Parasitol Res (2013) 112:595–601
DOI 10.1007/s00436-012-3171-8

ORIGINAL PAPER

Use of multiplex real-time PCR for detection of common diarrhea causing protozoan parasites in Egypt

John T. Nazeer · Khalifa El Sayed Khalifa · Heidrun von Thien · Mahmoud Mohamed El-Sibaei · Magda Youssef Abdel-Hamid · Ranya Ayman Samir Tawfik · Egbert Tannich

Received: 17 August 2012 / Accepted: 8 October 2012 / Published online: 1 November 2012

Abstract Diarrhea is an important cause of morbidity and mortality, worldwide. *Giardia intestinalis*, *Cryptosporidium* spp., and *Entamoeba histolytica* are the most common diarrhea-causing parasitic protozoa. Diagnosis of these parasites is usually performed by microscopy. However, microscopy lacks sensitivity and specificity. Replacing microscopy with more sensitive and specific nucleic acid based methods is hampered by the higher costs, in particular in developing countries. Multiplexing the detection of more than one parasite in a single test by real-time polymerase chain reaction (PCR) has been found to be very effective and would decrease the cost of the test. In the present study, stool samples collected from 396 Egyptian patients complaining of diarrhea along with 202 faecal samples from healthy controls were examined microscopically by direct smear method and after concentration using formol-ethyl acetate. Frozen portions of the same samples were tested by multiplex real-time for simultaneous detection of *E. histolytica*, *G. intestinalis*, and *Cryptosporidium* spp. The results indicate that among diarrheal patients in Egypt *G. intestinalis* is the most common protozoan parasite, with prevalence rates of 30.5 and 37.1 %, depending on the method used (microscopy vs. multiplex real-time PCR). *Cryptosporidium* spp. was detected in 1 % of the diarrheal patients by microscopy and in 3 % by real-time PCR. While *E. histolytica*/*dispar* was detected in 10.8 % by microscopy, less than one fifth of them (2 %) were found true positive for *Entamoeba dispar* by real-time PCR. *E. histolytica* DNA was not detected in any of the diarrheal patients. In comparison with multiplex real-time PCR, microscopy exhibited many false positive and negative cases with the three parasites giving sensitivities and specificities of 100 and 91 % for *E. histolytica*/*dispar*, 57.8 and 85.5 % for *G. intestinalis*, and 33.3 and 100 % for *Cryptosporidium* spp.

J. T. Nazeer · K. El Sayed Khalifa (✉) · M. M. El-Sibaei · M. Y. Abdel-Hamid · R. A. S. Tawfik
Department of Parasitology, Faculty of Medicine,
Ain Shams University,
Abbassia,
11566 Cairo, Egypt
e-mail: kelkhalifa@hotmail.com

H. von Thien · E. Tannich
Department of Molecular Parasitology,
Bernhard Nocht Institute for Tropical Medicine,
Hamburg, Germany

Introduction

Diarrhea is one of the main causes of morbidity and mortality in the world in particular in children in developing countries (WHO 2005). The etiological agents of diarrhea include viruses, bacteria, and parasites (Thielman and Guerrant 2004). *Entamoeba histolytica*, *Giardia intestinalis*, and *Cryptosporidium* species are the most common diarrhea-causing protozoan parasites, which induce at least in part indistinguishable clinical presentations (Walsh 1986). The WHO estimates that ~50 million people worldwide suffer from invasive amebic infection each year, resulting in 40,000–100,000 deaths annually (WHO 1997; Diamond and Clark 1993; Petri et al. 2000). *G. intestinalis* is the most common protozoan infection of the intestinal tract worldwide. Each year, 500,000 new cases are reported and about 200 million people develop symptomatic giardiasis (Minvielle et al. 2004). Cryptosporidiosis is a frequent cause of diarrheal disease in humans, in particular in immunocompromised patients. Moreover, *Cryptosporidium* infections occur in children younger than 5 years of age, with a peak in children younger than 2 years of age (Tumwine et al. 2003; Steinberg et al. 2004).

In Egypt, the three parasites are widely prevalent. Prevalence rates ranging from 16.2 to 57.1 % were recorded for *E. histolytica*/*dispar* (El-Kadi et al. 2006; Abd-Alla and Ravdin 2002; Abdel-Hafeez et al. 2012). Rates varying between 10.0 and 34.6 % were recorded for *G. intestinalis* (El Naggar et al. 2006; Foronda et al. 2008; Baiomy et al. 2010; Abdel-Hafeez et al. 2012). For *Cryptosporidium* species, several studies reported prevalence rates ranging from less than 5.0–31.1 % (Ibrahim et al. 1997; Abdel-Messih et al. 2005; Mousa et al. 2010; Abdel Kader et al. 2012).

