oocyst suspension were placed on a microscope slide, mixed with 10 μL fluorescein-labelled monoclonal antibodies (Cellabs), covered with a cover slide, grease-sealed and incubated at 37 °C for 30 minutes in a humid chamber. 600 oocysts were microscopically identified and their excystation status was recorded. The excystation rate was calculated as the percentage of excysted oocysts after application of the excystation protocol minus the percentage of empty oocysts before application of the excystation protocol. The excystation rate specified by the manufacturer for the date of production was 98.9%. At the date of delivery to the laboratory (37 days after production) the rate was 96.2%. When the experiments were performed the excystation rate was 95.0%. All experiments performed to calibrate the synthetic standard were performed within 3 weeks from the date of determination of the excystation rate and within 3 months from the date of production. Three weeks after the last experiment the excystation rate was still 94.0%.

In vitro excystation in combination with quantitative real time PCR

In vitro excystation in combination with quantitative real time PCR was performed exactly as described by Krüger *et al.* (2001).

For *in vitro* excystation and DNA extraction oocyst suspensions were placed onto the membrane of the filter unit of a Spin-X™ centrifuge tube filter (Costar). The tube was centrifuged for one minute at 8,000*g*. During centrifugation the oocysts remain on the surface of the filter membrane while excess fluid and any free DNA from already disintegrated oocysts, which might be present in the oocyst suspensions, passes through the membrane and is retained in the collection tube. The oocysts on the filter membrane were then washed with 100 μL sterile deionised water (1 minute, 8,000 *g*). For *in vitro* excystation 100 μL of HCl, pH 2.6, was pipetted onto the membrane and the tube was incubated at 37 °C for 30 minutes. After incubation the tube was centrifuged again to remove the HCl. The membrane was washed once with 100 μL excystation medium (1 minute at 8,000*g*), then 80 μL of excystation medium was added and the tube was incubated at 37 °C for 1 hour. After incubation the filter unit was placed in a second collection tube and centrifuged for one minute at 12,000 *g*. To further enhance the release of DNA from excysted sporozoites the membrane was washed once with 50 μL AMPUWA™ (sterile pyrogen free water; Fresenius, Germany) for 1 minute at 12,000 *g*. Another 50 μL was added and the tube was incubated for 5 minutes at 90 °C. After centrifugation (1 minute at 12,000 *g*) the membrane was washed again with 50 μL of AMPUWA™. DNA was extracted from the 230 μL collected fluid (80 μL excystation medium and 150 μL AMPUWA™) using a PCR template preparation kit (High-Pure, Roche) according to the manufacturer's instructions with the following modification: before the DNA was eluted, the spin column with the glass fibre fleece was dried at 72 °C for 5 minutes. DNA was eluted with 25 μL of elution buffer.

The primers and probes used in the LightCycler™ PCR assay were primer CRY1 (5′-CCT TTT GTA GCT CCT CAT ATG CCT TA-3′), primer CRY2 (5′-ACT TCA CGT GTG TTT GCC AAT G-3′), probe CRY-F (5′-GGT AAA AAG TAT AGA AAG CTC TCA TTA TTG ATC C-3′) and probe CRY-LC (5′-CCC TGA TAA GAC AAG TCA TGA AAA GGC TAG-3′). The primers were designed in the reverse complemented form on the minus strand. Probe CRY-F was labelled with fluorescein at the 3′-end. Probe CRY-LC was labelled with LightCycler-Red 640-N-hydroxysuccinimide ester at the 5′-end and linked to a terminal phosphate at the 3′ end in order to prevent elongation. The Gene Bank Accession number for the gene sequence is M59419. The underlined nucleotides in probe CRY-F and CRY-LC differ from the sequence at the Gene Bank. AT is a TA, *C* is a G and the two A's are missing in the sequence stored at the Gene Bank. A re-analysis of the sequence has shown that the Gene Bank sequence is erroneous at these locations (Krüger 2001).

The master mix was prepared according to the following recipe (n = number of reactions to be cycled): $2 \times (n + 1)$ μL PCR grade H_2O; $3 \times (n + 1)$ μL $MgCl_2$ stock solution (final concentration: 3.75 mM); $1 \times (n + 1)$ μL primer-mix (final concentration: 0.5 μM each primer); $2 \times (n + 1)$ μL probe-mix (final concentration: 0.2 μM each probe); $2 \times (n + 1)$ μL LightCycler™ DNA Master Hybridisation Probes (final concentration: 1 ×). 10 μL of DNA template and 10 μL of master mix were pipetted into each of the capillaries. The capillaries were sealed, centrifuged in a microcentrifuge (10 s at 3,000*g*) and placed in the LightCycler™ rotor.

