The importance of Real-Time Polymerase Chain Reaction method in diagnosis of intestinal parasites in cases with diarrhea

Koltas, I.S.[1*], Elgun, G.[1], Eroglu, F.[1] and Demirkazık, M.[1]
[1]Department of Parasitology, Faculty of Medicine, Çukurova University Balcalı, 01330, Sarıçam, Adana, Turkey
*Corresponding author e-mail: koltas@cu.edu.tr

Abstract. Diarrheal diseases cause significant morbidity in developing countries, and have a potential to cause severe complications. Stool samples collected from 272 patients with diarrhea in Adana, Turkey were screened using different laboratory methods such as the wet mount examination (WME), modified trichrome stain (MTS), modified acid-fast (MAF) stain, enzyme linked immunosorbent assay (ELISA) and real-time polymerase chain reaction (real-time PCR) for intestinal parasites. Intestinal parasites were detected in 25.4% (69/272) of the patients. Of the parasites detected, *Cryptosporidium* spp. was the commonest 10.3% (28/272) followed by *Blastocystis* spp., 5.9% (16/272), *Entamoeba dispar* 2.6% (7/272), *Giardia lamblia* 2.6% (7/272), *Entamoeba coli* 1.8% (5/272), *Entamoeba histolytica* 0.7% (2/272), *Isospora belli* 0.7% (2/272), *Trichuris trichiura* 0.4% (1/272) and *Strongyloides stercoralis* 0.4% (1/272). Our study shows that there are differences in results obtained using different laboratory methods; 22.1% (60/272) with real time-PCR, 10.3% (28/272) with ELISA, 9.9% (27/272) with MTS, 6.6% (18/272) with WME, and 5.9% (16/272) with MAF stain. The differences in the level of positivity using different laboratory methods were sequenced in the diagnosis of intestinal parasites ranging from highest to lowest, as real-time PCR>ELISA>MTS> WME>MAF stain. Molecular techniques are recommended as diagnosis of choice especially in patients suffering from chronic diarrhea.

INTRODUCTION

The symptoms of intestinal parasitic infections, such as severe diarrhea, abdominal pain, and malnutrition leading to severe anemia vary depending on the type of parasitic infection (Harp, 2003). Transmission is usually via the fecal-oral route. Sources of infection include infected humans and/or animals, contaminated water (the parasites are resistant to normal water disinfection), fruits, vegetables, food washed in contaminated water, or inadequate pasteurization of milk (Current & Garcia, 1991; Smith *et al.*, 2007). Cryptosporidiosis is a common cause of diarrhea in humans and animals (Tzipori, 1983). In immuno-competent persons, *Cryptosporidium* spp. may cause short term (3-20 day) diarrheal illness that resolves spontaneously (Current & Garcia, 1991). However, in young or immunocompromised patients, crypto-sporidiosis usually presents as a life-threatening, prolonged, cholera-like illness (Davies *et al.*, 2009; Cama *et al.*, 2008). *Blastocystis* spp., causes diarrhea and intestinal manifestations such as abdominal pain, vomiting and flatulence (Gregorio *et al.*, 2007; Kaneda *et al.*, 2001; Eroglu *et al.*, 2009). *Entamoeba histolytica* (*E. histolytica*) is the predominant causative agent of human amebiasis, a significant and common diarrheal disease in developing countries (Koltas *et al.*, 2007).

Wet mount examination (WME) and concentration methods are the most commonly used methods for detecting intestinal parasites from stool samples (Endris *et al.*, 2013). However both these methods may show low rate of parasites especially in light infections, which may be missed by using only a WME (Koltas *et al.*, 2014). Staining methods such as modified trichrome stain (MTS) can be used in the investigation of *E. histolytica*, *E. dispar* and *Blastocystis* spp., and modified acid-fast (MAF) stain can be used in the investigation of *Cryptosporidium* spp. and other coccidian parasites ((Koltas *et al.*, 2014; Kaushik *et al.*, 2008). In addition, antibody immuno-fluorescence assay (IF) and enzyme-linked immunosorbent assay (ELISA) for detecting antigens in stool samples or direct immuno-fluorescence assay (DIF) as serological tests can be used (Koltas *et al.*, 2014; Elgun & Koltas, 2011). In recent years, molecular techniques such as real-time polymerase chain reaction (real-time PCR) have been used for the detection of bacterial, parasitic, and viral agents from diarrheal patients (Goldfarb *et al.*, 2013). These techniques are very useful in unravelling the pathogenesis of human intestinal diarrheal parasites (Kaushik *et al.*, 2008).