Knowledge of the etiology of diarrhea is important for epidemiological surveillance and for correct treatment. Traditionally, the three parasites have been identified by simple microscopic methods. As expertise in stool microscopy is waning and multiple sampling, species-specific concentration and staining methods are needed to improve its performance, many of the infections were missed and some are overestimated due to similarities in morphologies as in the case of the *E. histolytica*/*dispar*/*moshkovskii* complex (Hamzah et al. 2010). Alternative approaches have been developed to improve the diagnosis of enteric parasitic diseases, including visualization by fluorescent-labeled antibodies and copro-antigen-detection assays, but many of these tests still lack sensitivity and specificity (Murray and Capello 2008; Ndao 2009). Meanwhile, polymerase chain reaction (PCR) methods for detecting intestinal parasites are increasingly available and exhibit excellent sensitivity and specificity compared to conventional methods such as microscopy and antigen detection assays (Webster et al. 1996; Sanuki et al. 1997; Haque et al. 1998; Fisher et al. 1998; Ghosh et al. 2000; Stark et al. 2008).

Real-time PCR (RT-PCR) is a very attractive technique for laboratory diagnosis of infectious diseases, as the methodology does not require post-PCR downstream analysis, leading to shorter turnaround times. Moreover, RT-PCR substantially reduces the risk of amplicon contamination of the laboratory and decreases the cost for reagents (Klein 2002). In addition, real-time PCR is a quantitative method that allows the determination of the parasite burden. Several real-time PCR assays have been developed to detect the common enteric protozoan *parasites* (Blessmann et al. 2002; Verweij et al. 2003; Qvarnstrom et al. 2005; Calderaro et al. 2010; Hadfield et al. 2011). A multiplex real-time PCR for the simultaneous detection of *E. histolytica*, *G. intestinalis*, and *Cryptosporidium parvum* in fecal samples was recently described (Verweij et al. 2004; Haque et al. 2007; ten Hove et al. 2007). The assay has been found to be quite sensitive and specific and is able to detect each parasite individually.

In this study, the multiplex real-time PCR, as a single test tube assay, was used for the detection of *E. histolytica*, *G. intestinalis*, and *Cryptosporidium* spp. among Egyptian patients complaining of diarrhea and in a representative control group. The results were compared with those obtained by routine microscopy.

Material and methods

Fecal specimens

A case–control study was conducted in which fresh stool samples were collected from 396 patients complaining of diarrhea and 202 apparently healthy individuals. The samples were collected over a period of 1 year from October 2010 to October 2011 in Cairo and the Egyptian governorates Fayoum and Benha, respectively. The patients and the healthy controls aged between 6 months and 60 years.

Microscopy

After collection, stool samples were divided into two portions; the first portion was preserved frozen at −20 °C for further processing by real-time PCR, the second portion was examined microscopically by direct saline and/or iodine mounts and after concentration by formol-ethyl acetate technique. Modified Ziehl–Neelsen was performed on direct fresh smears as well as on formol-ethyl acetate concentrates to detect *Cryptosporidium* oocysts.

DNA extraction

Of the stool sample, 0.2 g was used for extraction of DNA using the QIAamp DNA stool mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. *E. histolytica* control DNA was obtained from an axenic culture of the strain HM-1: IMSS. *G. intestinalis* DNA was isolated from purified cysts and *C. parvum* DNA was isolated from the purified oocysts. In each sample, 10^3 PFU of phocin herpesvirus 1 (PhHV-1) per milliliter was added to the isolation lysis buffer to serve as an internal control (Niesters 2002).

Primers and probes

The primers and probes described by Verweij et al. (2004) were used in the present study. They were all purchased from Eurofins MWG Operon, Germany. For *E. histolytica* and *Entamoeba dispar*, the primers Ehd-38F (5′-ATTGTCGTGGCATCCTAACTCA-3′) and Ehd-88R (5′-GCGGACGGCTCAT TATAACA-3′) targeting the small subunit of ribosomal RNA gene (SSU rRNA) of both *E. histolytica* and *E. dispar* such that to amplify a 172-bp fragment inside the gene. The Taqman probes; the minor groove binding (MGB) probe histolytica-96 T (JOE-5′-TCATTGAATGAATTGGCCATTT-3′-BHQ1) and the

MGB probe dispar-96 T (Cy5-5′-TTACTTACA-TAAATTGGCCACTTTG-3′-BHQ2; Verweij et al. 2003), specifically detect the *E. histolytica* and *E. dispar* amplification products, respectively.

