Original Article

Molecular Identification and Genotyping of *Blastocystis* Spp. In Children with Clinical Symptoms in Southeast Iran Using PCR-Sequencing Method

Mirahmadi, H[1,2], Rahmati-Balaghaleh, M[1,2], Darabi, E[3], Zarean, M[4,5], Sharifi, Y[4], Yousefnia, H[6], Etemadi, S[7*], Parandin, F[8], Askari, Z[9]

1. *Infectious Disease and Tropical Medicine Research Center, Research Institute of Cellular and Molecular Sciences in Infectious Diseases, Zahedan University of Medical Sciences, Zahedan, Iran.*
2. *Department of Parasitology and Mycology, Faculty of Medicine, Zahedan University of Medical Sciences, Zahedan, Iran.*
3. *Department of Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran.*
4. *Department of Parasitology and Mycology, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran.*
5. *Cutaneous Leishmaniasis Research Center, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran.*
6. *Department of Parasitology and Mycology, School of Medicine Hamadan University of Medical Sciences, Hamadan, Iran.*
7. *Department of laboratory sciences, Sirjan School of Medical Sciences, Sirjan, Iran.*
8. *Research Center for Environmental Determinants of Health (RCEDH), Health Institute, Kermanshah University of Medical Sciences, Kermanshah, Iran.*
9. *Department of Medical emergency, Sirjan School of Medical Sciences, Sirjan, Iran.*

How to cite this article: **Mirahmadi H, Rahmati-Balaghaleh M, Darabi E, Zarean M, Sharifi Y, Yousefnia H, Etemadi S, Parandin F, Askari Z. Molecular Identification and Genotyping of *Blastocystis* Spp. In Children with Clinical Symptoms in Southeast Iran Using PCR-Sequencing Method. Archives of Razi Institute. 2025;80(1):61-67.** DOI: 10.32592/ARI.2025.80.1.61

Article Info:
Received: 26 November 2023
Accepted: 20 October 2024
Published: 28 February 2025

Corresponding Author's E-Mail:
ssetemadi@gmail.com

ABSTRACT

Blastocystis spp. is a zoonotic anaerobic parasite that has been identified in the large intestine of humans and many vertebrates. It is predominantly encountered in individuals with frequent contact with animals. The present study aims to identify the prevalence of Blastocystis spp. and its common genotypes in children with clinical symptoms of diarrhea in the city of Zahedan, located in the southeast of Iran. A cross-sectional descriptive study was conducted on 60 children under ten years of age with gastrointestinal symptoms, especially diarrhea. Following the collection of samples, stool samples were subjected to direct stool testing for the initial diagnosis. Following this, a microscopic diagnosis was made, after which DNA was extracted and a Polymerase Chain Reaction (PCR) test with a small subunit ribosomal RNA (SSU rRNA) gene target was performed. The PCR products were then purified and sequenced. The resulting nucleotide sequences were then subjected to a thorough review using Chromas biotechnology software version 2.4 and CLC genomic work bench software 11. The alignment of the nucleotide sequences was subsequently facilitated by utilizing the BLAST database, and these sequences were then compared with the reference genotypes of Blastocystis spp. that are stored within the gene bank. The genotyping of the sequences was conducted using CLC genomic work bench software 11, and a phylogenetic tree was constructed using MEGA7 software with the Neighbor-Joining statistical method, which applied the Kimura 2-parameter method. Out of the 60 cases that were examined, 5 children (8.33%) were found to be positive by direct microscopic and PCR tests, where a 500 (479) bp fragment in the SSU-rRNA target was detected. Subsequent genetic analysis identified four distinct subtypes, including subtypes 1, 2, 3, and 5. The percentage of nucleotide identity with the sequences in the gene bank was found to be between 93 and 100%. Given the presence of subtypes 3 and 5 in the study and the evidence of their zoonotic nature, it can be concluded that examining parasite dynamics and epidemiological principles can be effective in the control strategy.

Keywords: *Blastocystis* spp., Children, PCR-Sequencing, Southeast Iran.