The program for PCR in the LightCycler™ instrument was set up as follows: denaturation (2 min at 95 °C); amplification (50 cycles; type = quantification; segment 1: target temperature = 95 °C, incubation time = 0 s, temperature transition rate = 20 °C/s, acquisition mode = none; segment 2: target temperature = 52 °C, incubation time = 15 s, temperature transition rate = 20 °C/s, acquisition mode = single; segment 3: target temperature = 72 °C, incubation time = 5 s, temperature transition rate = 20 °C/s, acquisition mode = none); melting curve analysis (1 cycle; type = melting curves; segment 1: target temperature = 95 °C, incubation time = 0 s, temperature transition rate = 20 °C/s, acquisition mode = none; segment 2: target temperature = 50 °C, incubation time = 10 s, temperature transition rate = 20 °C/s, acquisition mode = none; segment 3: target temperature = 95 °C, incubation time = 0 s, temperature transition rate = 0.2 °C/s, acquisition mode = continuous); and finally cooling for one minute at 40 °C. The fluorimeter gain for channel 1 was set to 1 and for channel 2 to 15.

Design and production of the synthetic standard

Primers for the production of the synthetic standard were designed using the HUSAR software package

(URL: www.genome.dkfz-heidelberg.de) with annealing sites 229 bp upstream and 79 bp downsteam of the PCR target site to yield a 486 bp product. The Gene Bank Accession number of the gene sequence is M59419. DNA extracted from commercially purchased *Cryptosporidium parvum* oocysts as described above in the section on *in vitro* excystation was amplified using the following conventional PCR protocol performed in a $T_{personal}$-Cycler (Biometra): 50 μL master mix (0.5 μL Taq-polymerase (PE Applied Biosystems), 1 μL nucleotide mix (Pharmacia), 5 μL 10x buffer (PE Applied Biosystems), 4 μL $MgCl_2$ (PE Applied Biosystems), 2.5 μL primer mix (10 pmol/μL of each primer CPS1: 5′-AGA ATA CTC AAA GCG AAG AT-3′ and CPS2: 5′-GCT TAT TTT TCT TGT TTT CT-3′), 27 μL H_2O) was pipetted into pre-cooled 0.5 mL reaction cups. 10 μL of DNA template was added. PCR conditions were 10 minutes denaturation at 94 °C, 45 amplification cycles (30 s at 94 °C, 45 s at 40 °C, 30 s at 72 °C (slope 20 °C/s)), final elongation for 3 minutes at 72 °C, and cooling to 4 °C. The product was split up in a 2% agarose gel, the gel was stained with ethidium bromide and the band with the amplification product was visualised under UV illumination, cut out and purified using a commercially available DNA extraction kit (QIAquick gel extraction kit, Qiagen, Catalog No. 28704) following the manufacturer's instructions.

The extracted DNA was quantified by spectrophotometry in a Ultrospec III spectrophotometer (Pharmacia) at 260 nm according to standard protocols. To achieve reliable results, DNA dilutions were prepared to yield absorptions between 0.1 and 1. The concentration was calculated according to the following formula using a conversion factor of 50 for double-stranded DNA:

$$\text{DNA concentration}(\mu g/mL) = 50 \times \text{absorption}_{260\,nm} \times \text{dilution factor}$$

Calibration of the synthetic standard by comparison to viable oocysts with a specified excystation rate

To determine the amount of synthetic standard (in ng) which corresponds to a certain number of excystable oocysts, DNA was extracted from a decimal dilution series of oocysts, as described above, and was used as the PCR template. The number of oocysts from which the DNA had been extracted at each of the dilution levels was multiplied with the excystation rate of the oocysts and the resulting numbers of oocysts were defined as standard concentrations in a LightCycler reaction. Decimal dilutions of a known amount of synthetic standard were defined as unknown sample concentrations in the same PCR run. Thus, as a result the LightCycler instrument calculated the number of excystable oocysts which corresponds to the amount of synthetic standard DNA applied at each of the dilution levels. To calculate the number of excystable oocysts which corresponds to 1 ng of synthetic standard DNA these results were divided by the amount in nanograms of synthetic standard DNA applied at each of the dilution levels.

