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Original paper

Phylogenetic tree of *Blastocystis hominis* in Iraqi children in Salah AL-Deen province, Iraq

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ABSTRACT. *Blastocystis hominis* is an intestinal protozoan that inhabits the large intestine of humans and a wide range of animals. *Blastocystis* species has a worldwide distribution. The current study aimed to determine the prevalence and the genetic variety of *Blastocystis* sp. in Iraqi children in Salah AL-Deen province, Iraq. 150 faecal samples were collected from children (5–10 years old) who attended the Salah AL-Deen hospital during the period from March to November 2020. The results revealed that 33.3% of children (50 out of 150) were found infected with *Blastocystis* sp., when the polymerase chain reaction (PCR) was used. The presence of ST3 gene was at a band of 526bp where this gene was observed in 11 samples out of 50 samples. The results also showed significant differences in the prevalence rate between rural and urban regions; between symptomatic and asymptomatic children, and between children who contacted domestic animals and those who did not contact animals ($P<0.05$). No significant differences in the prevalence rate were between different age groups ($P>0.05$). Regarding the genetic variation in subtype 3(ST3) revealed in phylogenetic tree analysis, there were three variations (transversion, deletion, and transition) which were detected through the sequence alignment, also the similarity was 97% with the sequences of *Blastocystis* sp. registered in GenBank. The Iraqi ST3 isolate was registered with ID: OL410286 in GenBank.

Keywords: *Blastocystis* sp., subtype 3(ST3), Iraqi children

Introduction

Blastocystis infection rates vary in developing and industrialized countries, reaching from 30–70% [1–3]. The different types of *Blastocystis* are widely distributed all over the world with a percentage of up to 100% in humans [4]. *Blastocystis hominis* is an intestinal protozoan that is endemic to humans and animals. It can be distinguished by different morphological forms in stool samples for one billion people in the world [5], as it has four morphological forms (central body, amoebic, granular, and cystic) [4]. These microorganisms are transferred via faecal-oral route, food, water and human-to-human contact [6].

Blastocystis have been associated with diarrhea, stomach pain, flatulence, colitis, irritable bowel syndrome, and dermatitis [7,8]. Asymptomatic spread is globally prevalent, but its pathogenicity is still a point of contention [9,10]. It appears to be able to produce cysteine proteins that interfere with the release of IL-8 from enterocytes, promoting intestinal cell death and increasing intestinal permeability while potentially avoiding differentiation via Toll-like receptors, thus, it has the potential to evade the immune system [11–13].

Blastocystis has many genotypes, at present, there are 22 different ST subtypes. Where there are subspecies of *Blastocystis* sp. including (ST1–ST17), ST21, and (ST23–ST26) in humans and various animals, these subtypes have been described depending on the 18S rRNA polymorphism [14]. Besides, the prevalent subtypes vary by countries and region in the same country. ST1–ST9 and ST12 were diagnosed in humans, and the most frequent subtype was ST3, moreover, ST9 was also detected in animals [5,15]. Regarding the epidemiology of *Blastocystis*, there are several studies that have shown this. Duda et al. [16] demonstrated in their study of the peacekeeping missions of Polish soldiers upon their return from Afghanistan and Iraq. Whereas intestinal parasites were examined in

1826 stool samples, the results were 15.3%, 1.0% and 0.7% for *Blastocystis hominis*, *Entamoeba coli* and *Giardia lamblia*, respectively. This indicates that the risk of parasitic infection is associated with countries that have tropical and subtropical climates [17]. It appeared that the prevalence of *Blastocystis* infection was 22.15% in children in Sulaymaniyah city, Iraq. Moreover, some studies were conducted on the prevalence of *Blastocystis hominis* in eastern and southern Baghdad, which was detected in 59 out of 250 samples with a percentage of 24.6% [18]. Also, other epidemiology research performed to detect *Blastocystis* in Baghdad it was reported 60 out of 267 patients with a rate of 22.5% [19]. The purpose of the current study was assessment the prevalence and the genetic variety of *Blastocystis* in Iraqi children in Salah AL-Din province, Iraq.

