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Parasitological diagnosis combining an internally controlled real-time PCR assay for the detection of four protozoa in stool samples with a testing algorithm for microscopy

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Abstract

Molecular detection of gastrointestinal protozoa is more sensitive and more specific than microscopy but, to date, has not routinely replaced time-consuming microscopic analysis. Two internally controlled real-time PCR assays for the combined detection of *Entamoeba histolytica*, *Giardia lamblia*, *Cryptosporidium* spp. and *Dientamoeba fragilis* in single faecal samples were compared with Triple Faeces Test (TFT) microscopy results from 397 patient samples. Additionally, an algorithm for complete parasitological diagnosis was created. Real-time PCR revealed 152 (38.3%) positive cases, 18 of which were double infections: one (0.3%) sample was positive for *E. histolytica*, 44 (11.1%) samples were positive for *G. lamblia*, 122 (30.7%) samples were positive for *D. fragilis*, and three (0.8%) samples were positive for *Cryptosporidium*. TFT microscopy yielded 96 (24.2%) positive cases, including five double infections: one sample was positive for *E. histolytica/Entamoeba dispar*, 29 (7.3%) samples were positive for *G. lamblia*, 69 (17.4%) samples were positive for *D. fragilis*, and two (0.5%) samples were positive for *Cryptosporidium hominis/Cryptosporidium parvum*. Retrospective analysis of the clinical patient information of 2887 TFT sets showed that eosinophilia, elevated IgE levels, adoption and travelling to (sub)tropical areas are predisposing factors for infection with non-protozoal gastrointestinal parasites. The proposed diagnostic algorithm includes application of real-time PCR to all samples, with the addition of microscopy on an unpreserved faecal sample in cases of a predisposing factor, or a repeat request for parasitological examination. Application of real-time PCR improved the diagnostic yield by 18%. A single stool sample is sufficient for complete parasitological diagnosis when an algorithm based on clinical information is applied.

Keywords: BOOM extraction, *Cryptosporidium*, *Dientamoeba*, *Entamoeba*, *Giardia*, PhHV, real-time PCR
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Introduction

In temperate, north-west European climates, a substantial percentage of diarrhoeal complaints are caused by protozoan pathogens, such as *Entamoeba histolytica*, *Giardia lamblia*, and *Cryptosporidium parvum/Cryptosporidium hominis*. Although the pathogenicity of *Dientamoeba fragilis* is still controversial, this organism has recently gained attention as a possible cause of gastrointestinal complaints and is therefore of relevance for differential diagnostic purposes [1]. These parasites are known to show day-to-day variation in faecal shedding of trophozoites and cysts. Moreover, the detection of *D. fragilis* necessitates preservation of the faecal specimen in preservatives such as sodium acetate–acetic acid–formalin (SAF). Therefore, the Triple Faeces Test (TFT) has been introduced, enabling a higher diagnostic yield [2]. However, the procedure requires considerable effort, both from the patient, in collecting three faecal samples, and from the microbiological laboratory, which has to examine all three samples using microscopy. In addition, detection of *C. parvum/C. hominis* requires additional staining to visualize the oocysts. Cysts and trophozoites of *E. histolytica* are morphologically indistinguishable from those of the non-pathogenic *Entamoeba dispar* and *Entamoeba moshkovskii* [3]. After microscopy, culture methodology or, preferably, molecular identification is still required for differentiation of these three species [3].

PCR-based methods have proved to be both specific and sensitive for the detection of protozoan infections [4–8], and

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allow high-throughput screening. Molecular diagnostics could therefore represent an attractive alternative to TFT, removing the need for cumbersome faecal sampling by the patient, as well as replacing time-consuming microscopy in the laboratory. It also eliminates the need for SAF, one component of which, formalin, contains the toxic carcinogen formaldehyde (5% or 10%).

In this study, TFT microscopy was compared with real-time PCR for the detection of *E. histolytica*, *G. lamblia*, *C. parvum/C. hominis*, and *D. fragilis*, in individual unpreserved stool samples. To enable high efficiency and an increased diagnostic yield of gastrointestinal parasites, a diagnostic algorithm was developed using a single faecal sample per patient.