The present study was undertaken to detect the intestinal parasites in diarrheal patients using real-time PCR and compare it with staining methods and ELISA techniques for the diagnosis of *Cryptosporidium* spp., *Blastocystis* spp., *E. histolytica*, *E. dispar* and *G. lamblia*.

MATERIALS AND METHODS

The collection of stool samples
A total of 272 stool samples were collected between April 2011 to March 2014 and received by the Department of Parasitology at the Faculty of Medicine, Çukurova University, Adana, Turkey. The study was approved by the Faculty of Medicine Ethics Committee, Çukurova University and informed consent was obtained from each patient. A questionnaire consisted of questions on the symptoms of diarrhoea (such as abdominal pain, nausea, acute diarrhea (more than three loose stools a day, present for <14 days), chronic diarrhea (diarrhea lasting for >14 days), weight loss, altered bowel habits (defined as a change in stool pattern other than diarrhea), vomiting and anal itching.

Patients with bacterial and/or viral-infection (such as *Shigella* spp., *Salmonella* spp., *Campylobacter* spp., *Clostridium difficile*, entero-invasive and entero-hemorrhagic *Escherichia coli*, Rotavirus, Adenovirus and Norovirus) were excluded from the study.

Microscopy methods (Wet mount examination (WME), Modified trichrome stain (MTS) and Modified acid-fast (MAF) stain)
Immediately after its collection, each stool sample was divided into two , one was mixed with polyvinyl alcohol (PVA) for preservation. The second portion (unpreserved subsample) was again divided into two portions, which were examined microscopically for the presence of intestinal protozoa trophozoite, cyst and/or helminth egg or larvae as a WME smear in 0.9% saline (one portion) and iodine (one portion). A double-blind procedure was performed on the stool samples for parasite examination. A portion of the sediment of the PVA was examined microscopically, after MTS, to check for trophozoites that had ingested erythrocytes or for cysts that each had four nuclei. In the comparison of test performance, such trophozoites and cysts were assumed to be those of *E. histolytica*, even though the cysts could have been those of *E. dispar* and/or *Blastocystis* spp. Smears were prepared from the unpreserved stool samples for staining with MAF. All the smears were examined with a 100X oil immersion objective for presence or absence of coccidian oocysts.

Serologic methods (Antigen detection)
Copro-antigen detection tests were used for *Cryptosporidium* spp., *E. histolytica* and *G. lamblia* in stool specimens (stored in –20°C) according to the manufacturer's instructions (*Cryptosporidium* spp. (Microwell ELISA, Catalog no: 8301-3,

Calabasas, California, USA), *E. histolytica* (*Entamoeba* CELISA Path test; Catalog no: KE1, Cellabs, Brookvale, New South Wales, Australia), and *G. lamblia* (Generic Assays GmbH commercial ELISA, Catalog no: 15827, Dahlewitz, Germany).

Molecular methods (DNA extraction and Real-time PCR)

Genomic DNA was extracted from all the stool samples by using the QIAmp DNA stool mini kit (QIAGEN, Catalog no: 51504, Hilden, Germany) according to the manufacturer's protocol.

The primers, probes and molecular targets are shown in Table 1. Genus-specific and/or species-specific real-time PCR assay amplification was carried out in a 20 µL total volume reaction mixture containing 10 ng of DNA. All of the real-time PCR reaction mixtures were carried out in a Rotor Gene RG-3000 (Corbett Research, San Francisco, CA, USA). The real-time PCR reaction mixture and thermal cycler programme for the diagnosis of *Cryptosporidium* spp., *Blastocystis* spp., *E. histolytica*, *E. dispar* and *G. lamblia* are shown in Table 2. A search for inhibitors was performed in samples that showed real-time PCR-negative results to assess possible real-time PCR failures to detect each parasite species.

All the reactions were analyzed using the software provided with the instrument. The average C_T values were determined and the standard curves were calculated using the Rotor-Gene 6.1.93 software.