For *G. intestinalis*, the primers Giardia-80F (5′-GACGGCTCAGGA CAACGGTT-3′) and Giardia-127R (5′-TTGCCAGCGGTGT CCG-3′) targeting SSU rRNA such that to amplify a 62-bp fragment inside the gene. The Taqman probe double-labeled Giardi-105 T (Fam-5′-CCCGCGGCGGTCCCTGCTAG-3′-Tamra) specifically detects the amplification products.

For *C. parvum*, the primers designed by Fontaine and Guillot (2002) were used. The primers CrF (5′-CGCTTCTCTAGCCTTTCATGA-3′) and CrR (5′-CTTCACGTGTGTTTGCC AT-3′) targeting the genomic DNA sequence such that to amplify a 138-bp fragment inside the *C. parvum*-specific 452-bp fragment. The double-labeled probe Crypto (Rox-5′-CCAATCACA-GAATCATCAGAATCGACTGGTATC-3′-BHQ2) specifically detects the amplification product.

As an internal control to detect possible PCR inhibition of amplification by stool contents, a specific primer and probe set, consisted of a forward primer PhHV-267s (5′-GGGCGAATCACAGATTGAATC-3′), a reverse primer PhHV-337as (5′-GCGGTTCCAAACGTACCAA-3′), and the specific double-labeled probe PhHV-305tq (Cy5.5-5′-TTTTTATGTGTCCGCCACCATCTGGATC-3′-BBQ), targeting PhHV-1 were included with each run.

PCR amplification and detection

Amplification reactions were performed in a volume of 25 μL with Qiagen HotstarTaq master mix, 5 mM $MgCl_2$, 3.125 pmol of each *E. histolytica*/*dispar*-specific primers, 3.125 pmol of each *G. intestinalis*-specific primers, 12.5 pmol of each *C. parvum*-specific primers, 1 pmol of each PhHV-1-specific primers, 4.375 pmol of *E. histolytica*-specific MGB-TaqMan probe, 4.375 pmol of *E. dispar*-specific MGB-TaqMan probe, 0.25 pmol of *G. intestinalis*-specific double-labeled probe, 4.375 pmol of *C. parvum*-specific double-labeled probe, and 1.25 pmol of PhHV-1-specific double-labeled probe. Amplification consisted of 3 min at 95 °C followed by 40 cycles of 30 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C. Fluorescence was measured during the annealing step of each cycle. Amplification, detection, and data analysis were performed with the Rotor gene 6000 real-time detection system.

Results

Table 1 shows the comparison of direct microscopy versus formol ethyl-acetate concentration technique for detection of *E. histolytica*/*dispar*, *G. intestinalis*, and *Cryptosporidium* spp. *among* 396 diarrheal fecal samples along with 202 fecal samples from healthy controls. While no significant difference was found between the two techniques for the detection of *G. intestinalis* and *Cryptosporidium* spp., formol ethyl-acetate concentration exhibited higher significant difference for detection of *E. histolytica*/*dispar* infections.

The results of examining diarrheal fecal samples along with fecal samples from healthy controls either by microscopy after formol ethyl-acetate concentration or multiplex real-time PCR for the simultaneous detection of *E. histolytica*, *E. dispar*, *G. intestinalis*, and *Cryptosporidium* spp. are shown in Table 2. Among diarrheal cases, microscopy detected *G. intestinalis* as mono infection in 110 (27.8 %) and as combined infection with *E. histolytica*/*dispar* in 11 cases (2.8 %). Sole *E. histolytica*/*dispar* and *Cryptosporidium* spp. infections were detected in 32 (8 %) and 4 (1 %) cases, respectively. Two hundred thirty-nine cases (60.4 %) were found negative for the three protozoan parasites

Table 1 Comparison of direct smear versus formol ethyl-acetate concentration technique for the detection of enteric protozoa among 396 patients with diarrhea and 202 control subjects

Organism	Direct smear				Formol ethyl-acetate concentration			
	Diarrheal cases		Control		Diarrheal cases		Control	
	No	%	No	%	No	%	No	%
E. histolytica/*dispar*	20	5.1	0	0	32	8.0	0	0
G. intestinalis	115	29	1	0.5	110	27.8	1	0.5
Cryptosporidium spp.	2[a]	0.5	0	0	4[a]	1.0	0	0
Combined *Giardia* and *E. histolytica*/*dispar*	3	0.76	0	0	11	2.8	0	0
Combined *Giardia* and *Cryptosporidium* spp.	0	0	0	0	0	0	0	0
Combined *E. hist*/*dispar Giardia*/*Cryptosporidium*	0	0	0	0	0	0	0	0
Negative for the three parasites	256	64.6	201	99.5	239	60.4	201	99.5

