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Comparison of microscopy, real-time PCR and a rapid immunoassay for the detection of *Giardia lamblia* in human stool specimens

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ABSTRACT

Giardia lamblia is one of the most common intestinal parasites worldwide, with microscopy being the diagnostic reference standard for use with human stools. However, microscopy is time-consuming, labour-intensive and lacks sensitivity when single stools are examined. In the present study, microscopy, real-time PCR and a rapid immunoassay were compared for the detection of *G. lamblia* in human stools. All three methods were highly sensitive, with values of 99%, 100% and 98%, respectively. Specificity and positive and negative predictive values were ≥97%, except when using real-time PCR, for which the specificity and positive predictive value were 92% and 93%, respectively. The lower specificity of real-time PCR was associated mostly with failure to detect specimens regarded as true positives for *G. lamblia* DNA, although cross-contamination was suspected in a minority of cases because of the large amount of *G. lamblia* DNA present in most positive specimens. It was concluded that microscopy should remain the primary diagnostic tool for identifying *G. lamblia* in human stools, mainly because of its ability to simultaneously detect other gastrointestinal parasites. However, the simple and rapid immunoassay is a valuable tool to decrease turn-around time. Real-time PCR provides additional sensitivity, although there is a risk of cross-contamination. Based on this observation, and the need for other real-time assays to be developed to detect other intestinal parasites, real-time PCR is currently useful only as an additional test supplementary to microscopy.

Keywords Diagnosis, faeces, *Giardia lamblia*, immunoasssay, microscopy, real-time PCR

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INTRODUCTION

Infectious gastroenteritis is still a major public health burden in developed countries, although the mortality rate is low [1]. In The Netherlands, the total number of cases of gastroenteritis in the population was estimated at 4.5 million in 1999 [2]. The intestinal protozoan *Giardia lamblia* (syn. *intestinalis* or *duodenalis*) is the most frequent pathogenic parasite involved in infectious gastroenteritis in The Netherlands [3]. Older children (aged 5–14 years) seem to be the predominant age group infected [3].

Classically, diagnosis of giardiasis is conducted by microscopical analysis of multiple stool specimens for the presence of *G. lamblia* cysts or trophozoites. Analysis of single stool specimens has been shown to be effective in only 70% of cases [4–6]. However, the sensitivity of microscopy is largely dependent on the skill of the microscopist [7–10], and this makes the technique time-consuming and expensive. In recent years, direct fluorescent-antibody staining tests and enzyme immunoassays have been shown to be sensitive and cost-effective alternatives to microscopical examination of stools [11], but these assays still require numerous reagent additions, washing procedures and incubation steps. Several

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immunochromatographic lateral flow immunoassays provide a rapid means of detecting *G. lamblia* and *Cryptosporidium parvum*, but are slightly less sensitive than the direct fluorescent-antibody staining test [10,12–14]. Nevertheless, rapid immunoassays might be useful for smaller hospital laboratories, as they do not require the use of specialised equipment or the skills of experienced microscopists. Real-time PCR methods for direct detection of *G. lamblia* in stools have also been described, with similar or improved sensitivity, when compared to microscopy and antigen detection, when used with single faecal specimens [15–18].

The present study describes the first direct comparison of the use of microscopy, a lateral flow immunoassay and real-time PCR for the detection of *G. lamblia* in human stool specimens.

MATERIALS AND METHODS

Bacteria and fungi

In total, 39 bacterial and fungal strains were used as negative controls, representing gastrointestinal pathogens and normal human gastrointestinal flora (Table S1, see Supplementary material). All organisms were grown using appropriate media and growth conditions for 16–48 h. After harvesting (*c.* 10^9 CFU) into 1 mL of physiological saline, chromosomal DNA was extracted from the strains by heat lysis for 10 min at 95°C.

