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Research Article

Comparison between Conventional PCR and SYBR Green based real-time PCR Assay for the Detection of *Blastocystis* from Human Fecal Specimens

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Abstract

Blastocystis is an atypical stramenopile that inhabits multiple hosts, including humans. Nine subtypes (STs) of the organism have been isolated from humans. This organism has a controversial pathogenicity status. Multiple detection methods are available to identify *Blastocystis* from human fecal specimens, but the molecular methods are the best for diagnosis. This study compares the ability and utility of conventional PCR with real-time PCR assay based on SYBR Green method for the detection of *Blastocystis* from human fecal specimens. From a total of 279 fecal samples, DNA was extracted and subjected to conventional PCR and SYBR Green based real-time PCR assay. Out of 279 samples screened, 105 were positive according to conventional PCR (37.63%). Furthermore, the positive samples were amplified in a conventional PCR using another set of primers to double check all the positive cases. The SYBR Green based real-time PCR assay was positive for 109 DNA samples (39.06%). The positive cases included all the positive cases detected by conventional PCR. Conventional PCR method was considered as the gold standard test for diagnosis. On comparing conventional PCR results with SYBR Green based real-time PCR assay, the latter was found to be 100% sensitive with 97.7% specific. A kappa agreement of 0.970 at 95% confidence interval was observed; hence, the strength of agreement between conventional PCR and SYBR Green based real-time PCR assay was considered to be "very good." Consequently, the assay can be effective in large-scale epidemiological studies for screening purpose because of its cost-effectiveness, capacity to detect all subtypes of *Blastocystis*, and reduction in detection time compared to conventional PCR. Additionally, melt peak analysis also provides a general perception of the different subtypes of *Blastocystis* available in the study population.

Keywords: *Blastocystis*; Subtypes; PCR; India

Introduction

Blastocystis, a lumen-dwelling human intestinal parasite, has been reported to inhabit multiple hosts. Till date, there are nine subtypes (STs) of *Blastocystis* that have been known to infect humans [1-3]. The role of *Blastocystis* in human health is questionable due to its presence in both healthy individuals as well as individuals with intestinal and other clinical manifestations [4,5]. There are many detection modalities available for identifying *Blastocystis* from human stool samples. Stool microscopy is considered as the most inaccurate detection modality among all existing techniques due to the polymorphic nature of the organism [6-8]. *Blastocystis* can be cultured from stool using various xenic and axenic culture media [9]. In the absence of molecular diagnostic facilities, cultures have proven to be the best alternative for detection. However, they consume 48–72 hours for detection and the culture morphology is not helpful for discriminating among the different subtypes of *Blastocystis* [8,10]. The subtype identification is very much necessary to unveil the association of a particular subtype

with a clinical condition. Molecular diagnosis involves using conventional PCR technique with various primer sets; the detection rate varies depending on the selection of the primer set [11]. Previous studies have found primers used by Scicluna., *et al*. [12] to be good for simultaneous diagnosis as well as subtyping of *Blastocystis*. However, in a large-scale epidemiological study, conventional PCR consumes additional time and detection rate would decrease if the parasite load is low. Hence, this study compares real-time PCR assay with conventional PCR. There are three real-time PCR assays available for the detection of *Blastocystis* from fecal samples [13-15]. Among these, two of the real-time assays use probes and are sensitive but expensive in resource-poor settings, especially during large-scale studies. Nevertheless, in spite of non-specific binding of the SYBR Green dye, the method developed by Poirier., *et al*. using SYBR Green assay was found to have a good sensitivity (100%) and specificity (95%) in a prospective epidemiological study involving two groups [14]. Hence, the real-time PCR assay based on SYBR Green acts as a low-cost, rapid assay with the capability to identify all relevant human subtypes of *Blastocystis* upon Sanger sequencing of the PCR product. Hence, this study uses the SYBR Green based real-time PCR assay for the detection of *Blastocystis* from clinical stool specimens and compares it with a convention PCR technique.

Material and Methods

Samples

This study was approved by the institute's ethics committee. A total of 279 clinical stool samples were collected during the study period (between 2014 and 2016) and processed at the Department of Microbiology, Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER), Puducherry, India. Initially, the samples were screened by macroscopy and microscopy techniques. Thereafter, for molecular analysis, a portion of the sample was stored at -80˚C without any added preservatives.

