The Molecular Prevalence of *Blastocystis* spp. in Patients with Diarrhea

İshalli hastalarda *Blastocystis* spp. Yaygınlığı

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ABSTRACT

Objectives: Gut microbiota plays an important role in human health. Its composition and diversity are the main factors that affect the metabolic, immunologic and physiologic conditions. Gut microbiota is mainly composed of bacteria; however, protozoa are also crucial part of this community. *Blastocystis* is also a prominent member of human microbiome. Although its pathologic potential is still unknown, its interaction with other populations and dysbiosis may induce gastrointestinal abnormalities such as diarrhea. The present study aimed to determine the molecular prevalence of *Blastocystis* in patients with diarrhea.

Material and Methods: A total of 120 fecal samples from patients with diarrhea were collected and the DNA were extracted. Genetic analyses were conducted on *Blastocystis* 18S RNA gene with real-time PCR.

Results: DNA from six samples were excluded due to the unsuccessful isolation and it was determined that the molecular prevalence of the *Blastocystis* was 26.3% (30/114). The positivity was found in male 28.8% and 24.2% in female patients. And the prevalence was detected as 25.5% in rural and 26.9 % in urban areas. The infection was detected in 61.1 % of the 45 and older age group and 16% of the 1-6 age group.

Conclusion: In this study, the prevalence of *Blastocystis* and the relationship between infection and diarrhea in children and adult patients were discussed. As a result, it is thought that Blastocystis should always be considered in diarrhea cases and it will be effective and beneficial to use DNA-based methods in routine diagnosis in order not to miss cases.

Keywords: Microbiota, *Blastocystis*, human, molecular, real-time PCR

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ÖZET

Amaç: Bağırsak mikrobiyotası insan sağlığında önemli bir rol oynar. Kompozisyonu ve çeşitliliği metabolik, immünolojik ve fizyolojik koşulları etkileyen ana faktörlerdir. Bağırsak mikrobiyotası esas olarak bakterilerden oluşur, ancak protozoa da bu topluluğun önemli bir parçasıdır. *Blastocystis* de insan mikrobiyomunun önemli bir üyesidir. Patolojik potansiyeli hala bilinmemekle birlikte, diğer popülasyonlarla etkileşimi ve disbiyoz, ishal gibi gastrointestinal anormalliklere neden olabilir. Bu çalışma ishalli hastalarda Blastocystis'in moleküler prevalansını belirlemeyi amaçlamaktadır.

Yöntem: İshalli hastalardan toplam 120 dışkı örneği toplandı. Hatslar yaş, cinsiyet ve yerleşim yerlerine göre gruplara ayrıldı. Gaita örneklerinin DNA'ları ekstrakte edildi ve *Blastocystis* 18S RNA geni üzerinde Real-time PZR uygulandı.

Bulgular: Başarısız izolasyon nedeniyle altı örnekten DNA elde edilemedi ve ishalli hastalarda Blastocystis'in moleküler prevalansının %26,3 (30/114) olduğu belirlendi. Erkek hastalarda pozitiflik %28,8, kadınlarda %24,2 bulundu. Yaygınlık kırsal kesimde yaşayanlarda %25,5, ve kentsel yerlerde %26,9 olarak tespit edildi. 45 yaş ve üstü grubun %61,1'inde, 1-6 yaş grubunun %16'sında enfeksiyon tespit edildi.

Sonuç: Bu çalışmada *Blastocystis* prevalansı ve çocuk ve yetişkin hastalarda enfeksiyon ve ishal arasındaki ilişki tartışıldı. Sonuç olarak, ishal vakalarında Blastocystis'in her zaman düşünülmesi gerektiği ve vakaların atlanmaması için rutin tanıda DNA tabanlı yöntemlerin kullanılmasının etkili ve faydalı olacağı düşünülmektedir.

Anahtar Sözcükler: Mikrobiyota, *Blastocystis*, insan, moleküler, real-time PZR

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INTRODUCTION

The human gut microbiota contains various microbial communities and it plays an important role in metabolic and immune conditions (1). Gut microbiota diversity was associated with healthy states, and dysregulation of this balance triggers several inflammatory diseases. In that case, protozoa population has crucial effects on the host organism. Although several protozoa could be found in healthy microbiota, they may lead to some diseases under certain conditions that are still unclear (2).

