A critical assessment of two real-time polymerase chain reaction assays targeting the (SSU) rRNA and gdh genes for the molecular identification of *Giardia intestinalis* in a clinical laboratory

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Abstract:

Introduction Giardiasis is an intestinal diarrhoeal illness caused by the flagellate protozoan parasite *Giardia intestinalis*. Molecular techniques for the identification of *Giardia intestinalis* have generally been shown to offer a better detection rate of the parasite than the traditional faecal concentration and microscopy techniques.

Aim The aim of this study was to critically assess the performance of a commercial and a published real-time PCR assay for their potential use as frontline tests for the diagnosis of giardiasis.

Methods A composite reference standard of enzyme immunoassay and rapid membrane test was used in a diagnostic accuracy study to assess the performance of Primerdesign Ltd. and Verweij et al. (2004) *Giardia intestinalis* real-time PCR assays comparing them with the traditional ova, cysts, and parasite microscopy test (OCP-M).

Results The Verweij real-time PCR used primers for the (SSU) rRNA gene and produced a diagnostic sensitivity of 93.4 % (95 % CI: 88.30 to 98.50 %) and an efficiency of 100 %. The Primerdesign Ltd. real-time PCR used primers for the glutamate dehydrogenase gene and produced a diagnostic sensitivity of 61.5 % (95 % CI: 51.50 to 71.50 %) and an efficiency of 203 %. The OCP-M sensitivity was 83.5 % (95 % CI: 75.87 to 91.13 %).

Conclusions The Verweij real-time PCR was robust and the most sensitive assay suited for use as a first line diagnostic test for giardiasis.

1. INTRODUCTION

Giardiasis is an intestinal diarrhoeal illness caused by the flagellate protozoan parasite *Giardia intestinalis* (synonymous with *Giardia lamblia* and *Giardia duodenalis*). Traditionally, faecal concentration technique described by Allen and Ridley [1-2] has been used to diagnose Giardiasis. Faecal concentration and microscopy techniques are cumbersome and rely heavily on the expertise of the person reading the slides. However, it is considered to be the gold standard for the diagnosis of giardiasis, even though it has relatively low sensitivity. Sensitivity has been reported to increase from 73 % to 85 % when multiple samples taken on different days were examined.[3] Molecular-based assays for the detection of *Giardia intestinalis* have been shown to be more sensitive than conventional methods. [4-8] However, their amplification efficiency (E) and correlation coefficient (linearity, R^2) have not been critically assessed for use in routine diagnosis. In this study, the Verweij et al. (2004) real-time PCR assay which targets the (SSU) rRNA gene,[8] and the Primerdesign Ltd. real-time PCR which targets the glutamate dehydrogenase gene (gdh) of *Giardia* assemblages A and B only (the subtypes known to infect humans), were compared with the traditional ova, cysts, and parasite microscopy (OCP-M) in a diagnostic accuracy study. The performances of the two PCR assays were also assessed for E and R^2.

2. MATERIALS AND METHODS

2.1 Samples

Stool samples (n = 213), from patients with suspected gastrointestinal infection, submitted for testing were archived from 30th March 2010 to 22nd July 2011 after performing the traditional faecal concentration technique (Parasep, DiaSys Europe Ltd.) on them. Before archiving, the faecal concentrates were examined microscopically using the x10 objective,

followed by the x40 objective with the application of a drop of iodine to aid in the identification of internal structures of cysts. When unformed or liquid stools were encountered, direct wet preparations and methanol fixed, rapid Field's (TCS Biosciences) stained faecal smears were also prepared to supplement the spun deposit to look for trophozoites of *Giardia intestinalis*. The samples comprised 98 *Giardia intestinalis* microscopy positive samples and 115 microscopy negative samples. Each sample was split and stored at 4-6 °C and at -20 °C to preserve the integrity of cysts for subsequent re-examination and the stability of parasite DNA and antigens for molecular studies. Forty three samples were excluded as they dried up in storage before testing began and repeated samples from the same patient were also discounted. One-hundred and seventy samples remained after applying the exclusion criteria.

2.2 Conventional methods

The samples were divided into true positive (TP) and true negative (TN) cases using a composite reference standard (CRS) of enzyme immunoassay (EIA), Techlab *Giardia* II, and a single one-step immunochromatographic membrane assay, Coris *Giardia* strip rapid membrane test (RMT). The manufacturers' recommended procedures were followed to test these samples blindly. The combined results of the EIA and the RMT defined four criteria for the determination of TP and TN cases (Table 1).

