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ORIGINAL PAPER

Is "dried stool spots on filter paper method (DSSFP)" more sensitive and effective for detecting *Blastocystis* spp. and their subtypes by PCR and sequencing?

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Abstract PCR and DNA sequencing are currently the diagnostic methods of choice for detection of *Blastocystis* spp. and their suptypes. Fresh or frozen stool samples have disadvantages in terms of several aspects such as transportation, storage, and existence of PCR inhibitors. Filter paper technology may provide a solution to these issues. The aim of the present study was to detect *Blastocystis* spp. and their subtypes by employing two different preservation methods: conventional frozen stool (FS) and dried stool spots on filter paper (DSSFP). Concentration and purity of DNA, sensitivity of PCR, and DNA sequencing results obtained from the two methods were also compared. A total of 230 fecal samples were included and separated into two parts: one part of the fecal samples were directly frozen and stored at −20 °C. The remaining portion of the specimens were homogenized with saline and spread onto the filter papers as thin layer with a diameter of approximately 3 cm. After air-dried, the filter papers were stored at room temperature. DSSFP samples were collected by scraping from the filter papers. DNA were extracted by EURx Stool DNA Extraction Kit from both samples. Concentration and purity were measured with NanoDrop, then PCR and sequencing were conducted for detection of *Blastocystis* spp. and its genotypes. Pure DNA was obtained with a A260/A280 ratio of 1.7–2.2 in both methods. DNA yield from FS was 25–405 ng/μl and average DNA concentration was 151 ng/μl, while these were 7–339 and 122 ng/μl for DSSFP, respectively. No PCR inhibition was observed in two methods. DNA from DSSFP were found to be stable and PCR were reproducible for at least 1 year. FS-PCR- and DSSFP-PCR-positive samples were 49 (21.3 %) and 58 (25.3 %), respectively ($p = 0.078$). The 43 specimens were concordantly positive by both FS-PCR and DSSFP-PCR. When the microscopy was taken as the gold standard, sensitivity of DSSFP-PCR and FS-PCR was 95.5 and 86.4 %, while specificity of both tests was 99.4 and 98.3 %, respectively. DNA sequencing results of 19 microscopically confirmed cases were strictly identical (concordance 100 %) in both methods, and ST2:6, ST3:8, ST4:3, and ST6:2 were the detected subtypes. Among the 230 fecal samples, the most predominant subtypes were ST3, ST2, ST4, and ST1 by both FS and DSSFP methods. Concordance of DNA sequencing results obtained from the two methods was noted to be 90.7 %. To our knowledge, this is the first study that demonstrates DNA extraction from DSSFP is more sensitive and effective than the FS method for diagnosis of *Blastocystis* spp. and their subtypes by PCR and DNA sequencing.

Keywords *Blastocystis* spp. · DSSFP · PCR

Introduction

Blastocystis spp. have been found as the most predominant single-cell protozoan in the gastrointestinal tract of humans (Wawrzyniak et al. 2013; Nithyamathi et al. 2016; Osman et al. 2016). Prevalence of *Blastocystis* spp. varies from 0.5–4 % in developed countries to 22.1–87.6 % in developing countries (Horiki et al. 1997; Stensvold et al. 2011; Abdulsalam et al. 2013;