DNA extraction

The stored stool sample was used for DNA extraction using QI Amp Stool Mini Kit (Germany) as per the manufacturer's instructions. The Nano Drop 2000C (Thermo Fisher, US) or 0.8% agarose gel electrophoresis was used to check the DNA's quality.

Conventional PCR

The following set of primer was used for conventional PCR detection: forward – RD5 5'-ATCGCCACTTCTCCAAT-3'; reverse – BhRDr 5'-GAGCTTTTTAACTGCAACAACG-3'. This primer amplifies the 600-bp barcoding region of the 18S small subunit ribosomal DNA (SSU-rDNA) of *Blastocystis* [12]. The PCR products were visualized on 1.5% agarose gel (Figure 1).

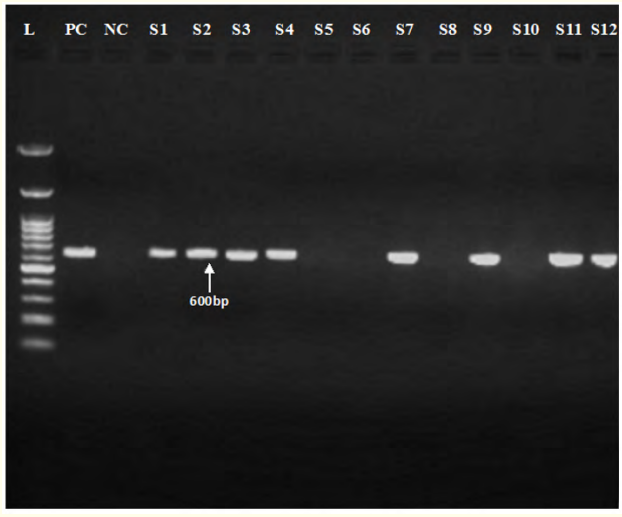

Figure 1: Representative Gel Depicting *Blastocystis* Screening with 600 bp Bar-coding Region of 18S SSU-rDNA Amplification. Notes: L – 100 bp ladder; NC – Negative control; PC – Positive control; and S1 to S12 – Stool DNA samples.

All *Blastocystis* positive samples were double checked for positivity using an additional set of primers (forward – 5'-GGAGGTAGTGACAATAAATC-3' and reverse – 5'-ACTAGGAATTCCTCGTTCATG-3'), which amplified the 1,112 bp of *Blastocystis* 18S SSU-rDNA [16]; 1.3% agarose gel was used to run the PCR amplicons. PCR inhibition was addressed by an internal process control PCR [17].

All three conventional PCR reactions were performed with 20 µL of reaction mixture. This mixture contained Amplicon III Red Taq master mix (2×), Tris HCL (pH 8.5), $(NH_4)_2$ SO4, $MgCl_2$ (4 mM), dNTPs (0.4 mM), Tween 20 (0.2%), ampliqon Taq DNA polymerase (0.2 units/µL), primers (0.4 µM), and DNA (2 µL). The final volume was made up using nuclease-free water.

SYBR Green based real-time PCR

SYBR Green based real-time PCR uses primers suggested by Poirier., *et al*. These primers are BL18SPPF1 (5'-AGTAGTCATACGCTCGTCTCAAA-3') and BL18SR2PP (5'-TCTTCGTTACCCGTTACTGC-3') [14]. Based on the presence of any one of the nine subtypes of *Blastocystis*, these primer sets amplify a DNA fragment of 320 to

342 bp. The PCR reaction mixture volume was 20 µl containing TaKaRa SYBR® Premix Ex Taq™ II (Tli RNase H Plus) ready mix (10 µL) with TAPS buffer (25 mM, pH 9.3, 25°C), $MgCl_2$ (2 mM), DTT (0.1 mM), and dATP, dGTP, and dCTP (each 200 µM), along with [^{3}H]-dTTP (100 µM), activated salmon sperm DNA (0.25 mg/mL), both primers (0.2 µL each), and DNA (2 µL). Nuclease-free water was used to make up the final volume.

