A critical assessment of two real-time PCR 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 *G. 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’s, and Verweij et al *G. 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 99.4% (95% CI 88.30% to 98.50%) and an efficiency of 100%. Primerdesign’s 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.

INTRODUCTION

Giardiasis is an intestinal diarrhoeal illness caused by the flagellate protozoan parasite *Giardia intestinalis* (synonymous with *G. lamblia* and *G. duodenalis*). Traditionally, faecal concentration technique described by Allen and Ridley1,2 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 *G. intestinalis* have been shown to be more sensitive than conventional methods.4-8 However, their amplification efficiency (E) and correlation coefficient (linearity, R²) have not been critically assessed for use in routine diagnosis. In this study, the Verweij et al8 real-time PCR assay which targets the small-subunit (SSU) rRNA gene, and Primerdesign’s 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 R².

MATERIALS AND METHODS

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 (Paraselp, DiaSys Europe) on them. Before archiving, the faecal concentrates were examined microscopically using the ×10 objective, followed by the ×40 objective with the application of a drop of iodine to aid in the identification of internal structures of cysts. When unfomed 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 *G. intestinalis*. The samples comprised 98 *G. 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.

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).
**Molecular methods**

**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 standardised quantity of *Escherichia coli* transformed with a green fluorescent protein (GFP) gene to serve as the extraction and internal control10 together with the extraction control provided by Primerdesign in the *G. intestinalis* PCR kit.

**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 *G. 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°C (10 min), 95°C (15 s), 60°C (60 s) for 45 cycles.

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 reamplification.

**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 amplics of *G. intestinalis* to investigate apparent false positive samples. A G-Storm Thermocycler (Kapa Biosystems Model GS00001) was used with the following protocol: heated lid 110°C; hot start 1 cycle for 95 min and 15 min; 95°C (45 s) and 60°C (90 s) repeated for a total of 45 cycles; 72°C for 1 cycle followed by a holding temperature of 10°C. The amplification protocol was repeated with the same set of primers as a two-step reaction to maximise the yield of 62 bp amplics for potential sequencing.

**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 130 bp fragment of the *G. intestinalis* (SSU) rRNA gene for visualisation 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 method12 using Biomix red (Bioline product). The amplification process consisted of 1 cycle at 95°C (2 min); 94°C (20 s), 59°C (20 s), and 72°C (30 s) repeated for a total of 40 cycles; 72°C (7 min) for 1 cycle. The nest 2 primers which amplified 130 bp fragment were: GiarF and GiarR13 (table 2). A Thermo Electron P×2 thermal cycler was used with the following amplification protocol: 1 cycle at 95°C (2 min); 94°C (20 s), 59°C (20 s), and 72°C (30 s) repeated for a total of 45 cycles; 72°C (7 min) for 1 cycle as per published protocol using Biomix red.12

**Analytical sensitivity and specificity**

The analytical potential of all the tests (index and reference) deployed in this study were verified by the estimation of their limit of detection (LOD) prior to the estimation of their diagnostic accuracy measures. The LOD of the five assays were determined using DNA extracted from serially diluted fivefold 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 *Giarda*-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,
Table 3  Determination of limit of detection (LOD) using *Giardia intestinalis* positive stool sample containing 71 000 cysts/mL

| Test          | Analytical sensitivity (cysts/mL of stool) | Analytical specificity (pooled *Giardia*-negative stool) |
|--------------|------------------------------------------|---------------------------------------------------------|
|              | 71 000                                   | 14 200                                                  | 2840 | 568 | 113.6 | 22.7 | 4.6 |
| RMT          | +                                       | –                                                       | –    | nt  | nt    | nt   | nt  |
| OCP-M        | +                                       | +                                                       | –    | –   | –     | –    | –   |
| EIA          | +                                       | +                                                       | +    | –   | –     | –    | –   |
| Primerdesign | +                                       | +                                                       | +    | +   | –     | –    | –   |
| Verweij real-time | +                                   | +                                                       | +    | +   | +     | –    | –   |

The cysts were counted using C-Chip counting chamber and diluted 1 in 5 down to 4.6 cysts/mL.

—, negative; +, positive; EIA, enzyme immunoassay; nt, not tested; OCP-M, ova, cysts, and parasite microscopy; RMT, rapid membrane test.