1. Introduction

Blastocystis spp. is a protozoan parasite that is both anaerobic and zoonotic. It has been found in the large intestine of humans and many other vertebrates. This parasite is isolated to a high extent from stool samples in parasitology laboratories. In the year 2000, using the SSU rRNA gene, Blastocystis spp. was included in the phylum Sarcomastigophora (1, 2). Despite its discovery in human feces approximately a century ago, it was long considered a non-pathogenic parasite. However, in recent years, the significance of Blastocystis spp., particularly in individuals with certain diseases, has come to the fore (3, 4). The pathogenic cysts of Blastocystis spp. have been shown to survive in water and to be transmitted directly between different hosts through contaminated water or food (5). The prevalence of Blastocystis spp. has been documented to range from 0.8% to 61.8% on a global scale (6). A preponderance of reports indicates that Blastocystis spp. is prevalent in tropical and subtropical regions. In developing countries, immigrants and refugees exhibit a higher rate of infection compared to the native population (7, 8). The literature has demonstrated the pathogenicity of Blastocystis spp., with associated symptoms including acute and chronic diarrhea, abdominal pain and bloating, constipation, nausea, irritable bowel syndrome, and intestinal inflammation. The validity of these indicators and pathological changes has been substantiated in laboratory animals and cell cultures. However, the debate surrounding the pathogenicity of Blastocystis spp. persists, particularly in the context of asymptomatic carriers, as further discussed below. According to the established morphological criteria, the Blastocystis spp. isolated from both animals and humans are morphologically indistinguishable. According to molecular studies, 17 subspecies of this parasite have been identified (6,10), with subtypes 1 to 9 and ST12 isolated from humans and mammals, and subtypes 10 to 17 found in animals. According to the findings of these studies, subtypes 1 to 4 are predominantly associated with humans, and types 3 and 4 are the most prevalent subtypes isolated from humans and are associated with gastrointestinal symptoms. Subtypes ST3 and ST4 have been observed to exhibit the highest and lowest levels of diversity, respectively, among the subtypes (11, 12). Blastocystis spp. is a mysterious and unique parasite with multiple evolutionary processes and various reproduction methods, including binary division, budding, endodyogeny, sporogony, schizogony, and various morphological forms (i.e., vacuolated, granulated, ameboid, and cystic forms with dimensions of 2-200 nm). This characteristic has led to a decline in the sensitivity and specificity of microscopy-based diagnostic methods (13). In the study of Funda Dogruman-Al, et al. in Turkey, the sensitivity of the Lugol's stain method, trichrome staining and IFA stain was of 36.7%, 50%, and 83%, respectively in contrast to the culture method (gold standard). PCR method has higher specificity and sensitivity compared to other methods (14). Identifying the diversity of parasite species in different geographical areas and identifying zoonotic strains can be useful in providing prevention strategies. *Blastocystis* spp. has a high genetic diversity and there is a possibility of transmission of animal species of the parasite to humans. So far, no study has been conducted in Zahedan city (southeast Iran) to determine the prevalence and genotypes of *Blastocystis spp.* in patients. Therefore, this study aimed at determining the prevalence of *Blastocystis* species in children with diarrheal stool under 10 years old in Zahedan using PCR-sequencing method.

2. Materials and Methods

2.1. The Target Community

This descriptive-cross-sectional study was conducted between 2019 and 2021. The inclusion criteria for the study comprised children under 10 years of age and children exhibiting gastrointestinal clinical symptoms, with a particular focus on diarrhea. Exclusion criteria included children without gastrointestinal clinical symptoms, especially diarrhea, and those who lacked consent to participate in the study.

2.2. Ethical Point of View

Prior to the conduction of any laboratory analysis or physical examination, written informed consent was obtained from the guardian (parents/guardians/LAR's= Legally Authorized Representative) of all participants. A unique code was assigned to each patient, and the data was kept confidential. The study protocol was approved by the Ethics Committee of Zahedan University of Medical Sciences (reference number: IR.ZAUMS.REC.1398.322).

2.3. Ethical Considerations

Ethical clearance was obtained from the ethical review committee of Zahedan University of Medical Sciences. Prior to the interview and stool examination, written consent was obtained from the children's parents/guardians/LARs.

2.4. Sampling

The samples were selected from children who met the inclusion criteria for the study (convenience sampling). An informed consent form regarding the collection of stool samples was prepared with the signature of the parents of children who met the inclusion criteria for the study. A total of 60 diarrheal stool samples were collected from children referred to Ali Asghar Children's Hospitals in Zahedan and the Reference Laboratory of Sistan and Baluchistan Province, Iran. The characteristics of each sample were recorded, and all stool samples were referred to these two centers. Therefore, the samples had a geographical distribution and were collected from all parts of the Sistan and Baluchistan Province, Iran. In the parasitology laboratory of Zahedan Medical School, Iran, daily collections were examined microscopically (i.e., direct examination of stool samples) for the presence of Blastocystis spp. Those samples that were microscopically positive were then entered into the molecular diagnosis stage.