Table 1: Classification of true positive and negative cases using EIA and RMT

Sample Group	Reference Tests		Composite Ref. Standard	No. of samples
	Enzyme Immunoassay(EIA)	Rapid Membrane Test(RMT)		
1	P or WP	N	TP	10
2	N	P or WP	TP	2
3	P or WP	P or WP	TP	79
4	N	N	TN	79
Total				170

P = Positive, WP = Weak positive, N = Negative, TP = True positive, TN = True negative.

2.3 Molecular methods

2.3.1 Extraction of DNA

DNA was extracted from 0.5 g of stool (500 µl when liquid) using a modified QIAamp DNA Mini kit protocol for tissue extraction. The tissue DNA extraction kit was chosen instead of the QIAamp DNA stool mini kit because, under these conditions, it yields higher quantities of parasite DNA and is more amenable to high throughput sample processing.[9] Prior to extraction, each sample was supplemented with a standardized quantity of *Escherichia coli* transformed with a green fluorescent protein (GFP) gene to serve as the extraction and internal control[10] together with the extraction control provided by Primerdesign Ltd. in the *Giardia intestinalis* PCR kit.

2.3.2 Real-time PCR assay

The Verweij et al. (2004) real-time PCR primers and probe set consisted of forward primer *Giardia*-80F and reverse primer *Giardia*-127R, and the *Giardia intestinalis*-specific double-labelled probe *Giardia*-105T (Biolegio, Malden, The Netherlands) (Table 2).

The primers were used at a final concentration of 400 nM each for the forward and reverse primers and 120 nM for the probe in 25 µl reactions containing 5 µl templates DNA. Amplification was performed in a Rotor-Gene Q 6000 (Corbett Life Sciences) using TaqMan(R) Environmental Master Mix 2.0 (Applied Biosystems). The cycling conditions were: 95 ^{o}C (10 min), 95 ^{o}C (15 s), 60 ^{o}C (60 s) for 45 cycles.

Table 2: Oligonucleotide primers and probes for real-time and conventional PCR assays for the detection of *Giardia intestinalis*

Target organism: Name of primers and probes	Oligonucleotide sequence 5' – 3'
Real-time PCR	
G. intestinalis	
Giardia-80F	5'-GACGGCTCAGGACAACGGTT-3'
Giardia-127R	5'-TTGCCAGCGGTGTCCG-3'
Giardia-105T	5'CY5-CCCGCGGCGGTCCCTGCTAG-3'BHQ2
Conventional PCR	
RH 11	5'-CATCCGGTCGATCCTGCC-3'
RH 4,	5'-AGTCGAACCCTGATTCTCCGCCAGG-3'
GiarF:	5'-GAC GCT CTC CCC AAG GAC-3'
GiarR:	5'-CTG CGT CAC GCT GCT CG-3'

A cycle threshold (CT) was assigned to a sample for each channel when its normalised fluorescence exceeded 0.1 units. Samples with a CT of ≤ 40 were deemed positive for *G. intestinalis* DNA. Samples with a CT of greater than 40 or with no CT were called negative only when their GFP CT values were below the run average for GFP CT values plus 1.23.

This is the value determined as the optimal cut off for a 5 % rejection rate through the analysis of multiple runs within the laboratory (data not shown). For negative samples whose GFP CT exceeded this value, the sample was repeated with a 1 in 10 dilution according to internal validation criteria to examine the effect of PCR inhibitors. Any diluted sample whose CT was greater than the run mean plus 5 CT was considered a technical failure, requiring re-extraction and re-amplification. The Primerdesign Ltd. assay did not have such a stringent criteria for determining positivity. According to the manufacturer's instructions (Genesig Advanced kit handbook, HB10.03.03), CT values of 31±3 are within the normal range for the internal extraction control, and any outside this range were treated as run failures and re-extracted and re-amplified.