Conservation, storage and monitoring of the stability of the synthetic standard

Aliquots of the synthetic standard in 1.5 mL microfuge tubes were frozen and stored at −20 °C and at −70 °C or lyophilised and stored at room temperature in the dark. For a period of one year samples preserved by each of the three methods were restored every month. Six decimal dilutions were all prepared in PCR-grade water and amplified in the same LightCycler™ run according to the described protocol. Crossing points were determined and monitored using the "second derivative maximum" method in order to achieve results independent from individual evaluation settings like noise bands or cut-off levels.

Statistics

Statistical evaluations were performed using the JMP 5.0 software package (SAS Institute Inc., USA).

RESULTS AND DISCUSSION

Calibration of the synthetic standard by comparison to viable oocysts with a specified excystation rate

Figure 1 illustrates the results of the amplification of DNA extracted from a dilution series of *Cryptosporidium* oocysts over a range of six orders of magnitude after application of

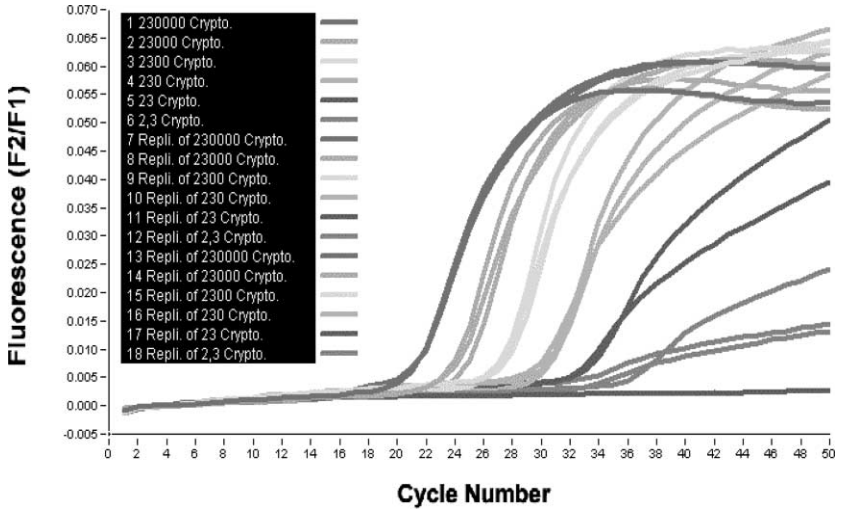

Figure 1 | Amplification of DNA extracted from viable *Cryptosporidium parvum* oocysts after *in vitro* excystation. Log_{10} dilution series starting with 230,000 oocysts, and ending with theoretically 2.3 oocysts per reaction. Reactions performed in triplicate. Numbers of oocysts are already corrected by the microscopically verified excystation rate of 95.0% in this experiment.

the *in vitro* excystation protocol. Each procedure was performed in triplicate for all six dilutions. The number of oocysts entered as the calibration standard into the LightCycler software is already corrected by multiplication with the microscopically verified excystation rate. Highly reproducible results were obtained for the replicates at each of the six dilution levels, yielding the standard calibration curve shown in Figure 2. Only at the second highest dilution level did amplification fail in one of the three parallel reactions. Figure 3 illustrates the corresponding results of the amplification of five dilutions of the synthetic standard performed in duplicate in the same LightCycler run. As the number of parallel reactions per run in the LightCycler is limited to 32, including controls with water and without template, this was the maximum number of replicates which could be realised in a single experiment. A total of six such experiments was performed to calculate how many nanograms of the synthetic standard correspond to a single excystable oocyst. Table 1 lists the raw results of these experiments, i.e. the number of excystable oocysts per