Blastocystis is one of the most common protists in human gastrointestinal tract and a member of healthy gut microbiota (3). Its prevalence depends on the geographical location and population. The prevalence is more common in developing and undeveloped countries especially located in tropical and subtropical regions (4). The infection concerns more than one billion people worldwide and the estimated prevalence is 11.2% (from varies 1.1 to 60.0%) depending on the climate and the sanitary conditions of the region (5, 6). Patients are commonly infected by fecal-oral transmission via contaminated water and food, and close contact with animals (7). Animal studies demonstrated that the environmentally resistant cyst stages are responsible for the transmission (8). Four major forms (vacuolar, granular, amoeboid, and cyst) of *Blastocystis* were described. Although, *Blastocystis* is routinely diagnosed with microscopic methods such as native-Lugol examination in fresh fecal specimens, this is not a reliable method due to morphological variations and its small size (9). In this way, accurate diagnosis cannot be done and the number of the cases are underestimated. Therefore, molecular approaches have been dramatically popular in detection and investigation of *Blastocystis* in recent years. Molecular studies demonstrated that this protozoan parasite is genetically diverse and until today 17 *Blastocystis* subtypes (ST1 to ST9 and ST12) were identified based on the variations of the small subunit ribosomal RNA gene (10). Ten out of 17 (ST1-ST9, ST12) *Blastocystis* subtypes were determined in human samples. Among these subtypes, the most frequent and predominant was ST3 in humans. ST1 and ST2 were also detected in human samples, however these are widely observed in animals such as monkeys, chickens, cattle, pigs, dogs and other non-human primates (11). Recent studies also demonstrated that subtypes isolated from humans varied based on different epidemiologic and demographic factors such as cultural habits and climate (11, 12).

Information on pathogenesis and molecular epidemiology of *Blastocystis* is still controversial and open to debate. Although increasing evidence that the parasite is one of the members of gut microbiota, it sometimes induces certain clinical conditions (13,14). Human infections are sometimes asymptomatic, however clinical studies demonstrated that it may lead to diarrhea, abdominal pain, dermatological problems, nausea and flatulence. Even so, there is still not sufficient evidence that demonstrated the exact correlation between clinical symptoms and *Blastocystis* infection (15-19). Certain researchers attributed the pathogenicity to the number of organisms. The parasite has also been reported frequently from patients with nonspecific gastrointestinal disorders like irritable bowel syndrome (IBS) and urticaria (9,16,20). The most common clinical feature induced by *Blastocystis* is diarrhea and it was reported that there was an association between diarrhea and *Blastocystis* infection (21). Also, it was suggested that ST3 subtype was likely responsible for the clinical symptoms (22). *Blastocystis* is also common in cancer patients and there is a strong correlation between chemotherapy and *Blastocystis* infection (23).

Subtropical regions such as Turkey provide advantages for proliferation of the parasite.[3] Van province is located in the Eastern part of Turkey and has border with Iran and Iraq. The parasite is more frequently observed in this region because of the socio-economic and demographic features of the population compared to western part of Turkey (21). Therefore, understanding the population-based clinical effects and prevalence of *Blastocystis* is important to clarify the pathogenesis and the importance of the parasite.

Based on the findings of the above-mentioned studies, the present study aimed to determine the prevalence of *Blastocystis* in patients with diarrhea in Van province by real-time PCR.

MATERIALS and METHODS

Collection of fecal samples

The present study protocol was reviewed and approved by Van Yüzüncü Yıl University Ethics Committee for Non-Interventional Clinical Research (no:2019/17-12). The study included 66 (55%) male and 54 (45%) female patients out of 120 individuals. Fecal specimens collected from patients were referred to the Parasitology Laboratory of Faculty of Medicine from different clinics with diarrhea during February 2016 until November 2018. The patients' age varied between 1 to 77 years and patients were divided into four age groups as: 1-6; 7-14; 15-44 and 45 and above. Forty-three, 20, 39 and 18 patients were inspected these ages group, respectively. Seventy-one (59.2%) stool samples were collected from urban area residents, and the remaining 49 (40.8%) stool samples were collected from patients who lived in rural areas.

