

## Detection of diarrhoea-causing protozoa in general practice patients in The Netherlands by multiplex real-time PCR

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### ABSTRACT

The diagnostic value of a multiplex real-time PCR for the detection of *Entamoeba histolytica*, *Giardia lamblia* and *Cryptosporidium parvum*/*Cryptosporidium hominis* was evaluated by comparing the PCR results obtained with those of routinely performed microscopy of faecal samples from patients consulting their general practitioner (GP) because of gastrointestinal complaints. Analysis of 722 faecal DNA samples revealed that the prevalence of *G. lamblia* was 9.3% according to PCR, as compared to 5.7% by microscopy. The number of infections detected was more than double in children of school age. Furthermore, *G. lamblia* infection was detected in 15 (6.6%) of 228 faecal samples submitted to the laboratory for bacterial culture only. *C. parvum*/*C. hominis* infections were not diagnosed by routine procedures, but DNA from these organisms was detected in 4.3% of 950 DNA samples. A strong association with age was noted, with *Cryptosporidium* being detected in 21.8% of 110 children aged <5 years. *C. hominis* was the most prevalent species. *E. histolytica* was not detected in this study population. Analysis of microscopy data revealed that the number of additional parasites missed by PCR was small. Overall, the study demonstrated that a multiplex real-time PCR approach is a feasible diagnostic alternative in the clinical laboratory for the detection of parasitic infections in patients consulting GPs because of gastrointestinal symptoms.

**Keywords** Cryptosporidiosis, diagnosis, giardiasis, intestinal parasites, microscopy, real-time PCR

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### INTRODUCTION

Diarrhoea is a major health problem worldwide, killing *c.* 3–4 million individuals each year. Those most affected by diarrhoea are children and immunocompromised individuals living in developing countries. Although the mortality rate from diarrhoea in developed countries has fallen considerably, morbidity remains high [1]. In most cases, the aetiologies of diarrhoea are related to viruses, bacteria and parasites. In The Netherlands, there are *c.* 4.5 million cases of gastroenteritis annually [2]. The intestinal parasite with the highest prevalence is *Giardia lamblia*, followed by *Cryptosporidium parvum*/*Cryptosporidium hominis* [3]. Infection with *Entamoeba histolytica*

is rare, but its high morbidity and, in particular, mortality make accurate diagnosis crucial. Classically, diagnosis of *Giardia*, *Cryptosporidium* and *E. histolytica* infections is achieved by microscopical examination of faecal samples. However, microscopy has several important disadvantages: (i) correct identification depends greatly on the experience and skills of the microscopist; (ii) sensitivity is low, and therefore examination of multiple samples is needed; (iii) *E. histolytica* cannot be differentiated from the non-pathogenic *Entamoeba dispar* simply on the basis of the morphology of cysts and small trophozoites; and (iv) in settings with relatively large numbers of negative results, e.g., The Netherlands, microscopy can be tedious, with relatively high costs for each case detected.

Although molecular methods such as PCR have proven to be highly sensitive and specific for the detection of *E. histolytica*/*E. dispar*, *G. lamblia* and *C. parvum*/*C. hominis* infections [4–8], their use in

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routine diagnostic laboratories is still very limited. The introduction of molecular methods has been hindered by time-consuming methods for the isolation of DNA from faecal specimens and the presence of inhibitory substances in such samples. Furthermore, amplification of DNA was previously laborious and expensive, and cross-contamination among samples was a notorious problem. However, newly developed methods have greatly reduced these obstacles [9–11]. Real-time PCR reduces labour time, reagent costs and the risk of cross-contamination, and offers the possibility of detecting multiple targets in a single multiplex reaction. A multiplex real-time PCR has been described for the simultaneous detection of the three most important diarrhoea-causing parasites, i.e., *E. histolytica*, *G. lamblia* and *C. parvum/C. hominis*, and has demonstrated high sensitivity and specificity with species-specific DNA controls and a range of well-defined stool samples [12]. However, the role of this assay as a diagnostic tool in a routine clinical laboratory requires further evaluation with respect to large-scale screening and improved patient diagnosis [10,13].