2.5. DNA Extraction and PCR

DNA extracted using an extraction kit (AccuPrep® stool DNA extraction kit Bioneer, Daejeon, South Korea) according to the manufacturer's instructions and genotyping was performed at the locations of *Blastocystis* spp. specific sequences (SSU rRNA gene). Before using the extraction kit, the freezing (-196˚c Liquid Nitrogen for 3 minutes) and thawing (95˚c) process was performed alternately (10 times) on the samples to break the parasite wall. The extracted DNA was kept at -20 ˚c until use. Polymerase chain reaction was performed using specific primers. The DNA extraction process was carried out in accordance with the manufacturer's instructions for the AccuPrep® stool DNA extraction kit (Bioneer, Daejeon, South Korea). Subsequently, genotyping was performed at the locations of Blastocystis spp. specific sequences (SSU rRNA gene). Prior to utilizing the extraction kit, a freezing (liquid nitrogen, -196˚C, 3 minutes) and thawing (95˚C) process was performed on the samples. This process was repeated ten times to disrupt the parasite wall. The extracted DNA was stored at -20˚C until use. Polymerase chain reaction (PCR) was subsequently performed using primers designed to target a specific region of the parasite's DNA.

Blast 505-532 (5′ GGA GGT AGT GAC AAT AAATC 3) forward, Blast 998-1017 (5′ TGC TTT CGC ACT TGT TCATC 3′; reverse (15). The PCR reaction mixture comprised the following components: 250 µM deoxynucleoside triphosphates (dNTPs), 1.5 mM $MgCl_2$, 1 U of Taq polymerase, 1 µM of a forward Blast primer, 1 µM of a reverse Blast primer, and 2 µL of DNA, yielding a final volume of 15 µL. The reaction mixtures were then subjected to incubation in a thermocycler (Flexcycler2, Germany) according to the following thermal cycling protocol: an initial denaturation step at 95°C for a period of 4 minutes, followed by 35 cycles of 95°C for 30 seconds, 54°C for 30 seconds, and 72°C for 30 seconds. The final extension step was conducted at 72°C for a duration of 5 minutes. Subsequent to the completion of the thermocycling protocol, the PCR products were subjected to electrophoresis in a 1.5% agarose gel. The positive control (Accession Number: MZ734401.1) was utilized. The expected PCR product size was determined to be between 479 and 500 base pairs (bp).

2.6. Sequencing and Phylogenic Analysis

Initially, the five samples of Blastocystis spp. sequences obtained from the new isolates were multi-aligned with ClustalW software against those of humans, which had been previously deposited in GenBank. Subsequent to this, the nucleotide sequences were examined using Chromas biotechnology software version 2.4 and CLC genomic work bench software 11. Thereafter, the sequences were aligned using BLAST database, and finally, they were compared with Blastocystis spp. reference genotypes in gene bank. The genotyping of the sequences was conducted using CLC genomic work bench software 11, and a phylogenic tree was constructed using MEGA7 software with the Neighbor-Joining statistical method, which applied the Kimura 2-parameter method based on nucleic acid. The ABI 310 automated fluorescent sequencing system was utilized to sequence high-quality PCR products, employing the indicated primers. The similarity between sequences of other Blastocystis spp. was performed by the Basic Local Alignment Search Tool (BLAST) software provided by the National Center for Biotechnology Information (NCBI) in GenBank. The sequences were then analyzed by CLC Genomics Workbench 12 software and manually edited. Maximum likelihood reconstructions were performed using the phylogenetic program MEGA software version X, with trees generated using the neighbor-joining method and the Kimura 2-parameter model. Bootstrap values were estimated using 100 replicate data sets at each node. Furthermore, a comparison was made between our samples and similar isolates from Iran and other regions of the world.

3. Results

3.1. Microscopic Examination and Molecular Diagnosis

A total of 60 stool samples from children were examined, and five samples were identified as positive for Blastocystis spp. This diagnosis was made based on the observation of morphological characteristics and the analysis of DNA. The presence of Blastocystis spp. was detected using the SSU rRNA gene. The PCR amplification product exhibited a fragment of 500 (479) base pairs.