Materials and Methods

An extended version of this section is included as Supporting Information.

Faecal samples

Samples from 406 patients with gastrointestinal complaints were collected between January 2007 and April 2007, according to the TFT protocol, on three consecutive days [2]. TFT sets consisted of two SAF-fixed samples (TFT1 on day 1 and TFT3 on day 3) and one unpreserved faecal sample (TFT2 on day 2). The complete set was sent by regular mail to the Laboratory of Medical Microbiology and Infectious Diseases of the Isala Clinics in Zwolle (The Netherlands). All samples were stored at 4°C upon arrival until all diagnostic tests had been performed.

Inclusion criteria were as follows: specific request for parasitological diagnosis because of gastrointestinal complaints, adoption or, in the case of diagnostic requests without specification, persistent diarrhoea for more than 7 days.

Microscopy

Microscopic examination of the TFT sets was performed as described previously [2]. Direct wet mounts of both SAF-preserved samples (TFT1 and TFT3) were examined to detect parasites. Positive or suspected direct smears were further examined by Chlorazol Black permanent staining. Microscopy for the presence of helminth ova and cysts was performed using a Ridley concentrate of the unpreserved TFT2 sample. Modified acid-fast staining for detection of *C. parvum/C. hominis* on the formol–ether concentrate was performed on specific request, on fluidic stool samples, and for samples from subjects under 16 years of age.

When indicators of helminth infection (e.g. eosinophilia) were present, or a specific request was made to test for helminth ova, the patients were requested to provide three unpreserved samples, which were concentrated separately and examined microscopically.

DNA extraction and real-time PCR

For real-time PCR, only unpreserved faeces samples (TFT2) were examined. Approximately 200 mg of unpreserved faeces was used for pretreatment (lysis and storage at −20°C) and DNA extraction. Prior to automatic DNA extraction, phocid herpes virus (PhHV) (laboratory strain, provided by the Erasmus MC Rotterdam, The Netherlands) was added as an internal control. DNA was stored at −20°C.

Real-time PCR: paraPCR

Detection of the four protozoa was performed in two reactions per DNA sample. *E. histolytica*, *G. lamblia* and *C. hominis/C. parvum* multiplex real-time PCR, including PhHV-1, was performed as described previously [4], with some modifications to the *C. hominis/C. parvum* oligonucleotides. *D. fragilis* DNA amplification was performed in a separate assay, also including PhHV-1 [6]. Primer and probe sequences and their optimized concentrations are listed in Table 1. The combination of multiplex and duplex reactions is hereafter referred to as 'paraPCR'.

Analytical performance and analysis of real-time PCR

The analytical sensitivity and specificity of the PCRs used have been validated by the Leiden University Medical Centre (The Netherlands) [4,6,9], and confirmed after the adjustments to the protocol. Standardized adjustments to the analysis parameters in the Applied Biosystems 7500 software were applied. Inhibition of the PCR reactions was measured by the cycle threshold (Ct)-value of the PhHV amplification. Negative extraction and positive DNA controls for each pathogen were included in all PCR runs.

Statistical analysis

The average Ct-values of PCR were compared between microscopy groups by the Mann–Whitney test, using SPSS 15.0, at a significance level of $p < 0.05$ (two-tailed).

Results

A total of 406 TFT sets were collected for parasitological examination. Of these, nine were excluded because of incomplete TFT sets or otherwise incomplete data, leaving 397 TFT sets for comparison.

Table 2 summarizes the comparison between the results of microscopy of the complete TFT set and the results of the paraPCR performed on the unpreserved (TFT2) faeces

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TABLE 1. Primers and probes used in real-time PCR assays

