High-throughput multiplex qPCR method for *Giardia lamblia* and *Cryptosporidium* species detection in stool samples

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ABSTRACT

Giardia lamblia and *Cryptosporidium* species belong to a complex group of pathogens that cause diseases hampering development and socio-economic improvements in the developing countries. Both pathogens are recognized as significant causes of diarrhea and nutritional disorders. However, further studies are needed to clarify the role of parasitic infections, especially asymptomatic infections in malnutrition and stunting. We developed a high-throughput multiplex qPCR method for *G. lamblia* and *Cryptosporidium* (spp.) detection in stool samples. The sensitivity and specificity of the method was ensured by analyzing confirmed positive samples acquired from diagnostics laboratories and participating in an external quality control round. Its capability to detect asymptomatic *G. lamblia* and *Cryptosporidium* (spp.) infections was confirmed by analyzing stool samples collected from forty-four asymptomatic 6-month-old infants living in an endemic region in Malawi. Five of these samples were found to be positive for *G. lamblia* and two for *Cryptosporidium* (spp.). In conclusion, the developed method is suitable for large-scale studies evaluating the occurrence of *G. lamblia* and *Cryptosporidium* (spp.) in endemic regions and for clinical diagnostics of these infections.

INTRODUCTION

G. lamblia and *Cryptosporidium* species (spp.) are among the most common parasites inflicting gastroenteritis and are recognized as significant causes of diarrhea and nutritional disorders.[1,2] Although numerous epidemiological studies have been conducted, there are still open questions regarding the contribution of parasitic infections, especially asymptomatic infections to malnutrition and stunting.[3--7] In order to clarify these questions there is a clear need for a sensitive and cost-effective *G. lamblia* and *Cryptosporidium* (spp.) detection method suitable for analyzing large number of clinical samples.

The traditional approach to diagnose *G. lamblia* and *Cryptosporidium* (spp.) infection is based on the detection of oocyst in stool samples. The most used diagnostic method is based on direct microscopy of the sample but it has downsides such as time-consuming and labor-intensive practice, high costs, and subjective interpretation of microscopic examination.[8--10] Furthermore, the difficulty of detecting low numbers of oocysts limits its sensitivity. Immunoassays have also been used to detect parasites in stool samples but their sensitivity has not reached optimal levels and non-specific cross-reactivity of detection antibodies have reduced their specificity.[11] Quantitative polymerase chain reaction (qPCR) is considered to be more sensitive method compared to microscopy but needs a well-equipped laboratory to avoid cross-contaminations between samples. High sensitivity of qPCR based detection technologies is a clear advantage in parasite diagnostics since the oocysts contain very small amount of DNA.[9] However, the firm structure of the parasite stages which are shed in stool make them resistant to detergents creating a challenge in DNA extraction for PCR. Different stool sample pretreatment strategies have to be applied to disrupt the oocyst wall in order to release the DNA for extraction. Various versions of freeze-thaw cycle protocols have been used as well as proteinases which have worked well for the oocyst wall layers of *Cryptosporidium* (spp.)[12--15] Heat shock is one additional commonly used treatment, and has usually been combined with other kinds of pretreatments.[10,16]

In the present study we compared various pretreatment methods and finally we developed a high-throughput DNA-extraction and PCR-based amplification protocol suitable for sensitive detection of *G. lamblia* and *Cryptosporidium* (spp.) in stool samples in a large-scale study setting.

MATERIALS AND METHODS

Samples. The method was optimized using formalin-fixed stool samples positive for *G. lamblia* or *Cryptosporidium* (spp.) UK Cryptosporidium Reference Unit (CRU; Public Health Wales Microbiology ABM, Singleton Hospital, Swansea, UK) provided four samples positive for *Cryptosporidium* (spp.) (one *Cryptosporidium parvum* and three *Cryptosporidium hominis* samples). Based on preliminary analysis, one *C. hominis*-positive sample was chosen for further optimization of the method. *G. lamblia* -positive sample was obtained from the Fimlab Laboratories (Pirkanmaa Hospital District, Tampere, Finland). In addition, the final evaluation of the optimized *G. lamblia* and *Cryptosporidium* (spp.) qPCR methods was done by participating in the external QCMD quality control round (Quality Control for Molecular Diagnostics: Parasitic Gastroenteritis panel 2013), containing unfixed *Cryptosporidium* (spp.) and *G. lamblia* positive samples in addition to negative control and *Entamoeba* (spp.) positive samples (http://www.qcmd.org). An epidemic stool sample confirmed to be *Cryptosporidium parvum* positive by microscopy (HUSLAB, Finland) was also tested.