In the present study, the diagnostic results obtained using a multiplex real-time PCR for the detection of *E. histolytica*, *G. lamblia* and *C. parvum/C. hominis* were compared with those obtained by routine microscopy of faecal samples from patients visiting their general practitioner (GP) because of gastrointestinal symptoms.

## MATERIALS AND METHODS

### Faecal specimens

DNA samples ( $n = 956$ ) were initially obtained as part of a study designed to evaluate the efficacy of molecular diagnosis of *Salmonella enterica* and *Campylobacter jejuni* (T. Schuurman *et al.*, manuscript in preparation), and were selected from c. 1900 faecal samples submitted between June and September 2005 to the Laboratory for Infectious Diseases, Groningen, The Netherlands, for routine bacterial culture and/or microscopical analysis. Faecal specimens from which DNA was extracted were submitted with a request from the GP for bacterial culture for *Salm. enterica*, *Shigella* spp. or *Camp. jejuni*, and contained a sufficient amount of faecal material for all tests. Other specimens received during this period were not subjected to DNA extraction.

All samples originated from patients who had visited their GP during the previous few days and who were suspected of having a gastrointestinal infection. According to the normal practice of the GP concerned, each patient submitted either an unpreserved faecal sample (classical method) or a set of three

faecal samples (Triple Faeces Test; TFT) collected on consecutive days; one sample was unpreserved and two samples were preserved with sodium acetate acetic acid formalin [14]. Upon arrival, the consistency of and the presence of mucus and/or blood in the unpreserved sample were recorded by a trained technician.

By definition, all 950 unpreserved faecal samples included for PCR analysis (six faecal samples were excluded because of inhibitory substances in the DNA extracts) were cultured routinely for *Campylobacter* spp., *Salmonella* spp. and/or *Shigella* spp., and in some cases for other pathogens, according to the request made by the GP or the clinical microbiologist. From this group, 722 samples were also examined by microscopy for the presence of parasites, according to the request made by the GP or the clinical microbiologist. For comparison, an additional 913 samples from the same group of 1900 cases who submitted faecal samples for the detection of parasites only were also examined by microscopy.

### Microscopy

Unpreserved samples were investigated for ova and cysts by microscopy of iodine-stained wet-mount preparations of a formalin–ether concentrate [15]. Sodium acetate acetic acid formalin-preserved samples were first screened by iodine-stained direct smears. Parasite-like structures were confirmed by microscopy of a chlorazol black dye permanent stain preparation [14]. Modified Ziehl–Neelsen staining for the detection of *Cryptosporidium* was only performed if cryptosporidiosis was suspected by the GP.

### DNA extraction

DNA was extracted from faecal suspensions (33–50% w/v) using the semi-automated NucliSens miniMAG instrument (bioMérieux, Boxtel, The Netherlands) in combination with NucliSens Magnetic Extraction Reagents (bioMérieux), according to the manufacturer's instructions. In brief, 100  $\mu$ L of faecal suspension was added to 2 mL of lysis buffer and incubated at room temperature for 10 min, after which an internal control (Phocin herpes virus-1 (PhHV-1); c. 6000 copies/sample) and 50  $\mu$ L of magnetic silica particles were added. The mixture was mixed and incubated for 10 min at room temperature. After centrifugation for 2 min at 1500 g, the supernatant was removed by aspiration and the pellet of silica–nucleic acid complexes was resuspended and washed in three washing buffers. Each washing step was conducted for 30 s on step 1 of the miniMAG instrument, with the exception of wash buffer 3 (15 s on step 1), after which the fluid was removed by aspiration. DNA was eluted in 100  $\mu$ L of elution buffer for 5 min at 60°C on a thermoshaker (Eppendorf, Hamburg, Germany) at 1400 rpm. The extracted DNA was stored at –20°C.

### PCR amplification and detection

The sample population was analysed by real-time PCR without reference to the initial microscopy results. Amplification and detection of *E. histolytica*, *C. parvum/C. hominis* and *G. lamblia* DNA, as well as the PhHV-1 internal control DNA, were performed on all samples using a multiplex real-time PCR, essentially as described previously [12], but with minor modifications.