3.2. Genotyping and Phylogenic Analysis

A subsequent analysis of the obtained sequences revealed the presence of four distinct subtypes, namely subtypes 1, 2, 3, and 5, among the existing isolates. The accession numbers of the five Blastocystis spp. isolates utilized in this study are documented in Gen Bank as follows: ON271191, ON271192, ON271193, ON271194, ON271195. The similarity percentage of the isolates was obtained by comparing the isolates available in the gene bank (Table 1). A comparison of the nucleotide changes in the sequences obtained from the studied isolates reveals the presence of significant nucleotide differences within the range of sequence 140 to 240, where deletions have occurred in the nucleotides of subtypes 1 and 2 compared to subtypes 3 and 5 within this range (Figure 1). Phylogenic analysis of the nucleotide sequence of the obtained isolates revealed a genetic affinity between subtypes 1 and 2. The same genetic affinity is also observed in the plotted tree between subtypes 3 and 5 (Figure 2).

4. Discussion

Small subunit ribosomal ribonucleic acid (SSU rRNA) constitutes the minor component of the two major RNA components of the ribosome. The SSU rRNA constitutes the small subunit of the ribosome and is an rRNA-encoding gene (16). The SSU-rDNA gene has a wide range of applications in fields such as phylogeny, taxonomy, and the analysis of evolutionary relationships among organisms. This is due to the fact that it is of ancient origin and is found in all known life forms (17). Due to the similarity of

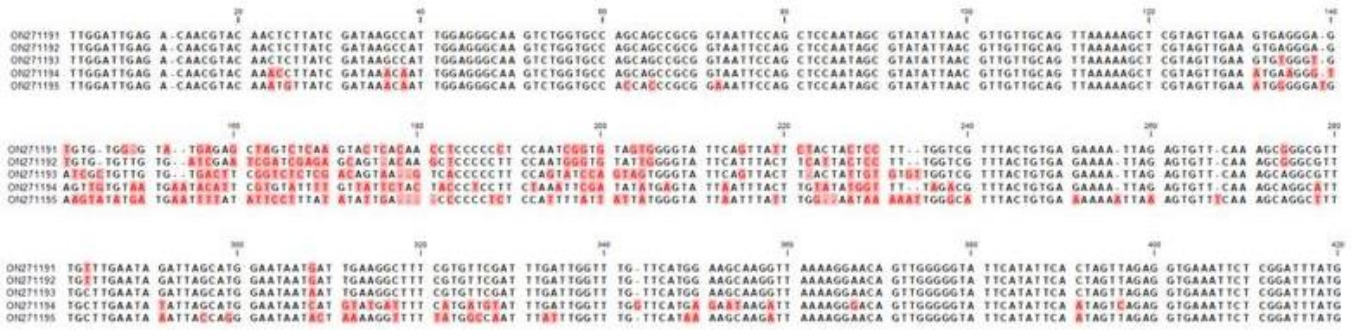

Figure 1. Alignment of 5 *Blastocystis* spp. sequenced isolates from Zahedan and a schematic representation of nucleotide changes in a small subunit rRNA gene fragment.