To find out whether the developed method could detect any asymptomatic infections in children living in high-risk region, stool samples collected from 44 6-month-old Malawian infants between January and June 2008 were analyzed. A short sedimentation step was added to the optimized protocol for these samples in order to prevent the clogging of extraction

columns by the large particles found in these samples (sand etc.). The children were participants in the Lungwena Child Nutrition Intervention Study (registration no.: NCT00524446) evaluating the influence of dietary interventions on early childhood growth.[17] The subjects were healthy 6-month-old children recruited from general population and forty-five per cent of them were male (20/44). Guardians collected a stool sample from the participants and brought it within 24 hours to the health center. The samples were then stored at –20°C and shipped later for long-term storage at –80°C. The guardians of all participating children gave a written informed consent and the trial adhered to Malawian regulatory guidelines and the principles of the Declaration of Helsinki. The trial protocol was approved by the College of Medicine's research and ethics committee (University of Malawi) and the ethical committee of the Pirkanmaa Hospital District, Finland.

Sample pretreatment. 10 % (w/v) stool suspensions were made in 0.2 % BSA in Hank's buffer. The sample was concentrated either by pelleting the oocysts by centrifugation followed by pellet resuspension or by sedimentation method which was based on sedimentation during a 1 hour incubation time in room temperature.[14,18] In the next step different methods were compared to disrupt the oocysts including exposure to combinations of proteinase K treatment, freeze-thaw cycles and heat shock treatment (10 min at +98 °C). The tested sample pretreatment protocols are summarized in Figure 1.

DNA extraction and qPCR. Three kits were tested for DNA extraction: QIAamp DNA Blood Mini Kit, QIAamp DNA Stool Mini Kit and QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany). The QIAamp DNA Stool Mini Kit was also tested without the "inhibit Ex" -tablet provided in the kit. DNA extraction was performed from 10-fold dilution series of non-pretreated parasite positive samples together with negative control samples which were added both in the extraction and PCR stages. Parasite DNA was detected in each sample dilution using qPCR and the efficiency of amplification was evaluated based on C_T-

values. The primers and probe used for *G. lamblia* detection were designed to recognize and amplify a 62-bp fragment of the SSU rRNA gene (GenBank accession no. M54878) and the *Cryptosporidium* (spp.) primer-probe set was targeted against a 70 bp fragment of DNAJ-like protein gene of both *Cryptosporidium parvum* and *Cryptosporidium hominis* (GenBank accession no. AF177278.1 / XM661034.1) (Table 2).[11,19] QuantiTect Probe PCR kit (Qiagen, Hilden, Germany) and Applied Biosystems 7900HT were used in all qPCR analysis (LifeTech, Paisley, UK). The primer and probe concentrations were first optimized according to the qPCR kit manufacturer's instructions separately for both parasites and combined later into a multiplex reaction. In the final test format both the DNA extraction and qPCR steps were done using 96-well plate format.

RESULTS

Optimization of the method. The ability of different stool sample pretreatments to release parasite DNA differed clearly from each other. The pretreatment protocol combining heat shock and over-night proteinase treatment and the protocol including pelleting, heat shock and 2 h proteinase treatment were the most efficient ones and their sensitivity to detect parasite DNA did not significantly differ (data not shown). The protocol with heat shock followed by over-night proteinase treatment was chosen for further development (Figure 1). QIAamp Viral RNA Mini Kit was found to be the most efficient method to extract parasite DNA for qPCR while the QIAamp DNA Stool Mini Kit was the least efficient (Table 2). The PCR inhibitor absorbing "InhibitEx" tablet provided in the QIAamp DNA Stool Mini Kit slightly improved its performance (Table 2). The ability of the optimized methods to detect both parasites was evaluated by participating in external quality control round (QCMD). Both parasites were detected in all QCDM panel samples correctly and no wrongly positive results

were obtained either (Table 3). The optimized assay detected *Cryptosporidium parvum* also in a stool sample confirmed to be positive by microscopy in a routine diagnostic laboratory (HUSLAB, Finland) (Table 3).