In brief, amplification reactions were performed in 25- $\mu$ L volumes containing PCR buffer (Hotstar mastermix; Qiagen, Venlo, The Netherlands), 5 mM MgCl<sub>2</sub>, 2.5  $\mu$ g of bovine serum albumin (Roche Diagnostics, Almere, The Netherlands), 3.125 pmol each of the *E. histolytica*- and *G. lamblia*-specific primers, 12.5 pmol of the *Cryptosporidium*-specific primer, 1.25 pmol of VIC-labelled MGB-*Taqman* probe (Applied Biosystems, Warrington, UK) for *E. histolytica*, 2.5 pmol of FAM-labelled double-labelled probe (Biolegio, Nijmegen, The Netherlands) for *G. lamblia*, 2.5 pmol of Texas-red-labelled double-labelled probe for *Cryptosporidium*, and 5  $\mu$ L of template DNA. The PhHV-1-specific primers and probe set consisted of 3.75 pmol of each PhHV-1-specific primer and 2.5 pmol of Cy5-labelled double-labelled probe.

Amplification comprised 15 min at 95°C, followed by 50 cycles of 15 s at 95°C and 30 s at 60°C. Amplification, detection and data analysis were performed using the I-cycler Real-Time PCR System and v.3.1.7050 software (Bio-Rad, Hercules, CA, USA).

Faecal DNA samples were considered to contain inhibitors if the PhHV-1 internal control was not detected, or if the expected cycle threshold (Ct) value of 32 cycles was increased by more than five cycles. Because of inhibition, six samples were excluded from the parasite analysis.

Ct values obtained for *G. lamblia*, *E. histolytica* or *Cryptosporidium* amplification are considered less reproducible at >36 cycles. Therefore, the real-time PCR was repeated for 17 faecal samples with an initial Ct value of 36.4–47.4 (median 40.7). For 12 samples, amplification was detected with comparable Ct values (range 33.0–39.1, median 38.0), while five samples showed no DNA amplification and were therefore considered to be negative.

The primers described by Morgan *et al.* [16] were used to differentiate between *C. hominis* and *C. parvum* in DNA samples positive for *Cryptosporidium* DNA. PCR was performed in 25- $\mu$ L volumes containing PCR buffer, 5 mM MgCl<sub>2</sub>, 2.5  $\mu$ g of bovine serum albumin, 12.5 pmol of forward primer (021F) annealing to both *C. hominis* and *C. parvum*, 6.25 pmol of *C. hominis*-specific reverse primer (CP-HR) and 6.25 pmol of *C. parvum*-specific reverse primer (CP-CR). Amplification comprised 15 min at 95°C, followed by 45 cycles of 30 s at 94°C, 30 s at 58°C and 30 s at 72°C, with a final extension for 5 min at 72°C. Amplification products (411 bp for *C. hominis* and 312 bp for *C. parvum*) were detected following electrophoresis in agarose 2% w/v gels stained with ethidium bromide.

#### Data analysis

Statistical analyses were performed using SPSS v.11.0.1 (SPSS Inc., Chicago, IL, USA). Proportions were compared among groups, and ORs and 95% CIs were calculated. Continuous variables were described by range and median values, and were compared among groups by the Mann-Whitney rank sum test, with  $p < 0.05$  considered to be statistically significant.

## RESULTS

### Study group

Table 1 summarises the patient characteristics of the 950 cases included for detection of intestinal

**Table 1.** Characteristics and outcome of faecal examination for parasites in 1863 patients who visited their general practitioner because of gastrointestinal complaints

