**Original Article**

**A novel genotype screening and phylogenetic analysis of Blastocystis hominis based on EF-1α**

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**Abstract:** Blastocystis hominis (B. h) is a kind of intestinal parasitic protozoa with the characteristic of worldwide distribution, morphology diversity, and diarrhea induced, etc. The traditional morphological classify was difficult to distinguish the genetic difference of B. h in different population and different geological strains. Recently, based on the small subunit ribosomal DNA sequence of B. h, the sequenced-tagged site (STS) primers was design, and successfully and widely applied to the distinguish the genotype of B. h, and however several B. h strains did not distinguish. To address it, the elongation factor-1 alpha (EF-1α) gene of B. h was screened due to its conservation here, and its specific primers were designed to distinguish the genotype of B. h. After epidemiological survey, the infection rate of B. h in boys was 14.74%, and that of girls was 15.05%, and the total infection rate of B. h was 14.93%. In total of 53 infection students, with the using of 7 pairs STS primers, 31 strains was validated by polymerase chain reaction (PCR), including 4 strains of Type 1, 17 strains of Type 3, 4 strains of Type 4, 1 strains of Type 6, and 5 strains of Type 7, and did not found the Type 2, Type 5 and mixture genotype. In the 23 unknown genotype strains of B. h, 15 strains were identified by PCR using EF-1α primers, and had a higher homology in the DNA sequence (70%), and was evolutionarily closer to the EF-1α sequence of S and H strains of B. h. This study indicated that STS primers could identify the genotype of B. h, and EF-1α primers as a novel diagnosis primers could auxiliary identify the unknown genotype strain of B. h, and exhibited a wide application on the identification of the genotype strain of B. h, and provided a significant reference on the study of B. h in clinic.

**Keywords:** Blastocystis hominis, distinguish, sequenced-tagged site, elongation factor-1 alpha, primer

**Introduction**

Blastocystis hominis (B. h) is a single-celled intestinal parasite, and is very common in both humans and animals [1-3], and firstly named as B. h in 1912 by Brumpt, et al. Based on the morphological study of it, it was divided into the intestinal parasite class in 1967 by Zerdt, et al, and was finally divided into the Blastocysta in 1993 by Jinbo Jiang, et al [4, 5]. It is one of the most common human parasites in the world and has a global distribution, especially in the developing country of the Southeast Asia and the South America [6-8]. In developing country, the infection rate was 30%-50%, and was much higher than that of the developed country (1.5%-10%) [9-11]. It is estimated that 25% of people are infected with this parasite [12-14]. For a long time, it was believed that B. h was harmless, and however of the 10 subspecies, 2 or 3 subspecies are known to cause complaints as recent study documented, including chronic intestinal symptoms and/or irritable bowel syndrome [15-17]. On the taxonomy, the traditional method based on the morphological structure of B. h does not clearly distinguish the genotype of B. h due to the diversity of it [18], and with the rapid developing of molecular technique, to determine the genetic difference and features of DNA in B. h exhibits a significant application value, especially several conserved genes, such as small subunit ribosomal DNA (SSU rDNA). In 1997, Clark, et al reported that the B. h had a genetic polymorphism, and exited at least 7 genotype after comparison of the SSU rDNA restriction fragment length polymorphism.
Genotype screening and phylogenetic analysis of Blastocystis hominis

Table 1. B. h infection rate

<table>
<thead>
<tr>
<th>Grade</th>
<th>Examination</th>
<th>Infection</th>
<th>Infection rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boys</td>
<td>563</td>
<td>83</td>
<td>14.74</td>
</tr>
<tr>
<td>Girls</td>
<td>870</td>
<td>131</td>
<td>15.05</td>
</tr>
<tr>
<td>Total</td>
<td>1433</td>
<td>214</td>
<td>14.93</td>
</tr>
</tbody>
</table>

(RFLP) in different regions [19]. Yoshikawa, et al has designed the sequenced-tagged site (STS) as a primer of polymerase chain reaction (PCR) based on the SSU rDNA specific fragment, and correctly distinguished the different genotype of B. h in different countries and regions, and still widely used to distinguish the genotype of B. h [20]. However, due to the diversity of B. h in different countries and regions, several genotype of B. h did not entirely identify just using the STS primers, and another primers based on a conserved gene fragment need to develop.