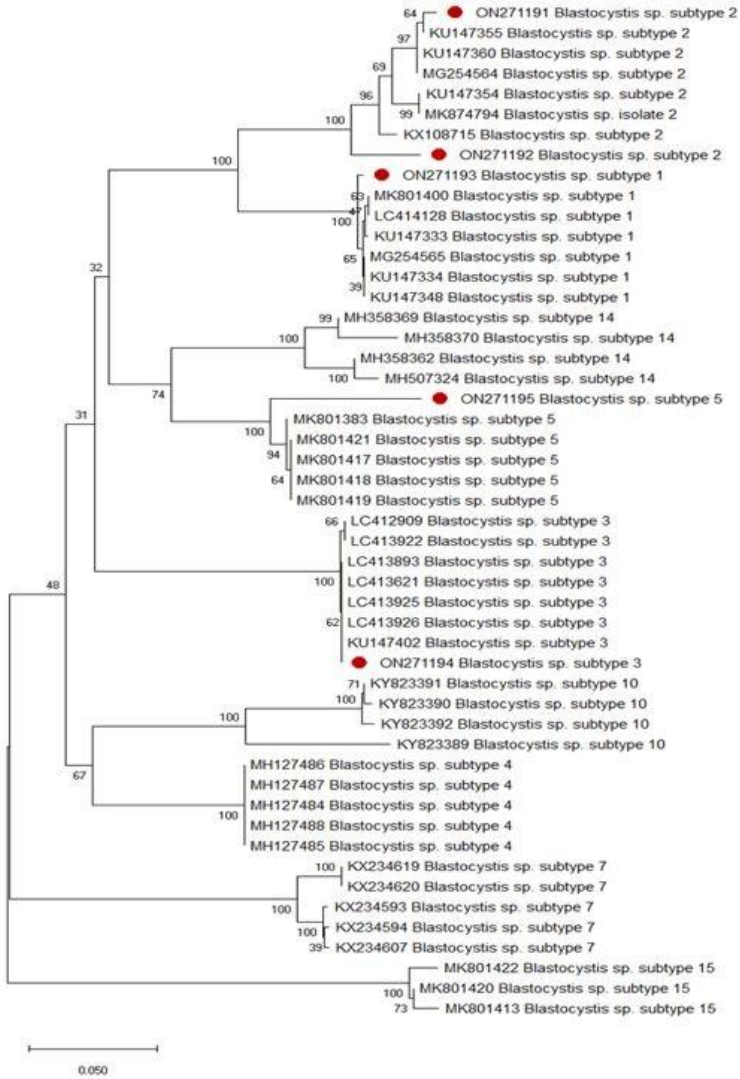

Figure 2. The phylogenetic position of *Blastocystis* spp., ST1,2,3 and 5 isolated from children's patients. Phylogenic tree was drawn using Neighbor-Joining statistical method applying Kimura 2-parameter method for different subtype *Blastocystis* spp.

Table 1. *Blastocystis* spp., subtype distribution, Homology/query coverage and Accession number among children's patients

Province	Area	Isolation source	Genus	subtype	Homology/query coverage	Accession number	host
Sistan and Baluchestan	Zahedan	stool	*Blastocystis*	subtype 2	99.77%	ON271191	Homo sapiens
Sistan and Baluchestan	Zahedan	stool	*Blastocystis*	subtype 2	99.53%	ON271192	Homo sapiens
Sistan and Baluchestan	Zahedan	stool	*Blastocystis*	subtype 1	99.31%	ON271193	Homo sapiens
Sistan and Baluchestan	Zahedan	stool	*Blastocystis*	subtype 3	100%	ON271194	Homo sapiens
Sistan and Baluchestan	Zahedan	stool	*Blastocystis*	subtype 5	93.55%	ON271195	Homo sapiens

Blastocystis spp. with yeast, fat globules, and cyclospora species, the prevalence of this parasite is usually reported higher using microscopic methods (15). The prevalence of Blastocystis spp. in different regions of the world varies significantly, ranging from 0.8% to 61%, with a predominance observed in tropical and subtropical regions, including Argentina, Egypt, the Philippines, Malaysia, China, Japan, Iran, Singapore, England, Spain, Italy, and Turkey (18–21). Despite the decline in the number of microorganisms employing the same transmission method, Blastocystis spp. has been identified as one of the most prevalent parasites in 38.9% of studies, suggesting an escalating prevalence (22, 23). Blastocystis spp. has been identified as an emerging parasite (24). Recent studies have documented a pooled prevalence of Blastocystis hominis of 3% in Iran (25). In the present study, 8.33% of the 60 samples examined were found to be infected, with the number of positive cases falling short of the reported cases. This discrepancy may be attributable to various factors, including weather conditions, low parasite concentrations, improper sample storage, inadequate sampling techniques, or the presence of fecal inhibitors. Notably, there was a 100% concordance between the microscopic and molecular results, underscoring the reliability of the diagnostic methods employed. A total of 17 subtypes have been identified in diverse mammalian, avian, and amphibian species based on the sequence of different genes and genetic analysis. Subtypes 1-9 have been isolated from humans, while subtypes 10-17 have been isolated from animals. It is noteworthy that subtypes 1-9 have been identified in other hosts in addition to humans. For instance, ST4 has been identified in rodents, ST6 in birds, and ST7-8 in birds. Additionally, ST1, ST2, ST5, ST8 have been detected in primates, pigs, and cattle, ST10 and ST15 in artiodactyls and non-human primates, ST11 in proboscideans, ST12 in artiodactyls and marsupials, ST13 in non-human primates and marsupials, ST14 in artiodactyls, and ST16 in marsupials. Finally, ST17 has been identified in rodents (26). As indicated by the findings of studies conducted on human subjects, the presence of ST1-9 and ST12 has been detected in certain cases (11,12). Subtype 5 is zoonotic and has been reported in Australia and China, which is one of the most common subtypes in animals (21,27-28). In the present study, subtypes 1, 2, 3, and 5 were identified in a total of five sequences examined from a sample of children exhibiting gastrointestinal clinical symptoms. This finding aligns with the prevalence of subtypes 1-4 reported in previous studies conducted in Iran and globally (29). The presence of subtypes 5 and 3 among the samples of children with diarrhea can be attributed to differences in lifestyle and reservoirs. Subtype 3 was isolated from 60% of Iranian cattle samples, and subtype 5 was found in pigs and cattle worldwide (30-32). The religious prohibition of pork in the Muslim population of Iran suggests the potential for zoonotic transmission of subtypes 3 and 5, highlighting the importance of public health measures to prevent the spread of these pathogens. A multitude of studies have reported subtype 1 as a pathogenic subtype in patients with gastroenteritis symptoms (33), and some studies have reported a high degree of infectivity in subtype 2. Subtype 3 has been identified as the most prevalent subtype in Iran and has been associated with AIDS and cancer patients in several studies (34). The presence of subtypes 1, 2, 3, and 5 in this study, as indicated by stool samples from children with diarrhea symptoms, aligns with the findings of previous studies (25). A previous study in this region reported the prevalence of intestinal protozoa in people with gastrointestinal discomfort as 2.2%, the average prevalence of this parasite based on common detection methods such as wet smear as 12.5%, and the low sensitivity of this method reducing the rate of parasite detection (35). The use of molecular methods directly on the stool sample can show the true prevalence of the parasite. The enigmatic nature of Blastocystis spp. may be a contributing factor to the observed increase in prevalence across most regions of