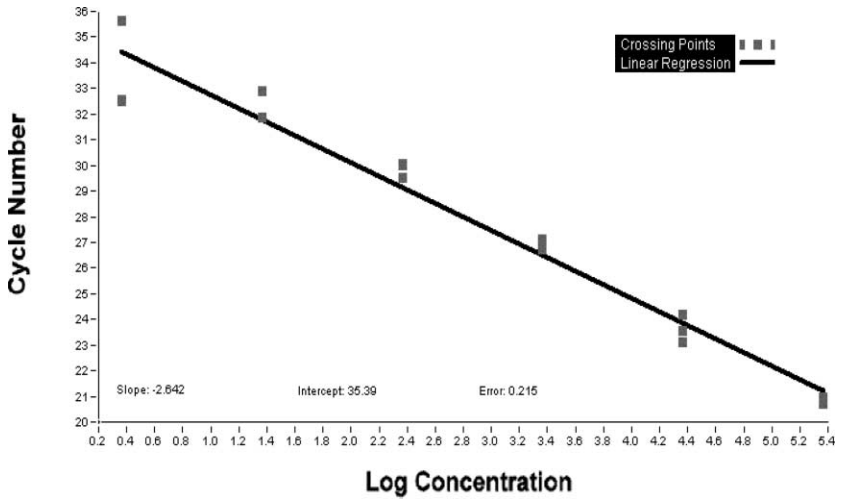

Figure 2 | Standard calibration curve calculated from the amplification results of DNA extracted from viable *Cryptosporidium parvum* oocysts after **in vitro** excystation as demonstrated in Figure 1.

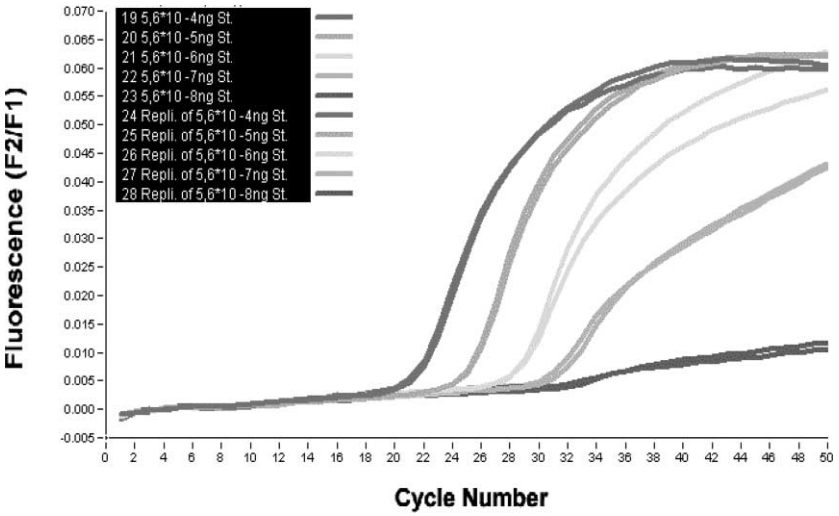

Figure 3 | Amplification results of the synthetic standard. Log_{10} dilution series starting with 5.6×10^{-4} ng and ending with 5.6×10^{-8} ng of synthetic standard DNA per reaction. Reactions performed in duplicate (Same PCR run as Figure 1).

Table 1 | Calculated number of excystable oocysts corresponding to the applied amount of synthetic standard DNA per reaction

		ng synthetic standard DNA				
		5.60E-04	5.60E-05	5.60E-06	5.60E-07	5.60E-08
Experiment	Replicate	Calculated number of excystable oocysts per reaction				
1	1	6.05E + 05	2.83E + 04	1.61E + 03	2.34E + 02	1.98E + 02
	2	6.85E + 05	3.57E + 04	1.59E + 03	2.02E + 02	1.03E + 02
2	1	2.15E + 05	1.07E + 04	5.45E + 02	5.00E + 01	8.80E + 01
	2	2.61E + 05	1.21E + 04	6.11E + 02	3.90E + 01	2.80E + 01
3	1	4.49E + 05	1.28E + 04	3.98E + 02	3.80E + 01	6.00E + 00
	2	4.57E + 05	1.92E + 04	6.75E + 02	4.00E + 01	9.00E + 00
4	1	2.09E + 05	3.33E + 04	1.33E + 03	1.29E + 02	0.00E + 00
	2	6.53E + 05	2.05E + 04	1.07E + 03	1.30E + 02	8.70E + 01
5	1	5.89E + 05	3.06E + 04	1.43E + 03	1.06E + 02	3.50E + 01
	2	9.18E + 05	3.29E + 04	1.41E + 03	1.51E + 02	6.20E + 01
6	1	1.80E + 05	9.35E + 03	4.00E + 02	1.50E + 02	1.30E + 01
	2	1.53E + 05	8.58E + 03	3.75E + 02	5.70E + 01	2.20E + 01