The elongation factor-1 alpha (EF-1α) is a highly conserved ubiquitous protein that is involved in translation and is desirable for use in phylogenetic studies on Blastocystis, an enigmatic intestinal parasite with a contentious taxonomic position [21]. Nakamura, et al has well documented that B. h did not belong to Eumycota based on the analysis of the EF-1α amino acid sequence of it [21]. Ho, et al has also documented that EF-1α gene also had genetic polymorphism, and constructed the phylogenetic relationship based on the DNA sequence and amino acid of 13 B. h strains [22]. All of the studies indicated that EF-1α as a conserved gene exhibited the importance on the classification of B. h. In this study, the EF-1α gene was chosen, and its PCR amplification primers was designed, and hoped to provide an auxiliary detection method of B. h genotype.

Materials and methods

Epidemiological survey and microscopic examination

A total of 1433 students who studied in the Guangxi Medical University were chosen as the object of this study, including 563 boys and 870 girls. All students were distributed the questionnaire to collect the general conditions, personal hygiene and environmental health, etc. After epidemiological survey, a total of 3-5 g faeces was collected, and treated with hydrochloric acid and diethyl ether according to acid ether centrifugal sedimentation method, and the nightsoil was smeared and analyzed by optical microscope.

B. h separation and culture in vitro

The above-validated positive faeces (2-3 g) was picked by bamboo stick, and placed in a 15 ml centrifuge tube, and added 10 ml of Locke’s to stir evenly, and removed the coarse particle. The turbid liquid was transferred to a new 15 ml centrifuge tube, and added 10 ml of Locke’s to naturally sediment 1-2 hrs, and then removed the supernatant. Repeated one time, and suck up 1 ml liquid nearby the nightsoil, and inoculated to Iscove’s Modified Dulbecco’s Media (IMDM) medium, and anaerobic cultured at 37°C for 48 hrs, and repeated one time. Then, centrifuged at 4,000 g for 2 min, and removed the supernatant, and added 10 ml of Locke’s to resuspended polypide, and centrifuged at 4,000 g for 2 min, repeated 3-4 times. Removed the supernatant, and added 4 ml of Locke’s to resuspended polypide, and centrifuged at 5,600 g for 3 min, repeated 3 times. Removed the supernatant, and added 1 ml of Locke’s to resuspended polypide, and counted using cell counting chamber.

Genomic DNA extraction of B. h

The above-separated B. h genomic DNA was extracted according to the manufacturers’ instructions of the blood/cell/tissue genomic DNA extraction kit (TIANGEN BIOTECH (BEIJING) CO. LTD). The B. h polypide was washed with double distilled water, and added 200 μl of GA buffer (pre-added 8 μl of Rnase A) to shaking at room temperature, and stewing for 5 min. Then, added 20 μl of proteinase K and 200 μl of GB buffer to reverse blending, and incubated at 70°C water bath. Added 220 μl of absolute ethyl alcohol to reverse blending, and then transferred to the CB3 column, and centrifuged at 12,000 rpm for 30 s, removed the elution. Added 500 μl of GD buffer, and centrifuged at 12,000 rpm for 30 s, removed the elution. Added 700 μl of PW buffer, and stewing at room temperature for 30 s, and removed the elution, repeated one time, and added 50 μl of TE buffer, and stewing for 2-5 min, and centrifuged at 12,000 rpm for 2 min. The elution was collected, and the concentration and the purity were detected by ultraviolet spectrophotometer.
Genotype screening and phylogenetic analysis of Blastocystis hominis

The above-extracted B. h genomic DNA (100 ng) was used as a template to perform PCR assay using EF-α primers as shown in Table 2. The reaction mixture, including 25 µL Taq Mix, 1 µL forward primer, 1 µL reverse primer, 2 µL genomic DNA, and 21 µL ddH2O, was prepared and the PCR was performed according to the following program: one cycle of 94°C for 5 min; 30 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 30 s. After PCR, products were identified by 1% agarose gel electrophoresis, and images were gathered by Gel Imaging (Bio-rad, USA). Subsequently, the DNA bands were cut, and extracted by Gel Extraction Kit (OMEGA, Japan), and then performed direct sequencing.

Identification of B. h different strains using EF-α primer by PCR

A total of 23 strains of unknown genotype B. h was chosen, including 11 strains of B. h of which did not identify using STS primers as above-mentioned, and 12 strains of unknown genotype B. h of which were come from the coastal region of the south of Guilin. As the above-mentioned, a total of 100 ng genomic DNA of B. h was used as a template to perform PCR assay using STS primers.

