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PROTOZOOLOGY - ORIGINAL PAPER

Development and evaluation of high-resolution melting curve analysis for rapid detection and subtyping of *Blastocystis* and comparison the results with sequencing

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Abstract

Blastocystis is a prevalent parasite that has a wide distribution. In order to design HRM real-time PCR, primers were selected from SSU rRNA gene to amplify specific fragment with different melting temperatures for each subtype of *Blastocystis*. Subsequently, HRM real-time PCR was performed and melting curve analysis was done by Rotor-Gene Q software. The results of HRM real-time PCR was then compared with sequence results of "barcoding region" of SSU rRNA gene of *Blastocystis*. To evaluate sensitivity of test, 10-fold serial dilutions of the parasite were prepared from ~ 10^6 to 1 parasite per mL of stool sample and were investigated by HRM real-time PCR. In order to determine specificity of method, HRM real-time PCR was done for some microorganisms and *Blastocystis*-negative stool samples. In silico analysis showed that all seventeen subtypes of *Blastocystis* were distinguish. In vitro analysis revealed that the test discriminated subtypes with specific melting temperatures.

Keywords *Blastocystis* · Subtypes · 18S ribosomal RNA gene · High-resolution melting curve analysis

Abbreviation

HRM	High-resolution melting curve
ST	Subtype
rRNA	Ribosomal RNA

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Introduction

Blastocystis genus, as a cryptic member of the group Stramenopiles, is frequently isolated from a broad spectrum vertebrate and invertebrate animals (Clark et al. 2013; Tan 2008; Yoshikawa et al. 2016). This parasitic eukaryote is a public health concern in almost all countries, particularly in undeveloped regions (Andersen and Stensvold 2016; Stensvold and Clark 2016). Although there are unclear features of the life cycle of *Blastocystis*, four morphological forms of the parasite, including vacuolar, granular, cyst, and amoeboid forms, have been characterized in stool samples, as well as in the medium culture (Stensvold and Clark 2016; Zhang et al. 2007). So far, several studies have evaluated the hypothesis that *Blastocystis* can cause intestinal and extra-intestinal manifestations (Alinaghizade et al. 2017; Basak et al. 2014; Kurt et al. 2016; Mirjalali 2017; Tan et al. 2010; Toro Monjaraz et al. 2017). However, there is not enough evidence to consider *Blastocystis* among enteric pathogens (Coyle et al. 2012; Jalallou et al. 2017; Sekar and Shanthi 2013). However, a high prevalence of this parasite alongside its unclear pathogenicity have convinced physicians to focus on the detection of this

parasite, particularly in *Blastocystis*-carriers, without specific gastrointestinal symptoms (Jones 2nd et al. 2008).

Over the past decade, the molecular characterization of *Blastocystis* has led to the classification and the consensus terminology of this parasite based on the amplification and sequencing of a discriminative fragment, known as "barcoding region," through small subunit of the ribosomal RNA (SSU rRNA) gene (Stensvold et al. 2007b). Accordingly, 17 subtypes (ST1–17) have been introduced so far that ST1–10 is considered the human-infecting subtype (Alfellani et al. 2013a; Alfellani et al. 2013c; Clark et al. 2013; Stensvold and Clark 2016).

In recent years, many studies have tried to establish a statistically significant linkage between certain subtypes and either gastrointestinal or extra-intestinal symptoms (El Safadi et al. 2013; Katsarou-Katsari et al. 2008; Taghipour et al. 2019; Vogelberg et al. 2010). Although this correlation was suggested by some studies, there is no convincing indication to show an association between certain subtypes and clinical manifestations (Jalallou et al. 2017; Salehi et al. 2017). However, molecular epidemiology studies indicated that ST1–4 are the most prevalent human-infecting subtypes with the majority being ST3 (Alfellani et al. 2013a, 2013b, 2013c; Rezaei Riabi et al. 2018). However, the distribution and prevalence of subtypes depend on the studied populations and geographical areas.

Apart from the studied populations and geographical areas, the prevalence and distribution of either *Blastocystis* or its subtypes vary, based on the method of detection. It seems that at least in developing countries, both/either direct examination or concentration of stool sample and then investigation using light microscopy and/or cultivation of stool in culture media are the common laboratory techniques (Eymael et al. 2010; Stensvold 2013a; Suresh and Smith 2004). In this regard, there are some limitations like either missing the parasite in mild infections or slow growth rate of some strains/subtypes in culture media (Stensvold et al. 2009; Stensvold 2015). Therefore, many molecular techniques have been developed and used to detect *Blastocystis* and distinguish its subtypes from stool samples or culture media such as conventional PCR using sequence-tagged site (STS) primers (Yoshikawa et al. 1996; Yoshikawa et al. 1998; Yoshikawa et al. 2000;Yoshikawa et al. 2004), PCR-RFLP (Clark 1997; Hoevers et al. 2000) and PCR/sequencing of SSU rRNA gene (Stensvold 2013b).