Materials and Methods

Samples collection

Total, 150 faecal samples were collected from children (5–10 years) attending Salah AL-Din hospital from March to November 2020.

Blastocystis detection

Stool samples were concentrated by sedimentation method and one drop of concentrated faeces was taken, then a drop of Lugol's solution was added to it and examined by optical microscope with magnification of 40× and 100×. Positive samples were used in genetic techniques

DNA extraction and genetic detection

DNA extracted from faeces samples by using the Quick-DNA MiniPrep Catalog number D3024 (Zymo Inc., USA). The genetic of *Blastocystis* was detected via PCR amplification of 526 bp fragment of ST3 subtype diagnostic primer that the forward sequence was 5'-TAGGATTTGGTGTTT GGAGA-3' and the reverse was 5'-TTAGAAGTGAAGG AGATGGAAG-3' where the characteristics primers were GC 40% and Tm 51°C for forward and GC40.9% and Tm 51.6°C for reverse. The mixture reaction contents with the final volume 25 µl were included Taq PCR PreMix(2X) 5 µl (iNtRON, Korea), forward primer10 picomols/µl (1 µl), reverse primer10 picomols/µl (1 µl), DNA sample 1.5 µl, free nuclease water 16.5 µl. The conditions' reaction was initial denaturation 94°C for 5 min., 1 cycle; final denaturation 94°C for 30 sec., 35 cycles; annealing 57°C for 30 sec., 35 cycles; initial extension 72°C for 1 min., 35 cycles; final extension 72°C for 7 min., 1 cycle. The electrophoresis (2% agarose gel) was utilized to visualize the products of PCR with Red safe staining (Intron, Korea).

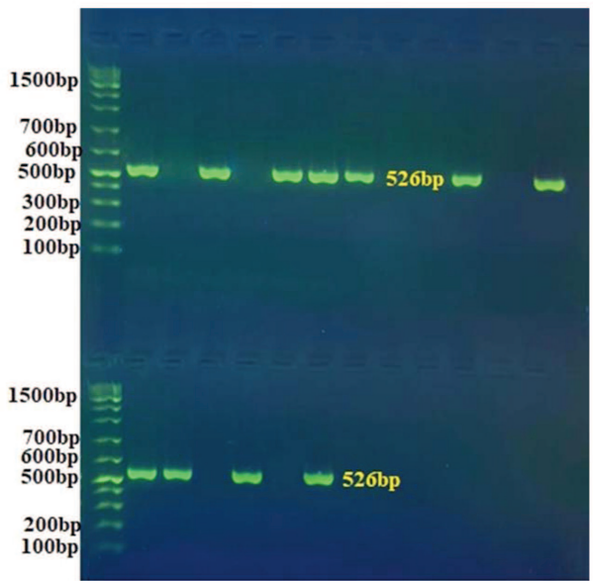

Figure 1. The product PCR for ST3 (526bp) for some samples. The electrophoresis on 2% agarose at 75 volt/15cm^2. 1× TBE buffer for 1:30 hours. DNA ladder (1000plus)

The sequence and phylogenetic assays

The sequence of PCR products was conducted for the gene by Macrogen Korea in both directions via the BigDye Terminator v3.1 Cycle Sequencing Kit on an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). The sequence of nucleotides was aligned in the nucleotide databases using the NCBI's Basic Local Alignment Search Tool Bio ID program to identify the sample and submit it to GenBank (ID). The related sequences of the sample were obtained from the NCBI's nucleotide database (www.ncbi.nlm.gov/nucleotide) and included in the multiple alignments using the Bio ID program [20]. The phylogenetic tree was inferred via the Neighbor-Joining method. The phylogenetic distance was computed using the Jukes-Cantor model by MEGA X [21].

Statistical analysis

Graphpad Prism version 8.0.1 for Windows (San Diego, California, USA) was used for statistical analysis. Fisher's exact test was used to compare *Blastocystis* prevalence depend on molecular detection. The results are significant when the *P*-value is less than 0.05.