Faecal specimens

Unpreserved (non-fixed) stool samples positive for *G. lamblia* ($n = 103$), and 97 stools negative for *G. lamblia* according to microscopy, were collected between August and October 2004. Specimens were labelled blindly, and stored at −20°C until further processing by real-time PCR and rapid immunoassay. All stools originated from patients with a suspicion of gastroenteritis, and were sent to the Laboratory for Infectious Diseases, Groningen, The Netherlands, for bacterial and parasitological examination. Among the 103 specimens positive for *G. lamblia* by microscopy, 23 also contained other (possibly pathogenic) parasites, pathogenic bacteria or yeast species that were detected by microscopy and culture methods, including: *Entamoeba coli* ($n = 9$), *Blastocystis hominis* ($n = 8$), *Candida albicans* ($n = 1$), yeast species ($n = 1$), *Endolimax nana* + *Ent. coli* + *B. hominis* ($n = 1$), *Dientamoeba fragilis* ($n = 1$), *B. hominis* + *Campylobacter jejuni* ($n = 1$), and *D. fragilis* + *B. hominis* ($n = 1$). Among the 97 specimens negative for *G. lamblia*, 17 contained other parasites, bacteria or yeast species, including *B. hominis* ($n = 6$), *Ent. coli* ($n = 2$), *Ent. coli* + *B. hominis* ($n = 2$), *D. fragilis* + *B. hominis* ($n = 2$), *D. fragilis* + *End. nana* ($n = 1$), *Campylobacter coli* + *B. hominis* ($n = 1$), *Camp. jejuni* ($n = 1$), *End. nana* ($n = 1$) and an unidentified yeast species ($n = 1$).

Microscopical examination for *G. lamblia*

Fifty-nine of the 103 specimens positive by microscopy were sent to the Laboratory for Infectious Diseases as single, unpreserved faecal specimens. The remaining 44 were sent as a triple faeces test set, containing one unpreserved specimen and two specimens fixed in sodium acetate–acetic acid–formalin [19]. Three of the 97 microscopy-negative faecal specimens were unpreserved single samples, while the remaining 94 were part of a triple faeces test set. Microscopy was conducted within 1 day of arrival at the initial diagnostic laboratory. Unpreserved faecal specimens were first concentrated using the method of Ridley *et al.* [20], and an iodine-stained wet-mount preparation was examined at ×400 magnification. Sodium acetate–acetic acid–formalin-preserved faecal specimens were initially examined using an iodine-stained wet-mount preparation, and this was followed by a chlorazol-black stain when a suspicion of vegetative-stage protozoa was raised by the iodine-stain examination.

Rapid immunoassay

The rapid immunoassay (ImmunoCard STAT! *Cryptosporidium/Giardia* Rapid Assay; Meridian Bioscience, Boxtel, The Netherlands) was used according to the manufacturer's instructions. In brief, faecal samples were thawed and diluted by adding three volumes of distilled water. Two drops of sample treatment buffer (contained in the assay kit) were added to the specimen dilution tube. Next, *c.* 60 μL of diluted faecal specimen was transferred to the specimen dilution tube and two drops of conjugate reagent A were added, followed by two drops of conjugate reagent B. After gentle mixing by manual swirling, the sample was transferred to the test device and the results were read after 10 min. A specimen was regarded as positive if both the 'organism' and 'control' lines showed a band, as negative if only the 'control' line showed a band, and as invalid if no band was visible at the 'control' line.

Faecal sample preparation and real-time PCR

Nucleic acid was extracted from faecal suspensions using the method described by Boom *et al.* [21,22] and Beld *et al.* [23] with minor modifications. In brief, 100 μL of faecal suspension (33–50% w/v) was added to a mixture containing 50 μL silica particles (SC-F, prepared as described previously [23], but with 2400 μL of HCl 32% v/v, rather than 600 μL as described by Beld *et al.* [23]) and 900 μL of lysis buffer L6, mixed and left at ambient temperature for 10 min. After centrifugation, the silica–nucleic acid complexes were washed as described previously [21]. The nucleic acids were eluted in 100 μL of 1 × TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and either processed for PCR immediately or stored at −20°C.