Analysis of stool samples from Malawian infants. Altogether five (11.4 %) of the stool samples collected from asymptomatic Malawian infants were positive for *Cryptosporidium* (spp.) and two (4.5%) were positive for *G. lamblia* (Table 3). None of the samples was positive for both parasites.

DISCUSSION

The aim was to develop a multiplex high-throughput qPCR method which is suitable for the detection of *G. lamblia* and *Cryptosporidium* (spp.) in stool samples in large-scale epidemiological studies and in diagnostic laboratories. All steps of the assay including sample pretreatment, DNA extraction and qPCR were systematically optimized to reach maximal sensitivity and to adapt the assay to a high-throughput 96-well format. The fully optimized assay detected these parasites in both external QC samples and in clinical sample series.

Two of the tested pretreatment protocols allowed equally sensitive and reproducible detection of these parasites by qPCR. The protocol with heat-shock and over-night proteinase treatment was chosen since it was more applicable to the high-throughput format compared to the other efficient protocol which included pelleting, heat shock and 2 h proteinase treatment. Four different DNA extraction methods were tested and surprisingly the QIAamp DNA Stool Mini Kit which is specifically designed for DNA extraction from stool samples and is often used as a default solution when performing stool sample DNA extraction was less efficient in purifying the parasite DNA in comparison to the other tested kits. In fact, similar results have been obtained by others as well. [15,20]

The high sensitivity of PCR is a clear advantage in the detection of parasites from stool samples. However, this makes PCR also prone to contamination that can happen if a positive sample contaminates negative samples which are analyzed in the same test run, or if the laboratory is contaminated by PCR products from previous amplifications. We used negative control samples in every step of the method (pretreatment, extraction and PCR) to detect if any contamination occurred but none was found. Also, no false negative results were detected in the external quality control samples (QCMD). In addition, the quality of the detection was also confirmed by using known *G. lamblia* and *Cryptosporidium* (spp.) positive samples as positive control in each test run. Even if our assay was not affected by contaminations, it is important to pay attention to this issue when organizing sample collection and handling as well as the laboratory work for PCR-based parasite detection in stools.

Relatively high proportion of stool samples obtained from asymptomatic Malawian infants were found to be positive for these parasites (11.4 % for *Cryptosporidium* (spp.) and 4.5 % for *G. lamblia)*. This finding, together with the result from external QCMD round, suggests that the method is sensitive. In fact, the sensitivity of PCR has been found to be superior compared to microscopy in several previous studies.[11,14,19,21] The detection of several parasite positive children who had no symptoms of cryptosporidiosis or giardiasis suggests that the method can be applied for epidemiological studies in high-risk and endemic areas making it possible to estimate the background frequency and circulation of these parasites in the population. However, one limitation of the present study is that we could not directly compare the detection rates by this PCR method to those using traditional microscopy. Therefore, further studies are needed to verify their relative sensitivity and specificity in different clinical materials including both symptomatic and asymptomatic patients.

The optimized protocol offers a multiplex high-throughput option for *G. lamblia* and *Cryptosporidium* (spp.) detection from human stool samples. The method is applicable for frozen and fixed samples, which eliminates the need to analyze the samples freshly. In addition, it is less labor-intensive compared to microscopy at least when large sample numbers need to be analyzed and the pretreatment phase is suitable for high-throughput format making it cost-effective for large screenings of *G. lamblia* and *Cryptosporidium* (spp.) and for clinical diagnostics. A qPCR machine is needed as a special equipment for setting up the method. The use of PCR technology also decreases the inter-individual variation in the sample analysis compared to microscopy. In addition, quantification makes it possible to use the method for monitoring the efficacy of antiparasitic treatments and estimating disease severity.