	Patient samples examined by		
	PCR and microscopy (n = 722)	PCR without microscopy (n = 228)	Microscopy without PCR (n = 913)
Gender, % male	41.8	41.2	43.4
Age range, years (median)	0–95 (36.0)	0–92 (41.5)	0–92 (27.0)
Travel history, n (%)			
Unknown	578 (80.1)	203 (89.0)	443 (48.5)
None	7 (1.0)	3 (1.3)	326 (35.7)
Low-risk areas	49 (6.8)	6 (2.6)	29 (3.2)
High-risk areas	88 (12.2)	5 (2.2)	93 (10.2)
Adopted child	0	11 (4.8)	22 (2.4)
Microscopy, n (%)			
TFT procedure	247 (34.2)	ND	637 (69.8)
Classical procedure	475 (65.8)	ND	276 (30.2)
<i>Giardia lamblia</i>	41 (5.7)	ND	44 (4.8)
<i>Cryptosporidium</i> spp.	0	ND	1 (0.1)
<i>Entamoeba histolytica</i> / <i>E. dispar</i>	0	ND	1 (0.1)
<i>Blastocystis hominis</i>	21 (2.9)	ND	70 (7.7)
<i>Dientamoeba fragilis</i>	8 (1.1)	ND	17 (1.9)
<i>Endolimax nana</i>	4 (0.6)	ND	14 (1.5)
<i>Entamoeba coli</i>	3 (0.4)	ND	20 (2.2)
<i>Iodamoeba butschlii</i>	0	ND	3 (0.3)
<i>Hymenolepis nana</i>	0	ND	1 (0.1)
Real-time PCR, n (%)			
<i>Giardia lamblia</i>	67 (9.3)	15 (6.6)	ND
<i>Cryptosporidium parvum</i> / <i>C. hominis</i>	36 (5.0)	5 (2.2)	ND
<i>Entamoeba histolytica</i>	0	0	ND

ND, not done; TFT, Triple Faeces Test.

protozoa by real-time PCR, and the 913 cases examined for parasites only by routine microscopy. The age range of the patients was 0–95 years (median 33 years). The group examined for parasites by PCR contained significantly fewer children aged <15 years as compared to the microscopy group (18.9% vs. 36.8%; OR 0.40, 95% CI 0.33–0.50).

Information concerning travel history was supplied by the GP for 639 (44.3%) patients, including 336 subjects for whom 'no travel history' was specifically noted. Eighty-four patients had travelled in areas considered to be low-risk areas for intestinal parasite infections (western Europe, North America and Australia), while 186 had travelled in areas considered to be high-risk areas (Africa, South America and Asia, including Turkey). No increased frequency of travel was reported for the subjects examined for parasites by microscopy only (Table 1). For 33 subjects (age 0–6 years, median 1 year), faecal specimens were sent to the laboratory for routine faecal examination as part of an adoption protocol. The countries of origin of these children were often not reported by the GP.

Information concerning the gastrointestinal symptoms of the patients was supplied by the GP for 65.8% of 950 cases tested by PCR. In most (88%) cases, diarrhoea was mentioned; other symptoms mentioned were watery (11.0%), bloody (5.9%) or slimy (4.8%) faeces. According to the laboratory description, most (90.4%) unpreserved faecal samples were unformed, and the number of watery (3.9%), bloody (1.7%) or slimy (0.8%) samples was limited. Examination for *Cryptosporidium* was specifically requested by the GP for seven patients.

### Microbiology

In 127 (13.4%) of 950 cases, a non-parasite faecal pathogen was demonstrated. Routine testing revealed *Campylobacter* spp. ( $n = 90$ ), *Salmonella* spp. ( $n = 29$ ), *Shigella* spp. ( $n = 2$ ), *Yersinia* spp. ( $n = 3$ ) and *Clostridium difficile* ( $n = 3$ ). Table 1 summarises the results of routine microscopy for faecal parasites. In total, 1635 subjects were examined, either by classical microscopy ( $n = 751$ ) or by TFT ( $n = 884$ ). Although significantly fewer TFT procedures were performed in the group analysed for parasites by PCR (34.2% vs. 69.8%; OR 0.23, 95% CI 0.18–0.28), the frequencies of detection of parasites by microscopy in both groups were comparable, with the exception of *Blastocystis hominis* (2.9% vs. 7.7%; OR 2.77, 95% CI 1.68–4.56) and *Entamoeba coli* (0.4% vs. 2.2%; OR 5.36, 95% CI 1.60–18.14). *G. lamblia* was the most common parasite detected ( $n = 85$ ; 5.2%), either by TFT ( $n = 48$ , 5.4%) or by classical microscopy ( $n = 37$ , 4.9%; OR 0.90, 95% CI 0.58–1.40). TFT also revealed 25 (2.8%) *Dientamoeba fragilis* infections. Except for one sample with *Hymenolepis nana*, no helminths were seen in any of the faecal samples examined, and only one patient infected with *Cryptosporidium* spp. was detected.