2.3.3 Conventional single round PCR

The real-time PCR primer pairs, *Giardia*-80F and *Giardia*-127R, were used but without the probe in a conventional PCR amplification and analysis by gel-electrophoresis for the 62 bp amplicons of *Giardia intestinalis* to investigate apparent false positive samples. A G-Storm Thermocycler (Kapa Biosystems Model GS00001) was used with the following protocol: heated lid 110 ^{o}C; hot start 1 cycle for 95 min and 15 min; 95 ^{o}C (45 s) and 60 ^{o}C (90 s) repeated for a total of 45 cycles; 72 ^{o}C for 1 cycle followed by a holding temperature of 10 ^{o}C. The amplification protocol was repeated with the same set of primers as a two step reaction to maximize the yield of 62 bp amplicons for potential sequencing.

2.3.4 Conventional nested PCR

Conventional nested PCR was run to investigate further apparent false positive results. The nested PCR used two different sets of primers to amplify a 130bp fragment of the *Giardia intestinalis* (SSU) rRNA gene for visualization using gel electrophoresis [11]. In the nest 1 reaction, the RH11/RH4 primers (Table 2) amplified a 292-bp region of the 5' end of the (SSU) rRNA gene. The PCR amplification was performed in 25 µl volumes with the final

mix containing 5-50 ng DNA as per published method [12] using Biomix red (Bioline product). The amplification process consisted of 1 cycle at 95 ^{o}C (2 min); 94 ^{o}C (20 s), 59 ^{o}C (20 s), and 72 ^{o}C (30 s) repeated for a total of 40 cycles; 72 ^{o}C (7 min) for 1 cycle. The nest 2 primers which amplified 130 bp fragment were: GiarF and GiarR[13] (Table 2). A Thermo Electron Px2 thermal cycler was used with the following amplification protocol: 1 cycle at 95 ^{o}C (2 min); 94 ^{o}C (20 s), 59 ^{o}C (20 s), and 72 ^{o}C (30 s) repeated for a total of 45 cycles; 72 ^{o}C (7 min) for 1 cycle as per published protocol using Biomix red [12-13].

2.4 Analytical sensitivity and specificity

The analytical potential of all the tests (both index and reference) deployed in this study were verified by the estimation of their limit of detection prior to the estimation of their diagnostic accuracy measures. The limit of detection (LOD) of the five assays were determined using DNA extracted from a serially diluted 5-fold dilutions of a *Giardia*-positive stool sample to provide the range of estimated cysts concentration of 71,000 cysts/ml to 4.6 cysts/ml of stool (Table 3). The diluent was a *Giardia*-negative stool liquefied with phosphate buffered saline (PBS) pH 7.2. The real-time assays were tested in triplicate.

Analytical specificity was ascertained by using a pooled *Giardia*-negative stool with various types of parasitic, bacterial, and fungal organisms including *Entamoeba coli*, *Endolimax nana*, *Entamoeba histolytica/dispar*, Yeasts, *Escherichia coli*, *Klebsiella* sp., *Citrobacter* sp., *Proteus* sp. and *Enterococcus faecalis*, *Cyclospora cayetanensis* and *Cryptosporidium* sp. Bacterial pathogens were not available but had already been tested in a previous study[7].

2.5 Real-time PCR performance evaluation

A set of 10-fold serial dilutions of a *Giardia intestinalis* DNA template solutions prepared from the 71,000 cysts/ml solution in Table 3 were tested in triplicate. The E and R^2 from the regression lines were compared with the equivalent values generated from the 5-fold serial

dilutions used for the LOD testing. The calculation of E was based the formula E = 10(-1/slope) – 1 for standard curve generated by a R^2 of the plotted points.[14]

2.6 Diagnostic accuracy

A composite reference standard (CRS) of RMT and EIA was used in a diagnostic study of the three index tests (OCP-M, Verweij real-time PCR, and Primerdesign Ltd. *Giardia* real-time PCR) as recommended by the Health Technology Assessment for diagnostic accuracy studies when there is no gold standard.[15] McNemar statistics was used in a pair wise comparison to establish the significance of any differences in the performance of the diagnostic tests before diagnostic accuracy measures were calculated using cross tabulation statistics (2x2-table): sensitivity = (number of true positives)/(number of true positives + number of false negatives) percent, and specificity = (number of true negatives)/(number of true negatives + number of false positives) percent.[16] Positive likelihood ratio (LR+) was calculated as (sensitivity)/(1 - specificity), and negative likelihood ratio (LR-) was calculated as (1 - sensitivity)/(specificity).[17]

3. RESULTS

3.1 Analytical sensitivity and specificity of diagnostic tests

The 5-fold serial dilutions of *Giardia intestinalis* positive stool were tested in triplicate for all five assays. A positive result was only recorded for each assay with a given dilution when all three of the repeats gave a positive result (Table 3). Since all the dilutions gave a positive result with the Verweij assay, the LOD for this assay was estimated to be 4.6 cysts/ ml or lower. By comparison, the LOD for the Primerdesign Ltd. PCR was 113.6 cysts/ml. Indeed, apart from an isolated positive result (CT 38.9) in one of the triplicate run in 22.7 cysts/ml

tube, the Primerdesign Ltd. assay did not detect any positivity in any replicates involving less than 113.6 cysts/ ml.