In conclusion, the results suggest that this method may besuitable for clinical diagnostics of *Giardia lamblia* and *Cryptosporidium* (spp.) infections after further validation and is sensitive enough to detect even asymptomatic infections in background population in endemic regions. It offers significant advantages compared to traditional microscopic methods in terms of costs and speed of the diagnosis. Thus, this method provides an efficient tool to study the outcomes of both symptomatic and asymptomatic *G. lamblia* and *Cryptosporidium* (spp.) infections in clinical studies.

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DISCLOSURES

The authors declare no conflict of interest.

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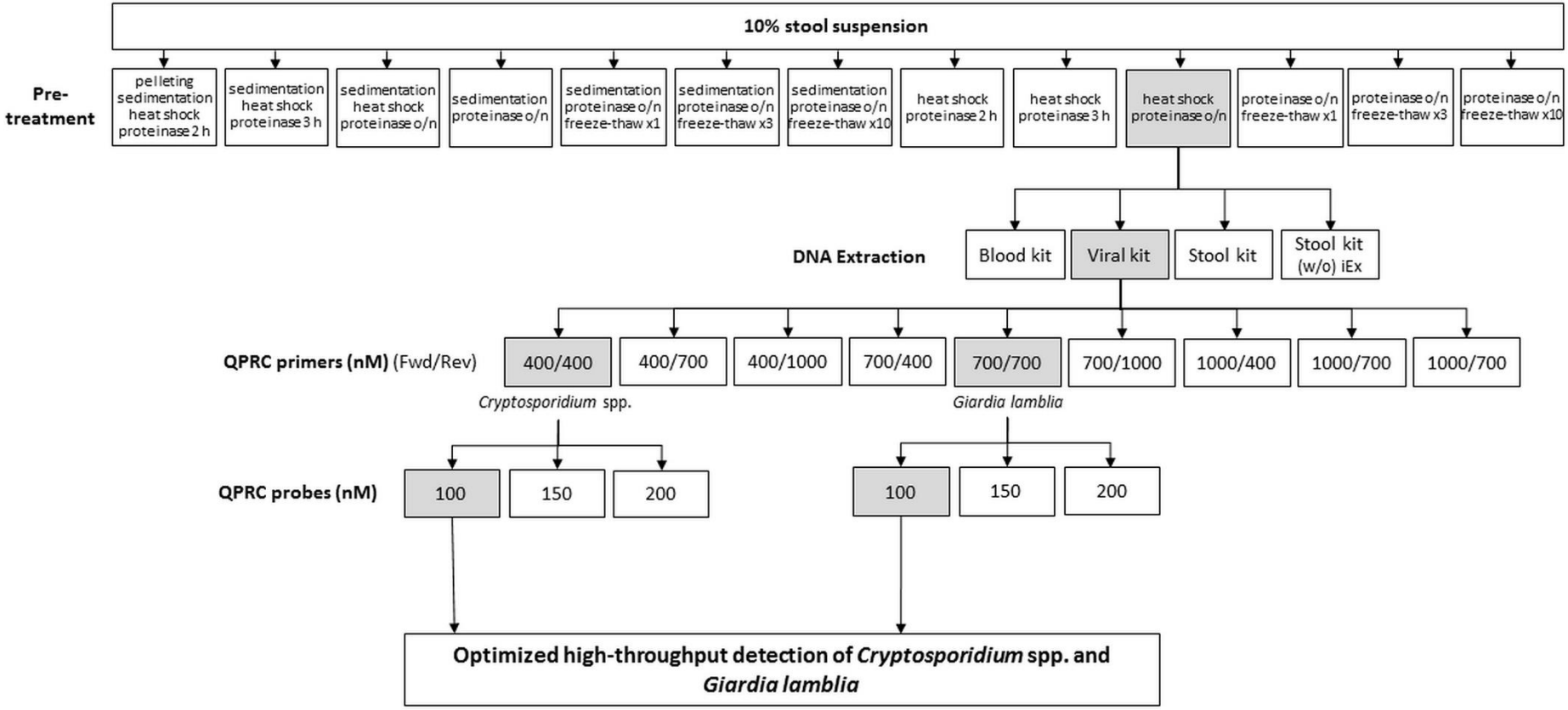

