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PCR-Based Subtyping of Blastocystis Isolates from Symptomatic and Asymptomatic Patients in North-West of Iran

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The pathogenic potential of *Blastocystis hominis* is still controversial. To find the pathogenicity of this organism determining the genotypic differences among symptomatic and asymptomatic isolates can be useful. To determine genetic diversity of *Blastocystis hominis* in North West of Iran, 57 isolates from 34 asymptomatic healthy individuals and 23 symptomatic patients were genotyped by polymerase chain reaction using seven pairs of known subtype specific sequence tagged site (STS) primers. Out of 57 isolates, ST3 as predominant (50.87%) followed by ST1 (40.35%) and ST2 (8.77%) were recognized. By comparing genotype of Blastocystis isolates among asymptomatic and symptomatic group, we found that in asymptomatic group subtype 3 was most dominant (29/34) and all patients in symptomatic group classify as subtype 1 (23/23).

Key words: *Blastocystis hominis*, PCR, Genotype, Iran.

Blastocystis hominis is recognized as one of the most frequently encountered parasite in human and animal fecal samples [1,2] . The prevalence of this organism is up to 10 % in the developed countries but these rates is about 50-60% in developing countries[3,4]. This parasite is commonly associated with gastrointestinal symptoms such as watery and mucous diarrhea, vomiting, abdominal cramp and bloating[5]. Epidemiological studies also suggest its role in irritable bowel syndrome[6]. Blastocystis is found in individuals with enteric symptoms and in clearly healthy and asymptomatic subjects[7, 8]. Blastocystis in humans have paying attention on genotypic analysis[9-12]. It has been proposed that genetically different genotypes (subtypes) may be associated with the pathogenic potential of Blastocystis and Polymerase chain reaction (PCR) can be used to discriminate strain, species, and pathogenic potential of *B. hominis* isolates[9, 10, 13-17]. To disclose which genotype of blastocystis will associate with pathogenic potential, comparative study and clinically distinct groups, symptomatic and asymptomatic groups, are essential [18]. Ribosomal RNA (rRNA) gene analyses have frequently been used to find out Phylogenetic and taxonomic relationships among of *B. hominis* isolates. Specifically, small-subunit (SSU) rRNA gene, relatively simple, rapid method, has increasingly being used for genetic comparison of various isolates of this parasite[11,17,19,20].In this study we try to find genotype of several *B. hominis* isolates from symptomatic and asymptomatic patients in Tabriz at the North-West of Iran by PCR with the

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seven pairs of sequenced-tagged site (STS) primers reported by Yoshikawa *et al* (2004) to this survey can be help to determining potential pathogenicity of this organism.

MATERIALS AND METHODS

Sources and isolates of B. hominis. From December 2011 to June 2012, 57 blastocystis positive samples collected at the central laboratory of west Azerbaijan province in North-West of Iran during parasitological examination of stool samples. Blastocystis sp. identified through direct light microscopy of native lugol's iodine smear and stool samples examined for the presence of other parasitic infection then we excluded fecal specimens involving other protozoan and then by a formalin-ethyl acetate technique were concentrated. Patients based on responses to a standard questionnaire were classified as symptomatic and asymptomatic group and their clinical histories have been gathered.

DNA extraction

Genomic DNA of *B. hominis* extracted according to the manufacturer's directions from about 200 mg of fecal samples using QIAamp DNA Stoll mini kit (Qiagen, Germany). DNA was eluted in 100 µl of AE buffer (Qiagen) to increase its concentration. To concentrate each DNA calculated with a spectrophotometer (Eppendorf bio photometer, Germany). Until PCR was carried out, extracted DNA stored at 20°C.

PCR with the sequenced-tagged site (STS) primers

To identify genotypes of *B. hominis*, PCR was carried out using seven pairs of subtype-specific sequenced-tagged site (STS) primers (SB83, SB155, SB227, SB332, SB340, SB336, and SB337) reported by Yoshikawa *et al.,* (2004) (Table I).