The PCR steps involved initial denaturation at 95°C for 30 seconds and repeated 40 cycles at 95°C for 5 seconds and at 60°C for 30 seconds. Real-time PCR amplifications were performed using a Biorad CFX 96 system. For data analysis, the melting curve and cycle threshold (CT) values were selected as the evaluation parameters. The samples showing CT values within 40 cycles and the melt curve ranging between 78°C and 84°C were considered positive.

Results and Discussion

Out of the 279 DNA samples screened by conventional PCR, 105 were found to be positive for *Blastocystis* (37.63%). The positivity of these samples was double confirmed by another PCR (Figure 2). The real-time PCR assay based on SYBR Green was positive for 109 DNA samples (39.06%) (Figure 3), which included all the positive cases detected by conventional PCR. Hence, this assay was found to be 100% sensitive. However, as mentioned by Poirier., *et al*. this assay did give false positive results with a specificity of 95% [14]. This false positivity could be because of low parasitic load in the stool sample or non-specific DNA amplification of genetically resembling eukaryotes found in the human gut [15]. These samples had a higher CT value (>35) and could not obtain the desired PCR product by running a 1.3% agarose gel as well. Hence, the 4 samples that were positive by quantitative PCR (qPCR) alone were unable to be sequenced.

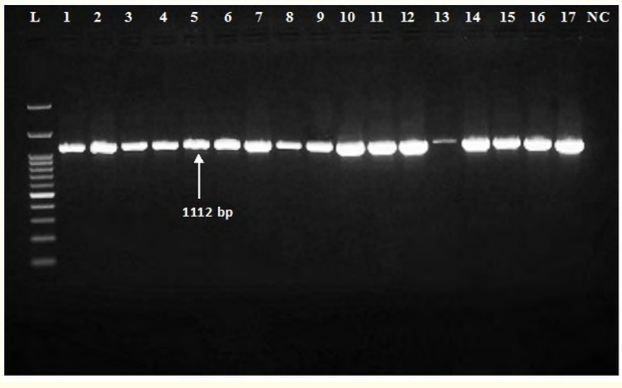

Figure 2: Representative Gel Depicting 1,112 bp Amplicons on a 1.3% Agarose Gel Confirming the Positive Results Obtained by Bar-coding PCR.

Notes: L – 100 bp ladder; 1–17 – Positive *Blastocystis* samples of PCR 1; NC – Negative control.

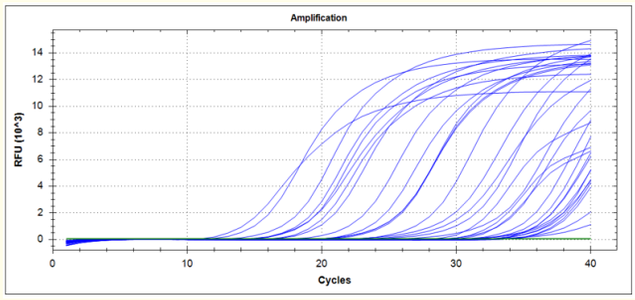

Figure 3: Real-time PCR based on SYBR Green Amplification Plots Showing *Blastocystis*-positive DNA.

The real-time assay based on SYBR Green method had 100% sensitivity and a specificity of 97.7%, with a Kappa agreement of 0.970 at 95% confidence interval. The strength of agreement between the conventional PCR and qPCR was considered to be "very good" (Table 1).

SYBR Green based Real-time PCR	Conventional PCR		Total
	Positive	Negative	
Positive	105 (TP)	4 (FP)	109
Negative	0 (FN)	170(TN)	170
Total	105	174	279
TP – True positive; FP – False positive; FN – False negative; TN – True negative.			
Sensitivity – 100%; Specificity – 97.7%; PPV – 96.33%; NPV – 100%; Kappa agreement = 0.970; Confidence interval – from 0.94 to 0.999.			
The strength of agreement is considered to be "very good."			

Table 1: Two-way Contingency Table for Comparing Real-time PCR based on SYBR Green method against Conventional PCR.

