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PCR-based molecular characterization of *Blastocystis hominis* subtypes in southwest of Iran

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ABSTRACT

Blastocystis hominis is the most common intestinal parasite found in humans and many other hosts. Pathogenicity of *Blastocystis* sp. remains controversial and it has been suggested that it may be associated with certain subtypes of organism. The aim of this study was to evaluate the molecular epidemiology of *B. hominis* and its subtype distribution in Ahvaz, southwest of Iran. During 2012–2014, a total of 481 samples were collected from patients referred to the medical laboratory centers in Ahvaz for stool examination. Samples were examined by wet mount, and genomic DNA was extracted from 50 positive samples. PCR was performed using seven primer pairs targeting the SSU rDNA gene and sequenced. 69 (14.35%) samples were found to be positive for *B. hominis* and the subtypes of 50 samples were identified. Five subtypes (STs) were identified, including: ST1 (22%), ST2 (6%), ST3 (40%), ST4 (2%), and ST5 (8%). 11 (22%) mixed infections were found, of which 5 were a mixture of ST3/ST4. Mixtures of ST1/ST3 and ST1/ST4 were 3, respectively. In this study people infected with ST3 showed the most gastrointestinal symptoms. This is the first study in the population of Ahvaz and indicates the high prevalence of ST3 in this area. The results suggest a possible association between this subtype and pathogenic potential of parasite.

Introduction

Blastocystis hominis is the most gastrointestinal eukaryotic parasite that exhibits low host specificity and is found in human and many animals fecal samples, with worldwide distribution [1–4]. Prevalence of infection varies widely from one region to another but in general it is higher in developing countries than developed countries. This has been due to poor hygiene, exposure to animals and consumption of contaminated food or water in developing countries [5–7]. In Iran the prevalence of *B. hominis* varies from 0.22% to 54.5% [8–12].

As *B. hominis* can be found in both symptomatic and asymptomatic patients, there are conflicting views about its pathogenicity [1,13,14]. *B. hominis*is is associated with a variety of symptoms, ranging from intestinal symptoms to cutaneous disorders. Non-specific intestinal symptoms that have been reported are nausea, anorexia, fatigue and flatulence [14–16]. In addition, an association between *B. hominis* and irritable bowel syndrome (IBS) is suggested [17,18]. Some studies indicated that the infection with *B. hominis* was more common in patient with chronic immunosuppressive disease [19,20]. However other studies suggested *B. hominis* as a commensal parasite in humans without any pathogenic effect [13,21]. An increasing number of new studies cited *Blastocystis* sp. as an emerging pathogen [1,22,23]. Recently, a 29-kDa parasite protein and a parasite associated protease have been noted as potential markers of pathogenicity [24,25].

Based on gene analysis of small-subunit ribosomal RNA (SSU-rRNA), *Blastocystis* sp. isolates were classified into subtypes [26]. It has been suggested that the pathogenesis of *Blastocystis* sp. may be dependent upon subtypes of organism [27–29]. 17 subtypes or species of *B. hominis* have been described, of which 9 have been reported in humans [2,30]. Some STs such as ST10 and ST14 are present only in cattle never in humans even the direct contact [2,31]. It is suggested that different subtypes have dif-

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Table 1
Primer sequences used in this study [33].

GenBank ACC, no	Sequences	Product size (bp)	Primers
AF166086	F:GAAGGACTCTCTGACGATGA R:GTCCAAATGAAAGGCAGC	351	SB83 Sub 1
AF166087	F:ATCAGCCTACAATCTCCTC R:ATCGCCACTTCTCCAAT	650	SB155 Sub 2
AF166088	F:TAGGATTTGGTGTTTGGAGA R:TTAGAAGTGAAGGAGATGGAAG	526	SB227 Sub 3
AF166091	F:GCATCCAGACTACTATCAACATT R:CCATTTTCAGACAACCACTTA	338	SB332 Sub 4
AY048752	F:TGTTCTTGTGTCTTCTCAGCTC R:TTCTTTCACACTCCCGTCAT	704	SB340 Sub 5
AY048751	F:GTGGGTAGAGGAAGGAAAACA R:AGAACAAGTCGATGAAGTGAGAT	317	SB336 Sub 6
AY048750	F:GTCTTTCCCTGTCTATTCTGCA R: AATTCGGTCTGCTTCTTCTG	487	SB337 Sub 7

ferent host, geographical distribution, and routes of transmission. Therefore, subtyping *B. hominis* is important for epidemiological studies because it helps to identify potential sources and routes of transmission of a specific subtype in a particular area and this new information can help us to complete the knowledge about pathogenicity of *B. hominis* [22]. So, the aim of this cross-sectional study was to estimate the infection prevalence and subtype distribution of human *B. hominis* by PCR and sequencing in Ahvaz, southwestern Iran.