PCR reaction mixtures (25 µl of total volume) containing of PCR buffer 1X (10 mM Tris-Hcl, PH 8.8, and 50 mM KCl), 1.5 mM $Mgcl_2$, 2.5 U/µl of Taq DNA polymerase(Fermantas), 1.25 µM of each dNTPs (fermantas), 20 pmol each primer, and 5 µl of the DNA sample. The PCR condition consisted of one cycle of initial denaturing at 94°C for 5min, followed by 40 cycles including denaturing at 94°C for 30s, annealing at 60°C for 30 s, and extending at 72°C for 1min, and additional cycle with a 5-min chain elongation at 72°C. For each primer pair PCR amplification at least twice repeated. The PCR products were electrophoresed in 1.5% agarose gels and Tris-borict-EDTA buffer. Gels were stained with ethidium bromide and visualized by using an ultraviolet gel documention system (uvitec, uk) and fragment sizes were confirmed with bands of a DNA length standard (50-1,000 bp DNA markers, fermantas).

Table 1. Subtype classification with the sequence-tagged site (STS) primer sets used in this study (Yoshikawa *et al*. 2004)

Subtype	STS primer sets	Product size (bp)	Sequences of forward (F) and reverse (R) primers (52 to 32)		Gen Bank accession no.
1	SB83	351	F	GAAGGACTCTCTGACGATGA	AF166086
			R	GTCCAAATGAAAGGCAGC	
2	SB155	650	F	ATCAGCCTACAATCTCCTC	AF166087
			R	ATCGCCACTTCTCCAAT	
3	SB227	526	F	TAGGATTTGGTGTTTGGAGA	AF166088
			R	TTAGAAGTGAAGGAGATGGAAG	
4	SB332	338	F	GCATCCAGACTACTATCAACATT	AF166091
			R	CCATTTTCAGACAACCACTTA	
5	SB340	704	F	TGTTCTTGTGTCTTCTCAGCTC	AY048752
			R	TTCTTTCACACTCCCGTCAT	
6	SB336	317	F	GTGGGTAGAGGAAGGAAAACA	AY048751
			R	AGAACAAGTCGATGAAGTGAGAT	
7	SB337	478	F	GTCTTTCCCTGTCTATTCTGCA	AY048750
			R	AATTCGGTCTGCTTCTTCTG	

RESULTS

In this study, stool samples collected from a total 57 patients (40 males and 17 females). The patient's age was between 2 to 81 years; their median age was 41.43 years old. In Fig. 1, the age distribution has been shown. The asymptomatic group involved of 23 patients (17 males and 6 females) presenting variously with abdominal pain, vomiting, flatulence, fever, diarrhea, nausea, and constipation. The asymptomatic group was comprised of 34 healthy individuals (23 males and 11 females) without any gastrointestinal symptoms. By PCR based on seven pair of STS primer genotype of total 57 isolates were obtained. All of the isolates with the seven kinds of STS primer were screened. Twenty three isolates from symptomatic patients were amplified by SB83 primer and their fragment size were about 350bp and these isolates identified as subtype 1 (17) (Fig. 2). We amplified 29 isolates from asymptomatic patients that their fragment size were about 526bp with SB227 primer. These isolates identified as subtype 3(17). Five asymptomatic isolates were positive amplification with SB155 primer and their fragment sizes were approximately 650 bp. These isolates identified as subtype2 (17)(Fig. 3 A and B).In this study, any isolates was not identified as subtype 4-7. Our data showed that in asymptomatic individuals most dominant genotype

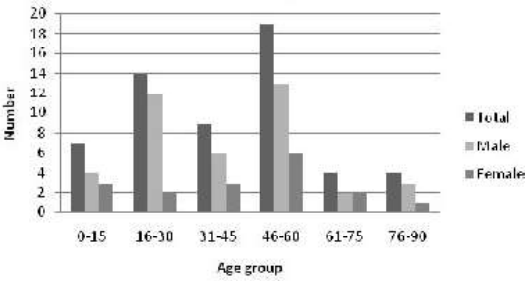
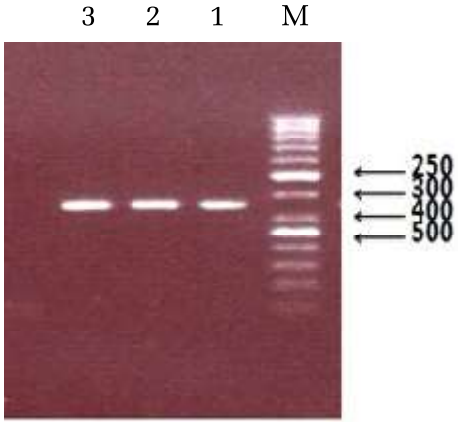