Nonetheless, some technical limitations raised from these approaches. It was shown that PCR using STS primers is only able to detect subtypes 1–7 (Stensvold 2013b). Furthermore, point mutations, particularly at the restriction site of the targeted fragment and mixed subtypes, may lead to misinterpretation of the results of PCR-RFLP (Moosavi et al. 2012; Stensvold et al. 2007b; Yoshikawa et al. 2004). Recently, SYBR green (Poirier et al. 2011) and TaqMan real-time PCR (Stensvold et al. 2012a) were experienced for the detection of *Blastocystis* from stool samples. Despite the high sensitivity of these methods, they were not able to discriminate the available subtypes. Therefore, the current study aimed to develop and evaluate a high-resolution melting curve analysis (HRM) by real-time PCR in order to directly detect and distinguish between existing subtypes from stool samples and compare the results with sequencing of the barcoding region of SSU rRNA gene of *Blastocystis*.

Materials and methods

Primer designing for HRM real-time PCR

Primers were designed based on their ability to amplify a fragment with specific melting temperature for each subtype. In addition, primers had to target all subtypes with minimum miss-amplification. Therefore, after in silico analysis of SSU rRNA gene, primers were designed from SSU rRNA gene of available subtypes of *Blastocystis*, which were previously deposited in GenBank database. In order to obtain different fragment melting temperatures (T_m), the available sequenced SSU rRNA gene of subtypes of *Blastocystis* were aligned using BioEdit software v.7.7.6.0. Afterward, primers forward (5′-CGAA TGGCTCATTATATCAGTT-3′) and reverse (5′-AAGC TGATAGGGCAGAAACT-3′) were selected from conserved region that amplified a ~ 220-bp fragment of SSU rRNA gene of the parasite containing subtype-specific parts for each subtype. To elucidate the specific T_m of amplified fragments for different subtypes in silico, the expected fragments were analyzed in online molecular analysis software, OligoCalc (http://biotools.nubic.northwestern.edu/OligoCalc.html).

Sample collection

In total, 72 stool samples, which were taken from humans (with/without gastrointestinal symptoms) (Rezaei Riabi et al. 2018) and domesticated animals (unpublished data) including 65 human samples and 7 animal samples, were included in our study. However, all of samples were subtyped using sequencing of the barcoding region of SSU rRNA gene.

Blastocystis cultivation

To cultivate *Blastocystis*, stool samples were inoculated in Dulbecco's modified Eagle medium (DMEM) (Gibco, Thermo Fisher Scientific, MA, USA), supplemented with penicillin-streptomycin (1000-unit penicillin and 4 mg/mL

streptomycin) and 20% inactivated fetal bovine serum (FBS) (Gibco). Cultivated samples were incubated at 37 °C under anaerobic conditions and examined every 48–72 h by iodine staining and direct microscopy observation. The samples without any growth of *Blastocystis* after 10 days were considered as negatives.

DNA extraction

In order to extract DNA from samples, 250 μL of *Blastocystis*-positive samples were transferred to sterile 1.5-mL tubes and centrifuged at 2,500×*g* for 5 min. Then, supernatant was discarded and DNA was extracted from the remained pellet using YTA stool DNA Extraction kit (Yekta Tajhiz Azma, Tehran, Iran). Finally, extracted DNA was kept out in − 20 °C until further investigation.

Primer efficacy

To evaluate the efficacy of designed primers, conventional PCR was assessed using thermal profile: 95 °C for 5 min followed by 35 cycle of 95 °C for 30 s, 59 °C/60 °C for 35 s, and 72 °C for 30 s and a final extension 72 °C for 5 min. Amplified fragments were electrophoresed on 1.2% agarose gel and visualized using ethidium bromide staining and UV transluminator.

Performing HRM real-time PCR

HRM real-time PCR was carried out using Rotor-Gene Q (QIAGEN, Germany) real-time instrument for *Blastocystis*-positive isolates. Each reaction was performed in total volume 20 μL of 2X Type-it HRM master mix (QIAGEN, Germany), 0.5 μL of 10 pmol of each primer, and template DNA. Thermal profile for amplification of the expected fragment was consisted of 95 °C for 5 min followed by 40 cycles: 95 °C for 30 s, 60 °C for 35 s, and 72 °C for 30 s and ramping from 70 °C to 85 °C at 0.02 °C s^{-1}. The melting profiles were analyzed using Rotor-Gene Q software. In order to confirm the results of HRM real-time PCR, barcoding region of SSU rRNA gene of *Blastocystis*-positive were amplified using primers: RD5 (5′-ATCTGGTTGATCCTGCCAGT-3′) and BhRDr (5′-GAGCTTTTTAACTGCAACAACG-3′) (Scicluna et al. 2006). The amplified fragments were sequenced using ABI sequencer 3130, and the generated results were compared in GenBank database to characterize subtypes.