During the analysis of results, three distinctive melt peaks and a corresponding melt curve in qPCR were detected (Figures 4 and 5). A representative sample from each peak was subjected for sequencing to discern the subtypes. The sequencing result confirmed the presence of the following subtypes *Blastocystis*: ST1, ST2, and ST3. It is mandatory to sequence all the positive samples to know the accurate subtype; however, a prediction may be made based on the melt peak analysis. In the study population, a large number of ST3 and ST1 isolates were detected, with very few ST2 isolates. Nevertheless, at this point of time, the presence of other subtypes was not that evident in the study population. *Blastocystis* genetic diversity studies from India have revealed the presence of ST3 and ST1 in human fecal specimens. However, this study reports for the first time in India the presence of ST2 in human stool samples.

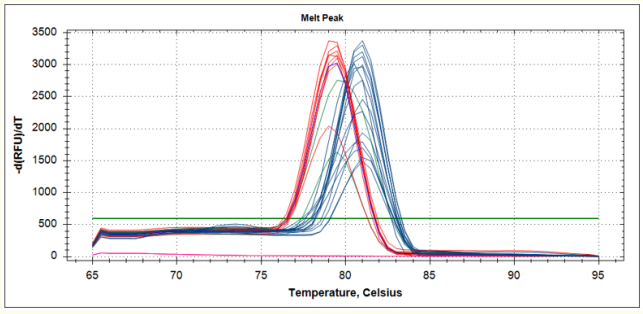

Figure 4: Melt Peak Differentiations of Subtypes of *Blastocystis*. Notes: Three distinct melt peaks were observed indicating the presence of three *Blastocystis* subtypes (ST1 – Blue peak; ST2 – Green peak; ST3 – Red peak).

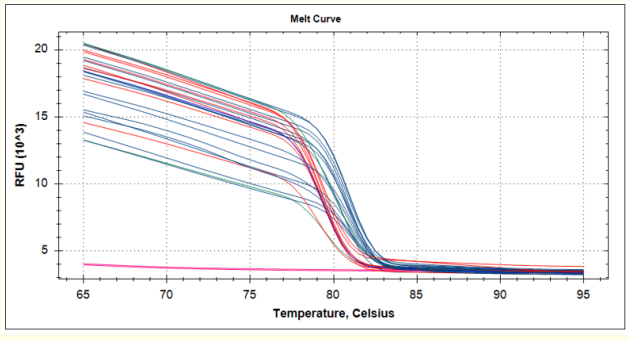

Figure 5: Melt Curve Analysis of SYBR Green based Real-time PCR Assay for *Blastocystis*.

The TaqMan assay developed by Stensvold., *et al*. is a superior detection method for the identification of *Blastocystis* from human fecal specimens [15]. However, one of the major disadvantages of this assay is its higher cost. During large-scale epidemiological or clinical studies, especially working on a neglected organism such as *Blastocystis*, having known for its controversial pathogenicity status, TaqMan assays were found to be expensive in resource-poor settings. Additionally, the assay cannot be used for accurate subtyping by sequencing of PCR products in spite of it targeting a hypervariable region. This is because the amplicon size is relatively smaller compared to the amplicon size usually recommended for the subtyping of *Blastocystis*.

The Light Cycler (LC) PCR real-time assay developed by Jones., *et al*. [13] targets an uncharacterized region of the *Blastocystis* genome and was able to amplify only certain subtypes of human relevance. This too is a probe-based method and was found to be expensive for large-scale studies in resource-poor settings.

In spite of the non-specificity associated with SYBR Green dye, as well as the length of the PCR product targeted, the real-time PCR assay based on SYBR Green method was able to identify all the positive samples of conventional PCR, indicating high sensitivity of the assay. However, to confirm the doubtful positive samples, sequencing of the PCR amplicon is necessary.

Conclusion

The SYBR Green based real-time assay was found to be highly sensitive with 97.7% specificity. The assay comes handy in large-scale epidemiological studies due to its cost-effectiveness, capacity to detect all subtypes of *Blastocystis*, and reduction in detection time compared to conventional PCR. Additionally, melt peak analysis also provides a general perception of the different subtypes present in the study population. Hence, the real-time PCR assay based on SYBR Green method may be used in large-scale epidemiological or clinical studies for screening *Blastocystis*.

Acknowledgments

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Conflicts of Interest

There are no conflicts of interest.

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