Table 2 | Calculated number of excystable oocysts corresponding to 1 ng of synthetic standard DNA

		ng synthetic standard DNA				
		5.60E-04	5.60E-05	5.60E-06	5.60E-07	5.60E-08
Experiment	Replicate	Calculated number of excystable oocysts per 1 ng DNA				
1	1	1.08E + 09	5.06E + 08	2.87E + 08	4.18E + 08	3.54E + 09
	2	1.22E + 09	6.37E + 08	2.83E + 08	3.61E + 08	1.84E + 09
2	1	3.83E + 08	1.92E + 08	9.73E + 07	8.93E + 07	1.57E + 09
	2	4.66E + 08	2.17E + 08	1.09E + 08	6.96E + 07	5.00E + 08
3	1	8.02E + 08	2.29E + 08	7.11E + 07	6.79E + 07	1.07E + 08
	2	8.16E + 08	3.42E + 08	1.21E + 08	7.14E + 07	1.61E + 08
4	1	3.74E + 08	5.94E + 08	2.38E + 08	2.30E + 08	*
	2	1.17E + 09	3.65E + 08	1.91E + 08	2.32E + 08	1.55E + 09
5	1	1.05E + 09	5.46E + 08	2.55E + 08	1.89E + 08	6.25E + 08
	2	1.64E + 09	5.88E + 08	2.51E + 08	2.70E + 08	1.11E + 09
6	1	3.21E + 08	1.67E + 08	7.14E + 07	2.68E + 08	2.32E + 08
	2	2.73E + 08	1.53E + 08	6.70E + 07	1.02E + 08	3.93E + 08

* Denominator is zero.

dilution level as calculated by the LightCycler using the calibration curves derived from oocysts after *in vitro* excystation. Table 2 lists the same data divided by the amount of synthetic standard added per reaction, i.e. the calculated number of excystable oocysts corresponding to 1 ng of synthetic DNA. Figure 4 is a normal quantile plot with 95% confidence intervals, and a distribution plot with a log normal distribution fit curve of the log 10 transformed data in Table 2. It demonstrates that the distribution of the data is consistent with the assumption of a log normal distribution (goodness-of-fit test (Shapiro-Wilk W test) $p = 0.15$) with narrow 95% confidence intervals for the mean and the standard deviation ($\mu = 8.50$ [8.40;8.61]; $\sigma = 0.42$ [0.35;0.51]), and with a low standard error of the mean (0.054). A log-normal distribution of results of a biochemical reaction which includes an exponential amplification of the initial amount of substance is highly plausible and is typical for many dose–response reactions in natural systems (Wissenschafliche Tabellen Geigy 1985). As a result the determined number of excystable oocysts per 1 ng of synthetic standard is antilog$_{10}$ (8.5) = 3.16×10^8. With a molecular weight of approximately 486 bp × 660 daltons/bp = 320,760 g/mol it can be calculated that 1 excystable oocyst corresponds to approximately 6 molecules, which would be consistent with an average yield of three sporozoites per excystable oocyst and the assumption that the target sequence is located on a double copy gene.

Comparison of the storage methods

As illustrated in Figures 5(a–c) no significant trend in the raw CP values could be observed for any of the three storage methods over a period of one year (bivariate fit of CP and storage days). However, when the methods were compared

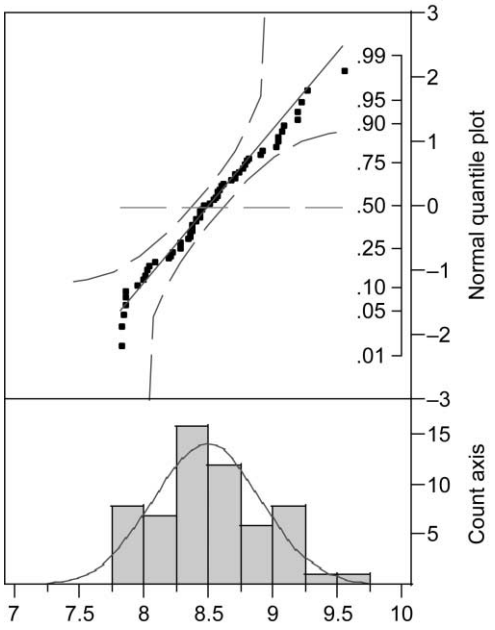

Figure 4 | Distribution and normal quantile plot of the $\log_{10}$ transformed calculated numbers of excystable oocysts corresponding to 1 ng of synthetic standard DNA ($n = 59$).

to each other by calculating the CP ratios (−20 °C/−70 °C; −20 °C/lyophilised; −70 °C/lyophilised) on the 10 monitoring dates (Figures 6(a–c)), the CP ratios −20 °C/−70 °C and −20 °C/lyophilised showed a highly significant positive trend ($p < 0.0001$) while the CP ratio −70 °C/lyophilised did not differ from the null hypothesis ($p = 0.53$).