Table 3: Determination of limit of detection (LOD) using *Giardia intestinalis* positive stool sample containing 71,000 cysts/ ml. The cysts were counted using C-Chip counting chamber and diluted 1 in 5 down to 4.6 cysts/ ml.

Test	Analytical sensitivity (cysts/ml of stool)							Analytical specificity (pooled *Giardia*-negative stool)
	71,000	14,200	2,840	568	113.6	22.7	4.6	
RMT	+	-	-	nt	nt	nt	nt	-
OCP-M	+	+	-	-	-	-	-	-
EIA	+	+	+	-	-	-	-	-
Primerdesign Ltd.	+	+	+	+	+	-	-	-
Verweij real-time	+	+	+	+	+	+	+	-

Keys: nt = not tested; + = positive; - = negative; RMT = Rapid Membrane Test; OCP-M = Ova, Cysts, and Parasite Microscopy; EIA = Enzyme Immunoassay.

The RMT and the EIA formed the constituent tests for the CRS with a combined LOD of ≤ 2,840 cysts/ml (Table 3). Analytical specificity was 100 % for each of the diagnostic tests evaluated as these all gave a negative result with the pooled *Giardia*-negative stool (Table 3).

3.2 Diagnostic accuracy measures

Using the four criteria listed in Table 1, the composite reference standard divided the 170 samples into 91 true positive and 79 true negative cases and this enabled diagnostic accuracy measures to be estimated for the OCP-M and the two real-time PCR assays (Table 4).

Table 4: Delineation of true positive and true negative cases

Index test	Composite reference standard of EIA and RMT (TP: 91, TN: 79)				
	TP (TPF)	FN (FNF)	TN (TNF)	FP (FPF)	TP +FN+ TN+FP
OCP-M	76 (83.5%)	15 (16.5%)	76 (96.2%)	3 (3.8%)	170
Primerdesign	56 (61.5%)	35 (38.5%)	78 (98.7%)	1 (1.3%)	170
Verweij	85 (93.4%)	6 (6.6%)	59 (74.7%)	20 (25.3%)	170

TP = true positive; TN = true negative; TPF = true positive fraction; TNF = true negative fraction, FPF = false positive fraction; FNF = false negative fraction.

McNemar test results produced p-values < 0.05 for each paired test (i.e OCP-M vs. Primerdesign; OCP-M vs Verweij; and Primerdesign vs Verweij) indicating significant differences in performance which warranted a further investigation with cross tabulation statistics (2x2 table) to calculate the diagnostic accuracy measures shown in Table 5. The Verweij assay was diagnostically more sensitivity [93.4 % (95 % CI: 88.30 to 98.50 %)] than the Primerdesign Ltd. assay [61.5 % (95 % CI: 51.50 to 71.50 %)] (Table 5).

Table 5: Comparative diagnostic performance results of real-time PCR assays and the traditional OCP-M method for the laboratory detection of *Giardia intestinalis*.

Index test	Sensitivity (SN)		Specificity (SP)		Likelihood ratios (LR)			
	SN	95 % CI	SP	95 % CI	LR+	95 % CI	LR-	95 % CI
OCP-M	83.5	75.87 to 91.13	96.2	91.98 to 100.00*	22.0	13.49 to 30.51	0.17	-0.74 to 1.08
Primerdesign	61.5	51.50 to 71.50	98.7	96.20 to 100.00*	47.3	37.04 to 57.56	0.39	-0.98 to 1.76
Verweij	93.4	88.30 to 98.50	74.7	65.11 to 84.29	3.7	-0.18 to 7.58	0.09	-0.57 to 0.75

LR+ = positive likelihood ratio; LR- = negative likelihood ratio; 95 % CI = 95 % confidence interval. * These values have been curtailed to 100 % since a specificity of greater than this value is biologically meaningless.