Real-time PCR was performed on an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) using the *Taq*Man Universal PCR Master Mix (Applied Biosystems) and the *G. lamblia* small-subunit rRNA gene-specific primers and probe described by Verweij *et al.* [18]. The reaction mixture (25 μL) consisted of 1 × *Taq*Man Universal PCR Master Mix, 300 nM each of primers Giardia F (Applied Biosystems) (5′-GACGGCTCAGGACAACGGTT) and Giardia R (Applied Biosystems) (5′-TTGCCAGCGGTGTCCG), 200 nM *Taq*Man probe Giardia T (Applied Biosystems) (5′-FAMCCCGCGGCGGTCC-

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CTGCTAG-TAMRA), 2.5 μg of bovine serum albumin (Roche Diagnostics, Almere, The Netherlands) and 5 μL of template DNA. Amplification comprised 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. PCR inhibition was monitored by running a duplicate reaction spiked with 50 pg of purified *G. lamblia* cyst DNA, equivalent to *c.* 160 *G. lamblia* cysts. Amplification data were analysed using Sequence Detector software v. 1.9.1 (Applied Biosystems).

RESULTS

Sensitivity and specificity of *G. lamblia* small-subunit rDNA real-time PCR

To assess the sensitivity of the *G. lamblia* real-time PCR, ten-fold serial dilutions of purified *G. lamblia* cyst DNA (250 ng to 250 fg) were tested in duplicate. All dilutions from 250 ng to 2.5 pg were detected in both tests. The dilution containing 250 fg was detected only once, and no signal was detected in the controls without any template. A *G. lamblia* cyst contains *c.* 313 fg of chromosomal DNA, and a trophozoite contains *c.* 144 fg [24]. Thus, in principle, the *G. lamblia* real-time PCR assay was able to detect DNA originating from a single *G. lamblia* cyst.

A stool specimen, negative for *G. lamblia* according to microscopy and real-time PCR, was spiked with ten-fold serial dilutions of purified *G. lamblia* cyst DNA (33 ng to 33 fg/faecal extraction) and DNA was isolated from these specimens. Real-time PCR was performed in duplicate on the faecal DNA extracts. In addition, *G. lamblia* cyst DNA serially diluted ten-fold was amplified in parallel in duplicate, using the amount that would be expected if recovery of DNA from the faeces was 100%. The purified *G. lamblia* cyst DNA and the faecal DNA extracts both showed nearly identical Ct values (the number of PCR cycles required to detect a positive reaction) for the specimens containing 1.7 ng to 1.7 pg of DNA/real-time PCR. Assuming 100% recovery, this corresponds to between 5000 and five *G. lamblia* cysts/real-time PCR. Further dilutions failed to yield positive results with either purified *G. lamblia* cyst DNA or the faecal DNA extracts. Thus, in a faecal background, the real-time PCR could detect the equivalent of five *G. lamblia* cysts, or 2000–3000 *G. lamblia* cysts/g of faeces, depending on the faecal suspension (i.e., 33–50% w/v), assuming 100% extraction and detection efficiency.

The specificity of the *G. lamblia*-specific real-time PCR has been documented previously for other intestinal parasites, including *Entamoeba histolytica*, *Entamoeba dispar*, *Enterocytozoon bieneusi*, *Encephalitozoon intestinalis*, *Cyclospora cayetanensis* and *Crypto. parvum* [17,18]. No cross-reactions were observed with the panel of 39 bacterial and fungal strains (Table S1) representing gastrointestinal pathogens and normal human flora.

Detection of *G. lamblia* in clinical samples by rapid immunoassay and real-time PCR

Ninety-eight of the 103 microscopy-positive faecal specimens were also found to be positive according to both rapid immunoassay and real-time PCR. Ct values for these specimens in real-time PCRs averaged 21.90 (range 14.53–31.16). Real-time PCR also detected *G. lamblia* DNA in two microscopy-positive faecal specimens (Ct values of 16.16 and 17.73) that were negative according to the rapid immunoassay. The three remaining microscopy-positive samples were detected neither by rapid immunoassay nor by real-time PCR. Of the 97 specimens negative for *G. lamblia* according to microscopy, one sample was positive for *G. lamblia* according to both rapid immunoassay and real-time PCR (Ct 33.30). Eight additional faecal specimens were also positive according to real-time PCR (Ct range 32.74–38.69), but were negative according to both rapid immunoassay and microscopy. When these samples were retested in quadruplicate using two new DNA preparations, three were negative and were regarded as false-positives caused by specimen cross-contamination. For the remaining five specimens, between one and eight of the replicates were positive.

The distribution of Ct values among all the real-time PCR-positive stool specimens is shown in Fig. 1, and indicates that most specimens contained large amounts of *G. lamblia* DNA. For 34.9% of the specimens, the Ct values were <20, and for 63.3% and 89.9% of the specimens, the Ct values were <25 and <30, respectively.