Table 1. The distribution of *B. hominis* in according demography data

Variables		Number of detected	Positive number	*P*-value
Ages	(3–6)	40	10(16.7%)	0.5
	(7–10)	110	40(36.4%)	
Habitation				
Rural		115	30(26.1%)	0.03
Urban		35	20 (57.1%)	
Clinical symptoms				
Yes		40	40(80%)	<0.001
No		10	10(20%)	
Domestic animals handling				
Yes		60	35(58.3)	0.0003
No		90	15(16.7%)	

Ethics approval

Ethics approval for the current study was properly gained from Committee Ethics of the Iraqi Ministry of Health.

Results

The results showed the prevalence of *B. hominis* in children was 33.3%. The stool samples were examined microscopically and 50 samples were positive out of 150 samples. Also, the presence of ST3 gene was diagnosed by PCR in a band of 526 bp where this gene was observed in 11 samples out of 50 samples (Fig. 1).

The results of demography data indicated significant differences in the habitation area, the urban habitation recorded a percentage of 57.1% more than rural which was 26.1% (*P*=0.03), the presence of clinical symptoms was recorded in the most children (40 patients out of all 50 patients)

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Query 16
TTGCTCGAGACGTTGCGA-TAGATCACTGACCACCAATGTCCCAAATGTTCAGATGAAAA
Sbjct 28
......C...AT......A......C.................G.....T.........
Query 75
CTACAAATAACAGCAGATGAATATCTCCTTCTTTTGGTAGTCCATTGAGAGAATCGAAAA
Sbjct 88
..............A......G.....................................
Query 135
GCCCATCGTGGGACTCACGTCGTCTGTCGTCTCATTTGTAAGTTTGTGGATAATCGTTGT
Sbjct148
....................T.....................................
Query 195
TTTTCCCGCATTGTCAAGACCTCTAGAGATTAGCATCACGGATCAGCCACCACAGAAACA
Sbjct  208
.........................T.................................
Query  255
GAACACGCATTTCGTGCTCCTTCCGCTTTGCTTTTCGGATAATCGTCAATAAACCCATGT
Sbjct  268
..............................C..............................
Query  315
ACAAACAAACAAGTGACATCAACTCAAACGACCTAGTTACTTACCTGTTCGCTTCAAATT
Sbjct  328
.....................................C.....................
Query  375
GTTCTGTTCAATCTTTTTCTGAATATTGCGATATAGCACTTGGTGCCGGTTCTACACATC
Sbjct  388
..................................G..........................
Query  435
AACGGTCCTCCTCTCACAAACGCGATCGCTCGCCAACCTCGCTTCCATCTC  485
Sbjct  448
...................................................  498
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Figure 2. Alignment analysis and location variation of ST3 for *B. hominis*

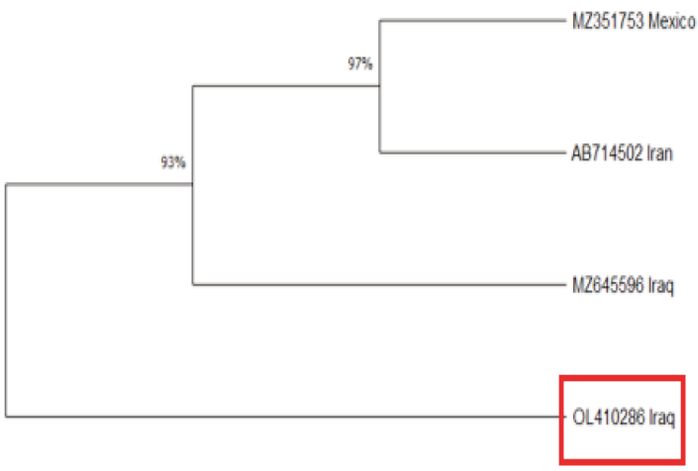

Figure 3. Phylogenetic tree for *B. hominis*

high percentage 80%, while 10 patients of children did not appear symptoms with percentage 20% ($P<$ 0.0001), and the percentage of handling with domestic animals was a high percentage 58.3% compared with non-handling at percentage 16.7% (P=0.0003). While there were no significant differences between the ages range where the range age (3–6) years recorded 16.7% as positive infection and the range age (7–10) years recoded 36.4% as positive infection with P=0.5 (Tab. 1).