Testing specificity of the primers

To analysis the specificity of designed primers, amplification of DNA of some parasites and bacteria was tested. The main reasons for selecting this microorganism were (1) presence in stool samples and (2) presence in stock bank of our laboratory. For this purpose, DNA of *Enterocytozoon bieneusi*, *Encephalitozoon* spp., *Fasciola* spp., *Giardia deodenalis*, *Helicobacter pylori*, *Clostridium difficile*, *Clostridium perfringens*, *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* was extracted. Indeed, to cover all other genera of bacteria and eukaryotes and also other materials that may be found in stool samples, total DNA of ten *Blastocystis*-negative stool samples were also extracted and tested for amplification of non-specific fragments using the primers (Table 1).

Testing sensitivity of HRM real-time PCR

In order to determine sensitivity of the test, vacuolar form of *Blastocystis* subtype 1 was harvested from a 3-day culture medium and counted by Neubauer chamber. After that, seven 10-fold serial dilutions (10^6 to one *Blastocystis* per mL of stool sample) of the parasite were prepared. Briefly, ≃ 10^6 of *Blastocystis* were suspended in 1 mL of *Blastocystis*-negative stool samples in a 1.5 mL tube. Afterward, 100 μL from the first tube was transferred to the second tube containing 900 μL of *Blastocystis*-negative stool suspension and it was continued to the seventh tube. Then, DNA extraction was performed for each of diluted stool sample as mentioned above. Finally, extracted DNA was used as template in a run of HRM real-time PCR.

Statistical analysis

To determine the mean ± standard deviation (SD) of temperature profile of each subtype, IBM SPSS Statistics for Windows, v23 (Chicago, IL, USA) was employed.

Table 1 Microorganisms that were tested for evaluation of specificity of primers

	Microorganisms
Bacteria	*Helicobacter pylori*
	Clostridium difficile
	Clostridium perfringens
	Escherichia coli
	Pseudomonas aeruginosa
	Staphylococcus aureus
Parasites	*Enterocytozoon bieneusi*
	Encephalitozoon spp.
	Fasciola spp.
	Giardia deodenalis

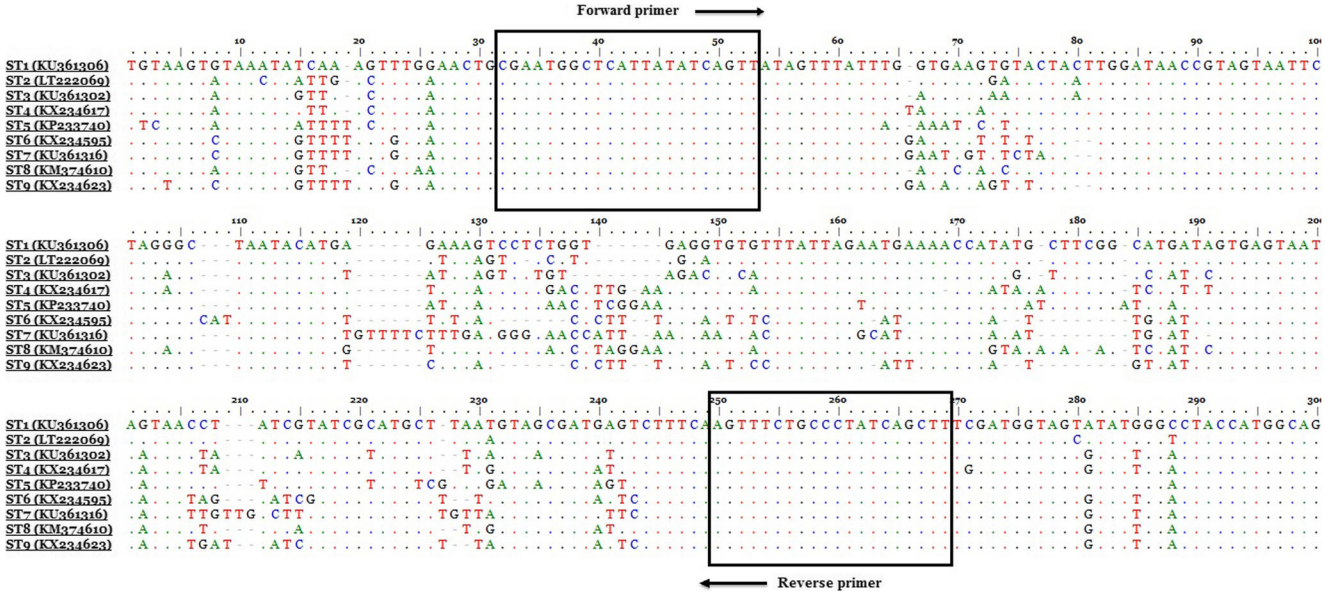

Fig. 1 Alignment of sequences of *Blastocystis* subtypes 1–9 using BioEdit software and the position of primers forward and reverse

Results

HRM assay and comparison the results with sequencing

According to the in silico analyzes, primers were designed from the same region of SSU rRNA gene for all subtypes of the parasite of which there were no any degenerative bases in designed primers. The results of in silico showed that designed primers were able to amplify all 17 subtypes of *Blastocystis* (Fig. 1).