DNA extraction and detection of Blastocystis

DNAs were extracted using QIAamp® DNA stool minikit (Qiagen, Germany) based on the manufacturer's instructions and stored at -20°C. Genetic analyses were conducted on *Blastocystis* 18S RNA gene with real-time PCR using the LightCycler System (Roche Diagnostics). Taqman hydrolysis probes and LightMix®Modular kits (TIB Molbiol, Germany) were used to quantify the presence of *Blastocystis* 18S RNA gene based on the manufacturer's instructions. Real-time PCR was conducted with a total volume of 15 µl that included 4 µl Roche Master mix, 0,5 ul Reagent mix and 10,5 nuclease free water and the DNA sample. PCR conditions were 5 minutes at 95°C, followed by 45 cycles of 95°C for 5 seconds, 60°C for 15 seconds, 72°C for 15 seconds, and one cycle of 40°C for 30 seconds. Roche LightCycler Nano System software was used to determine the threshold cycle (Ct) values.

RESULTS

A 148 bp long fragment of *Blastocystis* ribosomal 18S RNA gene was amplified. Amplification curves (Figure 1) and quantitative cycle threshold (Ct) values were recorded (Table 1). DNA from six samples were excluded due to unsuccessful isolation, four from male and two from female patients. A total of 30 out of 114 samples was identified as positive (26.3%) with real-time PCR. The positive isolates were found in male patients with the prevalence of 28.8% (15/52) and 24.2% (15/62) in female patients (Table 2). And the prevalence was detected as 25.5% (12/47) in rural and 26.9 % (18/67) in urban areas.

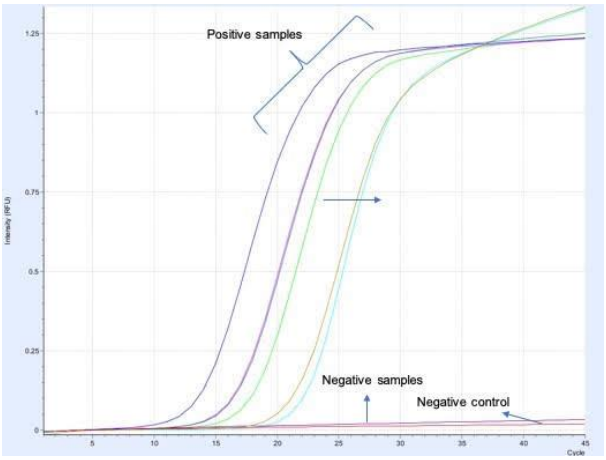

Figure 1. Amplification curves of positive and negative samples

Table 1. Representative Ct values of some *Blastocystis* spp. positive patients*

Number	Ct	Number	Ct
1	37,80	16	37,58
2	30,80	17	33,83
3	37,97	18	20,79
4	38,31	19	37,28
5	29,01	20	39,11
6	38,97	21	38,34
7	31,06	22	25,51
8	38,62	23	38,21
9	33,40	24	35,31
10	23,19	25	26,85
11	30,86	26	41,14
12	25,57	27	22,58
13	27,56	28	26,65
14	35,49	29	27,32
15	34,86	30	32,96

Table 2. Distribution of Real-Time PCR results based on gender*

	Male	Female	Total
Negative	48	36	84
Positive	15 (24.2%)	15 (28.8%)	30 (26.3%)
Total	62	52	114

*DNA of six samples could not extracted, four from male and two from female patients.

DISCUSSION

Intestinal microbiota plays an important role in the pathogenesis of diseases. Therefore, factors that affect the balance between the microorganisms in gut microbiota trigger pathologic changes in human body (24). In recent years, studies have demonstrated that parasites are as crucial as bacteria for the healthy microbiota. They can interact with other members of microbiota such as bacteria and they induce changes in intestinal flora. Due to its abundance in human gastrointestinal tract and its prevalence worldwide, *Blastocystis* is a prominent part of microbiota (25,26).