Target organism	Oligonucleotide	Sequence (5′–3′) and labels	Amount (pmol) added to 30 μL of reaction mix	Reference
Giardia lamblia	Sense primer	GAC GGC TCA GGA CAA CGG TT	3.7	[4]
	Antisense primer	TTG CCA GCG GTG TCC G	3.7	
	Probe	FAM-CCC GCG GCG GTC CCT GCT AG-BHQ	3.0	
Cryptosporidium parvum/ Cryptosporidium hominis	Sense primer	CTT TTT ACC AAT CAC AGA ATC ATC AGA	15.0	This publication
	Antisense primer	TGT GTT TGC CAA TGC ATA TGA A	15.0	
	Probe	NED-TCG ACT GGT ATC CCT ATA A-MGB	3.0	
Entamoeba histolytica	Sense primer	ATT GTC GTG GCA TCC TAA CTC A	3.7	[4]
	Antisense primer	GCG GAC GGC TCA TTA TAA CA	3.7	
	Probe	VIC-TCA TTG AAT GAA TTG GCC ATT T-MGB	1.5	
Dientamoeba fragilis	Sense primer	CAA CGG ATG TCT TGG CTC TTT A	4.5	[6]
	Antisense primer	TGC ATT CAA AGA TCG AAC TTA TCA C	4.5	
	Probe	FAM-CAA TTC TAG CCG CTT AT-MGB-BHQ	3.0	
PhHV	Sense primer	GGG CGA ATC ACA GAT TGA ATC	4.5	[9]
	Antisense primer	GCG GTT CCA AAC GTA CCA A	4.5	
	probe	Cy5-TTT TTA TGT GTC CGC CAC CAT CTG GAT C-BHQ	3.0	

PhHV, phocid herpes virus; BHQ, Black Hole Quencher; MGB, Minor Groove Binder. Fluorophores: Cy5, FAM, NED, and VIC.

TABLE 2. Results of microscopy of complete Triple Faeces Test sets and paraPCR for individual unpreserved stool samples (*n* = 397)

Target	Microscopy+/ PCR+	Microscopy+/ PCR−	Microscopy−/ PCR+	Microscopy−/ PCR−
Entamoeba histolytica	1	–	–	396
Giardia lamblia	28	1[a]	16	352
Cryptosporidium parvum/hominis	2	–	1	394
Dientamoeba fragilis	64	5	58	270

Microscopic re-examinations of discrepant samples are not included. Results of repeated paraPCR of initially inhibited samples are included. The protozoa in mixed infections are presented as separate detections.
[a]One PCR inhibited sample was positive for *G. lamblia* by microscopy and is included in the Microscopy+/PCR− results for *G. lamblia*.

sample. In total, 158 (40%) of 397 sets revealed one or two protozoa. *G. lamblia* was detected in 45 (11.3%) TFT sets and *D. fragilis* in 127 (32.0%) TFT sets. Mixed infections with *G. lamblia* and *D. fragilis* were detected in 18 patients by paraPCR; five of these were also detected with microscopy. In one case, *E. histolytica/E. dispar* was detected by microscopy, and *E. histolytica*-specific DNA was detected by real-time PCR. The *E. histolytica*-positive and the *C. parvum/ C. hominis*-positive TFT sets did not reveal other pathogens. Furthermore, microscopy revealed hookworm eggs in one sample, and the non-pathogenic protozoa *Blastocystis hominis*, *Endolimax nana* and *Entamoeba coli* were detected in 111, ten and eight cases, respectively.

In 78 TFT sets, discrepancies were found between microscopy and paraPCR results. SAF-preserved faeces samples were available for microscopic re-examination in 60 of 73 paraPCR-positive/microscopy-negative TFT sets. *G. lamblia* could be confirmed in eight of ten TFT sets and *D. fragilis* could be confirmed in 30 of 50 TFT sets after careful re-examination of several additional slides. Of five TFT sets initially found to be positive for *D. fragilis* by microscopy but negative by paraPCR, on re-examination by microscopy, three sets were found to be positive, whereas *D. fragilis* could not be confirmed in either TFT1 or TFT3 SAF-preserved samples of the two remaining sets. The paraPCR was also repeated for these five sets, and yielded one positive sample for *D. fragilis*. Hence, two microscopy-positive/paraPCR-negative samples remained discrepant.