The sensitivity of the Verweij assay was affected by 20 samples recorded as false positive cases. When these samples were investigated with gel-electrophoresis using the same primers as the real-time PCR, 14 samples gave an amplicon corresponding to the expected 62 bp product of this reaction, providing strong evidence for their consideration as TP cases (Figure 1).

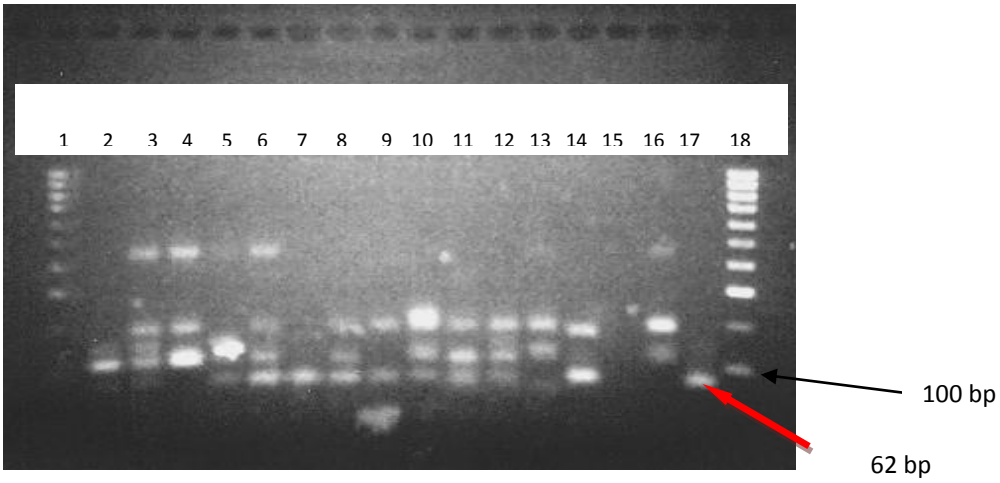

Figure 1: Conventional gel-electrophoresis analysis of Verweij real-time PCR. The figure shows a representative number of the 20 apparent false positive samples. Two % agarose was stained with Safeview and showed the results of four of the discrepant samples. Lanes 1 and 18 are 100 bp ladder (Gentaur); lanes 2, 3, and 5 showed diagnostic bands for 62 bp for Giardia intestinalis representing samples 103, 12, and 104 respectively; lane 4 is sample no. 44 and it was negative for 62 bp Giardia intestinalis amplicon. Lanes 14 and 17 were positive controls; lanes 15 and 16 were negative controls. (NB. lanes 6-13 were known Giardia intestinalis positive samples run alongside the discrepant samples on this gel). Sixty two bp bands were also seen on other gel (image not provided).

The absence of such a fragment would have almost certainly indicated a false positive case. In the real-time PCR, an additional level of specificity comes from the ability of the probe to specifically bind to the intended 62 bp fragment; hence it is unlikely that any of the larger sized bands are responsible for the positive CT in these samples. Indeed, one of the 14 samples was independently confirmed as *Giardia intestinalis* with 130 bp fragments when conventional nested PCR was performed. When these 14 samples were included in the CRS

as TP samples and used in a restricted comparison between the OCP-M and the Verweij real-time PCR, the re-calculated sensitivity of 94.3 % (95 % CI: 89.87 to 98.73) for the Verweij real-time (Table 6) resulted in an increase of 19.3 % (94.3 % - 75 %) in the detection of positive cases. More importantly the specificity was increased to 90.8 % (83.77 – 97.83).

Table 6: Adjusted diagnostic accuracy figures for OCP-M and Verweij real-time PCR resulting from the addition of the 14 confirmed TP cases to the CRS. Composite reference standard of EIA and RMT indicated TP: 91, TN: 79.

Adjusted Diagnostic accuracy	Diagnostic test			
	OCP-M		Verweij real-time PCR	
	%	95 % CI	%	95 % CI
Sensitivity	75	66.72 to 83.28	94.3	89.87 to 98.73
Specificity	98.5	95.55 to 100.00*	90.8	83.77 to 97.83
LR+	50.0	40.44 to 59.56	10.3	4.49 to 16.11
LR-	0.25	-0.96 to 1.46	0.06	-0.54 to 0.66

LR+ = Positive likelihood ratio; LR- = Negative likelihood ratio; OCP-M = Ova, cysts, and parasite microscopy. * These values have been curtailed to 100% since a specificity of greater than this value is biologically meaningless.