Regarding the results of genetic detection, there were three variations of the ST3 subtype which were detected through the sequence alignment, also the similarity was 97% with the sequences of *Blastocystis* (ID:AB714502.1) in GenBank. The variations types were six transversion in locations (22,26,27,41,225, and 410 nucleotide), one deletion in location (34 nucleotide), and seven transition in locations (60,66,90, 97,156,278, and 358 nucleotide), also their location was shown in figure 2.

Moreover, these variations of Iraqi ST3 isolate were registered with ID:OL410286 in GenBank. Figures 3 and 4 represent phylogenetic analysis for *Blastocystis* to match Iraq's isolate with the world isolates.

Discussion

The infection of *Blastocystis* was appeared to be spread in children in Salah AL-Deen hospital for the study region; the percent infection in this study of 33.3% was the raising than the recorded in children in Sulaimani hospital (22.15%) [17], also in Baghdad city recorded 22.5% [19], in Duhok city was the rate infection 22.79% [22]. Besides, previous studies indicated that *B. hominis* infection in children was higher than adults [23–25]. In addition, in the developing countries, such as Nigeria, the rate of infection was 83.9% at the age 2–14 years, and in Turkey was 38.0% at the age 3–13 years [26,27]. Also, in Europe, the average of *B. hominis* prevalence reached 20% and increased to 50% in Africa [28]. The rising of *B. hominis* prevalence is associated with consuming the water and food polluted and the absence of hygiene [29]. Further, in China (Guangxi Province) was recorded the highest rates of infection at 43.3% [30]. Consequently, some studies have pointed out that the difference in *Blastocystis* prevalence in humans was related to the host's age, immunological status, and geographical locations [31–33]. On the other hand, clinical research pointed, *B. hominis* that cause gastrointestinal disease including diarrhea, and colitis. Furthermore, it noted a possible relation between irritable guts syndrome and *Blastocystis* infection [8,34,35]. Shaker et al. [36] illustrated there were significant differences between *Blastocystis* infection prevalence and the habitation (P=0.007), where the rural people were risky to infect with *Blastocystis* (20.71%) due to loss of hygiene, food habits, agriculture, livestock husbandry, and contact with animals [37]. Consequently, these studies agreed with our study. Moreover, Lee et al. [6] demonstrated the differences in the prevalence of *Blastocystis* infection due to the deficiency of drinking water, the health system, and the loss of sewage system in rural areas versus urban.

In the present study conducted subtyping by molecular analysis, the ST3 has been diagnosed in