[a] As examined by modified Ziehl–Neelsen stain

Table 2 Results of microscopy and multiplex real-time PCR for the detection of enteric protozoa among 396 patients with diarrhea and 202 control subjects

Organism	Microscopy[a]				Multiplex real-time PCR			
	Diarrheal cases		Control		Diarrheal cases		Control	
	No	%	No	%	No	%	No	%
E. histolytica/*dispar*	32	8.0	0	0	5[b]	1.3	1[c]	0.5
G. intestinalis	110	27.8	1	0.25	141	35.6	4	2.0
Cryptosporidium spp.	4	1.0	0	0	8	2.0	3	1.5
Combined *Giardia* and *E. histolytica*/*dispar*	11	2.8	0	0	2[b]	0.5	0	0
Combined *Giardia* and *Cryptosporidium* spp.	0	0	0	0	3	0.75	0	0
Combined *E. hist*/*dispar Giardia*/*Cryptosporidium*	0	0	0	0	1[b]	0.25	0	0
Negative for the three parasites	239	60.4	201	99.75	236	59.6	194	96.0

[a] After formol ethyl-acetate concentration

[b] All the eight real-time positive-*Entamoeba* cases among the group of diarrheal patients were of the nonpathogenic *E. dispar*

[c] This is the only case found positive for *E. histolytica* by multiplex real-time PCR

suggesting other etiologies for diarrhea. In total, microscopy detected *G. intestinalis* in 30.5 % (121 out of 396), *E. histolytica*/*dispar* in 10.8 % (43 out of 396), and *Cryptosporidium* spp. in 1 % (4 out of 396) of the diarrheal cases. Among the control subjects, *G. intestinalis* was detected in only one case (0.25 %) whereas *E. histolytica*/*dispar* and *Cryptosporidium* spp. were not detected in any of the control subjects.

Within the 396 fecal samples from patients with diarrhea, multiplex real-time PCR revealed mono-infections with *G. intestinalis*, *E. dispar*, and *Cryptosporidium* spp. in 141 (35.6 %), 5 (1.26 %), and 8 (2.0 %) of the cases, respectively. In addition, six combined infections were identified comprising two (0.5 %) cases with *G. intestinalis* and *E. dispar*, three (0.75 %) cases with *G. intestinalis* and *Cryptosporidium* spp., and one (0.25 %) case containing all three protozoan parasites. It is noteworthy that *E. histolytica* was not detected in any of the diarrheal patients. Within the 202 controls, *G. intestinalis* was present in four (2.0 %), *E. histolytica* in one (0.5 %), and *Cryptosporidium* spp. in three (1.5 %) of the individuals investigated. It is worth noting that the only case of *E. histolytica* detected by real-time PCR was among the control subjects, whereas all the eight *Entamoeba* cases detected among the diarrheal patients were of the nonpathogenic *E. dispar*.

The validity of microscopy compared to multiplex real-time PCR for the detection of the three protozoan parasites is shown in Table 3. Sensitivities varied from 33.3 % for *Cryptosporidium* spp. to 57.8 % for *G. intestinalis* and 100 % for *E. dispar*. Specificities varied from 85.5 % for *G. intestinalis* to 91 % for *E. dispar*, and 100 % for *Cryptosporidium* spp. Positive and negative predictive values varied from 18.6 to 100 % as shown in Table 3. Out of the 43 microscopy-positive *E. histolytica*/*dispar* samples, only eight were true *E. dispar* by real-time PCR. All of the 353 cases microscopically negative for *E. histolytica*/*dispar* were also negative by real-time PCR. Out of the 121 microscopy-positive *G. intestinalis* samples, only 85 were true positive by real-time PCR. Out of the 275 cases microscopically negative for *G. intestinalis*, 62 were positive by

Table 3 Sensitivity, specificity, positive and negative predictive values of microscopy compared to multiplex real-time PCR for detection of *E. histolytica*/*dispar*, *G. intestinalis*, and *Cryptosporidium* spp. in the stool of 396 diarrheal patients

Microscopy[a]	Multiplex real-time PCR					
	E. histolytica/*dispar*		*G. intestinalis*		*Cryptosporidium* spp.	
	Positive	Negative	Positive	Negative	Positive	Negative
Positive	8	35	85	36	4	0
Negative	0	353	62	213	8	386
Sensitivity	100 %		57.8 %		33.3 %	
Specificity	91 %		85.5 %		100 %	
PPV	18.6 %		70.2 %		100 %	
NPV	100 %		77.5 %		98 %	

PPV positive predictive value, *NPV* negative predictive value

[a] After formol ethyl-acetate concentration

real-time PCR. Out of the 394 microscopically negative *Cryptosporidium* samples, eight were found to be true positive by real-time PCR. All the four microscopically positive *Cryptosporidium* samples were also positive by real-time PCR. Comparison of the prevalence of the different parasites between the three study sites within Egypt indicated no significant differences (Table 4).

