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Comparison of direct fecal smear microscopy, culture, and polymerase chain reaction for the detection of *Blastocystis* sp. in human stool samples

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ABSTRACT

Objective: To compare the sensitivity and specificity of direct fecal smear microscopy, culture, and polymerase chain reaction in the detection of *Blastocystis* sp. in human stool. **Methods:** Human stool samples were collected from a community in San Isidro, Rodriguez, Rizal, Philippines. These samples were subjected to direct fecal smear microscopy, culture and polymerase chain reaction to detect the presence of *Blastocystis* sp. **Results:** Of the 110 stool samples collected, 28 (25%) were detected positive for the presence of *Blastocystis* sp. by two or more tests. Culture method detected the highest number of *Blastocystis*-positive stool samples (*n*=36), followed by PCR of DNA extracted from culture (*n*=26), PCR of DNA extracted from stool (*n*=10), and direct fecal smear (*n*=9). Compared to culture, the sensitivity of the other detection methods were 66.7% for PCR from culture and 19.4% for both PCR from stool and direct fecal smear. Specificity of the methods was high, with PCR from culture and direct fecal smear having 97.3%, while PCR from stool at 95.9%. **Conclusions:** In this study, *in vitro* culture is the best method for detecting *Blastocystis* sp. in human stool samples.

1. Introduction

Blastocystis sp. is a strictly anaerobic, enteric parasite that inhabits the intestinal tract of many animal species, including humans. Optimal growth of this organism occurs at neutral pH and at a temperature of 37 ℃[1]. *Blastocystis* isolates show diversity by having four distinct morphologies[1] and at least thirteen unique subtypes[2] based on phylogenetic studies using small subunit ribosomal RNA sequences. It is believed to be the most frequent protozoan reported in human fecal samples, with a prevalence ranging between 30% and 50% in some developing countries[1]. The pathogenicity of this parasite is still an enigma, although the Centers for Disease Control and Prevention considers the organism as pathogenic[3]. *Blastocystis* cells are usually identified microscopically via direct examination of stool, fecal concentration techniques, or *in vitro* culture of fecal material. However, the polymorphic nature, size variation, and cell number of the parasite contribute to the factors that complicate detection of the parasite via microscopy[4]. Thus, other methods of detection have been developed, including immunoassays, and molecular techniques such as polymerase chain reaction (PCR). Many researchers have used PCR-based assays for *Blastocystis* detection[5-7]. In this study, we compared four commonly used methods for identifying presence of *Blastocystis* in human stool, namely, direct fecal smear, *in vitro* culture, PCR of DNA from stool, and PCR of DNA from culture.

2. Materials and methods

2.1. Collection of human stool samples

A total of 110 stool samples were collected from a

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community in San Isidro, Rodriguez, Rizal, Philippines. Samples were transported to the laboratory using a cooler with ice, and stored long-term in freezer prior to genomic DNA extraction.

2.2. Culture

Axenic *Blastocystis hominis* Brumpt SVM (ATCC number 50613) culture was used as positive control throughout the experiment. It was grown using *Blastocystis* egg medium (ATCC Medium 1671) with modified Locke's solution as overlay, supplemented with 25% horse serum. The organism was cultured anaerobically using a BBL Gas-Pak Jar and Anaeropack-Anaero (Mitsubishi Gas Chemical Co.) at 35.5 ℃. Subcultures were done every three to four days.

2.3. Detection of Blastocystis

2.3.1. Microscopy of direct fecal smear and culture from stool

Direct fecal smears stained with iodine were observed using light microscope to confirm the presence of *Blastocystis* cells. Meanwhile, approximately 100 μg of stool were inoculated onto egg medium and were incubated at 37 ℃ for three days. Prepared smears from cultures were also observed under 400× magnification of a bright field microscope to check for the presence of the characteristic vacuolar cells of *Blastocystis* when grown *in vitro*. Positive tubes were subcultured and subsequently observed after three days to confirm the presence of the parasite. Negative tubes, on the other hand, were incubated further and were observed everyday until after six days. Afterwards, all culture tubes were subjected to DNA extraction.

2.3.2. DNA extraction from stool

Genomic DNA extraction from parasitic cells in stool was performed using ZR Fecal DNA Mini-Prep Kit (Zymo Research, Irvine, CA, USA) following manufacturer's instructions.

2.3.3. DNA extraction from culture

Genomic DNA was extracted from cells grown in culture through the Chelex DNA extraction protocol[8]. Briefly, cells were harvested and washed thrice with phosphate buffered saline (PBS) via centrifugation. Afterwards, the pellet was resuspended by adding 200 μL of 5% Chelex mixture and was incubated at a 56 ℃ water bath for 30 min. The samples were then vortexed at high speed until the pellet was totally resuspended in the supernatant. The mixture was then incubated in boiling water for 8 min, followed by centrifugation at 14 000 *g* for 3 min. The remaining supernatant containing the genomic DNA was transferred to a new microcentrifuge tube and was stored in freezer until use.