Materials and methods

Study area

Ahvaz city, capital of Khuzestan province which is located in the southwest of Iran (31°50′N and 49°11′E), is ranked as the 7th largest city throughout the country and based on the latest census, its population is calculated about 1,395,184 in 352,128 families. Weather temperature is highly variable throughout the year so that in summer temperature exceeds 50 °C whereas in winter it falls to 5 °C. Also, annual average rainfall is approximately 230 mm [32].

Samples and parasitological examination

About 481 fecal samples were collected from persons (aged 8–79 yrs, mean age 42) who referred to the medical laboratory centers in Ahvaz. Before sampling, a questionnaire (including age, gender, symptoms, etc.) was completed by adult subjects and parent of children less than 18 years-old. They were tested for the presence of *B. hominis* by using saline wet mount method. Samples were stained by iodine techniques. A cover slip was placed on the surface and the sample was examined at a magnification of 40× and 100× for *B. hominis*. Samples with other pathogenic parasitic agents were excluded.

DNA extraction

The genomic DNA of 50 samples of *B. hominis* was extracted from positive feces with a DNA extraction kit, according to the manufacturer's protocol (QIAamp, QIAGEN Inc., Germany). The extracted DNA was stored at −20 °C until PCR amplification [14].

Subtyping

The PCR was performed using subtype-specific sequence tagged site primers to identify genotypes of *B. hominis* [33] (Table 1). PCR reaction mixtures (25 μl of total volume) consisted 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* polymerase (Fermentas), 1.25 μM of each dNTPs (Fermentas), 0.5 pmol of each primer and 5 μl of the DNA sample. The PCR conditions consisted of one cycle denaturing at 94 °C for 5 min, 40 cycles including annealing at 57 °C for 30 s, extending at 72 °C for 60 s, denaturing at 94 °C for 30 s, and additional cycle with a 5 min chain elongation at 72 °C (MyCycler, Bio-Rad, Hercules, CA, USA). Electrophoresis was performed by adding 5 μl of the PCR products to a 1.5% agarose gel and staining with ethidium bromide for 45 min at 100 V [14]. Bands were observed by ultraviolet transillumination (Uvidoc, Gel Documentation System, Cambridge, UK) [32,34].

Sequence analysis

PCR amplification of the gene from 20 randomly selected samples was sequenced by MWG (Germany), and the resulting data were analyzed using Chromas (Technelysium Pty Ltd. Australia) software (http://www.technelysium.com.au/Chromas.html). Sequences were individually compared with *B. hominis* SSU-rRNA gene sequences available in GenBank using the basic local alignment search tool (BLAST) algorithm. Subtypes were determined by an exact match or closest similarity with sequence data from known *B. hominis* subtypes. Each sequence was then aligned with a panel of reference sequences from GenBank using the ClustalW program to determine sequence similarity [14].

Statistical analysis

Statistical analysis was performed using PASW Statistics (version 18.0.0.) software. Data were presented as the prevalence of *B. hominis*. Prevalence of strains and other information were analyzed by descriptive statistical methods. Also variables analyzed by the chi-square test.

Results

Of 481 samples collected, 69 (14.35%) were positive for *B. hominis*. Prevalence of *B. hominis* in male and female was 15.26% and 13.36% respectively. The difference was not statistically significant ($P = 0.3$). Patients were divided into 6 groups based on their age (<20, 20–29, 30–39, 40–49, 50–59 and >60). The prevalence of *B. hominis* in age groups were 15.94%, 18.84%, 14.49%, 21.74%, 10.14% and 18.85%, respectively (Table 2). Out of 69 patients, 29 presented symptoms such as abdominal pain, diarrhea and vomiting, and 40 individuals were asymptomatic. Distribution of the symptoms

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Table 2
Distribution of *Blastocystis hominis* in patients with different age subgroups.