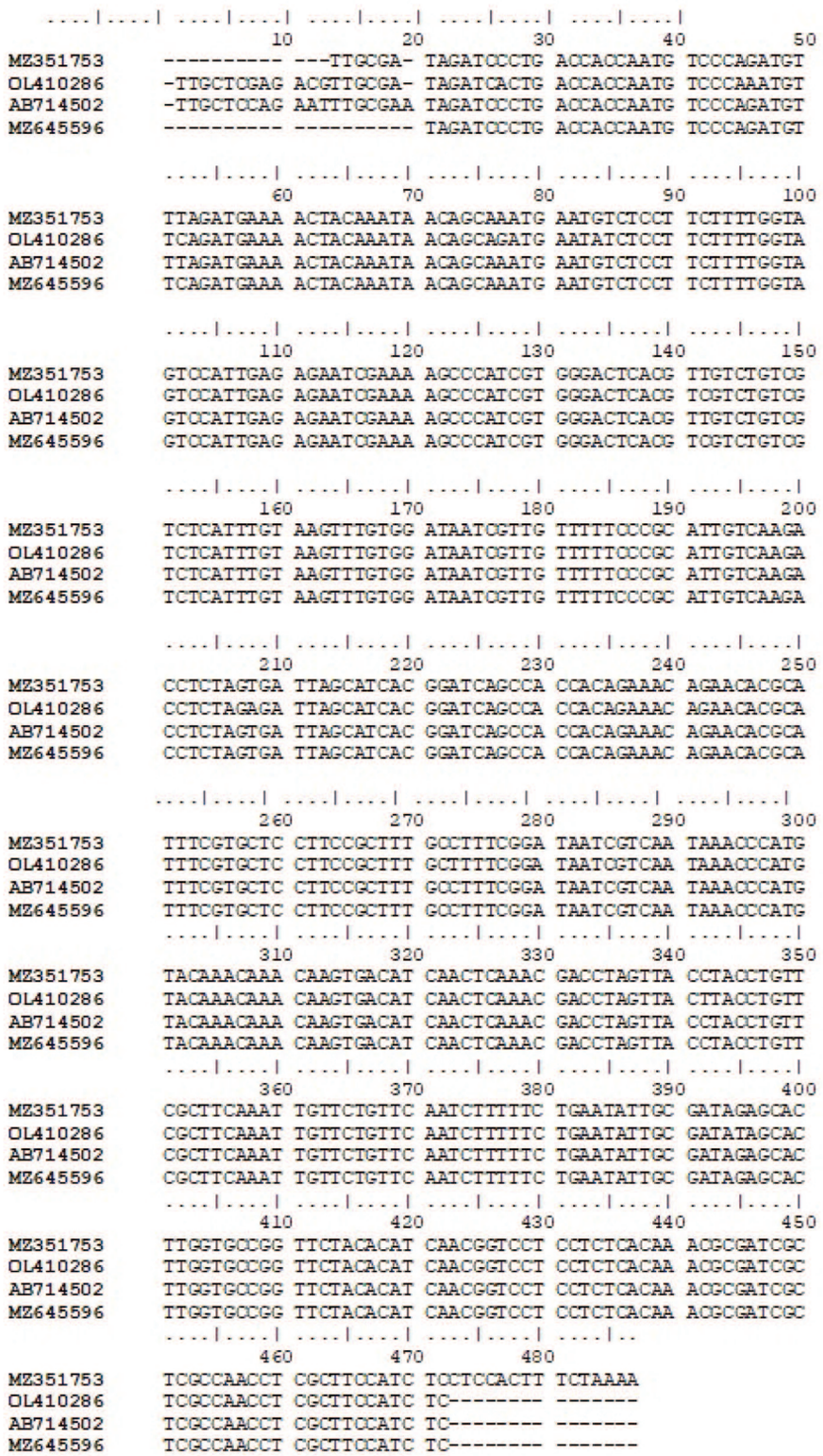

Figure 4. Match Iraq's isolate with the world isolates Mexico (ID:MZ351753); Iran (ID:AB7:14502); Iraq (ID:MZ645596) and OL410286 the registered isolate for this study

the most samples, therefore this study was approximately consistent with another study, where it recorded 62% for ST3 [38]. Besides, the epidemiological research worldwide notified that *Blastocystis* infection in humans is attributed to isolates of ST3 in various countries with rates (31.2–53%) [39–44]. Subtype 3 (ST3) was associated with the problems acute gastrointestinal in the studies

in Singapore, Malaysia, and the U.S [45,46]. While Dogruman-Al et al. [47] have illustrated that ST3 was predominant in both asymptomatic and symptomatic categories but no relation between *Blastocystis* subtypes and intestinal symptoms.

The prevalence rate of *Blastocystis* in children was the highest in rural than urban. Also, there were three genetic variations in subtype3 when sequences analysis. Phylogenetic tree investigation appeared to similarity was 97% with the sequences of *Blastocystis* registered in GenBank. Also, these variations for ST3 in Iraqi isolate were registered with ID:OL410286 in GenBank.

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