2.4. Polymerase chain reaction (PCR)

Genomic DNA extracted from *in vitro* culture and stool, respectively, were tested for the presence of *Blastocystis* via PCR. PCR was performed using specific primers, SR1F (5'-GCT TAT CTG GTT GAT CCT GCC AGT AGT -3') and SR1R (5'-TGA TCC TTC CGC AGG TTC ACC TA-3'), which target a conserved region of *Blastocystis hominis* small subunit rDNA[9]. The resulting amplicons have a length of about 1 780 base pairs. The PCR products were run in 1.5% agarose gel to check which samples are positive for *Blastocystis*. The gel was stained with SYBR Green to visualize the DNA fragments under UV light.

3. Results

3.1. Direct fecal smear

Of the 110 stool samples observed via direct fecal smear method, only 9 (8.2%) were positive for the presence of *Blastocystis* cells. Commonly observed in direct fecal smears were vacuolar forms of the parasite.

3.2. Culture

In vitro cultures inoculated with stool samples were observed under the microscope for the presence or absence of *Blastocystis* cells. Thirty-six (36) samples tested positive (32.7%) for the presence of the parasite. Initially, 5 out of the 36 culture-positive samples were recorded as negative. However, *Blastocystis* cells were seen after a continued incubation of six days. The vacuolar and granular forms of the parasite were frequently observed in culture-positive stool samples (Figure 1).

3.3. PCR from stool and culture

PCR assay of DNA extracted directly from stool showed 10 positive samples (9.1%) for *Blastocystis*. Meanwhile, a higher number of positive samples, 26 (23.6%), was detected by the PCR assay of DNA extracts derived from *in vitro*

cultures. Figure 2 shows the detection of representative PCR amplicons via agarose gel electropheresis from stool and culture DNA, respectively.

Figure 1. *Blastocystis* cells grown in *Blastocystis* egg medium.
A drop of culture suspension was observed at 40× magnification.

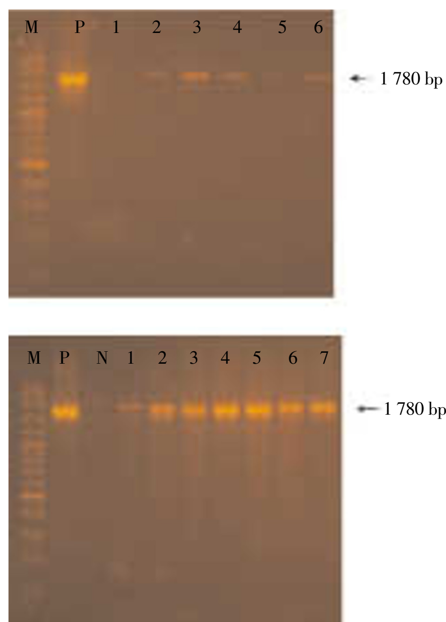

Figure 2. Detection of PCR amplicons via agarose gel electrophoresis in DNA extracted from (A) stool and (B) culture.
M – DNA ladder, P – positive control, N – negative control, 1–7 – samples positive for *Blastocystis*.

3.4. Sensitivity and specificity of the assays

Compared to culture, the sensitivity of the other detection methods were 66.7% for PCR from culture and 19.4% for both PCR from stool and direct fecal smear. Specificity of the methods was high, with PCR from culture and direct fecal smear having 97.3%, while PCR from stool at 95.9% (Table 1).

Table 1
Computed sensitivity and specificity of various methods for the detection of *Blastocystis* sp. in 110 stool samples.

Method	Sensitivity (%)	Specificity (%)
Direct microscopy	19.4	97.3
PCR from stool	19.4	95.9
PCR from culture	66.7	97.3

Culture method was used as gold standard.

4. Discussion

From a total of 110 stool samples collected, 28 were detected positive by two or more methods. Among all four methods compared, *in vitro* culture gave the highest number of positive samples. Culture was considered as the gold standard in this study. Previous studies have identified *in vitro* cultivation of stool as a more sensitive detection technique compared to fecal smears and concentration methods[10,11]. Culture is advantageous for the detection of *Blastocystis* sp. because it increases the number of cells that are initially present in the stool, thus significantly decreasing detection time[11], and allows succeeding assays to be performed, which may involve molecular analysis[12]. However, there are several factors which may affect detection of *Blastocystis* via culture method. Various media are available for cultivating *Blastocystis* namely, Locke-egg medium, Iscove's modified Dulbecco's medium, Robinson's medium, TYSGM-9, and Jones' medium. In this study, *Blastocystis* egg medium, a modification of Locke-egg medium, was used. The differences in composition among media suited for growing *Blastocystis*, and the protocols applied for its cultivation, may contribute to variation in sensitivity and specificity of the culture technique[10].

