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# Molecular Identification of human *Blastocystis* isolates in patients of Al-Diwaniyah Teaching Hospital, Iraq

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**Abstract**

**Introduction:** The human large intestine is inhabited by the anaerobic, eukaryotic, unicellular protozoan *B. hominis*, which is found worldwide. This study sought to examine the existence and molecular characteristics of this parasite in adult patients admitted to Al-Diwaniyah Teaching Hospital in Al-Diwaniyah Governorate from February to December 2025.

**Methods:** The parasitic infection was not recognized by microscopic examination at the hospital, but it was confirmed using PCR targeting the 16S rRNA gene as a DNA marker to assess the prevalence of *B. hominis*.

**Results:** The overall prevalence of the parasite was 3.1%. Hence, PCR analysis was more sensitive than microscopic analysis. The 18S rRNA gene of the local isolates was matched to the worldwide reference strains of *B. hominis* listed in the GenBank. Regarding mutation, the alignment analysis revealed the substitution mutations alignment similarity in the 18S ribosomal RNA gene between the local *B. hominis* H isolates and Global, where a (0.35%,0.29%,0.85%,0.85%) match was found between the local isolates and an earlier reported *B. hominis* isolates from Spain, China, Thailand, and Denmark, respectively. The local strains shared (99.65, 99.71, 99.15, 99.15) % of their identity with isolates from Spain, China, Thailand, and Denmark, respectively.

**Conclusion:** phylogenetic (evolutionary) analysis of a parasite is a vital tool for precisely identifying the microorganism and understanding its evolutionary relationships thereby enabling the development of effective therapeutic and preventive strategies based on the microbe's origins and genetic evolution.

**Keywords:** *Blastocystis sp.*, 18S rRNA gene, Phylogenetic tree, Iraq

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## Introduction

When a Russian scientist discovered the parasite *Blastocystis hominis* in 1870, it was initially disregarded because it lacked a formal taxonomic classification. In 1912, it was recognized in fecal samples as a benign yeast; even so, it remained overlooked for a long time. It is now recognized as the most widespread intestinal parasite and is considered unusual compared with others. An escalating number of reports indicate the isolation of *B. hominis* from human feces, especially in tropical countries (1). Infection is prevalent in tropical and subtropical regions and in developing nations, with rates between 30 and 50% in certain developing countries, compared with 1.5-10% in some developed nations (2-4). Additionally, prior research has shown that in developed nations, the number can reach up to 20% (5,6). The human large intestine is inhabited by the anaerobic, eukaryotic, unicellular protozoan *B. hominis*, which is found worldwide (7). Four distinct morphological forms—central body, amoebic, granular, and cystic—have been identified in stool specimens and/or through in vitro cultures (8,9). Its transmissible form is represented by the water-resistant infectious cyst (10). However, it has been proposed that the pathogenic form is the irregular amoeboid form (11). These parasites are spread through food, water, contact between humans, and the fecal-oral route (12), while the fecal–oral pathway is the primary mode of transmission of *B. hominis* (13). *Blastocystis* has been linked to dermatitis, colitis, stomach pain, flatulence, diarrhea, and irritable bowel syndrome (14,15). Although asymptomatic spread is common worldwide, its pathogenicity remains debatable (16, 17), and hypotheses regarding its pathogenicity have heightened awareness of it (1). Clinical signs of a *Blastocystis* infection include nausea, diarrhea, flatulence, anorexia, and abdominal pain; these symptoms are frequently linked to irritable bowel syndrome (IBD)(18). In immunocompromised patients, *Blastocystis* was also considered an opportunistic pathogen (19). As a result, *Blastocystis* has been identified as an emerging pathogen in several investigations (20).

The current study was among the few that examined the incidence of enteric infections in patients. The ability of the examiner determines whether parasites may be found in stool by microscopic examination, and culturing takes time and requires a qualified technologist (21); hence, in the present study, we attempted to use the 18S rRNA gene

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as a DNA marker to examine the prevalence of *B. hominis* in patients who visited the AL-Diwaniyah Teaching Hospital in Al-Diwaniyah Province, Iraq

## 2. Materials and Methods

### 2.1 Collection of samples

Of the 160 samples sent by specialists of the Diwaniyah Teaching Hospital to laboratory to diagnose the cause of diarrhea in patients for different ages during the period from February to December, 2025 where using the direct method or what is called direct wet mount without the use of dye but we took the positive samples of *Entamoeba histolytica* and examined by using polymerase chain reaction (PCR) to search for *Blastocystis hominis* parasite due to the phenotypic similarity between the two parasites.