The results of sequencing showed presence of ST1 (no. 20), ST2 (no. 14), ST3 (no. 28), ST6 (no. 2), ST7 (no. 3), ST10 (no. 1), and ST14 (no. 4) among the samples. The experimental analysis of HRM real-time PCR showed that seven subtypes including ST1 (no. 22), ST2 (no. 12), ST3 (no. 30), ST6 (no. 2), ST7 (no. 3), ST10 (no. 1), and ST14 (no. 4), were successfully discriminated based on different attributed amplified fragment T_m. Table 1 illustrates in silico and experimental T_m of amplified fragments for each subtype (Table 2). In addition, HRM real-time PCR indicated that the value of T_m of each subtype was repeatable for all studied samples (Figs. 2 and 3). Concerning the results of HRM real-time PCR, apart from two samples that were characterized as ST1 with HRM real-time PCR, while they were identified as ST2 with PCR/sequencing, the results of subtyping with HRM real-time PCR were the same with PCR/sequencing. In addition, HRM real-time PCR was detected in two mixed subtypes (ST1 and ST3) that were characterized as ST1 using PCR/sequencing (Table 3) (Fig. 4).

Primer efficacy

The results of primer efficacy test showed that the expected ~ 220-bp fragment was intensely amplified for all evaluated subtypes without non-specific PCR product at the annealing temperatures 59 °C and 60 °C (Fig. 5)

Table 2 In silico and experimental melting temperatures of the amplified fragments of each subtype

Subtypes	In silico temperature (°C)	Experimental temperature ± SD (°C)
ST1	77.4	78.55 ± 0.16
ST2	76.8–77	78.41 ± 0.13
ST3	75	76.32 ± 0.18
ST4	75.6–75.8	NA
ST5	75.2–75.4	NA
ST6	74–74.2	75.76 ± 0.02
ST7	75.3–75.4	76.62 ± 0.04
ST8	76–76.1	NA
ST9	74.2–74.3	NA
ST10	75.6	78.75
ST11	77.8	NA
ST12	77.8	NA
ST13	76.2	NA
ST14	75.4–75.8	76.86 ± 0.11
ST15	72.5	NA
ST16	75.2	NA
ST17	73.7	NA

NA not assigned (these subtypes were not detected in our study)