RESULTS

There were 272 patients in this study. The common gastrointestinal symptoms determined were abdominal pain 69.1% (188/272), nausea 21.0% (57/272) weight loss 36.0% (98/272), altered bowel habits 44.1% (120/272), vomiting 57.0% (155/272), and anal itching 12.9% (35/272). Intestinal parasites were detected in 27 of 169 patients (16.0%) with acute diarrhea, and 42 of 103 (41.0%) patients with chronic diarrhea. The frequency of intestinal parasites detected in diarrhea stool samples from 272 patients in Adana, Turkey are shown in Table 3. Intestinal parasites were detected in 25.4% (69/272) of patients; 10.3% (28/272) *Cryptosporidium* spp., 5.9% (16/272) *Blastocystis* spp., 2.6% (7/272) *Entamoeba dispar*, 2.6% (7/272) *Giardia lamblia*, 1.8 (5/272) *Entamoeba*

Table 1. The primers, probes and molecular targets

Intestinal parasites	Target Gene	Primer-Probes	Reference
Cryptosporidium spp.	SSU rRNA	F: 5'-GGAAGGGTTGTATTTATTAGATAAAG-3' R: 5'-AAGGAGTAAGGAACAACCTCCA-3' P1: 5'-CCGTCTAAAGCTGATAGGTCAGAAACTTGAATG flourescein-3' P2: 5'-LCred705-GTCACATTAATTGTGATCCGTAAAG-3'	(Limor *et al.*, 2002)
Blastocystis spp.	SSU rRNA	F: 5'-GGTCCGGTGAACACTTTGGATTT-3' R: 5'-CCTACGGAAACCTTGTTACGACTTCA-3' P: 5'-FAM-TCGTGTAAATCTTACCATTTAGAGGA-3'	(Stensvold *et al.*, 2012)
E. histolytica	SSU rRNA	F: 5'-ATTGTCGTGGCATCCTAACTCA-3' R: 5'-GCGGACGGCTCATTATAACA-3' P: 5'-FAM-TCATTGAATGAATTGGCCATTT-3'	(Verweij *et al.*, 2003a)
E. dispar	SSU rRNA	F: 5'-ATTGTCGTGGCATCCTAACTCA-3' R: 5'-GCGGACGGCTCATTATAACA-3'	(Verweij *et al.*, 2003a)
G. lamblia	SSU rRNA	F: 5'-GACGGCTCAGGACAACGGTT-3' R: 5'-TTGCCAGCGGTGTCCG-3' P: FAM-5'CCCGCGGCGGTCCCTGCTAG-3'-TAMRA P: 5'-HEX-TTACTTACATAAATTGGCCACTTTG-3'	(Verweij *et al.*, 2003b)

Table 2. Real-time PCR reaction mixture and thermal cycler programme for diagnosis of *Cryptosporidium* spp., *Blastocystis* spp., *E. histolytica*, *E. dispar* and *G. lamblia*

Intestinal parasites	Real-time PCR mixture	Thermal cycle programme
Cryptosporidium spp.	2XPCR buffer*, 400 nM primers 200 nM probes, 0.5 µL BSA	50ºC 10s, 95ºC 3min, 50 cycles (94ºC 2s, 50ºC 10s, 72ºC 15s), 72ºC 10 min
Blastocsytis spp.	2XPCR buffer, 1 µM primers, 300 nM probes, 0.1 µL DMSO	50ºC 10s, 95ºC 2min, 50 cycles (95ºC 15s, 60ºC 1min, 72ºC 15s), 72ºC 10 min
E. histolytica	2XPCR buffer, 2.5 pM primers 2.5 pM probes, 2.5mM MgCl$_2$	50ºC 10s, 95ºC 2min, 50 cycles (95ºC 15s, 59ºC 10s, 72ºC 30s), 72ºC 10 min
E. dispar	2XPCR buffer, 2.5 pM primers 2.5 pM probes, 2.5 mM MgCl$_2$	50ºC 10s, 95ºC 2min, 50 cycles (95ºC 15s, 60ºC 10 s, 72ºC 30s), 72ºC 10 min
G. lamblia	2XPCR buffer, 2.5 pM primers 2.5 pM probes, 2.5 mM MgCl$_2$	50ºC 10s, 95ºC 2min, 50 cycles (95ºC 15s, 60ºC 30s, 72ºC 30s), 72ºC 10 min

*All of the real-time PCR mixture contained 2X PCR buffer; QuantiFastSYBRGreen PCR kit (Qiagen, catalog no: 204052, Valencia, CA) and 10 ng of stool DNA.