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Abu-Madi et al. 2015; Nithyamathi et al. 2016). The *Blastocystis* genus consists of at least 17 subtypes (ST), and nine of these STs have been reported in humans (Stensvold et al. 2007a, 2009; Tan 2008; Parkar et al. 2010). It has been hypothesized that certain genotypes of this parasite may contribute to pathogenicity (Dogruman-Al et al. 2008; Eroglu et al. 2009). Recent investigations reported that infection developed by *Blastocystis* spp. is associated with nonspecific gastrointestinal disorders such as abdominal pain, diarrhea, constipation, flatulence, bloating, vomiting, and weight loss (Abdulsalam et al. 2013; Wawrzyniak et al. 2013). These protozoans are considered to be potential pathogens especially in immunocompromised patients (Libre et al. 1989; Ok et al. 1996; Tasova et al. 2000; Karasartova et al. 2015). The common diagnostic approaches for the detection of *Blastocystis* spp. consist of light microscopic examination of native-Lugol or trichrome-stained fecal smears or in vitro culture of fecal samples. Generally, stained and direct smear methods have lower sensitivity (Dogruman-Al et al. 2010; Elghareeb et al. 2015) and in vitro culture is considered as the gold standard technique with high sensitivity; however, it is time-consuming and laborious (Leelayoova et al. 2002; Stensvold et al. 2007b). In recent years, there is an increase use of PCR for diagnosis and epidemiological studies of *Blastocystis* infection. Additionally, this method allows genotyping by using DNA obtained from fresh or frozen stool samples (Forsell et al. 2012; Stensvold 2013). However, PCR has several drawbacks such as being expensive and needing complex equipments. Also, there are difficulties in preservation and transportation of feces for diagnosis of the etiologic agent by PCR. Besides, PCR inhibitors in the fecal DNA may decrease the sensitivity of these assays. Filter paper-based methods are known for storing the biological materials with improved stability, integrity, and purity (Rajendram et al. 2006; Smit et al 2014). These methods have been shown to have high sensitivity and specificity for DNA detection by PCR (Rajendram et al. 2006; Nuchprayoon et al. 2007). These methods are commonly used in drug monitoring (Lindström et al. 1985) and diagnosis of viral and bacterial agents (Rajendram et al. 2006; Picard-Meyer et al. 2007). Besides, filter papers are frequently used for blood samples in terms of detection of the blood-associated parasites (Smit et al. 2014). There are a few studies that applied this method in human fecal samples for detection of parasites such as *Giardia* and microsporidia (Carnevale et al. 2000; Subrungruang et al. 2004; Nantavisai et al. 2007). However, the efficiency of filter paper methods for detecting *Blastocystis* spp. and their suptypes from fecal specimens has never been investigated. The aim of this study was to determine *Blastocystis* spp. and their subtypes in human fecal samples by employing two different preservation methods: frozen stool (FS) and dried stool spots on filter paper (DSSFP). Concentration and purity of DNA, sensitivity of PCR, and DNA sequencing results obtained from the two methods were also compared.

Materials and methods

Stool samples

A total of 230 fresh fecal samples were obtained from human volunteers in North Cyprus. For evaluation of the results obtained from conventional FS-PCR and DSSFP-PCR methods, and confirmation of DNA sequencing analysis of two methods, all samples were examined microscopically by using both native-Lugol and trichrome-stained smears (Wheatley 1951). Microscopic examination of the fecal samples was performed double-blinded by two different experts from the Near East University, Faculty of Medicine, North Cyprus. All samples were exposed to two different preservation methods, FS and DSSFP.

Preparation of DSSFP and FS

For the FS method, a total of 70 mg from the fecal samples were filled into cryovials and directly stored at −20 °C as described previously (Roberts et al. 2011; Forsell et al. 2012).

For the DSSFP method, firstly, 70 mg from the stool samples were diluted with 300 μl of saline. After homogenization, the samples were spread onto the ordinary filter papers (which are used in laboratories for general purposes such as basic filtration and sample preparation) as thin layer with a diameter of approximately 3 cm. After air-dried, the filter papers containing the stool spots were stored individually in sealed polyethylene bags at room temperature until the DNA extraction procedure.

Additionally, five samples which were negative for PCR and microscopy were kindly provided by the National Reference Laboratory and all procedures were also applied for these negative control samples.

DNA extraction from DSSFP and FS samples

Maximum duration of storage was 3 months for both methods. DNA extraction was conducted according to the manufacturer's protocol (EURx Stool DNA Extraction Kit). A total of 70 mg from frozen fecal samples were used for the extraction of DNA with a final elution volume of 100 μl. The kit's procedure was based on bead-beating methods, while horizontal vortexing at the highest speed was preferred for achieving the optimal DNA yield.

Dried stool samples on the filter papers were scraped very carefully under a biosafety cabinet class II, and disposable materials were used for each sample in order to avoid contamination of DNA between the specimens and infection risk for the researchers. Powdery stool particles collected from the scrapings were extracted by using the same DNA extraction kit with a final DNA elution volume of 100 μl. For the control of possible presence of DNA on the filter papers, the

extraction protocol (EURx Stool DNA Extraction Kit) was also applied to an additional filter paper which did not contain any stool sample.