None of the 200 faecal specimens showed evidence of PCR inhibition. However, two microscopy-negative samples gave invalid results with the rapid immunoassay; both samples showed valid negative results upon retesting, and these results were used for the comparative analysis.

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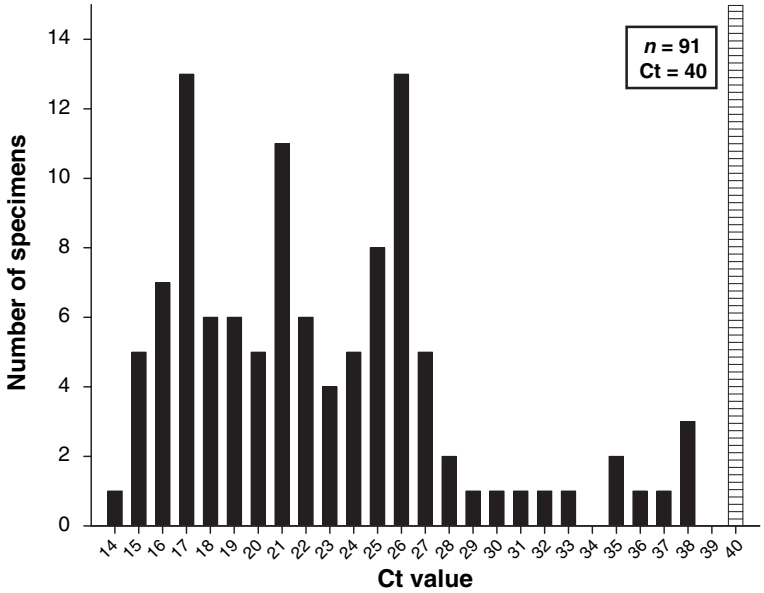

Fig. 1. Distribution of Ct values for the *Giardia lamblia* faecal specimens that were positive according to real-time PCR. The Ct value indicates the number of PCR cycles that were required to detect a positive reaction. Closed bars represent the number of faecal specimens for a given Ct range. The dashed bar represents faecal specimens with no detectable amplification signal (Ct 40.00). Ct value groups cover the following ranges: Ct value group 14 represents Ct values from 14.00 to 14.99; Ct value group 15 represents Ct values from 15.00 to 15.99; etc.

Independent analysis of the performance of all methods

To independently compare all three methods for the detection of *G. lamblia*, an extended reference standard was used for true-positive stool specimens. A true-positive result was defined as a specimen with at least two positive results among the three available tests. Table 1 shows the results of all three methods in comparison with the extended reference standard. The sensitivity, specificity, positive predictive value and negative predictive value were 99%, 97%, 97% and 99% for microscopy, 100%, 92%, 93% and 100% for real-time PCR, and 98%, 100%, 100% and 98% for rapid immunoassay, respectively.

Detection of *Crypto. parvum* by rapid immunoassay

Although the present study was aimed at detecting *G. lamblia*, the rapid immunoassay was also capable of detecting *Crypto. parvum* in stool specimens. Of the 200 specimens tested, three samples showed a *Cryptosporidium*-specific band in the rapid immunoassay. This resulted in the identification of two probable mixed infections with *G. lamblia* and *Crypto. parvum*.

DISCUSSION

Real-time PCR, rapid immunoassay and microscopy were all highly sensitive (98–100%) when compared with an extended reference standard for the detection of *G. lamblia* in human stools. As recommended previously, a composite standard was used in the absence of a good reference standard test [25,26]. Specificity and positive and negative predictive values were also ≥97%, except when using real-time PCR, where the specificity and positive predictive value were 92% and 93%, respectively.

Fourteen faecal specimens showed discrepant results with one or two of the methods. However, both the real-time PCR and the rapid immunoassay might generate false-negative results because of intermittent shedding of *G. lamblia* cysts in stools. Also, the amount of specimen analysed may have influenced the results, as the amount of faeces analysed by microscopy is *c.* 100–200 mg, which is then concentrated further using the method of Ridley and Hawgood [20] for unpreserved specimens. For real-time PCR and rapid immunoassay, 1.67–2.5 mg and 15 mg of faeces are analysed, respectively, assuming that 1 mg equals *c.* 1 μL.