Age	Male (n = 249) No (%)	Female (n = 232) No (%)	Total (n = 481) No (%)
<20	8 (11.59%)	3 (4.34%)	11 (15.94%)
20–29	7 (10.14%)	6 (8.69%)	13 (18.84%)
30–39	5 (7.24%)	5 (7.24%)	10 (14.49%)
40–49	7 (10.14%)	8 (11.59%)	15 (21.74%)
50–59	3 (4.34%)	4 (5.79%)	7 (10.14%)
60–79	8 (11.59%)	5 (7.24%)	13 (18.85%)
Sum	38 (55.07%)	31 (44.93%)	69 (100%)

Table 3
Distribution of the intestinal symptoms according to gender.

	Male (n = 31)	Female (n = 38)	*P*-value
Symptomatic	20 (64.51%)	9 (23.69%)	0.01
Asymptomatic	11 (35.49%)	29 (76.31%)	

according to gender is shown in Table 3. The symptoms were significantly more prevalent in men compared to women ($P = 0.01$, Table 3).

Subtypes of *B. hominis* in 50 positive samples was identified using PCR method. ST3 was the most prevalent genotype (40%) and ST4 was the least (2%). Two subtypes including ST6 and ST7 were not detected in the samples. 11 (22%) isolates were detected to be a mix of two different subtypes (Table 4). Distribution of *B. hominis* subtypes was compared in symptomatic and asymptomatic patients. There was no difference in distribution of *B. hominis* genotypes between symptomatic and asymptomatic patients ($P = 0.1$) (Table 4).

Comparative molecular analysis of seven *B. hominis* sequences with other GenBank sequence subtypes showed high similarity to homologous sequences from previously reported *B. hominis* isolates stored in GenBank (Fig. 1).

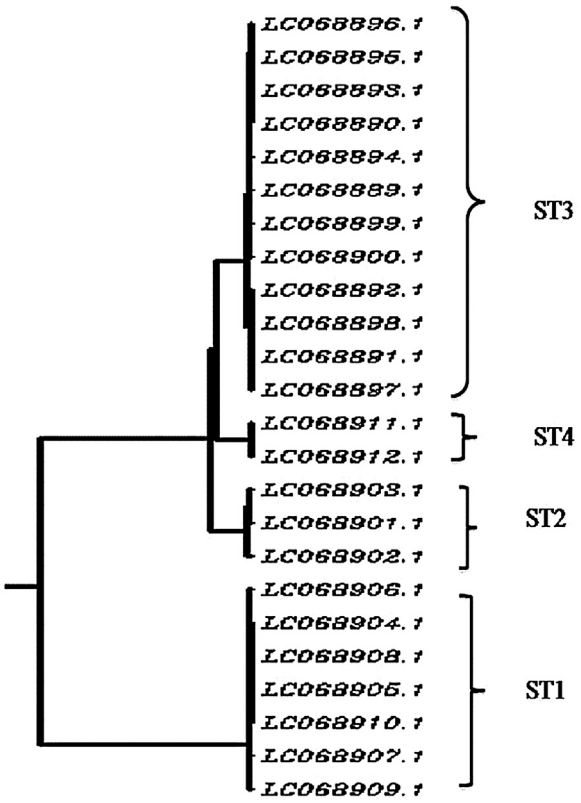

Fig. 1. Rooted phylogenetic tree showing the relationships of the subtypes of the-rRNA gene fragment for the isolates of *Blastocystis hominis* using PAUP.