### PCR results

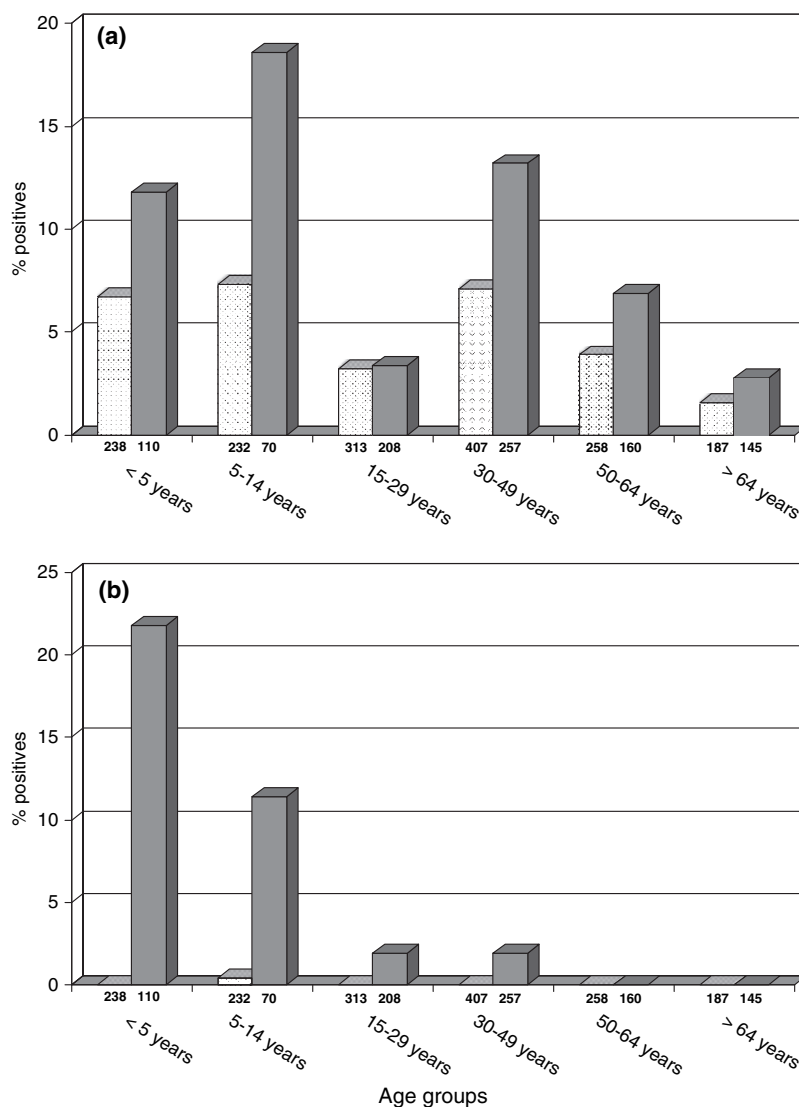
Amplification of the PhHV-1 internal control was, by definition, detected within the correct Ct range for all 950 DNA samples (Ct 29.1–36.9, median 31.7). No *E. histolytica*-specific amplification products were detected.