### 2.2 DNA extraction and genetic detection

DNA was extracted from stool samples with the Presto™ Stool DNA Extraction Kit, following the manufacturer's instructions. The genomic DNA isolated from feces samples was analyzed using a Nanodrop spectrophotometer (THERMO, USA), which assesses DNA purity by measuring absorbance at 260/280 nm. The PCR master mix was made using (GoTaq® Green PCR Master Mix), following the manufacturer's instructions. This study developed Nested PCR primers for the detection of Blastocystis hominis, targeting the small subunit ribosomal gene, using sequences from NCBI GenBank and Primer3 Plus software. These primers were supplied by Scientific Researcher Co., Ltd., Iraq, as follows: PCR primer (their sequence and product size) is shown in Table 1.

The primer was supplied at a stock concentration of 100 pmol/µL after being dissolved in nuclease-free water as per the manufacturer's instructions. After that, primers were prepared using the conventional dilution procedure (C1V1 = C2V2) to achieve a final primer concentration of 10 pmol/µL. The PCR conditions were as follows: Initial denaturation at 95 °C for 5 minutes; 35 cycles of (initial denaturation at 95 °C for 30 seconds, annealing at 58 °C for 30 seconds, extension at 72 °C for 1 minute); and final extension at 72 °C for 5 minutes. PCR results were conducted on an ethidium bromide-pretreated 1.5% agarose gel. A UV imager was used to visualize the product separation.

### 2.3 DNA sequencing method

The DNA sequencing technique was used to investigate the genetic relationship between local *Blastocystis hominis* isolates and NCBI-submitted global isolates. The PCR and Nested PCR products from some positive small subunit ribosomal RNA gene samples were sent to Macrogen for DNA sequencing. NCBI BLAST was used for the DNA sequencing study, and Molecular Evolutionary Genetics Analysis version 6.0 (Mega X) was used for the phylogenetic tree analysis and ClustalW alignment. The evolutionary distances were calculated using the UPGMA technique on the phylogenetic tree, with the Maximum Composite Likelihood method. Finally, to obtain a GenBank accession number, the identified isolates were submitted to NCBI GenBank.

## Results and Discussion

This parasite has not been diagnosed microscopically by laboratory technicians at Al-Diwaniyah Teaching Hospital. Initially, all positive samples were incorrectly identified as *Entamoeba histolytica* because they were not stained with laboratory dyes and were examined only by direct microscopy, and because of the significant similarity between the two parasites, which share some morphological characteristics. Of the 160 samples, 28 tested positive for *Entamoeba histolytica*. However, when we took and examined these positive samples by using (PCR) and parasite primer of *Blastocystis hominis*, five of them tested p.ositive for this parasite (3.1%), as shown in Figure 1

The *B. hominis* parasite under study was not diagnosed in patients with gastrointestinal symptoms admitted to Al-Diwaniyah Teaching Hospital. All positive samples were misdiagnosed as *Entamoeba histolytica*. This misdiagnosis is attributed to confusion between the two parasites for several reasons, including: Similarity in appearance: *Blastocystis* and *Entamoeba* share some morphological

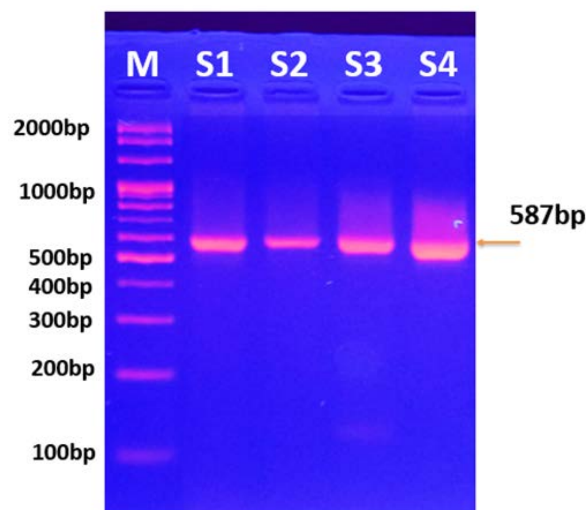


**Figure 1.** Agarose gel electrophoresis image displaying the small subunit ribosomal gene PCR product analysis in *Blastocystis hominis* from Human stool samples. Where M: marker (2000-100bp). Lanes (S1-S4) show some positive *B. hominis* samples at the (587bp) PCR product.