the world. This increase could be attributed to either hitherto unknown modes of transmission or a lack of consensus among medical professionals regarding the pathogenesis and treatment of the condition. Conversely, it is imperative to acknowledge the rarity of this condition in clinical laboratories, where its presence must be systematically documented and reported. The presence of 7-8 Blastocystis spp. in each microscopic field indicates a need for treatment (36). The most prevalent subspecies in humans and animals are ST3 and ST5, respectively. These two subspecies have been documented in cattle in Iran, suggesting the potential for zoonotic transmission of these subspecies. Given the transmission of the parasite cyst via the fecal-oral route, further research is necessary to elucidate the precise mechanisms of transmission among humans, animals, and different hosts. This investigation should prioritize regions within Iran to obtain a comprehensive understanding of the pathogenicity and transmission dynamics of these subspecies.

Acknowledgment
We would like to express our profound gratitude to all the family members for their participation and cooperation in this study.

Authors' Contribution
The conceptualization of the study was initiated by HM and MRB, who also played a pivotal role in its design. MRB and HY were responsible for the collection of samples and the execution of tests. MZ and YSH contributed to the analysis of data and the interpretation of results. ED and SE were responsible for the drafting of the manuscript and the review of the literature. All authors have read and approved the final manuscript.

Ethics
Prior to the administration of any laboratory analysis or physical examination, written informed consent was obtained from the legal guardian of each participant. A unique identifier was assigned to each patient, and all data were kept confidential. The study protocol was approved by the Ethics Committee of Zahedan University of Medical Sciences (reference number: IR.ZAUMS.REC.1398.322).

Conflict of Interest
The authors declare that they have no conflict of interest.

Funding
The research leading to these results has received funding from the project titled "Molecular Diagnosis and Genotype Determination of Blastocystis hominis in Children by Sequencing-PCR in Zahedan, South-East Iran." This research was supported by Zahedan University of Medical Sciences (Grant agreement number "9219"). The research supervisors of Mansour Rahmati-Balaghaleh were entrusted with the management and oversight of the study.

Consent for Publication
We obtained fully informed written consent from parents of minor subjects for the publication of their clinical data.

Consent to Participate
Prior to the administration of any laboratory analysis or physical examination, written informed consent was obtained from the subject or their legal guardian. A unique identifier was assigned to each patient, and all data were kept confidential.

Data Availability
The authors affirm that the data substantiating the study's findings are accessible within the article itself and its supplementary materials.