Discussion

Diarrhea is an important cause of morbidity and mortality in the world. *G. intestinalis*, *Cryptosporidium* spp., and *E. histolytica* are the most common diarrhea-causing parasitic protozoa (Pierce and Kirkpatrick 2009). Microscopy is the most commonly used method for the routine diagnosis of these parasites in developing countries, however, microscopy lack sensitivity and specificity (Utzinger et al. 2010). Replacing microscopy with the more sensitive and specific nucleic acid-based methods is hampered by the cost in developing countries. Multiplexing the detection of more than one parasite in a single test by real-time PCR has been found to be very effective (Verweij et al. 2004; Haque et al. 2007; ten Hove et al. 2007; Amar et al. 2007; Stark et al. 2011; Bruijnesteijn van Coppenraet et al. 2009; Taniuchi et al. 2011). Adopting such approached in developing countries, at least in reference laboratories, would decrease the cost and ensure rapid and accurate diagnosis of diarrheal causing protozoa. In the present study, stool samples collected from 396 patients complaining of diarrhea and 202 apparently healthy controls were tested by microscopy in Egypt. Frozen portions of the same samples were tested by multiplex real-time PCR in the Department for Molecular Parasitology at The Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany, which represents the German National Reference Centre for tropical infections.

The results indicate that among diarrheal patients in Egypt *G. intestinalis* is the most common protozoan parasite, with prevalence rates of 30.5 and 37.1 %, depending of the method used (microscopy vs. multiplex real-time PCR). This is in accordance with many studies form developing and developed countries indicating that *G. intestinalis* is the most common protozoan parasite-causing diarrhea (Thompson et al. 2000; de Wit MAS et al. 2001; Stark et al. 2009; Foronda et al. 2008; El Naggar et al. 2006; Sabry et al. 2009). Comparing microscopy with real-time PCR revealed that microscopy resulted in many false-negative and positive results, giving lower sensitivity, specificity as well as positive and negative predictive values. The lower sensitivity and specificity of microscopy compared to conventional and real-time PCR has already being reported by a number of other studies (Ghosh et al. 2000; Verweij et al. 2003; ten Hove et al. 2007; Calderaro et al. 2010; Stark et al. 2011).

Microscopy showed lower sensitivity of 33.3 % when compared with real-time PCR for detection of *Cryptosporidium* spp. The parasite was detected in 1 % of the diarrheal patients by microscopy and in 3 % by real-time PCR. Several studies have previously demonstrated that PCR has superior sensitivity for detection of *Cryptosporidium* spp. when compared to conventional staining and microscopy (Morgan et al. 1998; Kaushik et al. 2008; Stark et al. 2011).

While *E. histolytica*/*dispar* was detected in 10.8 % by microscopy. Less than one fifth of them (2 %) were found true positive for *E. dispar* by real-time PCR. *E. histolytica* DNA was not detected in any of the diarrheal patients. As *E. dispar* is nonpathogenic, the presence of this DNA in the stool of diarrheal cases is indicative for an association rather than being the etiologic agent. Comparison between microscopy and real-time PCR for *E. histolytica*/*dispar* indicates that microscopy exhibited many false positive results giving a low positive predicative value of 18.6 %. This may be due to misdiagnosis of other *Entamoeba* species such as *Entamoeba coli*, *Entamoeba hartmanni*, or the morphologically identical *Entamoeba moshkovskii*. (Verweij et al. 2003; González-Ruiz et al. 1994; Tanyuksel and Petri 2003; Hamzah et al. 2010). These findings confirm the limitation of microscopy for the differentiation of the various *Entamoeba* spp. and calling into question prevalence rates of *E. histolytica* previously recorded on the basis of microscopy.