Blastocystis is commonly found in human intestines, however its pathogenicity is still debated. The infections sometimes can be asymptomatic or have clinical manifestations. In the past decade, studies on the prevalence, pathogenicity and genetic diversity of *Blastocystis* have increased considerably (18). And these researches have shown that many people are infected with the Blastocystis and carry the agent, regardless of clinical symptoms. Anderson et al. (27), showed that there is a significant association between *Blastocystis* and gut microbiota and *Blastocystis* colonization is associated with other microorganisms which are responsible for eubiosis. *Blastocystis* may show its pathogenicity by changing the intestinal permeability which is caused by disruption of balance in gut microbiota and colonization (28). Perturbation in intestinal permeability leads gastrointestinal disorders that are common clinical symptoms seen in *Blastocystis* infection such as diarrhea (22,28). Therefore, clarifying the relationship between *Blastocystis* infection and diarrhea is important. In this perspective, in our study *Blastocystis* was detected in 26.3% of samples with diarrhea. A study by Zulfa et al. (22) reported the prevalence of 44.23% *Blastocystis* infection in Indonesian children with diarrhea. In another study, *Blastocystis* infection was observed in 4.7% of 580 pediatric patients with chronic diarrhea and it was mentioned that *Blastocystis hominis* is one of the common parasites that cause diarrhea in pediatric patients (29). In this study, low *Blastocystis* infection was reported with the prevalence of 26.3% could be due to certain factors such as the age group of patients and the geography as the prevalence of *Blastocystis* varies depending on the country and the population and it shows variations even in the same country within different locations because of life styles such as westernized and agrarian life (12, 30). In Turkey, previous studies reported differences in the prevalence between 4.38% to 51%. Dogan et al. (30) detected *Blastocystis* in 21.4% of children patients with diarrheal disease in Eskişehir province.

In our study, we did not find significant changes in the levels of *Blastocystis* positive patients with diarrhea living in rural areas compared to urban areas probably due to limited patient samples or the lifestyles and habits of the patients.

Several factors may affect the determination of the exact role of *Blastocystis* in human diseases and one of the most important factors is the methodology. Microscopic methods such as Lugol and Giemsa have low sensitivities and specificities to detect *Blastocystis* when compared to molecular methods (9,31). Thus, molecular approaches such as Real-Time PCR and next generation DNA technologies play a pivotal role in understanding the impacts of parasites on pathogenesis of diseases and our lives. Numerous studies compared microscopic analysis results with molecular findings, and they demonstrated higher efficiency of molecular approaches in diagnosis of *Blastocystis* (30, 32). Sari et al. (33) also showed PCR is more sensitive and specific compared to culture method for diagnosis and identification of *Blastocystis*. However, culture has the advantage of multiplying a small number of parasites and detecting forms after excitation, and also microscopy is inexpensive compared to other methods and allows the detection of other parasites (18, 31). Thus, in the present study, only Real-Time PCR was used as a molecular approach in determination of the prevalence of *Blastocystis* in patients with diarrhea. It was possible to determine low *Blastocystis* genome content in feces even when parasites were degenerated. In the present study, sample cycle threshold (Ct) values varied between 20.8 and 41.1. This finding demonstrated that the samples contained lower or degenerated *Blastocystis* genome content, and microscopic examinations may have a higher risk of giving false negative results in these patients. In these cases, culture and microscopic methods are time consuming and have disadvantages such as misdiagnosis. Therefore, Real-Time PCR may be one of the gold standards in diagnosis of *Blastocystis*.

Consequently, the present study was discussed the prevalence of *Blastocystis* infection in child and adult patients in Eastern part of Turkey. Although the frequency of *Blastocystis* spp. in patients without diarrhea was not revealed because control stool samples were not used in the current study, *Blastocystis* was detected as high as 26% in patients with diarrhea. Therefore, it is thought that *Blastocystis* must be consider in diarrhea cases. Real-Time PCR plays a crucial role in determination of *Blastocystis* genome in stool samples and routine diagnosis of protozoan-induced diarrhea. Thus, the present study findings should be confirmed with future studies conducted with larger patient groups and the *Blastocystis* still needs to be investigated.

Conflict of interest

No conflict of interest was declared by the authors.

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