Evaluation of DNA concentration, purity, and PCR inhibitors

DNA yield was measured by Thermo Scientific™ NanoDrop™ 2000 Spectrophotometers. One microliter from the extracted DNA was evaluated in duplicate. Control of possible PCR inhibitors was done according to the method of Stensvold et al. (2006). Briefly, 1 μl from PCR-positive DNA and 4 μl from PCR-negative DNA were mixed up and the standard PCR was evaluated for the presence of expected amplicons.

PCR assay

For detection of SSU-rDNA of *Blastocystis* spp., primers BhRDr (GAGCTTTTTAACTGCAACAACG) and RD5 (ATCTGGTTGATCCTGCCAGT) (Scicluna et al. 2006) were used in Touch-Down PCR assay. Two microliters of DNA solution were added into the standard PCR mixture with a total volume of 25 μl. Touch-down PCR assay was conducted under the following conditions: 10 cycles of denaturation at 94 °C for 45 s, annealing at 65–55 °C (the temperature was decreased by 1 °C step by step) for 45 s and the extension at 72 °C for 1 min and then 30 cycles of denaturation at 94 °C for 45 s, annealing at 61 °C for 45 s and extension at 72 °C for 1 min. Five microliters of PCR product were visualized under UV transilluminator.

In order to determine the stability of DNA and reproducibility of DSSFP-PCR method in terms of DNA extractions and PCR results, the samples of 22 microscopically confirmed cases were selected. During a year, DNA extraction and PCR were performed every 2 months and the same results were obtained in each attempt.

DNA sequencing of the PCR products

The positive PCR samples were purified and sequenced in one direction at MACROGEN (Laboratory Company in Amsterdam, Netherlands). Sequencing of small subunit rRNA gene (SSU-rDNA) of the PCR products were analyzed by using the *Blastocystis* suptype (18S) and sequence typing database (MLST) (http://pubmlst.org/blastocystis/) online software.

Statistical analyses

For comparison of DNA concentrations which obtained by DSSFP and FS methods, Mann-Whitney *U* test was used. PCR results of both methods in 230 fecal samples were statistically analyzed by McNemar's test.

Results

In both of the preservation methods, 70 mg of fecal samples were used. A260/A280 ratio of pure DNA was 1.7–2.2 in both methods. DNA yield from FS was 25–405 ng/μl and average DNA concentration was 151 ng/μl, while these were 7–339 and 122 ng/μl for DSSFP, respectively. There was no statistically significance of DNA concentration between FS and DSSFP preservation methods ($p = 0.549$ by Mann-Whitney *U* test). None of the negative control samples were found positive by both of the methods. No DNA was detected from the control filter paper which did not contain any stool sample. No PCR inhibition was observed in two methods.

PCR results of the 230 specimens were shown in Table 1. FS-PCR- and DSSFP-PCR-positive samples were 49 (21.3 %) and 58 (25.3 %) respectively (McNemar's test for paired observations, $p = 0.078$). Forty-three specimens were concordantly positive by both FS-PCR and DSSFP-PCR. The number of total positive specimens was 64 by two methods. Fifteen of 64 samples were positive by DSSFP-PCR but negative by FS-PCR, and conversely 6 of 64 samples were positive by FS-PCR but negative by DSSFP-PCR.

When the microscopy was taken as the gold standard, sensitivity of DSSFP-PCR and FS-PCR was found 95.5 and 86.4 %, while specificity of both tests was 99.4 and 98.3 %, respectively (Table 2).

The reliability of DNA sequencing results was evaluated according to the microscopically confirmed cases. However, three specimens among the 22 microscopically confirmed cases were excluded from the analysis due to the negative FS-PCR results. DNA sequencing results of the remaining 19 microscopically confirmed cases were strictly identical (concordance 100 %) in both methods, and ST2:6, ST3:8, ST4:3, and ST6:2 were the detected subtypes.

DNA sequencing results of the 230 fecal samples obtained from the FS and DSSFP methods were demonstrated in Table 3. The most predominant subtypes were ST3, ST2, ST4, and ST1 by both FS and DSSFP methods. Concordance of DNA sequencing results obtained from the two methods was noted to be 90.7 %.