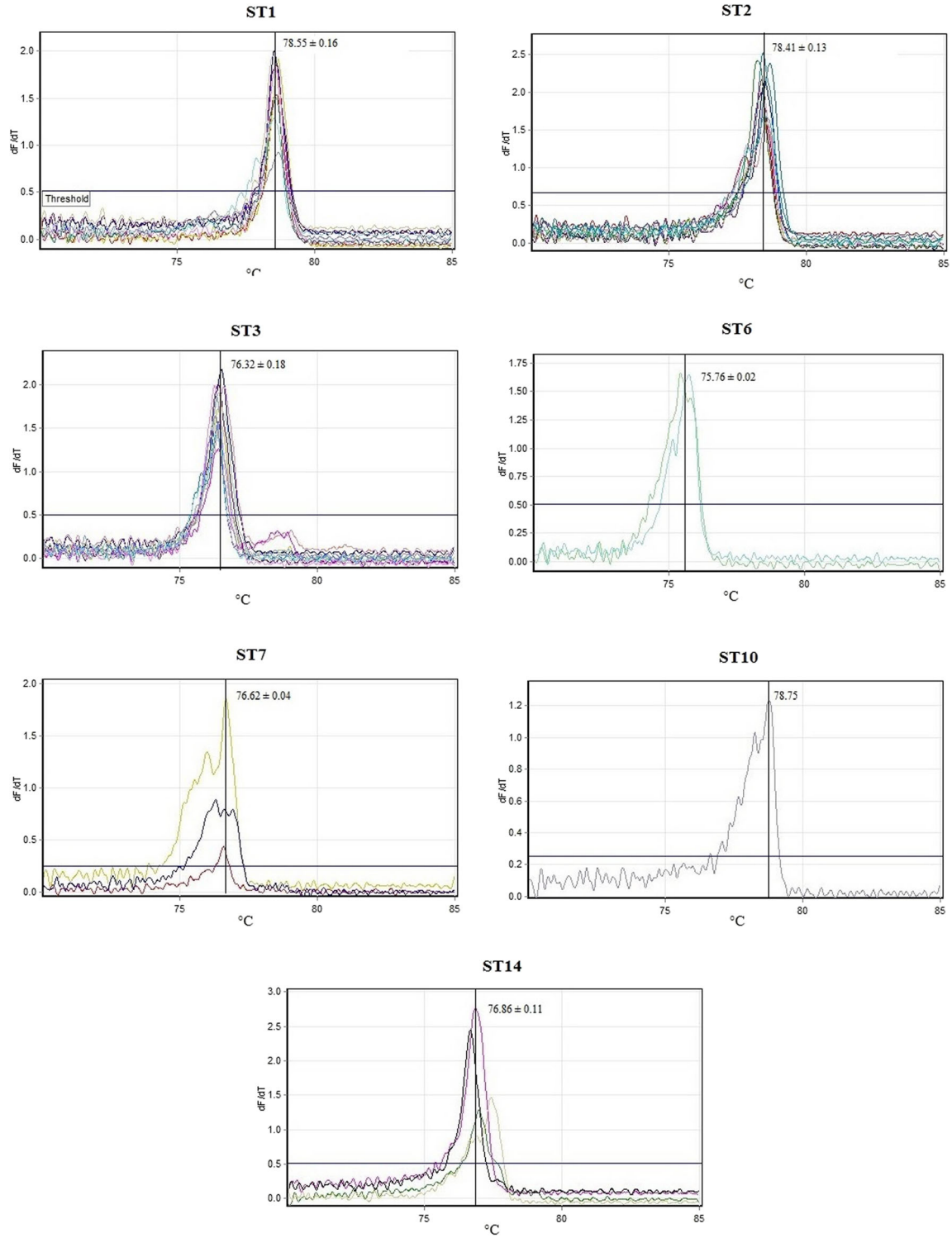

Fig. 2 Melting T_m peaks indicate different melting temperatures for relevant subtypes

Specificity and sensitivity tests

In order to evaluate the specificity of the primers, HRM real-time PCR was performed using DNA template of other microorganisms mentioned in the "Materials and methods" section that showed no amplification of DNA among tested microorganisms. Furthermore, the results of the sensitivity test revealed that real-time PCR coupled

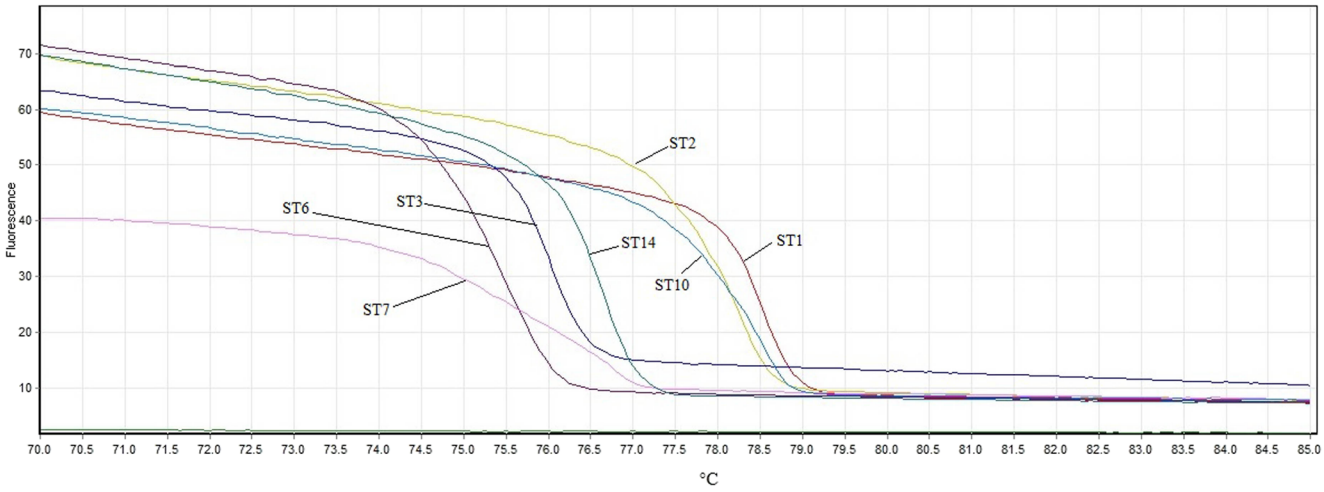

Fig. 3 High-resolution normalized melting curves of subtypes 1-3, 6, 7, 10, and 14 in a single run

with HRM was able to detect 10^6 to even one *Blastocystis* in 1 mL of stool sample with cycle threshold (Ct) from 14 to 24.5, respectively (Fig. 6).