Figure 1. Summary of the *Cryptosporidium* (spp.) and *Giardia lamblia* detection method optimization. The selected options are highlighted with gray. QPCR primer and probe concentrations were tested separately for both parasites and combined into a multiplex reaction. Stool kit (w/o iEx) = Stool kit without the inhibit Ex –tablet included in the kit, Fwd= Forward (primer), Rev= Reverse (primer).

Table 1. QPCR primers and probes. Both primer-probe set sequences have been published earlier (Verweij et al., 2004; Bruijnesteijn van Coppenraet et al., 2009) FAM= fluorescein amidite, MGB=minor groove binder, VIC is a proprietary name of Lifetech.

Giardia lamblia	
Forward primer	GAC GGC TCA GGA CAA CGG TT
Reverse primer	TTG CCA GCG GTG TCC G
Probe	FAM - CCC GCG GCG GTC CCT GCT AG - MGB

***Cryptosporidium* (spp.)**	
Forward primer	CTT TTT ACC AAT CAC AGA ATC ATC AGA
Reverse primer	TGT GTT TGC CAA TGC ATA TGA A
Probe	VIC - TCG ACT GGT ATC CCT ATA A - MGB

Table 2. The ability of different kits to extract *Giardia lamblia* DNA for qPCR. The extraction kits were tested with 10 times dilution series of *Giardia lamblia* DNA. All dilutions were processed as three parallel samples following three qPCR reactions per sample. The results are presented as mean CT values and standard deviations. For negative samples the CT value of 45 was used for the calculations based on the number of cycles used in the PCR reaction. Blood kit = QIAamp DNA Blood Mini Kit, Viral kit = QIAamp Viral RNA Mini Kit, Stool kit = QIAamp DNA Stool Mini Kit. The stool kit was also tested without the InhibitEx tablets included in the kit (NT).

Sample dilution (log10)	**Blood kit**		**Viral kit**		**Stool kit**		**Stool kit (NT)**	
	C_T value	SD	C_T value	SD	C_T value	SD	C_T value	SD
-1	27.8	0.2	26.7	0.6	32.1	0.3	35.0	0.3
-2	32.5	0.7	29.2	0.1	39.0	1.5	38.2^	3.9^
-3	37.7*	0.1*	34.1	0.8	nd	–	nd	–
-4	nd	–	40.2*	3.0*	nd	–	nd	–
-5	nd	–	nd	–	nd	–	nd	–
-6	nd	–	nd	–	nd	–	nd	–

The mean Ct and SD values are calculated from the reactions that the product was detected in.
* in 33% of the reactions no product was detected.
^ in 66% of the reactions no product was detected.

Table 3. *G. lamblia* and *Cryptosporidium* (spp.) positivity in external quality control samples (QCMD), an epidemic *Cryptosporidium parvum* sample confirmed to be positive by microscopy and samples collected from asymptomatic six-month-old Malawian infants analyzed with the optimized qPCR method.

Sample content	*Giardia lamblia* PCR	*Cryptosporidium* spp. PCR
External quality control round (QCMD)[1]		
Negative control	Negative	Negative
Entamoeba histolytica	Negative	Negative
Cryptosporidium parvum/hominis	Negative	Positive
Cryptosporidium parvum/hominis (low conc.)	Negative	Positive
Entamoeba dispar	Negative	Negative
Entamoeba histolytica	Negative	Negative
Giardia lamblia	Positive	Negative
Giardia lamblia	Positive	Negative
Epidemic sample positive in microscopy		
Cryptosporidium parvum	Negative	Positive
Forty-four stool samples from endemic region (Malawi)		
Prevalence	4.5%	11.4%

[1] Quality Control for Molecular Diagnostics: Parasitic Gastroenteritis panel 2013, QAP124154. The sample analysis was blinded.