Discussion

Blastocystis spp. is considered as one of the common intestinal protozoans worldwide. PCR diagnosis of *Blastocystis* spp. depends on the direct DNA extraction from fresh or frozen fecal samples, and genotyping should also be performed (Roberts et al. 2011; Forsell et al. 2012; Stensvold 2013). However, in stool specimens, inhibitors such as bile salts, complex carbohydrates, bilirubins, and other limiting factors including bacterial nucleases and oxidation affect the PCR sensitivity negatively (Nadkarni et al. 2009; Schrader et al.

Table 1 Comparison of FS-PCR and DSSFP-PCR results for detection of *Blastocystis* spp. in the stool samples

		FS-PCR		
DSSFP-PCR		Positive	Negative	Total
	Positive	43	15	58
	Negative	6	166	172
	Total	49	181	230

$p = 0.078$

2012). Also, molecular diagnosis is restricted by the difficulties in preservation and transport of feces. Thus, effective DNA extraction methods are needed for improving the sensitivity of PCR. Filter paper-based methods may provide solution to these problems. The number of studies that applied this method in human fecal samples for detection of parasites is very limited (Carnevale et al. 2000; Subrungruang et al. 2004; Nantavisai et al. 2007). Therefore, this study was conducted to detect *Blastocystis* spp. and their subtypes in human fecal samples which were exposed to FS and DSSFP methods. Results obtained from the two methods were also compared in terms of concentration and purity of DNA, sensitivity of PCR, and DNA sequencing results.

In recent years, filter paper-based method has been commonly used for detection of different types of DNA from humans (Sirdah 2014; Chen 2016), plants (Drescher and Graner 2001), animals (Smith and Burgoyne 2004), viruses (Picard-Meyer et al. 2007), bacteria (Rajendram et al. 2006), insects (Harvey 2004), and parasites (Nantavisai et al. 2007). Different kinds of filter papers such as Whatman filter paper, common filter paper, and Flinders Technology Associates (FTA) filter paper were used in the studies (Mutwewingabo et al. 1984; Harvey 2004; Smit et al. 2014). Whatman filter paper consists of 100 % cellulose and varies in thickness and pore size. Common filter paper which also consists of 100 % cellulose has smooth surface and normal hardness. This kind of paper is used for routine laboratory procedures such as basic

Table 2 Comparison of FS-PCR and DSSFP-PCR results with microscopy for detection of *Blastocystis* spp. in the stool samples

		Microscopy		
		Positive	Negative	Total
FS-PCR	Positive	19	30	49
	Negative	3	178	181
	Total	22	208	230
Sensitivity, 86.4 %; specificity, 98.3 %				
DSSFP-PCR	Positive	21	37	58
	Negative	1	171	172
	Total	22	208	230
Sensitivity: 95.5 %, specificity: 99.4 %				

filtration and sample preparation. On the other hand, FTA paper is treated with a proprietary mix of chemicals which provide DNA extraction from the organisms collected on the filter paper (Rajendram et al. 2006). FTA paper has the advantage of inactivating highly pathogenic organisms. On the contrary, in Whatman-type and common filter papers, contamination risks are higher regardless of pathogens; thus, samples must be processed as potentially infectious agents and all biosafety regulations should be followed (Smit et al. 2014). For detection of protozoans, disadvantage of FTA paper is the use of limited sample volume. Diluted fecal sample applied on the FTA paper is restricted to 15 μl (Subrungruang et al. 2004; Nantavisai et al. 2007). If the costs of methods are compared, our method with common filter paper which even needs using extraction kit is at least twice times cheaper than FTA.

Use of common filter paper for preservation of stool samples was described. In that article, the efficiency of this method for sending dysenteric feces samples to laboratory was demonstrated. In 1984, Mutwewingabo et al. showed that temperature and time did not affect the survival of Shiga bacillus in stool samples collected on the common filter paper (Mutwewingabo et al. 1984).

In our DSSFP methods, 300–350 μl of diluted stool samples were spread as thin layer on the common filter papers. DNA yields obtained from DSSFP method (122 ng/μl) were comparable with those of FS method (151 ng/μl), and the difference was not statistically significant. Also, no DNA inhibitors were detected in two methods. Stability of DNA extracted by DSSFP method and reproducibility of DSSFP-PCR method were compatible with those of FS extraction and FS-PCR methods. The main drawback of DSSFP method is the high contamination risk of DNA during the scrabing process; hence, this procedure should be performed in biosafety cabinets with moderate airflow. Use of bead-beating protocol-based stool DNA extraction kit and horizontal vortex system produced better results for both of the DSSFP and FS methods.