A *G. lamblia*-specific amplification product was seen for 82 (8.6%) of 950 samples (Ct 21.1–39.1, median 28.6). In the subgroup analysed by both microscopy and PCR, the prevalence of *G. lamblia*

increased from 5.7% to 9.3% (Table 1). All but one of 41 samples in which *G. lamblia* was detected by microscopy showed a *G. lamblia*-specific amplification product (Ct 21.1–32.8, median 27.5). The discrepant sample (from a male aged 61 years with watery diarrhoea) contained cysts of both *G. lamblia* and *Endolimax nana* when investigated by classical microscopy, with no other pathogens being detected following culture. A *G. lamblia*-specific amplification product was obtained for 16 (3.6%) and 11 (4.8%) of the samples in which *G. lamblia* was not found by classical microscopy or the TFT procedure, respectively. Ct values were significantly higher for those samples in which *G. lamblia* was not detected by microscopy (Ct median 33.0, range 23.2–39.1;  $p < 0.001$ ). Furthermore, 15 (6.5%) samples yielded a *G. lamblia*-specific amplification product, despite the fact that no microscopical examination for intestinal parasites was requested (Table 1). No significant differences in the Ct values were noted for those samples that were examined microscopically and those that were not.

Compared with microscopy, an increased prevalence of *G. lamblia* infection was revealed by PCR for all age groups, with the exception of patients aged 15–30 years (Fig. 1a). The same pattern was seen when the microscopy and PCR data for *G. lamblia* were compared for the 720 subjects analysed by both procedures (data not shown). No associations were seen between *G. lamblia* infection and stool consistency or travel history, with the exception of 11 children who were screened according to the adoption protocol, five (45.5%) of whom yielded a *G. lamblia*-specific amplification product. In comparison, *G. lamblia* infection was indicated in five (22.7%) of 22 children examined by microscopy only.

A *Cryptosporidium*-specific amplification product was obtained from 41 (4.3%) of 950 DNA samples (Ct 24.5–37.9, median 31.0), including two samples in which *G. lamblia* and *Cryptosporidium* were detected simultaneously. *Cryptosporidium* was also detected in five (2.2%) cases for which no microscopical examination was requested by the GP (Table 1). Infections with *Cryptosporidium* were associated strongly with the age of the patients, with detectable *Cryptosporidium* DNA in 21.8% of children aged <5 years (Fig. 1b). No association was seen between *Cryptosporidium*



**Fig. 1.** Prevalence of (a) *Giardia lamblia* and (b) *Cryptosporidium parvum/Cryptosporidium hominis* in different age groups, detected either by microscopy (hatched bars;  $n = 1635$ ) or real-time PCR (solid bars;  $n = 950$ ). The number of cases examined in each category is indicated below each bar.

infection and travel history. *Cryptosporidium* was detected more often in patients complaining of watery diarrhoea (seven of 72; 9.7%), but this trend was not significant.

Additional PCRs for differentiation of *C. parvum* and *C. hominis* identified 29 samples that contained *C. hominis* and nine samples that contained *C. parvum*; no amplification product was obtained from three samples. *C. parvum* and *C. hominis* were distributed almost equally among the positive samples collected in July, while *C. hominis* was detected exclusively in the samples collected during September (data not shown).

Double infections with parasites and non-parasitic pathogens involved *G. lamblia* and *Campylobacter* spp. ( $n = 3$ ), and *Cryptosporidium* and *Campylobacter* spp. ( $n = 1$ ).

## DISCUSSION

A multiplex real-time PCR has been described previously for the simultaneous detection of *E. histolytica*, *G. lamblia* and *Cryptosporidium* spp. DNA in faecal samples [12]. In the present study, the results obtained using this multiplex real-time PCR assay were compared retrospectively with the results obtained by routine microscopy in clinical laboratory practice for patients with diarrhoea who consulted their GP. Faecal DNA samples examined in the present study were isolated initially from a selected number of patients, based on the results of bacterial culture. To evaluate the possibility of selection bias, the microscopy data were compared with those for 913 additional subjects, also suffering from