In conclusion, the present study has shown that the implementation of multiplex real-time PCR for the simultaneous detection target DNA in a closed-tube system would be beneficial for the rapid and accurate diagnosis of

Table 4 Prevalence of *E. histolytica*/*dispar*, *G. intestinalis*, and *Cryptosporidium* spp. in diarrheal patients form three Egyptian Governorates

Governorate (number examined)	*E. histolytica*/*dispar*		*G. intestinalis*		*Cryptosporidium* spp.	
	Microscopy (%)[a]	PCR (%)	Microscopy (%)[a]	PCR (%)	Microscopy (%)[a]	PCR (%)
Cairo (306)	28 (9.2)	4 (1.3)	96 (31.4)	83(27.1)	4 (1.3)	11 (3.6)
Benha (36)	4 (11.1)	0 (0)	7 (19.4)	6 (16.7)	0 (0)	1 (2.8)
Fayoum (54)	11 (20.4)	4 (7.4)	18 (33.3)	16 (29.6)	0 (0)	0 (0)

[a] After formol ethyl-acetate concentration

common diarrhea-causing protozoa. For developing countries, although the reagents costs are still high compared to microscopy, multiplex real-time PCR not only simplifies the detection of several enteric pathogens, but also reduces the cost of unnecessary treatment following misdiagnosis. Pooling of samples to a reference laboratory would reduce the running cost of the test.

Acknowledgments This work was done for partial fulfillment of the MD in Medical Parasitology of John T. Nazeer. John T. Nazeer was financially supported by the Institute for Quality Assurance in Laboratory Medicine "INSTAND e.v.", Dusseldorf, Germany.

References

Abd El Kader NM, Blanco MA, Ali-Tammam M, Abd El Ghaffar Ael R, Osman A, El Sheikh N, Rubio JM, de Fuentes I (2012) Detection of *Cryptosporidium parvum* and *Cryptosporidium hominis* in human patients in Cairo, Egypt. Parasitol Res 110(1):161–166

Abd-Alla MD, Ravdin JI (2002) Diagnosis of amoebic colitis by antigen capture ELISA in patients presenting with acute diarrhoea in Cairo, Egypt. Trop Med Int Health 7(4):365–370

Abdel-Hafeez EH, Ahmad AK, Ali BA, Moslam FA (2012) Opportunistic parasites among immunosuppressed children in Minia District, Egypt. Korean J Parasitol 50(1):57–62

Abdel-Messih IA, Wierzba TF, Abu-Elyazeed R, Ibrahim AF, Ahmed SF, Kamal K, Sanders J, Frenck R (2005) Diarrhea associated with *Cryptosporidium parvum* among young children of the Nile River Delta in Egypt. J Trop Pediatr 51:154–159

Amar CF, East CL, Gray J, Iturriza-Gomara M, Maclure EA, McLauchlin J (2007) Detection by PCR of eight groups of enteric pathogens in 4,627 faecal samples: re-examination of the English case–control Infectious Intestinal Disease Study (1993–1996). Eur J Clin Microbiol Infect Dis 26:311–323

Baiomy AM, Mohamed KA, Ghannam MA, Shahat SA, Al-Saadawy AS (2010) Opportunistic parasitic infections among immunocompromised Egyptian patients. J Egypt Soc Parasitol 40(3):797–808

Blessmann J, Buss H, Nu PA, Dinh BT, Ngo QT, Van AL, Alla MD, Jackson TF, Ravdin JI, Tannich E (2002) Real-time PCR for detection and differentiation of *Entamoeba histolytica* and *Entamoeba dispar* in fecal samples. J Clin Microbiol 40:4413–4417

Bruijnesteijn van Coppenraet LE, Wallinga JA, Ruijs GJ, Bruins MJ, Verweij JJ (2009) Parasitological diagnosis combining an internally controlled real-time PCR assay for the detection of four protozoa in stool samples with a testing algorithm for microscopy. Clin Microbiol Infect 15:869–874

Calderaro A, Gorrini C, Montecchini S, Peruzzi S, Piccolo G, Rossi S, Gargiulo F, Manca N, Dettori G, Chezzi C (2010) Evaluation of a real-time polymerase chain reaction assay for the laboratory diagnosis of giardiasis. Diagn Microbiol Infect Dis 66:261–267

de Wit MAS, Koopmans MPG, Kortbeek LM, van Leeuwen NJ, Vinje J, van Duynhoven YTHP (2001) Etiology of gastroenteritis in sentinel general practices in The Netherlands. Clin Infect Dis 33:280–288

Diamond LS, Clark CG (1993) A redescription of *Entamoeba histolytica* Schaudinn 1903 (emended Walker 1911) separating it from *Entamoeba dispar* (Brumpt 1925). J Euk Microbiol 40:340–344

El-Kadi MA, Dorrah AO, Shoukry NM (2006) Patients with gastrointestinal complains due to enteric parasites, with reference to *Entamoeba histolytica*/*dispar* as detected by ELISA *E. histolytica* adhesion in stool. J Egypt Soc Parasitol 36(1):53–64

El-Naggar SM, El-Bahy MM, Abd Elaziz J, El-Dardiry MA (2006) Detection of protozoal parasites in the stools of diarrheic patients using different techniques. J Egypt Soc Parasitol 36(1):7–22