Identification of B. h different strains using STS primers by polymerase chain reaction (PCR)

The above-extracted B. h genomic DNA (100 ng) was used as a template to perform PCR assay using STS primers as shown in Table 2. The reaction mixture, including 25 µL Taq Mix, 1 µL forward primer, 1 µL reverse primer, 2 µL genomic DNA, and 21 µL ddH2O, was prepared and the PCR was performed according to the following program: one cycle of 94°C for 5 min; 30 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 30 s. After PCR, products were identified by 1% agarose gel electrophoresis, and images were gathered by Gel Imaging (Bio-rad, USA).

Table 2. STS primers used in this study

<table>
<thead>
<tr>
<th>Subtypes</th>
<th>STS primer</th>
<th>Product size (bp)</th>
<th>Sequences of primers</th>
<th>GenBank</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SB83</td>
<td>351</td>
<td>5’-GAAGGACTCTGACGATGA-3’/5’-GTCCAAATGAAAGGCAGC-3’</td>
<td>AF166086</td>
</tr>
<tr>
<td>2</td>
<td>SB155</td>
<td>650</td>
<td>5’-ATCAGCCTAATCCCTCCT-3’/5’-ATCGCCACTTCTCCAAT-3’</td>
<td>AF166087</td>
</tr>
<tr>
<td>3</td>
<td>SB227</td>
<td>526</td>
<td>5’-AGGATTTGTTTGAAAGGA-3’/5’-TTAGAAGGAGGAGTGGGA-3’</td>
<td>AF166088</td>
</tr>
<tr>
<td>4</td>
<td>SB332</td>
<td>338</td>
<td>5’-GCATCCCACTATCCAAGAT-3’/5’-CCATTTCAGACAAACCTTA-3’</td>
<td>AF166091</td>
</tr>
<tr>
<td>5</td>
<td>SB340</td>
<td>704</td>
<td>5’-GTGCTGTCGGACACGTC-3’/5’-TTCTTCCACCTCCGGTCAT-3’</td>
<td>AY048752</td>
</tr>
<tr>
<td>6</td>
<td>SB336</td>
<td>317</td>
<td>5’-GTGGGTCAGAGGAGAAAC-3’/5’-AGAAACTCGAGATGAAGTGA-3’</td>
<td>AY048751</td>
</tr>
<tr>
<td>7</td>
<td>SB337</td>
<td>487</td>
<td>5’-GTCTTTCCCTGCTAATCC-3’/5’-AATCGGCTCTTCTCTCT-3’</td>
<td>AY048750</td>
</tr>
</tbody>
</table>

Multiple sequences alignment using Cluster W/X and phylogenetic analysis

The above-validated EF-α DNA sequences of B. h were performed multiple sequences alignment to the DNA sequence of B. h different strains using Cluster W/X software, including C strain, E (G) strain, S strain and H strain. After then, the phylogenetic tree was imaged using the function of “Phylogeny” in the MEGA 7.0.21 software (http://www.megasoftware.net/download_form).
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Figure 2. Identification of the PCR products of STS in 53 samples by agarose gel electrophoresis. The image indicated that the feature STS DNA bands were amplified by PCR.

Figure 3. Identification of the PCR products of EF-1α in 23 samples by agarose gel electrophoresis. The image indicated that the feature EF-1α DNA bands were amplified by PCR.

Statistical analysis

All data expressed as the mean ± standard deviation (SD). Statistical analysis was performed with one-way ANOVA using SPSS software (version 21.0, http://spss.en.softonic.com/; Chicago, IL, USA), and Student’s t-tests were performed in a group of two sample, and P < 0.05 and P < 0.01 were considered to indicate significant differences and highly significant differences, respectively.

Results

Epidemiological survey and microscopic examination

As exhibiting of Table 1, the B. h infection rate of boys was 14.74%, and the B. h infection rate
### Table 3. DNA sequence of EF-1α in different strains of B. h after sequencing

<table>
<thead>
<tr>
<th>Number</th>
<th>DNA sequence</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>&gt; 6</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>&gt; 7</td>
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</tr>
<tr>
<td>57</td>
<td>&gt; 57</td>
<td></td>
</tr>
</tbody>
</table>

**Note:**
- The table provides the DNA sequence of EF-1α in different strains of Blastocystis hominis.
- Each row represents a different strain with its corresponding DNA sequence.
- The sequences are compared for variations and similarities.
- The note column contains additional information about each sequence.