Table 3. The frequency of intestinal parasites detected in diarrhea stool samples from 272 patients in Adana, Turkey with the methods such as wet mount examination (WME), modified trichrome (MTS) stain, modified acid-fast (MAF) stain, ELISA and real-time PCR

Identified parasite	WME	MTS	MAF	ELISA	Real-time PCR
Cryptosporidium spp.	–	–	5.1% (14/272)	7.4% (20/272)	10.3% (28/272)
Blastocystis spp.	1.5% (4/272)	4.0% (11/272)	–	ND	5.9% (16/272)
Entamoeba dispar	0.7% (2/272)	1.5% (4/272)	–	–	2.6% (7/272)
Giardia lamblia	1.5% (4/272)	2.2% (6/272)	–	2.6% (7/272)	2.6% (7/272)
Entamoeba coli	1.5% (4/272)	1.8% (5/272)	–	ND	ND
Entamoeba histolytica	0.4% (1/272)*	0.4% (1/272)*	–	0.4% (1/272)	0.7% (2/272)
Isospora spp.	0.4% (1/272)	–	0.7% (2/272)	ND	ND
Trichuris trichiura	0.4% (1/272)	–	–	ND	ND
Strongylodies stercoralis	0.4% (1/272)	–	–	ND	ND

*Trophozoites of *Entamoeba histolytica* with ingested erythrocystes.
ND: Not done.

coli, 0.7% (2/272) *Entamoeba histolytica*, 0.7% (2/272) *Isospora belli*, 0.4% (1/272) *Trichuris trichiura*, and 0.4% (1/272) *Strongyloides stercoralis* by WME, MTS, MAF, ELISA and real-time PCR methods.

Intestinal parasite species were detected in 22.1% (60/272) of patients using real time-PCR, 10.3% (28/272) with ELISA, 9.9% (27/272) with MTS, 6.6% (18/272) with WME and 5.9% (16/272) with MAF stain.

A total of 5.1% (14/272) of patients with diarrhea were positive for *Cryptosporidium* oocycts when the MAF staining technique was used and 7.4% (20/272) of the patients were positive in the results obtained by the ELISA technique. On the other hand, the real-time PCR assay detected *Cryptosporidium*

DNA in 10.3% (28/272) of the diarrhea patients. A total of 4.0% (11/272) of patients with diarrhea were positive for *Blastocystis* spp., in granular, vacuolar or amoeboid forms using the MTS technique and 5.9% (16/272) of patients were positive by real-time PCR. All positive stools examined by WME, MTS, MAF and ELISA techniques were also positive by real-time PCR as shown in Table 3.

The real-time PCR assay revealed a diagnostic sensitivity and specificity of 100% for diagnosis of intestinal parasites. These results led us to consider the real time PCR as the reference test for diagnostic sensitivity and specificity; while microscopy (WME, MTS, and MAF) showed the lowest sensitivity

Table 4. The results of positive values and sensitivity-specificity of laboratory methods

Method	No. of samples	No. of positive samples				Sensitivity (%)[a]	Specificity (%)[b]
		Cryptosporidium spp.[c]	*Blastocystis* spp.[d]	*Giardia lamblia*[e]	*Entamoeba histolytica*[f]		
Real-time PCR	272	28	16	7	2	100	100
ELISA	272	20	ND	7	1	50–100	100
Microscopy	272	14	11	6	1	50–86	100

[a]Calculated as follows: (number of true positives/number of true positives + number of false negatives) X100.
[b]Calculated as follows: (number of true negatives/number of true negatives + number of false positives) X 100.
[c]Sensitivity, 71% (ELISA)-50% (Microscopy); Specificity, 100%.
[d]Sensitivity, ND (ELISA)-69% (Microscopy); Specificity, ND (ELISA)-100%.
[e]Sensitivity, 100% (real-time PCR)-100% (ELISA)-86% (Microscopy); Specificity, 100% (real-time PCR)-100%.
[f]Sensitivity, 50%(ELISA)-50% (Microscopy); Specificity, 100%.

varying between 50.0% and 86.0% (Table 4). In addition, no cross-reactivity was detected in 69 stool samples containing various other bacterial, viral, and protozoan species. There were no inhibitors and no false negative result in real time PCR methods in this study.

DISCUSSION

Of the 272 diarrheal patients, 22.1% (60/272) were positive by the use of real-time PCR-method. *Cryptosporidium* spp. was the commonest, detected in 10.3% (28/272) of the patients, followed by *Blastocystis* spp. in 5.9% (16/272) of patients. In this study, the sensitivity and specificity of real-time PCR method was found to be 100% when compared to ELISA and microscopy (WME, MTS, and MAF).