Fischer P, Taraschewski H, Ringelmann R, Eing B (1998) Detection of *Cryptosporidium parvum* in human feces by PCR. Tokai J Exp Clin Med 23(6):309–311

Fontaine M, Guillot E (2002) Development of a TaqMan quantitative PCR assay specific for *Cryptosporidium parvum*. FEMS Microbiol Lett 214:13–17

Foronda P, Bargues MD, Abreu-Acosta N, Periago MV, Valero MA, Valladares B, Mas-Coma S (2008) Identification of genotypes of *Giardia intestinalis* of human isolates in Egypt. Parasitol Res 103:1177–1181

Ghosh S, Debnath A, Sil A, De S, Chattopadhyay DJ, Das P (2000) PCR detection of *Giardia lamblia* in stool: targeting intergenic spacer region of multicopy rRNA gene. Molecular and Cellular Probes 14(3):181–189

González-Ruiz A, Haque R, Aguirre A, Castañón G, Hall A, Guhl F, Ruiz-Palacios G, Miles MA, Warhurst DC (1994) Value of microscopy in the diagnosis of dysentery associated with invasive *Entamoeba histolytica*. J Clin Pathol 47(3):236–239

Hadfield SJ, Robinson G, Elwin K, Chalmers RM (2011) Detection and differentiation of *Cryptosporidium* spp. in human clinical samples by use of real-time PCR. J Clin Microbiol 49(3):918–924

Hamzah Z, Petmitr S, Mungthin M, Leelayoova S, Chavalitshewinkoon-Petmitr P (2010) Development of multiplex real-time polymerase chain reaction for detection of *Entamoeba histolytica*, *Entamoeba dispar*, and *Entamoeba moshkovskii* in clinical specimens. AmJTrop Med Hyg 83(4):909–913

Haque R, Ali IKM, Akther S, Petri WA Jr (1998) Comparison of PCR, isoenzyme analysis, and antigen detection for diagnosis of *Entamoeba histolytica* infection. J Clin Microbiol 36:449–452

Haque R, Roy S, Siddique A, Mondal U, Rahman SMM, Mondal D, Houpt E, Petri WA Jr (2007) Multiplex real-time PCR assay for detection of *Entamoeba histolytica*, *Giardia intestinalis*, and *Cryptosporidium* spp. AmJTrop Med Hyg 76(4):713–717

Ibrahim MA, El-Ganzoury M, Khalifa KE, Gazala W (1997) Cryptosporidiosis among children presenting with summer diarrhoea. Egypt J Paediatr 14(4):81–594

Kaushik K, Khurana S, Wanchu A, Malla N (2008) Evaluation of staining techniques, antigen detection and nested PCR for the diagnosis of cryptosporidiosis in HIV seropositive and seronegative patients. Acta Trop 107:1–7

Klein D (2002) Quantification using real-time PCR technology: applications and limitations. Trends Mol Med 8:257–260

Minvielle M, Pezzani B, De Luca M, Apezteguia M, Basualodo J (2004) Epidemiological survey of *Giardia* sp. and *Blastocystis hominis* in an Argentinian rural community. Korean J Parasitol 42:61–66

Morgan UM, Pallant L, Dwyer BW, Forbes DA, Rich G, Thompson RC (1998) Comparison of PCR and microscopy for detection of *Cryptosporidium parvum* in human fecal specimens: clinical trial. J Clin Microbiol 36:995–998

Mousa KM, Abdel-Tawab AH, Khalil HH, El-Hussieny NA (2010) Diarrhea due to parasites particularly *Cryptosporidium parvum* in great Cairo, Egypt. J Egypt Soc Parasitol 40(2):439–450

Murray TS, Cappello M (2008) The molecular diagnosis of parasitic diseases. Pediatr Infect Dis 27:163–164

Ndao M (2009) Diagnosis of parasitic diseases: old and new approaches. Interdiscip Perspect Infect Dis 2009:278246

Niesters HG (2002) Clinical virology in real time. J Clin Virol 25(suppl 3):S3–S12

Petri WA Jr, Haque R, Lyerly D, Vines RR (2000) Estimating the impact of amebiasis on health. Parasitol Today 16:320–321

Pierce KK, Kirkpatrick BD (2009) Update on human infections caused by intestinal protozoa. Curr Opin Gastroenterol 25:12–17

Qvarnstrom Y, James C, Xayavong M, Holloway BP, Visvesvara GS, Sriram R, da Silva AJ (2005) Comparison of real-time PCR protocols for differential laboratory diagnosis of amebiasis. J Microbiol 43(11):5491–5497