Applications and limitations

Possible applications may include all methods which utilise *in vitro* excystation as a surrogate marker for viability or infectivity of *Cryptosporidium parvum*, such as detection methods for *Cryptosporidium* oocysts in water and food, disinfection experiments, studies of the survival of oocysts under various conditions and the screening of antiprotozoal drugs.

The set of primers and probes of the assay described by Krüger *et al.* (2001) has been designed to meet the specific requirements of the LightCycler system. It has been tested with DNA extracted from *C. parvum* genotypes 1 and 2 (kindly donated by SPDL, Scottish Parasite Diagnostic Laboratory, Glasgow, UK), and works for both genotypes. Since the two genotypes have been reclassified it can be

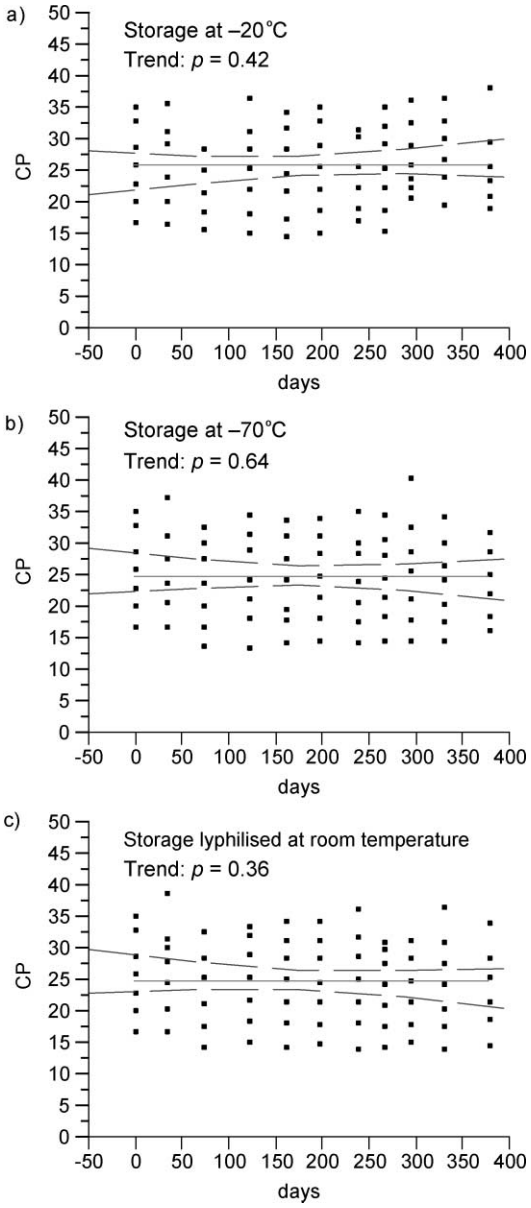

Figure 5 | Crossing points of six decimal dilutions of synthetic standard DNA monitored over a period of approximately one year of storage with three different conservation methods. Grand mean (null hypothesis) and 95% confidence limits of the linear regression line (bivariate fit of CP by days).

stated that this set of primers and probes works with *C. hominis* and *C. parvum*. However, it has not yet been evaluated whether the complete assay, including *in vitro* excystation, works as effectively for *C. hominis* or for other *Cryptosporidium* species or for mixtures of species found in the environment. The primer set also works with TaqMan™-PCR and the TaqMan™ probe FAM-TCA AAC GCT TCT