References

1. Markell & Voge's Medical Parasitology - 10th SEA ed - 10th Edition. Print Book & E-Book. ISBN 9789814865012, 9789814865029.
2. Javaherizadeh H, Khademvatan S, Soltani S, Torabizadeh M, Yousefi E. Distribution of haematological indices among subjects with Blastocystis hominis infection compared to controls. Przeglad gastroenterologiczny. 2014;9(1):38.
3. Souppart L, Sanciu G, Cian A, Wawrzyniak I, Delbac F, Capron M, et al. Molecular epidemiology of human Blastocystis isolates in France. Parasitology research. 2009;105(2):413
4. Zhang X, Qiao J, Zhou X, Yao F, Wei Z. Morphology and reproductive mode of Blastocystis hominis in diarrhea and in vitro. Parasitology research. 2007;101(1):43-51
5. Rivera WL. Phylogenetic analysis of Blastocystis isolates from animal and human hosts in the Philippines. Veterinary parasitology. 2008;156(3-4):178-82.
6. C.R. Stensvold, C.G. Clark, Current status of Blastocystis: a personal view, Parasitol.
7. Int. 65 (2016) 763–771.
8. Lee I, Tan T, Tan P, Nanthiney D, Biraj M, Surendra K, et al. Predominance of *Blastocystis* spp. subtype 4 in rural communities, Nepal. Parasitology research. 2012;110(4):1553-62.
9. Jafari R, Sharifi F, Bagherpour B, Safari M. Prevalence of intestinal parasites in Isfahan city, central Iran, 2014. Journal of Parasitic Diseases. 2016;40(3):679-82.
10. Khademvatan S, Masjedizadeh R, Rahim F, Mahbodfar H, Salehi R, Yousefi-Razin E, et al. Blastocystis and irritable bowel syndrome: Frequency and subtypes from Iranian patients. Parasitology international. 2017;66(2):142-5.
11. Alfellani MA, Stensvold CR, Vidal-Lapiedra A, Onuoha ESU, Fagbenro-Beyioku AF, Clark CG. Variable geographic distribution of Blastocystis subtypes and its potential implications.Acta Trop. 2013;126:11–18.
12. Ramírez JD et al. Geographic distribution of human blastocystis subtypes in South America Infection. Genet Evol. 2016;41:32–35.
13. Stensvold CR, Alfellani M, Clark CG. Levels of genetic diversity vary dramatically between Blastocystis subtypes infection.Genet Evol. 2012;12:263–273.