3.3 Real-time PCR assay performance

The E and R^2 for the 1 in 5 serially diluted stool samples were E = 100 % (the slope was -3.326), R^2 = 0.99 for the Verweij real-time PCR and E = 203 % (the slope was -2.076) and R^2 = 0.93 for the Primerdesign Ltd. real-time PCR. A 10-fold serial dilutions of *G. intestinalis* DNA produced an efficiency of 100 % (the slope was -3.315) and R^2 = 0.95 with the Primerdesign Ltd. real-time PCR and 96.3 % (the slope was -3.414) and R^2 = 0.99 for the Verweij real-time PCR, respectively. Generally efficiency between 90 % and 110 % is considered acceptable and an R^2 value > 0.99 provides good confidence in correlating two values (Life Technologies, Real-time PCR: Understanding C_t, 2011). None of the 170 Verweij real-time PCR reactions had an internal control CT value greater than the run mean

CT plus 1.23. Using our rejection criteria, this means than none of the samples were considered to show levels of inhibition that would identify the sample as a run failure. The Primerdesign Ltd. real-time PCR however showed 48 out of the 170 samples (28.2 %) to have CTs higher than manufacturer's quoted range of 31±3.

4. DISCUSSION

The aim of this study was to use a composite reference standard (CRS) to critically assess the diagnostic accuracy of both OCP-M and real-time PCR methodology for use as a frontline test for the laboratory diagnosis of giardiasis. The failure of the OCP-M to detect *Giardia* in at least 16.5 % *Giardia intestinalis* positive stool samples (as determined by the Composite Reference Standard - CRS) may explain why symptoms highly indicative of chronic giardiasis (include diarrhoea and malabsorption) persist in some patients despite repeatedly negative stool microscopy results. Indeed, many of such patients when treated empirically for giardiasis using tinidazole show clinical resolution of their symptoms (data not shown), strongly supporting the clinical need for implementation of new diagnostic approaches with increased sensitivity.

The Verweij assay has proven to be both sensitive and robust when applied to clinical stool samples submitted for routine diagnosis of giardiasis. The initial low specificity of the assay (74.7 %) compared to OCP-M (96.2 %) could be a potential draw back with the assay, leading to unnecessary treatment of patients for giardiasis. However, there is good evidence to suggest that 70 % of the Verweij real-time PCR apparent false positive cases (14/20) were true positives because of the presence of appropriately sized amplicons (62 bp) (Figure 1). These amplicons did not yield enough DNA for sequencing when they were excised and

extracted from the conventional electrophoresis gel, thus limiting our investigations to visual interpretation of gel images. An increased sensitivity with the Verweij assay is also supported by the dramatic differences in the LOD for this assay (< 5 cysts/ml), OCP-M (14,200 cysts/ml) and CRS (≤ 2,840 cysts/ml). Re-evaluating these 14 samples as true positives gives a specificity of 90.8 %. The effects of the sensitivity and specificity can be seen by examining the likelihood ratios[18], calculated in diagnostic accuracy studies to determine the presence or absence of an abnormality[17]. The lower specificity of the Verweij assay compared to OCP-M resulted in a decrease in LR+ of 50.0 to 10.3, meaning patients diagnosed as being positive are less likely to have active giardiasis. The improvement in sensitivity resulted in a reduction of LR- from 0.25 (achieved with OCP-M) to 0.06 (Table 6). Thus patients who are negative with this assay can be classified as true negatives with a far higher certainty than can be achieved with conventional diagnostic approaches. Tinidazole is a well-tolerated drug with few side effects. Using a regret theory approach to decision curve analysis, implementation of a diagnostic test with a very high level of sensitivity but lower level of specificity may therefore be associated with a far lower level of regret compared to existing diagnostics with higher specificity but lower sensitivity. The inclusion of these 14 likely additional true positive cases in the CRS would result in a 19.3 % increase in the detection of positive cases compared to OCP-M (Tables 6). There was only one apparent amplification failure where cysts were seen on microscopy but the Verweij real-time PCR failed to give a positive result even though the GFP extraction control was positive. The problem was most likely a sampling issue caused by the uneven distribution of the *Giardia intestinalis* parasite in the stool sample. The issue was resolved when the stool (study case no. 142) was re-extracted and the PCR repeated. The intermittent excretion and uneven distribution of the parasite in faecal samples are confounding problems with stool microscopy, which a very sensitive test can only partially mitigate it would seem.