Direct fecal smear is one of the most commonly used methods for the detection of *Blastocystis* sp. in human stool, because it takes less time and resources compared to other methods. However, morphology-based diagnosis has several disadvantages, including the challenge posed by the diversity in cellular forms of *Blastocystis*. Smears may often mistakenly associate vegetative stages of the parasite as lipid globules or other contaminants[11]. As an alternative to direct fecal smear, other microscopy-based methods may be employed, such as indirect fluorescence assay (IFA). It was previously reported that IFA staining was more sensitive compared to conventional staining of chemically-preserved stool specimen[12]. In this study, direct fecal smear detected only 9 positive samples compared to *in vitro* culture, which detected 36 positive samples. This corresponded to a low sensitivity value of 19.4%. This finding is similar to a

previous report comparing sensitivity of fecal smear with *in vitro* culture[10]. The sensitivity of direct fecal smear is greatly affected by the cell number in the stool samples. A very low cell count in the specimens may have led to an increased number of false negative data. In a clinical setting, a direct consequence of such false negative findings will be mismanagement of the infection, especially if the only method of detection available is direct fecal microscopy.

PCR has been widely used as a tool to detect *Blastocystis* sp., and subsequently determine other molecular information such as genomic characterization and subtyping[13–20]. In this study, we used conventional PCR to detect the presence of *Blastocystis* DNA extracted directly from stool, and from *in vitro* culture of fecal samples. PCR–based assays that detect *Blastocystis* directly from fecal samples offer numerous advantages. These methods do not rely on the viability of organisms and do not require preparation of various reagents for culture. Subtype identification may also be performed after sequencing of nucleotide samples. However, between the two PCR assays, PCR of culture DNA detected more positive *Blastocystis* samples (*n*=26), compared to PCR from stool DNA (*n*=10).

The computed sensitivity values for the PCR–based assays, when compared to *in vitro* culture were low. It is possible that the primer set used in this study was not able to amplify the target sequence in various *Blastocystis* subtypes that may be present in the stool samples collected. The sensitivity of PCR of culture DNA and PCR of stool DNA were 66.7% and 19.4%, respectively. This finding is similar to a report by Termmathurapoj *et al* in 2004, showing PCR of culture DNA had a significantly higher sensitivity compared to PCR of stool DNA[21]. The generally low sensitivity values of the PCR–based assays may be attributed to some factors including, the protocol of genomic DNA extraction applied for both stool and *in vitro* culture samples. In 2007, Parkar *et al* reported a higher detection rate by PCR of DNA from stool, compared to *in vitro* culture, suggesting that the sensitivity of the PCR assay is largely affected by the DNA extraction procedure[16]. In 2011, Yoshikawa *et al* analyzed different commercial DNA elution kits for fecal samples. It was determined that the commercial kits have wide ranging detective sensitivities, suggesting that *in vitro* culture is a more superior approach for *B. hominis* diagnosis, in terms of rate of detection and economy[17]. Another factor that affects PCR sensitivity is the set of primers used to amplify target *Blastocystis* nucleotide sequences. The high genetic variation of the organism has been the major consideration in the design of primers for its detection. In a large–scale *Blastocystis* prevalence study, Roberts *et al* in 2011 reported significant variation in the sensitivity of PCR when different primers sets were used[7]. Also in 2011, Santin *et al* developed a highly sensitive PCR protocol that was able to amplify target sequences from various *Blastocystis* subtypes[18]. In this study, the primer set used might not have recognized other existing *Blastocystis* subtypes, thus, the sensitivity of the method was low. Recently, quantitative real–time PCR assays have been developed and reported to be highly sensitive compared to direct microscopy and culture methods[19,20]. However, although PCR assays present many advantages, the cost of the assay serves as barrier in routine clinical detection of the parasite, especially in remote and underprivileged areas of developing countries. Meanwhile, the specificity values of the three methods, when compared to culture were generally high, with both direct fecal smear, and PCR from culture DNA having 97.3% and PCR from stool having 95.9%.

The pathogenicity of *Blastocystis* sp. continues to be debated. However, there are strong evidences which suggest a link between *Blastocystis* and various clinical symptoms which include abdominal pain, diarrhea, constipation, fatigue, and skin rash[4]. It is also closely associated with irritable bowel syndrome[22]. The implication of *Blastocystis* sp. in these health conditions makes it important to improve routine diagnosis of the parasite in fecal samples. In this study, we recommend the use of *in vitro* cultivation as an effective method for detecting *Blastocystis* in human stool.

Conflict of interest statement

We declare that we have no conflict of interest.

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