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of girls was 15.05%, and the total infection rate of B. h was 14.93%. After separation of B. h in faeces with the 3 generation anaerobic culture in vitro, the medium was orange red and translucent and floccule sediment was observed in the bottom of tube. After microscopic examination, B. h protozoon was well breed, and was appeared gemmipary (Figure 1).

Five genotypes of B. h were identified using STS primers

As PCR results exhibiting of Figure 2, in 53 samples, five genotypes of B. h were identified using 7 pairs STS primers (Table 2), including 4 strains of Type 1, 17 strains of Type 3, 4 strains of Type 4, 1 strains of Type 6, and 5 strains of Type 7, and did not found the Type 2, Type 5 and mixture genotypes, and appeared several obvious DNA bands, such as the 351 bp band of Type 1 in sample 27, 35, 44 and 46, the 526 bp band of Type 3 in sample 4, 8, 10, 13, 18, 19, 20, 21, 24, 25, 30, 31, 14, 32, 33, 36, 39 and 40, the 338 bp band of Type 4 in sample 16, 41, 42 and 43, the 317 bp band of Type 6 in sample 22, and the 487 bp band of Type 7 in sample 26, 29, 38, 47 and 48. In addition, a total of 22 strains of B. h had no any DNA bands, and belonged to the unknown genotypes B. h.

EF-α had a higher homology in 13 strains of B. h, and highly conserved

After multiple sequence alignment (Figure 4), the 13 DNA sequences of B. h strains had a higher homology (70%). Evolutionarily, the EF-α DNA sequence of 13 samples was closer to that of the S and H strains of B. h (Table 4) as phylogenetic analysis (Figure 5).

Discussion

This study demonstrated that B. h had a higher infection rate in the students of Guangxi Medical University, and firstly identified 5 genotype of B. h using STS primers, including Type 1, 3, 4, 6, 7, and did not found Type 2, 5, and any mixture genotypes. Based on it, in 23 strains unknown genotype B. h, 13 strains was identified by PCR using EF-1α primers, and exhibited a closed evolutionary relationship to the EF-1α sequence of the S and H strains of B. h, and exhibited a significant application value on the phylogenetic analysis of B. h.

B. h is a single-celled intestinal parasite and widely distributed all over the world [23-26], and existed multiple stages in its life cycle with the morphological diversity [17, 27, 28], and therefore did not clearly distinguish its genetic difference in different population and different regions just based on the morphological structure of B. h, and a novel approach need to design. Based on the rapid developing of molecular technology, the genotype classification according to the genetic difference of gene or protein has been raised much more attention. With the STS primers used in the PCR in 1997 by Clark, et al, several genotypes of B. h were found [19]. In this study, seven features primers of STS were designed to distinguish the genotype of B. h in 53 samples, and 31 strains of B. h were amplified the features DNA bands,
Figure 4. Multiple sequence alignment of the 13 DNA sequences of B. h strains. The images indicated that the 13 DNA sequences of B. h strains had a higher homology (70%).
and belonged to 5 genotypes, including Type 1, 3, 4, 6, 7, and did not found Type 2, 5, and any mixture genotypes, and wherein the Type 3 was identified in 13 strains, and these sequences had a higher homology (70%). In addition, after phylogenetic analysis, the EF-1α primers in 23 strains unknown genotype were amplified with the 421 bp length features bands. After PCR amplification using the EF-1α primers to amplify the 421 bp length feature, the 421 bp features bands was amplified in 13 strains, and these sequences had a higher homology (70%). In addition, after phylogenetic analysis, the EF-1α primers in 23 strains unknown genotype was used another phylogeny biomarker.

The elongation factor-1 alpha (EF-1α) is a highly conserved ubiquitous protein that is involved in translation and is desirable for use in phylogenetic studies on Blastocystis, an enigmatic intestinal parasite with a contentious taxonomic position [21]. Therefore, the EF-1α full length fragment was chosen, and designed a pair of novel amplified primers.
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Figure 5. Phylogenetic analysis of the 13 DNA sequences of B. h strains after sequencing and 4 strains of B. h strains. The images indicated that the EF-α DNA sequence of 13 samples was closer to that of the S and H strains of B. h.

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Disclosure of conflict of interest

None.

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