For detection of *Blastocystis* spp., sensitivity of DSSFP-PCR and FS-PCR were found to be 95.5 and 86.4 %, while specificity of both tests were 99.4 and 98.3 %, respectively. Nantavisai et al. (2007) conducted PCR for diagnosis of *Giardia duodenalis* by using the FTA filter paper method and found a sensitivity and specificity of 97.3 and 100 %, respectively. The authors indicated that the sensitivity and specificity of immunofluorescence assay performed by using the stool samples were 91.9 and 100 %, respectively. In a study conducted by Subrungruang et al. (2004), the sensitivity of FTA filter paper for the detection of *Enterocytozoon bienuesi* in stool specimens by PCR was documented as 100 %. Another study demonstrated that a double PCR method for *E. bienuesi* using the stool specimens in different preservation solutions which were spotted on filter paper disks was effective for sample collection, mailing, and diagnosis of this pathogen (Carnevale et al. 2000). Chu et al. (2004), effectively applied FTA filters for

Table 3 DNA sequencing results obtained from the FS and DSSFP methods

Sampling methods	DNA sequencing results							
	ST1 *n* (%)	ST2 *n* (%)	ST3 *n* (%)	ST4 *n* (%)	ST6 *n* (%)	ST7 *n* (%)	Non-ST *n* (%)	Negative
FS (49 positives)	5 (7.8)	13 (20.3)	19 (29.7)	6 (9.4)	2 (3.2)	2 (3.1)	2 (3.1)	15 (23.4)
DSSFP (58 positives)	7 (10.9)	15 (23.5)	20 (31.3)	7 (11)	3 (4.7)	2 (3.1)	4 (6.2)	6 (9.4)
FS + DSSFP[a] (39 positives)	5 (7.8)	8 (12.5)	14 (21.9)	6 (9.4)	2 (3.1)	2 (3.1)	2 (3.1)	NA

NA not applicable

[a] Concordance, 90.7 %

detection of *Cyclospora cayetanensis* in animal fecal isolates. Dried stool preparation appears to protect DNA from oxidation, damage caused by nucleases or ultraviolet light (UV) and contamination with microorganisms (Thacker et al. 1999; Salvadore and De Ungria 2003). Therefore, sensitivity and specificity of PCR is high when filter paper-based methods are used for the preparation of stool samples. On the other hand, based on personal communications, it was indicated that after storing at −20 °C, positive fecal samples could convert to negative (Boorom et al. 2008). Thus, comparison of fresh and frozen fecal samples is needed.

Roberts et al. (2011) used two different primer sets in PCR method for detecting *Blastocystis* in stool samples, and they found the sensitivities as 66 and 94 %. This study expressed that the right set of primers and reaction condition was important. Several authors found that conventional PCR was not significantly more sensitive than xenic in vitro culture (XIVC) (Stensvold et al. 2007a, 2007b). Nevertheless, in two different studies, XIVC was compared with real-time PCR and sensitivity was 79 and 52 %, respectively (Poirier et al. 2011; Stensvold et al. 2012). In our study, we found that DSSFP-PCR method had better results for detection of *Blastocystis* spp., but statistically there was no significance with conventional FS-PCR methods. DNA sequencing of SSU-rDNA gene of the PCR products obtained from two methods were analyzed for detection of *Blastocystis* subtypes. DNA sequencing results of the 19 microscopically confirmed cases were strictly identical (concordance 100 %) in both methods. On the other hand, detection rates of ST1, ST2, ST3, ST4, and ST6 were slightly higher in DSSFP method, and ST7 was identified at equal percentages by two methods. Thus, the concordance in clinical samples was 90.7 %.

In conclusion, to our knowledge, this is the first study which clearly showed that dried stool spots on filter paper method is highly sensitive and effective for PCR-based detection and sequencing analysis of *Blastocystis* spp. Also this method facilitates collection, transport, and preservation of the stool samples. This filter paper method which is cost-effective maintains high yields of pure and stable DNA for long periods.

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Compliance with ethical standards

Ethics committee approval The study was approved by the Clinical Research Ethics Board of Ankara Numune Training and Research Hospital, Turkey, No/Year: E.Kurul-E-15-446/06.04.2015.

Informed consent Written informed consent was obtained from each patient who participated in this study.

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