The Verweij real-time PCR did not appear to be affected by PCR inhibitors because all CTs for the GFP internal control were within the acceptance criteria of the mean CT plus 1.23. The resilience of the Verweij assay to PCR inhibition is also supported by the tight concordance of the efficiencies generated from DNA extracted from the stool dilution series and a dilution of purified *G. intestinalis* DNA in molecular grade water. With both of these standards, efficiencies remained in the acceptable range of 90-110 %. The Primerdesign PCR did not appear to fare so well with PCR inhibition: About 28 % of the internal control CTs for the Primerdesign PCR were greater than 34, the higher end of the normal range given by the manufacturer. This may well explain the high discrepancy between the efficiency of amplification achieved with the two sets of *G. intestinalis* DNA standards. In addition our data support a lower sensitivity for assays targeting the gdh gene compared to those targeting the (SSU) rRNA [19-20], suggesting an optimal downstream deployment for the Primerdesign PCR reserved for the molecular characterization of *G. intestinalis* parasites into assemblages or subspecies.

Real-time PCR technology has become an appealing alternative to conventional methods for diagnosing infectious diseases[21] and has already brought improvement in laboratory workflows and turnaround times for epidemiological typing of clinical isolates of *G. intestinalis*.[22] A future extension of this study will be analysis of *G. intestinalis* genotypes using a multi-locus approach to explore host specificity, transmission patterns and possible targets for drug resistance. The increase in sensitivity offered by this technique opens up the possibility of using real-time PCR to monitor patients post treatment as a test of cure. Before this can be done, however, it will be critical to determine the length of time DNA from non-viable *G. Intestinalis* can be detected in patients following successful treatment. Such work will also require the ability to differentiate between viable and non-viable parasites, possibly using reverse transcriptase PCR.

Take home messages

1. The Verweij real-time PCR protocol with (SSU)-rRNA primers detected approximately 10 % more *Giardia intestinalis* parasites than the traditional ova, cysts, and parasite microscopy (OCP-M) method and 32 % more than the Primerdesign Ltd. real-time *Giardia* PCR assay.

2. The Primerdesign Ltd. *Giardia* real-time PCR assay was more suited for epidemiological studies involving the molecular characterization of *Giardia intestinalis* into assemblages A and B.

3. The Verweij assay was shown to be more optimised and robust in the harsh environment of stool samples with reaction efficiency well within the acceptable range of 90 – 110 % and a $R^2 \geq 0.99$ making it more applicable as a frontline diagnostic test.

4. Likelihood ratios (LR+ and LR-) were better with the (SSU)-rRNA primers at 95 % CI.

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Contributors

All authors contributed to the design of the study and revision of the manuscript. **SB** performed the bulk of the laboratory work and drafted the manuscript. **SDP** made substantial contribution to the statistical analysis and interpretation of the experimental data. **SK** and **GM** reviewed the manuscript critically for important intellectual and sound scientific content. **PLC** contributed to the conception of the study and critically revised the manuscript for sound clinical content. As the guarantor, **PLC** is responsible for the overall content of this study. All the authors approved the final version of the manuscript for submission.

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Competing Interest: None declared.

Ethics approval: The stool samples were submitted as part of the usual clinical practice for diagnostic purposes. Ethical approval was not required under National Health Service (NHS) research governance arrangements (NHS-Research Ethics Committees (NHS REC) 12 November 2009).

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REFERENCES

1. **Allen AVH, Ridley DS.** Technical Methods: Further observations on the Formol-ether concentration technique for faecal parasites. *J Clin Pathol*. 1970;**23**:545-546.

2. **Ridley DS.** The value of formol-ether concentration of faecal cysts and ova. *J Clin Pathol*. 1956;**9**:74-76.

3. **Goka AK, Rolston DD, Mathan VI, *et al.*** The relative merits of faecal and duodenal juice microscopy in the diagnosis of giardiasis. *Trans R Soc Trop Med Hyg*. 1990;**84**:66-7.