### Real-time PCR performance evaluation

A set of 10-fold serial dilutions of a *G. 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 fivefold serial dilutions used for the LOD testing. The calculation of $E$ was based on the formula $E=10^{(-1/slope)}$—1 for standard curve generated by a $R^2$ of the plotted points.14

### Diagnostic accuracy

A CRS of RMT and EIA was used in a diagnostic study of the three index tests (OCP-M, Verweij real-time PCR, and Primerdesign’s *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 pairwise 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 (2×2 table): sensitivity=\((\text{number of } TP)/(\text{number of } TPs+\text{number of false negatives})\) percent, and specificity=\((\text{number of } TN)/(\text{number of } TNs+\text{number of false positives})\) percent.16 Positive likelihood ratio (LR+) was calculated as (sensitivity)/(1—specificity), and negative likelihood ratio (LR−) was calculated as (1—sensitivity)/(specificity).17

### RESULTS

#### Analytical sensitivity and specificity of diagnostic tests

The fivefold serial dilutions of *G. 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 Primerdesign’s PCR was 113.6 cysts/mL. Indeed, apart from an isolated positive result (CT 38.9) in one of the triplicate runs in 22.7 cysts/mL tube, Primerdesign’s assay did not detect any positivity in any replicates involving less than 113.6 cysts/mL.

The RMT and the EIA formed the constituent tests for the CRS with a combined LOD of ≤2840 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).

#### Diagnostic accuracy measures

Using the four criteria listed in table 1, the CRS divided the 170 samples into 91 TP and 79 TN cases and this enabled diagnostic accuracy measures to be estimated for the OCP-M and the two real-time PCR assays (table 4).

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 (2×2 table) to calculate the diagnostic accuracy measures shown in table 5. The Verweij assay was statistically more sensitive (93.4% (95% CI 88.30% to 98.50%)) than Primerdesign’s assay (61.5% (95% CI 51.50% to 71.50%)) (table 5).

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).

The absence of such a fragment would have almost certainly indicated a false positive case. In the real-time PCR, an

### Table 4  Delineation of TP and TN cases

| Index test   | TP (TPF) | FN (FNF) | Composite reference standard of EIA and RMT (TP: 91, TN: 79) |
|--------------|----------|----------|-----------------------------------------------------------|
|              | 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                                 |

EIA, enzyme immunoassay; FN, false negative; FNF, false negative fraction; FP, false positive; FPF, false positive fraction; RMT, rapid membrane test; TN, true negative; TNF, true negative fraction; TP, true positive; TPF, true positive fraction.
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 *G. 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 recalculated 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% to 75%) in the detection of positive cases. More importantly the specificity was increased to 90.8% (83.77% to 97.83%).

### Real-time PCR assay performance

The E and R² for the 1 in 5 serially diluted stool samples were E=100% (the slope was −3.326), R²=0.99 for the Verweij real-time PCR, and E=203% (the slope was −2.076) and R²=0.93 for Primerdesign’s real-time PCR. A 10-fold serial dilutions of *G. intestinalis* DNA produced an efficiency of 100% (the slope was −3.315) and R²=0.95 with Primerdesign’s real-time PCR, and 96.3% (the slope was −3.414) and R²=0.99 for the Verweij real-time PCR, respectively. Generally efficiency between 90% and 110% is considered acceptable, and an R² value >0.99 provides good confidence in correlating two values (Life Technologies, Real-time PCR: Understanding Cₛ, 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 was considered to show levels of inhibition that would identify the sample as a run failure. Primerdesign’s 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.

### DISCUSSION

The aim of this study was to use a CRS to critically assess the diagnostic accuracy of 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% *G. Intestinalis*-positive stool samples (as determined by the 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 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 drawback 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 TPs because of the presence of
appropriately sized amplicons (62 bp) (figure 1). These amplicons did not yield enough DNA for sequencing when they were excited 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 (≤2840 cysts/mL). Re-evaluating these 14 samples as TP gives a specificity of 90.8%. The effects of the sensitivity and specificity can be seen by examining the likelihood ratios18 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 TNs 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 TP cases in the CRS would result in a 19.3% increase in the detection of positive cases compared to OCP-M (table 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 *G. 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 these standards, efficiencies remained in the acceptable range of 90–110%. Primerdesign’s PCR did not appear to fare so well with PCR inhibition: About 28% of the internal control CTs for Primerdesign’s 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. Additionally, 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 Primerdesign’s PCR reserved for the molecular characterisation of *G. intestinalis* parasites into assemblages or subspecies.

Real-time PCR technology has become an appealing alternative to conventional methods for diagnosing infectious diseases21 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 multilocus 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 that 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.

### 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

| 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 |

*Composite reference standard (CRS) of EIA and RMT indicated TP: 91, TN: 79.

*These values have been curtailed to 100% since a specificity of greater than this value is biologically meaningless.

EIA, enzyme immunoassay; LR−, negative likelihood ratio; LR+, positive likelihood ratio; OCP-M, ova, cysts, and parasite microscopy; RMT, rapid membrane test; TN, true negative; TP, true positive.

### Take-home messages

- 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 Primerdesign’s real-time *Giardia* PCR assay.
- Primerdesign’s *Giardia* real-time PCR assay was more suited for epidemiological studies involving the molecular characterisation of *G. intestinalis* into assemblages A and B.
- 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.
- 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 provided samples. SK and GM performed the stool collection 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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