Fig. 1. The age distribution of patient positive for blastocystis



Lane M, 50-1000 bp DNA marker and lane 1-3, subtype 1 (350bp).

Fig. 2. Genotyping of *B. hominis* isolates from symptomatic patients

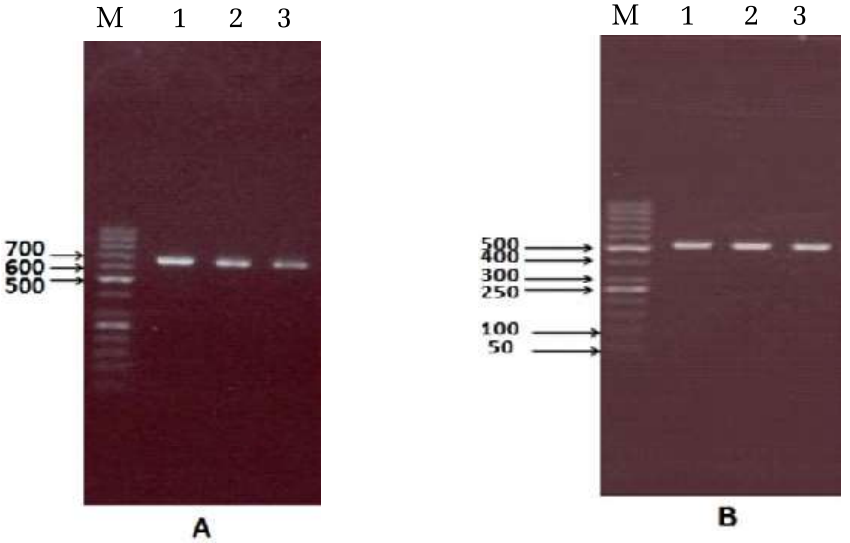


Fig. 3. A and B Genotyping of *B. hominis* isolates from asymptomatic individuals. A Lane M, 50-1000 bp DNA marker and Lanes 1-3, subtype2 (650bp). B Lane M, 50-1000 bp DNA marker and Lanes 1-3, subtype3 (526bp)

was subtype 3 (29/34) and subtype 2 was in second rank while all of symptomatic patients identified as subtype1 (23/23). Overall, among all patients subtype 3 was the most common (29/57) and subtype 1 was in second rank.