4. **Calderaro A, Gorrini C, Montecchini S, *et al.*** Evaluation of a real-time polymerase chain reaction assay for the laboratory diagnosis of giardiasis. *Diagn Microbiol Infect Dis*. 2010;**66**:261-7.

5. **Haque R, Roy S, Siddique A, *et al.*** Multiplex real-time PCR assay for detection of Entamoeba histolytica, Giardia intestinalis, and Cryptosporidium spp. *Am J Trop Med Hyg*. 2007;**76**:713-7.

6. **Schuurman T, van Zwet A, Lankamp P, *et al.*** Comparison of microscopy, real-time PCR and a rapid immunoassay for the detection of Giardia lamblia in human stool specimens. *Clin Microbiol Infect*. 2007;**13**:1186 - 1191.

7. **Verweij JJ, Schinkel J, Laeijendecker D, *et al.*** Real-time PCR for the detection of Giardia lamblia. *Mol Cell Probes*. 2003;**17**:223-225.

8. **Verweij JJ, Blangé R, Templeton K, *et al.*** Simultaneous detection of Entamoeba histolytica, Giardia lamblia, and Cryptosporidium parvum in fecal samples by using multiplex real-time PCR. *J Clin Microbiol*. 2004;**42**:1220-3.

9. **Polley SD, Boadi S, Watson J, *et al.*** Detection and species identification of microsporidial infections using SYBR Green real-time PCR. *J Med Microbiol*. 2011;**60**:459-66.

10. **Murphy NM, McLauchlin J, Ohai C, *et al.*** Construction and evaluation of a microbiological positive process internal control for PCR-based examination of food samples for Listeria monocytogenes and Salmonella enterica. *Int J Food Microbiol*. 2007;**120**:110-9.

11. **Nantavisai K, Mungthin M, Tan-ariya P, *et al.*** Evaluation of the sensitivities of DNA extraction and PCR methods for detection of Giardia duodenalis in stool specimens. *J Clin Microbiol*. 2007;**45**:581-3.

12. **Hopkins RM, Meloni BP, Groth DM, *et al.*** Ribosomal RNA sequencing reveals differences between the genotypes of Giardia isolates recovered from humans and dogs living in the same locality. *J Parasitol*. 1997;**83**:44-51.

13. **Read C, Walters J, Robertson ID, *et al.*** Correlation between genotype of Giardia duodenalis and diarrhoea. *Int J Parasitol*. 2002;**32**:229-231.

14. **Lee C, Kim J, Shin SG, *et al.*** Absolute and relative QPCR quantification of plasmid copy number in Escherichia coli. *J Biotechnol*. 2006;**123**:273-80.

15. **Rutjes AWS, Reitsma JB, Coomarasamy A, *et al.*** Evaluation of diagnostic tests when there is no gold standard. A review of methods. *Health Technol Assess*. 2007;**11**:1-72.

16. **Sloan LM.** Real-time PCR in clinical microbiology: verification, validation, and contamination control. *Clin Microbiol Newsl*. 2007;**29**:87-95.

17. **Deeks JJ, Altman DG.** Diagnostic tests 4: likelihood ratios. *BMJ*. 2004;**329**:168-169.

18. **Cook C, Cleland J, Huijbregts P.** Creation and Critique of Studies of Diagnostic Accuracy: Use of the STARD and QUADAS Methodological Quality Assessment Tools. *J Man Manip Ther*. 2007;**15**:93-102.

19. **Boontanom P, Siripattanapipong S, Mungthin M, *et al.*** Improved sensitivity of PCR amplification of glutamate dehydrogenase gene for detection and genotyping of Giardia duodenalis in stool specimen. *Southeast Asian J Trop Med Public Health*. 2010;**41**:280-4.

20. **Feng Y, Xiao L.** Zoonotic potential and molecular epidemiology of Giardia species and giardiasis. *Clin Microbiol Rev*. 2011;**24**:110-40.

21. **Espy MJ, Uhl JR, Sloan LM, *et al.*** Real-time PCR in clinical microbiology: applications for routine laboratory testing. *Clin Microbiol Rev*. 2006;**19**:165-256.

22. **Elwin K, Fairclough HV, Hadfield SJ, *et al.*** Giardia duodenalis typing from stools: a comparison of three approaches to extracting DNA, and validation of a probe-based real-time PCR typing assay. *J Med Microbiol*. 2014;**63**:38-44.