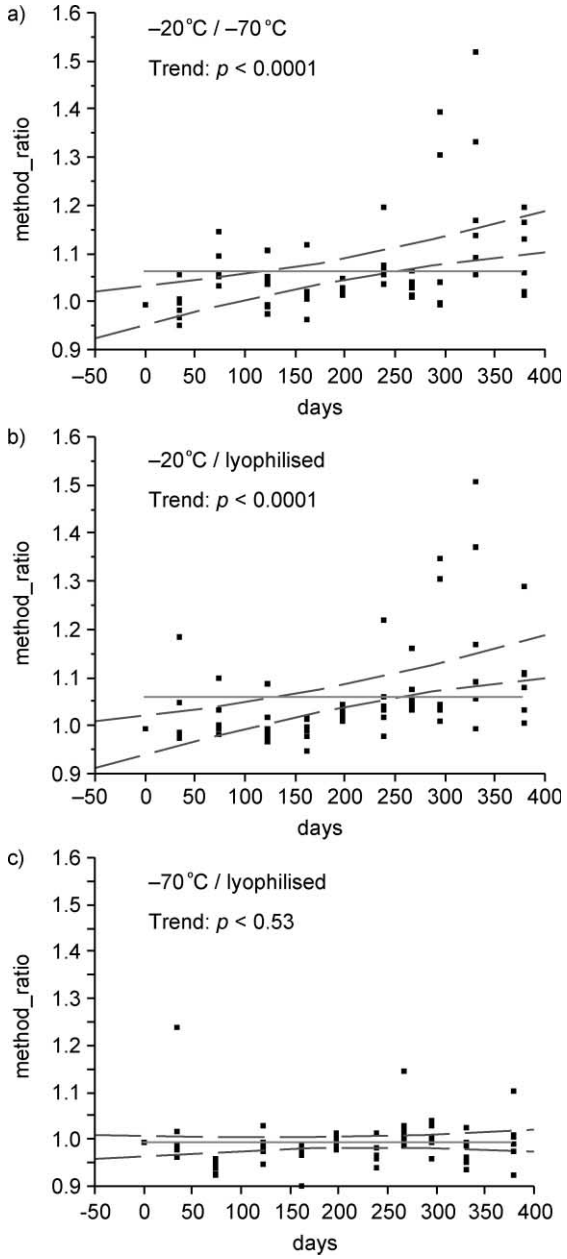

Figure 6 | Crossing point quotients of three different conservation methods at six decimal dilution levels of synthetic standard DNA monitored over a period of approximately one year. Grand mean (null hypothesis) and 95% confidence limits of the linear regression line (bivariate fit of CP by days).

CTA GCC TTT CAT GAC TTG TCT-TAMRA, where FAM is the reporter and TAMRA is the quencher dye (Krüger *et al.* 1998).

The sensitivity of the assay has been evaluated for concentrates from drinking water in a round robin trial with three participating laboratories, two in Germany and one in the UK (Krüger 2001). In this trial approximately 10 oocysts were spiked into sediments which had been produced by filtration of 100 L equivalents of finished drinking water through compressed foam filters according to UK standard methods (DWI 1999). The oocysts were recovered from the spiked sediments by immunomagnetic separation and quantified in parallel by immunofluorescence microscopy and by *in vitro* excystation in combination with LightCycler PCR. As recommended by the UK Drinking Water Inspectorate (DWI 1999) both a hard and a soft water quality was used in these experiments. In general, the quantitative PCR results corresponded well with the microscopic results. However, immunomagnetic separation was clearly less effective in the sediments from hard water. In the sediments from soft water oocysts could be detected in all 12 experiments (four at each of the three laboratories) with immunofluorescence microscopy. In 10 of the 12 experiments oocysts could also be detected by LightCycler PCR after *in vitro* excystation. In the experiment presented in this paper (Figure 1), 23 viable oocysts could be detected in two of three parallel reactions. 2.3 oocysts could be detected in all three parallel reactions. It has to be kept in mind that the results with such low numbers of oocysts are subject to the statistical uncertainties encountered in the preparation of decimal dilution series.

With the capability of detecting excystable oocysts over a concentration range of at least 5 $\log_{10}$ units within a single LightCycler run the theoretical potential of this method to monitor the effect of conditions which reduce the excystation rate of *Cryptosporidium parvum* is clearly superior to the potential of the microscopical method. For example, to demonstrate a reduction of the excystation rate from 95% to 0.0095% (4 log reduction) with the microscopical method more than 10,000 oocysts would have to be counted. Research to evaluate the method for this purpose is currently taking place.

In vitro excystation in combination with PCR is one of several possible surrogate markers for the viability and/or infectivity of *Cryptosporidium* oocysts. It has to be compared to *in vitro* excystation in combination with immunofluorescence microscopy, differential interference contrast microscopy or flowcytometry as well as alternative methods like the inclusion or exclusion of vital dyes, the combination of *in vitro* excystation with cell culture infection and mouse