The two false-negative results obtained with the rapid immunoassay may have been caused by an overload of the rapid immunoassay with *G. lamblia* antigen, leading to the prozone effect. Both of these specimens showed high *G. lamblia* DNA levels according to real-time PCR (Ct values of 16.16 and 17.73, respectively), and it is therefore likely that high antigen levels were also present in the stools. For the eight specimens that were

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Table 1. Comparison of the results obtained according to microscopy, real-time PCR and rapid immunoassay in comparison with the extended reference standard

Test result				
Microscopy	**Real-time PCR**	**Rapid immunoassay**	**No. of specimens**	**Extended reference standard interpretation[a]**
Positive	Positive	Positive	98	True positive
Positive	Positive	Negative	2	True positive
Positive	Negative	Positive	0	True positive
Negative	Positive	Positive	1	True positive
Positive	Negative	Negative	3	True negative
Negative	Positive	Negative	8	True negative
Negative	Negative	Positive	0	True negative
Negative	Negative	Negative	88	True negative
Total			200	

[a]A true-positive result was defined as a specimen with at least two positive results among the three available tests; a true-negative result was defined as a specimen with at least two negative results among three available tests.

positive only according to real-time PCR, there was a strong suspicion of cross-contamination, based on the large number of strong positive specimens (Fig. 1). The source of contamination could be related to the extremely large amount of *G. lamblia* DNA present in most positive specimens, with contamination occurring during DNA extraction or during the addition of template DNA to the real-time PCR assays. The latter seems most likely to be the case in the present study, as additional amplification of the original DNA extracts for the three specimens regarded as false-positive did not yield any positive real-time PCR results. These results emphasise the need for great care when performing PCR, and weak positive results should always be interpreted in relation to the clinical data. It is not clear whether the presence of small amounts of DNA in a patient's stool correlates with disease, or indicates asymptomatic infection, which has been described previously for *G. lamblia* in a case-control study in The Netherlands [3]. Further studies are needed to elucidate the relevance of the positive real-time PCR results with high Ct values.

The performance of the rapid immunoassay was better than has been reported previously [10,13], and was in line with the performance reported for a similar type of rapid assay [12,14]. Although the detection of *G. lamblia* in stools was the focus of the present study, the rapid immunoassay also detected *Cryptosporidium* antigen in three specimens. Of these, one was negative for *G. lamblia* according to all three detection methods, but the other two samples were positive for *G. lamblia* according to all three methods. Re-testing of these three specimens with a different batch of rapid immunoassay reagents yielded identical results, thereby providing clear evidence of co-infection by both parasites. However, Ziehl–Neelsen staining or PCR analysis is needed to confirm *Cryptosporidium*-positive rapid immunoassay results, especially as mixed infections with *G. lamblia* and *Crypto. parvum* are rare. The three samples in the present study were also positive for *Crypto. parvum* according to a specific real-time PCR assay (A. Bergmans, personal communication).

In conclusion, all three methods investigated were sensitive for the detection of *G. lamblia* in stools. Microscopy remains the primary diagnostic method for detecting gastrointestinal parasites because of its ability to detect parasites other than *G. lamblia*. However, the rapid immunoassay is a valuable tool for the routine clinical microbiology laboratory because of its speed and simplicity, especially when microscopical examinations of stools are not performed in the laboratory. When using the rapid immunoassay, specimens still need to be processed by microscopy in order to detect other parasites and the small number of false-negative *G. lamblia* stools, and for confirmation of rapid immunoassay *Cryptosporidium*-positive stools. Although real-time PCR seems to be the most sensitive method for detection of *G. lamblia* in human stools, it does not, as yet, provide additional advantages when used as a single test, mainly because of the risk of false-positive results and the fact that it detects only one of the clinically relevant gastrointestinal parasites. However, because of its higher sensitivity, it may eventually become the method of choice once it has been developed to detect all gastrointestinal parasites in one or a few reactions.

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SUPPLEMENTARY MATERIAL

The following Supplementary material is available for this article online at http://www.blackwell-synergy.com:

Table S1. Bacterial and fungal negative control strains used in this study.

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