Discussion

Blastocystis is a prevalent protozoan parasite of which 17 subtypes have been characterized from humans and animals. However, this is the first study that developed and assessed real-time PCR coupled with HRM in order to directly detect *Blastocystis* and characterized its subtypes from stool samples.

Early detection and subtyping of *Blastocystis* is very important not only for epidemiological studies but also for the treatment of patients with gastrointestinal disorders, particularly when there are no specific symptoms. Some studies indicated and proposed the pathogenic role of some subtypes in gastrointestinal and extra-intestinal disorders (Bart et al. 2013; El Safadi et al. 2013). Indeed, epidemiological data have shown the importance of characterization of *Blastocystis* subtypes in order to investigate the source and route of transmission (Alfellani et al. 2013a, 2013b; Stensvold et al. 2012b). Therefore, techniques with high sensitivity for direct detection of *Blastocystis* and the subtyping of this parasite from stool samples can be used for clinical, as well as epidemiological studies.

Several studies have shown a higher sensitivity and specificity of molecular methods when compared with conventional microscopical examination and even cultivation (Jalallou et al. 2017; Poirier et al. 2011; Stensvold et al. 2012a; Stensvold et al. 2007a). Although most of studies have indicated that the cultivation of *Blastocystis* has a higher sensitivity for the detection of the parasite compared to traditional parasitological examinations, it was stated that competition between subtypes could lead to the overgrowth of some subtypes compared to others (Parkar et al. 2007; Stensvold 2015). However, this phenomenon can result in an underestimation of the prevalence and distribution of some subtypes. Besides, it has been proven that only the viable forms of *Blastocystis* are able to grow in culture media (Poirier et al. 2011; Stensvold et al. 2012a). Therefore, in patients who because of the consumption of antibiotics are negative for viable forms of the parasite, the results of cultivation would be falsely negative.

During the last two decades, several parasitological and molecular techniques have been used for the detection of *Blastocystis*. It seems that Clark and his colleagues (Clark 1997) firstly employed molecular techniques for the detection and molecular analysis of *Blastocystis*. For this purpose, they

Table 3 The number of *Blastocystis* isolates identified by PCR/sequencing and HRM real-time PCR

Subtypes	No. of samples identified by PCR/sequencing	No. of samples identified by HRM
ST1	20	22
ST2	14	12
ST3	28	30
ST6	2	2
ST7	3	3
ST10	1	1
ST14	4	4
Total	72	74

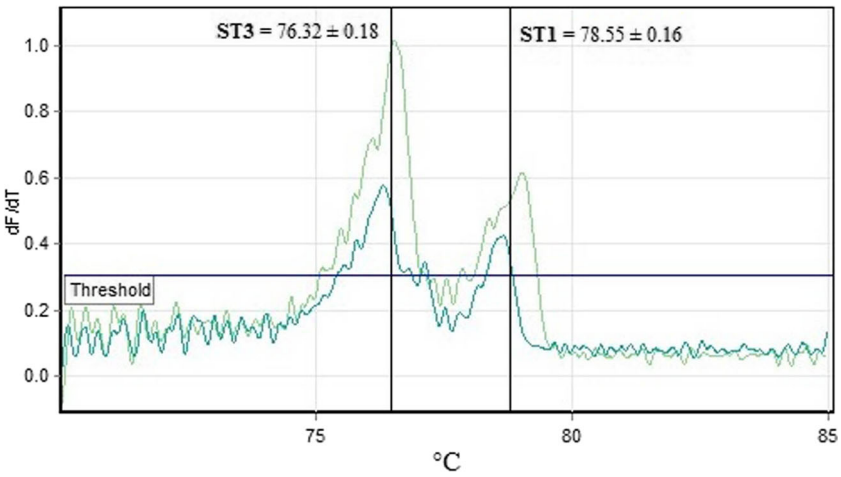

Fig. 4 Two peaks of melting T_m show mixed subtypes with ST1 and ST3

classified *Blastocystis* into seven ribodems using PCR-RFLP. Although this method was used for at least the next 10 years, the limitations of PCR-RFLP such as mutation at the site of enzyme restriction, as well as mixed subtypes (Moosavi et al. 2012; Stensvold et al. 2007b) led to a dramatic increase in the number of studies that used STS primers (Yoshikawa et al. 1996; Yoshikawa et al. 1998; Yoshikawa et al. 2000; Yoshikawa et al. 2004) for the detection and subtyping of *Blastocystis* from stool samples.