infectivity assays. All these methods have specific limitations in terms of their evidence, feasibility, time requirements, costs and ethical considerations. One of the known limitations of *in vitro* excystation as a surrogate marker for infectivity is that, after certain disinfection procedures, e.g. ultraviolet irradiation, the ability of sporozoites to excyst may still be partially retained while the organisms have already lost their ability to infect cell cultures or to fully replicate in the gut of an infected vertebrate. *In vitro* excystation therefore provides a conservative estimate of infectivity and will rather underestimate the effect of inactivating treatments than overestimate them. The opposite effect has been described by Neumann *et al.* (2000) who investigated the infectivity of *C. parvum* oocysts which before had failed to excyst in two different *in vitro* excystation assays. They found that unexcysted oocysts which were separated from excysted oocysts by flow cytometry retained their infectivity in neonatal CD-1 mice. However, these authors have applied incomplete *in vitro* excystation protocols without a preincubation step in an acidified salt solution. They obviously were not aware of previous work by Campbell *et al.* (1992) who demonstrated, that acidic preincubation is essential to achieve maximum excystation rates. Campbell *et al.* (1992) have also shown that the portion of oocysts which require acidic preincubation may vary from isolate to isolate. This may be explained by the fact that, during the life-cycle of *Cryptosporidium* in an infected host, thick-walled and thin-walled oocysts are produced in variable concentrations. While thin-walled oocysts are primarily produced to enhance multiplication within the infected host by auto-infection, thick-walled oocysts are only produced to be shed with the faeces and to withstand various pressures in the environment until they are ingested by a second host (Current & Garcia 1991). While sporozoites in thin-walled oocysts must be able to excyst in the intestines without a passage through the stomach, thick-walled oocysts must be capable to withstand such a passage, and it is plausible that they require exposure to acidic conditions as an initial trigger for excystation. This is also in accordance with the finding that, in the beginning of a *Cryptospridium* infection, the majority of oocysts which are shed can be stained with an acid-fast staining procedure, while towards the end of an infection the faeces contain more and more "ghosts" which do not take up the dye (Bronsdon 1984).

The synthetic standard was developed to replace the quantitative *in vivo* standard which is required to produce a standard curve for the quantification of samples with unknown concentrations of viable oocysts by rapid cycle real time PCR. The synthetic standard was calibrated by comparison to viable oocysts with a specified excystation rate, and the stability of this standard for a period of one year was demonstrated. Thus, the repeated microscopic determination of numbers of viable oocysts and the calculation of their excystation rate which was necessary to produce a standard curve for the quantitative PCR assay described by Krüger *et al.* (2001) can be avoided, once a calibrated standard is available. In accordance with good laboratory practice for PCR assays it goes without saying that every LightCycler run should include a no-template control (water), an extraction control and a positive control. The positive control should consist of live oocysts from a trustworthy supplier. Suppliers normally deliver oocyst suspensions with a certificate stating their origin, date of production, concentration, excystation rate and a guaranteed minimum time period for which the oocysts retain their viability. While the no-template and extraction controls are used to demonstrate the absence of laboratory contamination, the positive control demonstrates the success of the complete assay including *in vitro* excystation, DNA extraction and amplification. Today, commercially available LightCycler PCR kits are often provided with an internal control sequence, which is detected by a different set of hybridisation probes at a different wavelength. An internal control would give proof of the absence of any inhibitory substances which might interfere with the amplification reaction in every individual sample. The protocol described by Krüger *et al.* (2001) does not include an internal control. The design of an internal control would therefore be a worthwhile attempt to further improve the reliability of the method.

The gene sequence used in the protocol of Krüger *et al.* (2001) was the first one ever published (Laxer *et al.* 1991) and is not patented. In the US the combination of *in vitro* excystation and PCR to detect viable *Cryptosporidium* oocysts for commercial purposes was protected by patent no. 5,556,774 at the time when the manuscript was submitted. No patents exist or are pending in other countries.

CONCLUSIONS

The gene sequence described in this publication could be successfully calibrated to an *in vivo* standard over a wide concentration range of at least five orders of magnitude. It can be stored at −70 °C or, when lyophilised, at room temperature in the dark over a period of at least one year without any loss of usability. The availability of a synthetic standard makes the quantification of viable *Cryptosporidium* oocysts by *in vitro* excystation and LightCycler™ PCR, as described by Krüger *et al.* (2001), practicable for routine applications such as detection in water samples or examination of inactivation procedures.

ACKNOWLEDGEMENTS

This project was funded by a grant from "Grimminger-Stiftung für Zoonosenforschung". We thank Dr Petra Krüger for assistance and Dr Manfred Baier, 4base lab GmbH, Reutlingen, Germany, for technical advice with the primer design. We thank Prof. Hew V. Smith and Brian M. Campbell, Scottish Parasite Diagnostic laboratory, Stobhill NHS Trust, Glasgow, UK, and Dr Despina Trougianidou, 4base lab GmbH, for participation in the round robin trial, and Diane Blaurock, Tübingen, for the linguistic revision of the manuscript.

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