**Table 1.** PCR primer (their sequence and product size)

| Primers | Sequence 5′-3′ | | Product size | GenBank Reference code |
|---|---|---|---|---|
| *Blastocystis hominis* | F | ACCCCCTTCCAGTATCCAGT | 587bp | OQ594924.1 |
| | R | CATGCACCACCACCCATAGA | | |

characteristics, such as size and a rounded shape, Lack of experience: Inexperienced examiners may confuse the two parasites due to a lack of expertise in distinguishing subtle differences, Sample quality: Sample quality can affect diagnostic accuracy. If the sample is poor quality or contaminated, it may be difficult to identify the parasite, Failure to use appropriate dyes: Failure to use appropriate dyes may make it difficult to distinguish the two parasites, atypical morphology: Some atypical morphologies of the two parasites can confuse diagnosis but However, *Blastocystis* usually larger than *Entamoeba*, and their shape is also different, *Blastocysts* are typically round or oval, while *entamoeba* are irregularly shaped.

However, microscopic examination alone is usually insufficient; additional tests, such as PCR or serological tests, are necessary to confirm the diagnosis (22). Although the percentage of B. hominis H. present was small, “Blastocystosis or Zierdt-Garavelli Disease” is a self-limited “pathogenic” course that can develop in a tiny percentage of cases (23).

The current study’s findings conflict with previous studies that sought to determine the extent of this parasite’s spread, such as in Salah al-Din Governorate. Their study results for the year (2022) showed that 50 out of 150 children, or 33.3% of the total, had *Blastocystis sp.* infections (24,25). This may be because most of those arriving at the teaching hospital in Diwaniyah Governorate are adults. In a study of 25, the B. hominis infection rate was 34 (34%) in both the adult and pediatric groups in Wasit Province, Iraq, for the year 2021. The age group 14 to 35 years had the highest infection rate, at 12 (35.29%). This is consistent with our research findings, in which 3 of the positive samples were from patients in their twenties, and 2 were from patients in their thirties. In Iran, Iraq’s neighbor, a study found that out of 1,878 stool samples collected from northeastern Iran patients during the period from January to December 2017, 152 samples (8.1%) were detected as *Blastocystis hominis* using microscopy (26) while in another study between 2012 and 2014, 481 samples were taken from patients in the southwest of Iran who were referred to Ahvaz’s medical laboratory institutions for stool analysis where samples were analyzed by wet mount, 69 (14.35%) samples were found to be positive for *B. hominis* (27). And in South Khorasan province, microscopic examinations identified 118 (6.5%) cases of Blastocystis sp. among 1800 randomly selected stool specimens from the province’s medical laboratories in 2025 (28). Factors more common in underdeveloped nations, such as unsanitary conditions, poor personal hygiene, close contact with animals, and consumption of contaminated food or water, were responsible for the variations in prevalence (29).

The percentage of homologous sequence identity (NCBI-BLAST) between the local *Blastocystis hominis* sequences and the NCBI-BLAST sequences deposited from other countries is shown in Table 2.

In this research, the worldwide reference strains of *B. hominis* cataloged in GenBank were compared with the

**Table 2.** NCBI-BLAST Homology Sequence Identity between the gene bank’s local B. hominis sequences and those from other countries

| ***B. hominis* isolate and accession number** | **Accession number** | **Country** | **Homology sequence identity (%)** | |
|---|---|---|---|---|
| | | | **Mutation (%)** | **Identity (%)** |
| H.No.1(PZ222155) | EF680767.1 | Spain | 0.35% | 99.65% |
| H.No.2(PZ222156) | KR262937.1 | China | 0.29% | 99.71% |
| H.No.3(PZ223918) | AY618267.1 | Thailand | 0.85% | 99.15% |
| H.No.4(PZ223919) | AM275346.1 | Denmark | 0.85% | 99.15% |