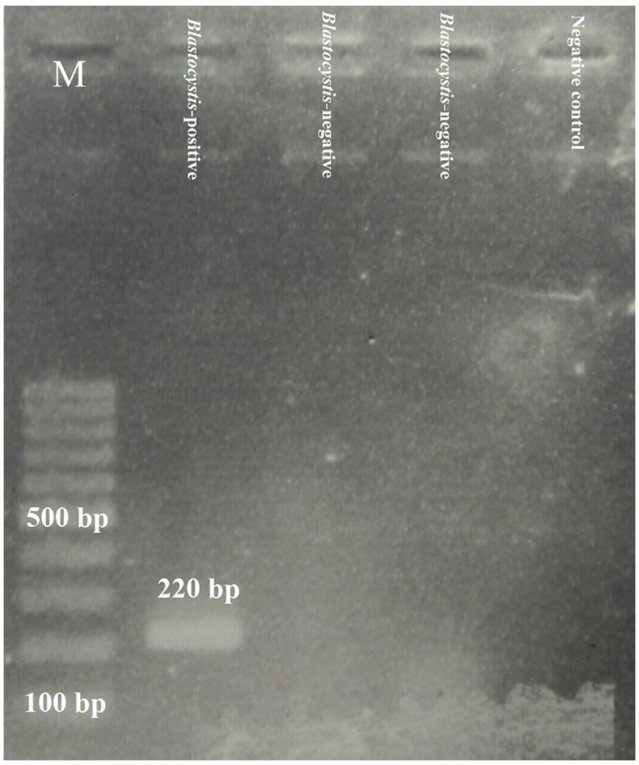

Fig. 5 Agarose gel electrophoresis shows that ~ 220-bp fragment of SSU rRNA gene was amplified using designed primers for *Blastocystis*-positive sample, while non-specific fragments were not seen. M, 100 bp ladder

As the literature show, although amplification of the targeted fragment of SSU rRNA gene using STS primers overcomes the limitations resulting from PCR-RFLP, these primers are not able to amplify subtypes other than ST1–7. In addition, it was shown that in spite of high specificity of STS primers, the sensitivity of these primers for amplification of *Blastocystis* DNA was low (Stensvold 2013b). However, in recent years, amplification and sequencing of the targeted fragment of SSU rRNA gene using primers introduced by Scicluna et al. (2006) and Stensvold et al. (2007a) were the most popular techniques to detect and characterize *Blastocystis* subtypes in both clinical and epidemiological studies. Although this technique provides interesting data on inter- and intra-subtype genetic diversity, it looks that mixed subtypes is the main limitation of PCR/sequencing. In another words, in the cases of mixed subtypes sequencing results mainly shows the dominantly amplified subtype, thus, another available subtype may be misdiagnosed. This subject was highlighted by the results of the current study, showing that the designed HRM real-time PCR was able to distinguish 2/72 (2.77%) of the sequenced subtypes as mixed (ST1 and ST3), while they were characterized as ST1 using PCR/sequencing.

Previous studies designed, developed, and executed qReal-time PCR (Poirier et al. 2011) and TaqMan real-time PCR (Stensvold et al. 2012a) for detection of *Blastocystis* from stool samples. Poirier et al. (2011) could detect *Blastocystis* and showed a melting curve of all nine human-infecting subtypes from stool samples using SYBR green real-time PCR, while the obtained melting temperatures were not discriminative enough to separate different available subtypes. The next study by Stensvold et al. (2012a) was performed based on the