gastrointestinal problems, but with suspected parasitic infections. As expected, the latter group included more children. However, no significant differences in travel history or the number of parasitic infections detected by microscopy were revealed, with the exception of two non-pathogens (*B. hominis* and *E. coli*). In general, pathogenic parasites were detected during routine microscopy in a low number of cases, and with the exception of a single case involving *H. nana*, no helminths were seen. With a detection rate of 5.2% ( $n = 1635$ ), *G. lamblia* was the most common pathogenic enteric parasite found in patients diagnosed using microscopy. This finding agrees with other studies of *G. lamblia* infection in The Netherlands, including data from a large Dutch case-control study in which *G. lamblia* was detected in 5.4% of patients who consulted their GP for gastroenteritis [3,17]. Using real-time PCR ( $n = 950$  cases), the rate of detection of *G. lamblia* increased to 8.6%, and the number of infected cases was more than double in children of school age. In addition, very high rates of *G. lamblia* infection were found in adopted foreign children, who had presumably been exposed in their country of origin [18].

Although no specific request for the diagnosis of *Cryptosporidium* infection was made by the GP, an overall prevalence of 4.3% was detected by PCR, with one in five children aged <5 years being *Cryptosporidium*-positive. Among the infected children, no predisposition towards *C. parvum* or *C. hominis* was noticed. However, a sudden increase in cases positive for *C. hominis* was detected in September 2005, whereas *C. parvum* was detected only in July and August. Seasonal variation in the incidence of cryptosporidiosis related to travel or environmental factors has been suggested [19–21], but further investigation is needed to unravel the specific underlying factors explaining this complex epidemiology.

*E. histolytica* was not detected by real-time PCR in the present study. However, cases of amoebiasis in The Netherlands are very limited, and are always directly or indirectly related to travel in high-risk areas. Nevertheless, accurate diagnosis of *E. histolytica* infection is vital in order to interrupt transmission of the parasite and to avoid any progression into invasive disease. Furthermore, PCR enables specific detection of *E. histolytica*, thereby differentiating this species

from the non-pathogenic species *E. dispar*, which is more common and is morphologically identical to *E. histolytica*.

No relationship was found between specific pathogens and the patient's symptoms, as the latter tended to be non-specific and vague, making appropriate diagnostic requests difficult. In faecal samples for which only bacterial culture was requested, real-time PCR revealed 20 parasitic infections. Screening all faecal specimens for parasites in a routine diagnostic laboratory would therefore be appropriate.

The specificity of PCR obviously restricts the number of different pathogens that can be detected, in contrast to the broad range of different parasites that can be detected using microscopy. Nevertheless, microscopy showed limited additional value in this patient population, even with the use of the TFT procedure, as most of the additional parasites detected were non-pathogenic. These included *D. fragilis* and *B. hominis*, two organisms whose pathogenicity is still unresolved. *D. fragilis* is one of the priority candidates for inclusion in an expanded multiplex real-time PCR, as such detection would also provide a valuable tool to further elaborate its possible pathogenicity.

The present study focused on the detection of *E. histolytica*, *G. lamblia* and *C. parvum*/*C. hominis* in subjects with community-acquired diarrhoea in a developed country. The diagnostic value of this multiplex real-time PCR should also be evaluated in additional patient groups, particularly travellers returning from the tropics and immunocompromised individuals in whom additional intestinal parasites might be expected. The possibility of combining alternative parasite targets or panels within a multiplex real-time PCR should be investigated, e.g., the detection of *Cyclospora cayentanensis*, *E. histolytica* and *G. lamblia* in travellers, or the detection of microsporidia, *Cryptosporidium* and *Isospora belli* in immunocompromised patients. Used in combination with a similar approach for the detection of diarrhoea-causing viruses and bacteria, this would give a completely new alternative for the laboratory diagnosis of diarrhoeal disease. However, until these approaches have been further evaluated and validated, conventional diagnostic techniques, e.g., culture, microscopy and antigen/antibody detection, will still have a prominent role in the diagnosis of diarrhoeal disease.

In conclusion, the present study revealed that significant numbers of *G. lamblia* and *Cryptosporidium* infections remain undetected by microscopy in patients with gastrointestinal symptoms who consult their GP. Furthermore, the number of additional parasites detected with microscopy was shown to be limited in this population. Therefore, the introduction of real-time PCR for the routine detection of diarrhoea-causing protozoa would improve the diagnostic efficiency of laboratories dealing with faecal samples from this patient group.

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