DISCUSSION

The main controversy about the *B. hominis* is its role in human disease[6]. There is increasing evidence to propose that immune compromised individuals, predominantly patients with AIDS are more susceptible to suffer from Blastocystis-related diarrheal illness[21-23]. There are some reports about the prevalence of Blastocystis sp. among cancer patients[24-26]. Several studies showed an association between Blastocystis with irritable bowel syndrome (IBS) [27-29]. On the other hand, this parasite has been found in many healthy people without showing any symptoms. In previous studies have been reported Blastocystis infection showing itself with fever, chills, abdominal pain and diarrhea[30] and Morris *et al*. found blastocystis infection in patient who presented with fatigue, depression, skin rash, joint pain, constipation, abdominal pain, and diarrhea[31]. In our study, we found this organism in the symptomatic group involved of 23 patients (17 males and 6 females) presenting variously with abdominal pain, vomiting, flatulence, fever, diarrhea, nausea, and constipation. The extensive genetic diversity that has been demonstrated among *B. hominis* isolates from humans and animals[17, 32-36] it has been assumed that certain distinct genotype may exhibit pathogenicity. Yoshikawa *et al* in recent years from random amplified polymorphic DNAs (RAPDs) have developed seven pairs of STS primer that can be used to classify the genotypes[35, 37-39]. These primers can be used to identify the genotypes that correspond to phylogenetically different clades inferred from the small-subunit rRNA genes (SSU rDNA)[36, 37,39]. We realize the value of identification subtypes using STS primers, for a more accurate classification of Blastocystis subtypes[17]. Because there is no appropriate animal model existing for *B. hominis* at present, the pathogenic potential of *B. hominis* cannot be confirmed experimentally[7]. Therefore, to disclose a possible association between definite genotype and pathogenic potential of this parasite, So far, several comparative studies of human *B. hominis* population from clinically symptomatic and asymptomatic patient by PCR with STS primers were performed. Yan et.al reported subtype 1 was detected among symptomatic patients and subtype 3 was the most common among symptomatic patients[10]. Tan *et al*. have found that all symptomatic patients belonged to the subtype 1 and all asymptomatic patients belonged to subtype3[8]. Eroglu et.al observed genotype of *B. hominis* isolates from asymptomatic and symptomatic patients and they found that subtype 3 was the most dominant genotype in asymptomatic individual and determined all of symptomatic patients as subtype1[14]. In previous study in Iran Moosavi et.al in Tehran investigated genetic variability of *B. hominis* by PCR with STS primers and they were identified subtype 3 as dominant (53%), followed by subtype 1 (48%), subtype 5 (33%) and subtype 2 (7%) (40). Up to now conflicting reports about the relationship between subtypes of the *B. hominis* sp. and the symptoms presented. Recent molecular studies have focused on clarifying what specific subtype of this organism may be a potential pathogen. It is still matter of debate whether distinct Blastocystis subtypes correlate with the pathogenic potential of this parasite[32]. In some cases, no relation between subtype and symptoms has been observed[15, 39, 41]. In contrast, some studies suggest an association between subtype and symptoms. Dogruman-Al *et al*. demonstrated that subtype 1 was associated with elevated pathogenicity[18]. Blastocystis subtype 1 and subtype 4 have also been suggested to be responsible in causing disease in patients[13,39]. In the present study, 57 Blastocystis isolates were amplified with STS primers, and we analyzed and compared the possible role of different genotype of these organisms in two group of patients with symptomatic and asymptomatic. The results show that in group of symptomatic patients subtype1 is main subtype even though subtype 3 is predominant genotype in asymptomatic patients followed by subtype 2. The results of the present study are similar to data reported by Yan *et al*. (2006) in China, Hussein *et al*. (2008) in Egypt, and Tan *et al*. (2008), Eroglu *et al*. (2009) in Turkey [8, 10, 14, 15]. In some studies, subtype 3 have related with

pathogenicity such as Katsarou-katsari *et al* and Hameed *et al*. asserted this subtype was associated with acute urticaria[42,43]. Or in some studies conducted in Malaysia, Singapore and USA that subtype 3 was reported to be predominant with chronic gastrointestinal disease[8, 44, 45], whereas we found this subtype just in asymptomatic group that our results is similar to data reported by Yan *et al*. and Eroglu *et al.,*[10, 14] .In present study, subtype 2 was detected in five isolates of asymptomatic group. Dogruman-Al *et al*. suggested subtype 2 is most likely the one that is non pathogenic[18], however Subtype 2 in some studies reported as the second rank subtype[13, 46, 47].In this study none of isolates classified as subtypes 4-7. These results also suggest geographical dissimilarity in genotype because these subtypes were reported in other studies[12 15,17,28]. Hussein *et al*. in their study of experimental infection in animals with human Blastocystis isolates, confirmed that subtype 1 was associated with elevated pathogenicity[15]. Lately, Yan *et al*. and Eroglu *et al*. also demonstrated predominant subtype 1 in a group of symptomatic patients[10,14]. Moreover, Yakoob *et al*. was found subtype 1 commonly in patients with IBS-D[28]. Based on results of our study and previous studies, once more, data indicate the possible correlation between subtype 1 and a pathogenic potential of this parasite. However, more studies are necessary to verify this issue.

Declaration of interest

The authors declare no conflict of interest.

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