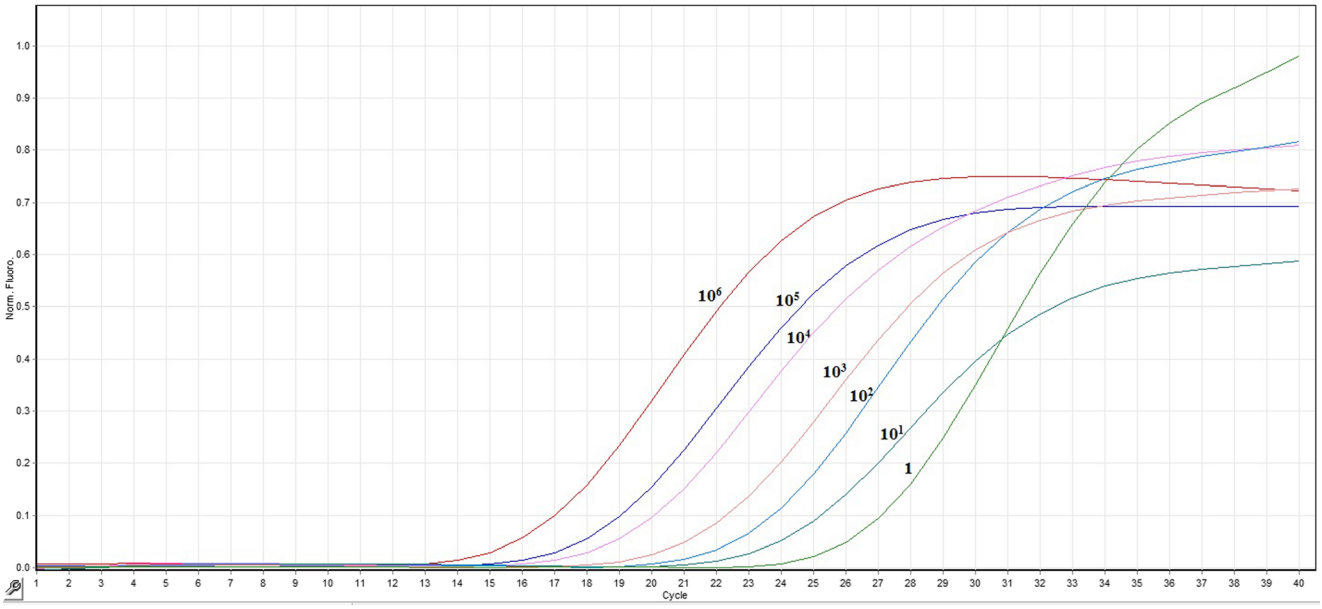

Fig. 6 The results of sensitivity assay showed that HRM real-time PCR using designed primers was able to detect 10^6 to 1 *Blastocystis* in 1-mL stool sample

TaqMan real-time PCR using specific primers and probe and claimed that not only the sensitivity of the method was significantly higher than previously designed real-time PCR but also because of using probe, the specificity of the test was more than the other introduced real-time PCR techniques for the detection of *Blastocystis*. However, this method was not able to separate and nominate subtypes of the parasites. In the current study, real-time PCR coupled with HRM was employed to detect and characterize subtypes of *Blastocystis* from stool samples. In this study, the designed HRM real-time PCR was able to discriminate all 17 subtypes of *Blastocystis* using in silico analysis, although, due to the lack of access to all subtypes, only 7 available subtypes (ST1–3, ST6, 7, 10, and 14) were successfully tested.

The primers used for the HRM real-time PCR amplified the ~ 220 bp fragment of the SSU rRNA gene. It is stated that molecular approaches that provide shorter PCR products provide enough sensitivity for use in diagnostic laboratories (Bustin and Huggett 2017; Debode et al. 2017; Stensvold et al. 2012a). In the current study, the designed HRM real-time PCR was able to detect approximately one parasite in 1 mL of stool suspension. Therefore, besides the results of the sensitivity test and the length of targeted product, it seems that our method has enough sensitivity to detect *Blastocystis* in stool samples. On the other hand, concerning the negative results of the specificity test, it seems that the HRM real-time PCR using these primers is suitable for the detection of *Blastocystis* from stool samples.

Although it looks that HRM real-time PCR was able to simultaneously detect and characterize *Blastocystis* and its subtypes in stool samples, because of the short amplified fragment in this technique, this method is not able to provide enough information about nucleotide variation in comparison with PCR/sequencing. Therefore, presence of unexpected mutation through the targeted fragments may lead to misinterpretation of subtyping. Furthermore, HRM real-time PCR needs specific instrument (real-time PCR machine with HRM canal) that may not be available in all laboratories, particularly in undeveloped regions.

However, early detection and subtyping of *Blastocystis* using new methods were claimed to be useful and helpful for both clinical and epidemiological studies, particularly when compared with those methods that are not able to detect and distinguish either samples with a small number of *Blastocystis* or mixed subtypes. Nonetheless, it seems that unexpected nucleotide variability across the amplified fragment using the HRM real-time PCR is the main limitation of this method.

Conclusion

HRM real-time PCR was able to detect even only one parasite in 1 mL of stool suspension, while DNA of *Blastocystis*-negative stool samples together with a number of other microorganisms were not amplified using the designed primers. Furthermore, in silico analysis showed that this test was able to discriminate all 17 subtypes of the parasites and all tested *Blastocystis* subtypes were distinguished based on melting curve analysis of the targeted fragment. Therefore, we believe that this method could be employed as a rapid, sensitive, and specific method for simultaneous detection of *Blastocystis* and its subtypes in both clinical and epidemiological practices.

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Availability of data and materials The data associated with this manuscript consisted of normalized melting curves are included in the article. Furthermore, we used from available isolates in our laboratory that were previously sequenced and subtyped.

Authors' contributions HM conceived and designed the experiments. HMR performed the experiments. HM and HMR analyzed the data. MN, AH, HAA, and MRZ contributed reagents/materials/analysis tools/positive samples.HM, HMR, and MN wrote the paper. All authors read and approved the final version of the manuscript.

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Compliance with ethical standards

Ethics approval and consent to participate All procedures performed in this study were in accordance with the ethical standards (IR.SBMU.RIGLD.REC.1396.163) released by Ethical Review Committee of the Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran. Consent is not applicable.

Informed consent Not applicable.

Competing interest The authors declare that they have no conflict of interest.

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