14. Elwakil HS, Hewedi IH. Pathogenic potential of Blastocystis hominis in laboratory mice. Parasitology research. 2010;107(3):685-9.
15. Dogruman-Al F, Simsek Z, Boorom K, Ekici E, Sahin M, Tuncer C, et al. Comparison of methods for detection of Blastocystis infection in routinely submitted stool samples, and also in IBS/IBD Patients in Ankara, Turkey. PloS one. 2010;5(11): 15484
16. Saleh Khoshnood, Abdollah Rafiei,, Jasem Saki, and Kobra Alizadeh. Prevalence and Genotype Characterization of Blastocystis hominis Among the Baghmalek People in Southwestern Iran in 2013 – 2014. undishapur J Microbiol. 2015; 8(10): e23930.
17. Martin Kolisko, Olga Flegontova, Anna Karnkowska, Gordon Lax, Julia M Maritz, Tomáš Pánek, Petr Táborský, Jane M Carlton, Ivan Čepička, Aleš Horák, Julius Lukeš, Alastair G B Simpson, and Vera Tai. EukRef-excavates: seven curated SSU ribosomal RNA gene databases. Database (Oxford). 2020.
18. hundi Wang, Tengteng Zhang, Yurui Wang, Laura A. Katz, Feng Gao and Weibo Song. Disentangling sources of variation in SSU rDNA sequences from single cell analyses of ciliates: impact of copy number variation and experimental error. Proc. R. Soc. B 284: 20170425.
19. Mehlhorn H, Tan KSW, Yoshikawa H. Blastocystis: pathogen or Passenger? spp.ringer Berlin Heidelberg. 2012. 225 p.
20. Sarinee J, Porntip P, Kookwan S, Somchai P, Arunnee S, Chotechana W, Wachanan W, Hisao Y. Subtype identification of *Blastocystis* spp. isolated from patients in a major ho spp.ital in northeastern Thailand. Parasitology Research. 2013;112:1781–1786.
21. Souppart L, Moussa H, Cian A, Sanciu G, Poirier P, Alaoui HE, Delbac F, Boorom K, Delhaes L, Dei-Cas E. Subtype analysis of Blastocystis isolates from symptomatic patients in Egypt. Parasitology Research. 2010;106:505–511.
22. Lei Deng, Yijun Chai, Ziyao Zhou, Haifeng Liu, Zhijun Zhong, Yanchun Hu, Hualin Fu,Chanjuan Yue, and Guangneng Peng. Epidemiology of *Blastocystis* spp. infection in China: a systematic review. Parasite. 2019;26:41.
23. Basak S, Rajurkar MN, Mallick SK. Detection of Blastocystis hominis: a controversial human pathogen. Parasitol Res. 2014;113(1):261–5.
24. Dagci H, Kurt O, Demirel M, Ostan I, Azizi NR, Mandiracioglu A, et al. The prevalence of intestinal parasites in the province of Izmir, Turkey. Parasitol Res. 2008;103(4):839–45.
25. Scanlan PD. Blastocystis: past pitfalls and future per spp.ectives. Trends Parasitol. 2012;28(8):327–34.
26. Ebrahim Badparva, Behrouz Ezatpour, Hossein Mahmoudvand, Masoud Behzadifar, Meysam Behzadifar, and Farnaz Kheirandish. Prevalence and Genotype Analysis of Blastocystis hominis in Iran: A Systematic Review and Meta-Analysis. Arch Clin Infect Dis. 2017;12(1):36648.
27. Ivan Wawrzyniak, Philippe Poirier, Eric Viscogliosi, Meloni Dionigia, Catherine Texier, Fre´de´ric Delbac and Hicham El Alaoui. Blastocystis, an unrecognized parasite: an overview of pathogenesis and diagnosis. Ther Adv Infect Dis. 2013;1(5):167178.
28. Moosavi A, Haghighi A, Mojarad EN, Zayeri F, Alebouyeh M, Khazan H, Kazemi B, Zali MR. Genetic variability of *Blastocystis* spp. isolated from symptomatic and asymptomatic individuals in Iran. Parasitology Research. 2012;111:2311–2315.
29. Stensvold CR, Alfellani MA, Nørskov-Lauritsen S, Prip K, Victory EL, Maddox C, Nielsen HV, Clark CG. Subtype distribution of Blastocystis isolates from synanthropic and zoo animals and identification of a new subtype. International Journal for Parasitology. 2009;39:473–479.
30. Giti Shirvani, Majid Fasihi‑Harandi, Omid Raiesi, Nasrin Bazargan and et al. Prevalence and Molecular Subtyping of Blastocystis from Patients with Irritable Bowel Syndrome, Inflammatory Bowel Disease and Chronic Urticaria in Iran. Acta Parasitologica. 2019;10:24.
31. Noel C, Dufernez F, Gerbod D, Edgcomb VP, Delgado-Viscogliosi P, Ho LC, et al. Molecular phylogenies of Blastocystis isolates from different hosts: implications for genetic diversity, identification of species, and zoonosis. *J Clin Microbiol*. 2005;43(1):348–55.
32. Rajah Salim H, Suresh Kumar G, Vellayan S, Mak JW, Khairul Anuar A, Init I, et al. Blastocystis in animal handlers. Parasitol Res.1999;85(12):1032–3.
33. Forsell J, Granlund M, Stensvold CR, Clark CG, Evengard B. Subtype analysis of Blastocystis isolates in Swedish patients. Eur J Clin Microbiol Infect Dis. 2012;31(7):1689–96.
34. Abdel-Hameed DM, Hassanin OM. Proteaese activity of Blastocystis hominis subtype3 in symptomatic and asymptomatic patients. Parasitol Res. 2011;109(2):321–7.
35. Tan TC, Ong SC, Suresh KG. Genetic variability of *Blastocystis* spp. isolates obtained from cancer and HIV/AIDS patients. Parasitol Res. 2009;105(5):1283–6.
36. Ali Haghighi, Alireza Salimi Khorashad, Ehsan Nazemalhosseini Mojarad, Bahram Kazemi and etal. Frequency of enteric protozoan parasites among patients with gastrointestinal complaints in medical centers of Zahedan, Iran. Trans R Soc Trop Med Hyg. 2009;103(5):452-4
37. Anahita Razavi Piranshahi, Mehdi Tavalla, Shahram Khademvatan. Genomic analysis of Blastocystis hominis isolates in patients with HIV-positive using locus SSU-rDNA. J Parasit Dis. 2018;42(1):28–33.