Ct-values were significantly higher for DNA samples from TFT sets in which *D. fragilis* was not found by microscopy (*n* = 58, median Ct 31.0, range 21.3–40.1) than for those from sets in which *D. fragilis* was detected by microscopy (*n* = 64, median Ct 25.8, range 18.4–37.3) (Mann–Whitney test; $p < 0.0001$). A statistical difference was also found between the Ct-values of the sets in which *D. fragilis* trophozoites were detected in both SAF samples (*n* = 36, median Ct 23.5, range 18.4–35.0) and those of the sets in which trophozoites were detected in only one of the two SAF samples (*n* = 28, median Ct 26.3, range 19.4–37.3) (Mann–Whitney test; p 0.037).

The Ct-values of the *G. lamblia*-specific PCR showed a similar distribution to the Ct-values of the *D. fragilis* PCR: Ct-values were higher in DNA samples from TFT sets in which *G. lamblia* was not found by microscopy (*n* = 16, median Ct 35.4, range 29.6–42.8) than in those from sets in which *G. lamblia* was detected by microscopy (*n* = 28, median Ct 26.0, range 19.1–37.8) (Mann–Whitney test; $p < 0.0001$).

Inhibition was observed in the paraPCR in 20 stool samples. Twelve samples were inhibited completely (no PhHV signal or a Ct value >40), and eight samples were partially inhibited (only one of two PhHV signals per sample yielded a Ct value >40). A small pilot study was performed

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to investigate the influence that different starting points in the protocol had on inhibition. Only a freeze–thaw step on the inhibited DNA extract, a new DNA extraction from pretreated faeces and a new DNA extraction from the original stool sample were compared (*n* = 20). The results of this pilot study showed that inhibiting factors were already present in the pretreated sample, and only repeating the extraction protocol from the original stool sample was successful in diminishing inhibition. Eighteen of the 20 samples showed no inhibition, whereas two samples showed inhibition again when DNA was re-extracted and real-time PCR was repeated (one sample was positive for *G. lamblia* by microscopy, and one sample was negative by microscopy).

Analysis of the microscopy results of TFT sets (*n* = 2887) over a 10-month period (May 2006 to March 2007) revealed nine positive results for non-protozoan parasitic infections: *Enterobius vermicularis* (*n* = 2), *Ascaris lumbricoides*, *Trichuris trichiura*, *Strongyloides stercoralis*, *Taenia* sp. (ova), *Taenia saginata* (proglottid), *Hymenolepis nana*, and a hookworm. Four diagnoses resulted from specific requests for a single pathogen: *S. stercoralis* (*n* = 1), *Taenia* sp. (*n* = 2), and *Enterobius vermicularis* (*n* = 1). In three cases, a link with specific risk factors could be made: travelling to risk areas or showing eosinophilia. One case was not related to specific risk factors (*H. nana*) and one case appeared to be a coincidental diagnosis without any relation to the complaints (*Enterobius vermicularis*).

Discussion

The comparison of microscopy with paraPCR for the detection of gastrointestinal protozoa yielded 152 (38.3%) positive faecal samples by paraPCR vs. 96 (24.2%) positive TFT sets detected by microscopy, for one or more pathogenic protozoa. When the faecal samples that were either positive by microscopy, positive by paraPCR or positive by both were defined as 'true positives', the sensitivity of paraPCR was clearly superior to that of microscopy (96% vs. 54% for *D. fragilis* and 98% vs. 64% for *G. lamblia*, respectively). Moreover, the Ct-values of *D. fragilis*-specific and *G. lamblia*-specific signals, reflecting the amount of parasite-derived DNA in the samples, correlated significantly with the microscopy results. This is fully in accordance with earlier comparisons of microscopy and the more sensitive molecular approaches [8–10]. In our study, one patient was found to be positive for *E. histolytica*, which causes a potentially life-threatening infection. Although *E. histolytica*/*E. dispar* was detected by microscopy, *E. histolytica* had to be confirmed by molecular examination. An interesting observation in the present study was the low prevalence of *C. parvum*/*C. hominis* when compared with previous findings in The Netherlands [10]. This may be explained by the different time of year in which the samples were collected, as it is known that the incidence of *C. parvum*/*C. hominis* infections is highly seasonal.