18S rRNA gene of the local isolates. Regarding mutation, the alignment analysis revealed the substitution mutations alignment similarity in the 18S ribosomal RNA gene between the local *B. hominis* H isolates and Global, where a (0.35%,0.29%,0.85%,0.85%) match was found between the local isolates and an earlier reported *B. hominis* isolates from Spain, China, Thailand, and Denmark, respectively. It is worth noting that the second sample was registered in the gene bank as having the highest rate of mutational heterogeneity. The local strains shared (99.65, 99.71, 99.15, 99.15) % of their identity with isolates from Spain, China, Thailand, and Denmark, respectively. This striking genetic homology suggests slight genetic variation among B. hominis H populations (regardless of location) and that the 18S rRNA gene is highly conserved and a sound candidate for species hunting.

Phylogenetic tree analysis based on the partial sequence of the 18S ribosomal RNA gene in local *B. hominis H.* Isolates (green circles) is shown in Figure 2. The UPGMA method in MEGA 6.0 was used to create the phylogenetic tree. At total genetic alterations (0.0010%), the *B. hominis H.*No1-No4 isolates had genetic variants related to NCBI-BLAST *B. hominis* global isolates.

Compared to the results of our study, and according to the constructed phylogenetic tree of SSU rRNA gene sequences of Blastocystis from a study in Duhok, Iraq (30), all isolates showed that their sequences were closely related to other identified strains from GenBank of the same species from Germany, the Philippines, Argentina, Singapore, Japan, Poland, and Thailand. In contrast, a study conducted in Tikrit, Iraq, showed that the sequence of its isolate was 97% similar to other strains of the same species identified in Mexico, Iran, and Iraq in the gene bank when matched with world isolates, based on the results of genetic testing for the ST3 subtype of *Blastocystis hominis* (24). The data from the current inspection effort may more definitively show how the local strains evolved genetically, increasing the likelihood that they were relatives or sisters of the global strains.

The multiple sequence alignment analysis of the 18S ribosomal RNA gene in local *Blastocystis hominis H* isolates acquired here and global *Blastocystis hominis* isolates of NCBI-Genbank is shown in Figure 3. The ClustalW alignment tool (online) was used to construct the multiple alignment analysis. The alignment analysis revealed substitution mutations in the 18S ribosomal RNA gene between isolates and nucleotide alignment

**Figure 2.** Phylogenetic tree for *B. hominis*

**Figure 3.** Multiple sequence alignment analysis of 18S ribosomal RNA gene in local B. *hominis H* isolates and global NCBI-GenBank related *B. hominis* isolates

similarity as (*).

The similarity between local strains of *B. hominis* and global strains in the gene bank can be attributed to the parasite's global distribution. It is a common intestinal parasite infecting humans and animals worldwide, leading to the spread of its strains across geographical boundaries. The zoonotic character of B. hominis is supported by evidence found in the excrement of birds and mammals, suggesting that these animals may cause several human infections (31, 32). Furthermore, the parasite can be transmitted between humans and animals, facilitating the spread of local and global strains. In addition, *Blastocystis hominis* exhibits significant genetic diversity, with 22 genotypes identified, enabling it to adapt to diverse environments. Based on research on small subunit (SSU) RNA genes, these subtypes may indicate distinct species (33-35).

## Conclusion

The microscopic examination of stool is the usual diagnostic technique for identifying protozoa and helminths, owing to its availability and the reduced time required. However, this study validated the significance of employing PCR in the diagnosis of *Blastocystis hominis*. Although Blastocystis does not pose a significant health risk, it is widespread throughout the country. Further research is required to determine its potential as a pathogen in humans. Lastly, a crucial method for accurately identifying a parasite and comprehending its evolutionary relationships is phylogenetic (evolutionary) study. This aids in identifying the infection's origin, forecasting its pathogenic behavior, and understanding its antibiotic resistance. Based on the microbe's origins and genetic evolution, such analysis enables the development of effective treatment and prophylactic strategies.

### Ethical Approval

All participants selected for the study were apprised of its objectives and methodologies.

### Competing Interests

The authors disclose no conflicts of interest.

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This study was conducted without external financial support.

### Author Contribution

Writing–original draft: Rana Saleh Al-Difaie
Writing–review & editing: Khawla Hussien Sabbar
Methodology: Hiba Shehab Ahmed

### Availability of Data and Materials

Data are provided upon request.

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