Discussion

Classification of *B. hominis* into several species on the basis of molecular data may explain the variations in symptoms and epidemiological differences in different area [35]. 17 subtypes were previously recognized by PCR and sequencing [2]. The routine diagnostic method for identification of *B. hominis* is microscopic observation. Since direct observation has some limitations including: high expertise, unable to differentiate *Blastocystis* subtypes, low sensitivity, etc. Thus, molecular techniques with rapidly feature, high specificity and sensitivity are prefered. In this study, detection of *B. hominis* subtypes in Ahvaz, southwest of Iran was investigated by PCR and sequencing. ST3 has been reported to be the most prevalent subtype in the human population from a number of symptomatic and asymptomatic people, which was followed by ST1, as was true for our study [36–38]. Our results are similar to a study performed in Lorestan province (West of Iran) (ST3 = 56%) [39] and Tehran province (North of Iran) (ST3 = 30.5%) [29], but in a previous study in Hamadan province (West of Iran) (ST1 = 56.1%) (West of Iran), China and Brazil ST1 was the most prevalent [40–42]. ST3 is the only subtype of human origin [26], even if it can also be found in primates, pigs, dogs, cattle and rodent [37]. Predominance of ST3 in Ahvaz might be explained by large scale human to human transmission in this area of Iran. ST1 has many animal reservoirs and is found in different animal hosts such as pigs, monkeys, chickens that can pass this subtype on to humans [43]. In Ahvaz chickens probably have an important role in transmission of this subtype to human.

Previously relationship of pathogenicity and the presence of pathogenic and nonpathogenic genotypes was reported [44]. In this study, prevalence report of ST5 (8%) after ST3 and 1 is interesting. ST5 is often isolated from pig and cattle and is rarely found in human stool [45]. In Iran cow is the main source of meat production. So in Ahvaz zoonotic cycle of infection should be considered. In Spain, Nepal and France ST4 have been identified as the predominant subtype [5,46,47] but in our study it exhibits the lowest prevalence. In previous studies in Iran this subtype was not detected [39,40]. In different studies, mix infection was between 1.1–14.3% [40] but in our research the rate of mix infection was higher than the other reports, 22%. Prevalence of mix infection with ST3/ST4 was reported 10% in this study, with ST1/ST4 and ST1/ST3 being the same, 6%. In another study the most mix infection was for the ST1/ST3 [40]. Mix infection resulted from various sources of infection so the difference between prevalence of mix infection in Ahvaz with other places is probably due to higher prevalence of subtype 5 in contrast with other regions.

In this study, 44.83% of patients with ST3 noted some kinds of gastrointestinal disorder. Results of a study from Malaysia indicated pathogenic potential of ST3 [48]. In another study this

Table 4
Prevalence and subtype distribution from symptomatic and asymptomatic (n = 50).

Subtypes	N (%)	ST1	ST2	ST3	ST4	ST5	ST1/ST3	ST1/ST4	ST3/ST4
Symptomatic	29 (58)	9 (18)	2 (4)	13 (26)	0 (0)	1 (2)	2 (4)	0 (0)	2 (4)
Asymptomatic N (%)	21 (42)	2 (4)	1 (2)	7 (14)	1 (2)	3 (6)	1 (2)	3 (6)	3 (6)
Total no. of samples	50 (100%)	11 (22%)	3 (6%)	20 (40%)	1 (2%)	4 (8%)	3 (6%)	3 (6%)	5 (10%)

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subtype was also found in symptomatic patients [42]. In addition extra-intestinal manifestations such as chronic urticaria have been reported previously [49–51]. In a case report correlation between ST3 and acute urticaria and gastrointestinal symptoms was reported in a 19-year-old Caucasian male with rash and itchy wheals over his body and extremities [52]. From our results 31.03% of individuals with ST1 were noted as having gastrointestinal disease. In a previous study it was suggested that ST1 is pathogenic [42] but a different study disagreed with the results of this study [52]. In one study in Iran, ST1 was significantly higher in gastrointestinal patients [29]. Based on our results it seems that there is no association between other subtypes that were found in this study and pathogenicity.

In conclusion, it was found from this study that ST3 is the predominant subtype found in Ahvaz. This study also described the pathogenic potential of this subtype in this area. Understanding the prevalence of *B. hominis* and its subtypes in certain areas may contribute to a better understanding of the risk factors and the route of transmission in different ecological areas. Therefore, data on *B. hominis* could be helpful in designing intervention methods to reduce the burden of Blastocystosis in Ahvaz, Iran.

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Conflicts of interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical statement

Ethical approval was obtained from the Committee of Research, Publications and Ethics of the Faculty of Medicine, Ahvaz Jundishapur University of Medical Sciences. Before collecting stool samples a written informed consent was obtained from adult subjects and parent of children less than 18 years-old.

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