Sabry MA, Taher ES, Meabed EMH (2009) Prevalence and genotyping of zoonotic *Giardia* from Fayoum Governorate Egypt. Res J Parasitol 4(4):105–114

Sanuki J, Asai T, Okuzawa E, Kobayashi S, Takeuchi T (1997) Identification of *Entamoeba histolytica* and *E. dispar* cysts in stool by polymerase chain reaction. Parasitol Res 83(1):96–98

Stark D, van Hal S, Fotedar R, Butcher A, Marriott D, Ellis J, Harkness J (2008) Comparison of stool antigen detection kits to PCR for diagnosis of amebiasis. J Clin Microbiol 46(5):1678–1681

Stark D, Barratt JL, van Hal S, Marriott D, Harkness J, Ellis JT (2009) Clinical significance of enteric protozoa in the immunosuppressed human population. Clin Microbiol Rev 22:634–650

Stark D, Al-Qassab SE, Barratt JLN, Stanley K, Roberts T, Marriott D, Harkness J, Ellis JT (2011) Evaluation of multiplex tandem real-time PCR for detection of *Cryptosporidium* spp., *Dientamoeba fragilis*, *Entamoeba histolytica*, and *G. intestinalis* in clinical stool samples. J Clin Microbiol 49(1):257–262

Steinberg EB, Mendoza CE, Glass R, Arana B, Lopez MB, Mejia M, Gold BD, Priest JW, Bibb W, Monroe SS, Bern C, Bell BP, Hoekstra RM, Klein R, Mintz ED, Luby S (2004) Prevalence of infection with waterborne pathogens: a seroepidemiogic study in children 6–36 months old in San Juan Sacatepequez, Guatemala. AmJTrop Med Hyg 70:83–88

Taniuchi M, Verweij JJ, Noor Z, Sobuz SU, van Lieshout L, Petri WA Jr, Haque R, Houpt ER (2011) High throughput multiplex PCR and probe-based detection with luminex beads for seven intestinal parasites. AmJTrop Med Hyg 84(2):332–337

Tanyuksel M, Petri WA Jr (2003) Laboratory diagnosis of amoebiais. Clin Microbiol Rev 6(4):713–729

ten Hove R, Schuurman T, Kooistra M, Moller L, van Lieshout L, Verweij JJ (2007) Detection of diarrhoea-causing protozoa in general practice patients in The Netherlands by multiplex real time PCR. Clin Microbiol Infect 13:1001–1007

Thielman NM, Guerrant RL (2004) Clinical practice. Acute infectious diarrhea. N Engl J Med 350:38–47

Thompson RCA, Hopkins RM, Homan WL (2000) Nomenclature and genetic groupings of *Giardia* infecting mammals. Parasitol Today 16:210–213

Tumwine JK, Kekitinwa A, Nabukeera N, Akiyoshi DE, Rich SM, Widmer G, Feng X, Tzipori S (2003) *Cryptosporidium parvum* in children with diarrhea in Mulago Hospital, Kampala, Uganda. AmJTrop Med Hyg 68:710–715

Utzinger J, Botero-Kleiven S, Castelli F, Chiodini PL, Edwards H, Koehler N, Gulletta M, Lebbad M, Manser M, Matthys B, N'Goran EK, Tannich E, Vounatsou P, Marti H (2010) Microscopic diagnosis of sodium acetat–acetic acid–formalin-fixed stool samples for helminths and intestinal protozoa: a comparison among European reference laboratories. Clin Microbiol Infect 16:267–273

Verweij JJ, Oostvogel F, Brienen EA, Nang-Beifubah A, Ziem J, Polderman M (2003) Prevalence of *Entamoeba histolytica* and *Entamoeba dispar* in northern Ghana. Trop Med Int Health 8:1153–1156

Verweij JJ, Blange RA, Templeton K, Schinkel J, Brienen EA, van Rooyen MA, van Lieshout L, Polderman AM (2004) Simultaneous detection of *Entamoeba histolytica*, *Giardia lamblia*, and *Cryptosporidium parvum* in fecal samples by using multiplex real-time PCR. J Clin Microbiol 42:1220–1223

Walsh JA (1986) Problems in recognition and diagnosis of amebiasis: estimation of the global magnitude of morbidity and mortality. Rev Infect Dis 8:228–238

Webster KA, Smith HV, Giles M, Dawson L, Robertson LJ (1996) Detection of *Cryptosporidium parvum* oocysts in feces: comparison of conventional coproscopical methods and the polymerase chain reaction. Vet Parasitol 61:5–13

World Health Organization (1997) Amoebiasis. WHO Weekly Epi Rec 72:97–100

World Health Organization (2005) World Health Report. Making every mother and child count. World Health Organization, Geneva