The number of samples in which PCR was inhibited (*n* = 20; 5%) might be considered to be high. As earlier studies have shown [4,11], further optimization of the DNA extraction protocol should be possible to increase the yield of uninhibited extracts to nearly 100% of samples. However, by repeating the pretreatment and DNA extraction protocols for the inhibited samples, a simple practice that is suitable for high throughput, we managed to decrease the number of inhibited samples to a mere 0.5%. The superior sensitivity of the paraPCR allows a diagnosis using an individual faecal sample without the need for preservative. Microscopy requires SAF preservative for the detection of *D. fragilis*, whereas PCR detection of *D. fragilis* DNA is as sensitive with unpreserved faeces, after several weeks of storage, as with a fresh sample [6]. Therefore, with the application of the more sensitive paraPCR, only a single stool sample, without preservation fluid, is sufficient for the diagnosis of gastrointestinal protozoa.

Although studies have been published on the clinical relevance of *D. fragilis* [12–17], the pathogenic properties of *D. fragilis* have not yet been completely elucidated. The *D. fragilis* prevalence found in the present study with microscopy alone (17.4%) is higher than reported elsewhere, ranging from <1% [15,18] to 16.8% [19]. Positive microscopy results were confirmed by PCR in 64 of 69 samples (92.7%). PCR has shown a specificity approaching 100% [4,19]. Comparison of our results with those of previous publications remains difficult, because of differences in almost every aspect, including definitions of signs and symptoms, clinical protocols for diagnosis, and laboratory methods.

To date, no genetic differences between *D. fragilis* isolates from clinical cases and isolates from controls have been described. A future case–control study would be necessary to prove the clinical significance of *D. fragilis* in this patient population, together with a genotyping study to establish type distribution in this geographical area.

Molecular methods will detect the specified target organisms only. A disadvantage of the paraPCR approach, therefore, is that other organisms, for which microscopy is essential for diagnosis, e.g. helminths, escape detection. Microscopy will thus remain necessary for the diagnosis of other pathogenic parasites that are not included in the PCR assay used. Unfortunately, detection of these additional pathogens cannot always be predicted from specific signs and symptoms or other information conveyed by the clinician requesting the microbiological investigation. Nevertheless, microscopy is necessary to minimize the chance of missing

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other parasitic pathogens, even when the prevalence of non-protozoal parasites is low, which may be the case in particular diagnostic settings or patient populations. For example, in our laboratory, only nine helminth infections have been diagnosed in 2887 patients over a 10-month period. We have therefore designed an algorithm that includes microscopy in the parasitological examination for helminths or protozoa such as *Isospora belli* or *Cyclospora cayetanensis* only when necessary. Criteria for the use of microscopy to detect these parasitic pathogens are: an explicit request for specific parasitological investigation, eosinophilia, increased IgE level (>120 U/mL), urticaria, a history of recent travel to the (sub)tropics, adoption, an impaired immune system, or, finally, persistent or recurrent complaints after a previous analysis by paraPCR, within a period of 2 months. If microscopy for the detection of helminths had been performed according to one or more of these criteria, seven of the nine cases would have been correctly identified. The only two cases missed through the lack of an initial diagnostic request, the two remaining helminth cases, would have been detected in the repeat sample.

In our laboratory, using microscopy, a single technician is able to process TFT samples from 15 patients per day (8 h of hands-on time), as opposed to processing a maximum of about 90 patient samples by paraPCR in same time period. Routine application of the proposed algorithm for parasitological diagnostics in our laboratory has reduced the number of microscopic examinations by about 90%. The remaining 10% of the examinations were triggered by one or more criteria for the performance of microscopy. Because a single sample is now sufficient for adequate diagnosis, including microscopy, a significant reduction in the need for scarce technician time has been accomplished per examination.

In conclusion, implementation of paraPCR for the diagnosis of parasitic gastrointestinal infections yields the benefits of less hands-on time, higher sensitivity, direct differentiation of *E. histolytica* from other *Entamoeba*, and a shorter turnaround time, and requires only one unpreserved stool sample.

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Transparency Declaration

All authors declare no conflict of interest.

Supporting Information

Additional Supporting Information (extended version of Materials and Methods) may be found in the online version of this article.

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