Diarrheal diseases are extremely common in developed and developing countries and are a major cause of morbidity and mortality, affecting millions of individuals each year (Verweij *et al.*, 2003a). *Crytosporidum* spp., *Blastocystis* spp., *Entamoeba* spp., and *G. lamblia* are diarrhea-causing intestinal parasites with almost similar clinical presentations (Verweij *et al.*, 2004; Stensvold *et al.*, 2012). The often non-specific clinical presentation of the infections makes it difficult to determine a more specific and sensitive method for the detection of these pathogens (Verweij *et al.*, 2004). A number of studies have demonstrated that molecular methods have superior sensitivity

for the detection of intestinal parasites compared to conventional staining methods, microscopy and ELISA (Verweij *et al.*, 2004; Stensvold *et al.*, 2009; Xiao, 2010). Molecular studies on the diagnosis of intestinal parasites are rarely used in Turkey (Eroglu *et al.*, 2009; Tamer *et al.*, 2007; Dagci *et al.*, 2007). To our knowledge, this is the first study to evaluate the sensitivity and specificity of real-time PCR in diarrheal patients in Adana, Turkey.

Cryptosporidium spp. is known as one of the major enteropathogenic parasites of both immunocompetent and immuno-compromised vertebrate hosts worldwide. Currently, the detection of *Cryptosporidum* spp. and genotypes is made mostly by PCR-restriction fragment length polymorphism or sequencing analysis of antigen, structural, and housekeeping genes (Rolando *et al.*, 2012). These procedures are usually time-consuming (Rolando *et al.*, 2012; Limor *et al.*, 2002). Therefore, we used real-time PCR method for the determination of *Cryptosporidium*. Calderaro *et al.* have reported that; *Cryptosporidium* spp. was detected in 3.0% (31/1040) of the sample by immuno-cromatographic assay (IC) and immuno-fluorescence assay (IF); the real-time PCR assay revealed *Cryptosporidium* spp. DNA in 5 additional samples for a total of 3.5% (36/1040) sample (Calderaro *et al.*, 2011). In our study, we detected only 5.1% (14/272) positive samples using the modified acid-fast technique but 10.3% (28/272) positive samples were detected by real-time PCR technique.

Lebbad *et al.*, 2001, reported that of the 52 adult patients with chronic diarrhea, 37 were found to be HIV-positive and fulfilling the clinical criteria of AIDS (five HIV-1, 28 HIV-2 and four dually infected with HIV-1 and HIV-2). Twenty five percent of the HIV-2 positive patients were infected with *C. parvum*, 11.0% with *I. belli* and 11.0% with microsporidia, all these three parasites are usually seen only in HIV-positive patients (Lebbad *et al.*, 2001). We found 75.0% (6/8) *Cryptosporidium* spp. in 8 HIV-positive patients.

Based on molecular analysis of the small subunit (SSU) rRNA gene, *Blastocystis* isolates from man, mammals and birds can be assigned to one of at least ten subtypes. (Stensvold *et al.*, 2009). In our study, we used small subunit (SSU) rRNA real-time PCR for *Blastocystis* spp. diagnosis in which similar molecular method used by Stensvold *et al.*, 2009 and Poirier *et al.*, 2011.

Since microscopy is unable to differentiate between *E. histolytica* and *E. dispar* with 60.0% sensitivity at best, it can be confounded by false positive and false negative results due to the misidentification of macrophages and other species of *Entamoeba* (Ali *et al.*, 2008). We used microscopy, copro-antigen ELISA and real-time PCR methods for diagnosis of *Entamoeba* spp. The most sensitive method to determine *Entamoeba* spp. was determined to be the real-time PCR method in this study. According to the result of our study, in order to prevent the patients from receiving unnecessary treatment, it is best that the patients stool be examined using the objective, high sensitive, and high specific copro-antigen ELISA, PCR and/or real-time PCR methods in every parasitology laboratory, as well as using microscopy.

Real-time PCR are advantageous in many ways, primarily due to its high sensitivity and specificity (Limor *et al.*, 2002). Concluding from the result of this study, real-time PCR would be the most accurate tool for the diagnosis of intestinal parasites in patients with diarrhea. Thus, in clinical laboratories real-time PCR can be an ideal screening tool for the detection and genotyping of intestinal parasites in especially diarrhea stool samples. Its application should be evaluated individually by every single laboratory, depending on the availability of trained personnel, financial resources and the cost/effectiveness related to the prevalence of the infection.

The main limitation of the study is that real-time PCR analysis cannot be used to determine *Microsporidia* spp., No subtype analysis was performed for *Blastocystis* spp.

In conclusion, this study highlights the lack of sensitivity demonstrated by WME; as such, real-time PCR method would be better considered as the diagnostic method of choice especially in chronic diarrhea for the detection of intestinal parasites.

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