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Short Report: Molecular Epidemiology of *Blastocystis* in Lebanon and Correlation between Subtype 1 and Gastrointestinal Symptoms

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Abstract. *Blastocystis* is the most common eukaryotic parasite in the intestinal tract of humans. Because of its potential impact in public health, we acquired the first data concerning the prevalence of this parasite and the frequency of the *Blastocystis* subtypes (STs) in the Lebanese population. In this study, fecal samples from 220 Lebanese symptomatic and asymptomatic patients were collected and a total of 42 patients (19%) were identified as positive for this parasite by direct-light microscopy of smears. Among these, 36 *Blastocystis* isolates were genotyped using partial small subunit ribosomal RNA gene sequencing. The ST distribution in the present Lebanese population was as follows: ST3 (33.3%), ST2 (33.3%), ST1 (30.6%), and ST4 (2.8%). These data were compared with those available in other Middle Eastern and neighboring countries. Finally, ST1 was significantly more prevalent among symptomatic patients of this Lebanese population.

Blastocystis is the most common intestinal parasite of humans and a wide range of animals with a worldwide distribution[1]; its prevalence can reach 30–60% in developing countries and 1.5–20% in industrialized countries.[1] Even if the clinical significance of this parasite remains controversial, *Blastocystis* has been correlated with various gastrointestinal symptoms. It may also play a significant role in irritable bowel syndrome (IBS) and has been linked with urticaria.[2–6] According to recent *in vivo* and *in vitro* studies as well as genomic data, a model for pathogenesis of this parasite was proposed, and mainly involves cysteine proteases secreted by the parasite.[5–7] *Blastocystis* organisms found in different hosts are morphologically indistinguishable. However, this genus exhibits an extensive genetic diversity and at least 13 subtypes (STs) have been described on the basis of molecular data,[8–10] which showed sufficient genetic divergence to be classified as separate species.[11] Moreover, nine of these STs (ST1–ST9) have been isolated from human fecal samples highlighting both the low host specificity of the parasite and its zoonotic potential.[9–11] In the recent literature, it is still in debate whether distinct *Blastocystis* STs correlate with the development of gastrointestinal symptoms caused by the parasite.[1,4,5,12,13] Moreover, information on the distribution of STs in some geographic locations including Middle Eastern countries is only starting to emerge. Therefore, the aim of this study was to acquire the first epidemiological data regarding the prevalence of *Blastocystis* in the Lebanese population together with the frequency of STs in symptomatic and asymptomatic patients.

To conduct this study, fecal specimens were randomly collected at six hospitals in North Lebanon (Nini Hospital, Governmental Hospital of Tripoli, Tripoli Center for Medical Analysis, Hamidi Medical Center, Monla Hospital, and Saydet Zgharta Hospital) from 220 patients living in or in the vicinity of Tripoli during the period of March-April 2011. These patients were followed up for different pathologies such as gastrointestinal symptoms or presented for routine medical checkups. Stool samples were subsequently examined by direct-light microscopy (DLM) of smears for the presence of *Blastocystis* at the Center AZM of Tripoli. No information was available on potential viral or bacterial infections. Genomic DNA was directly extracted from fecal samples positive for *Blastocystis* by DLM using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany). Each sample was amplified by non-quantitative polymerase chain reaction (non-qPCR) as previously described using two independent pairs of *Blastocystis*-specific primers,[14,15] both targeting the small subunit (SSU) rDNA coding region. The respective 600 bp- and 520 bp-amplified domains have been shown to provide sufficient sequence information to discriminate between *Blastocystis* STs[14,15]; for each DNA sample, the non-qPCR product with the highest intensity on agarose gel was purified and cloned as previously described.[16] Two clones containing inserts of approximately the expected size were arbitrarily selected for each sample and sequenced. The DNA samples negative by non-qPCR were subsequently amplified using the highly sensitive real-time qPCR assay developed by Poirier and others[17]; the expected 320 bp-amplified variable region of the SSU rRNA gene was directly sequenced for subtyping. To compare the subtyping data obtained by molecular methods, 7 DNA samples were amplified by both non-qPCR and qPCR methods. The SSU rRNA gene sequences obtained in this study have been deposited in GenBank under accession nos. KC294143 to KC294196. These new sequences were aligned with the use of the BioEdit v7.0.1 package (http://www.mbio.ncsu.edu:BioEdit/bioedit.html), and then compared with all the *Blastocystis* SSU rRNA gene sequences available from the National Center for Biotechnology Information (NCBI) using the basic local alignment search tool (BLAST) program. Subtypes were identified by determining the closest similarity against all known *Blastocystis* STs according to the last classification by Stensvold and others.[8]

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A total of 42 patients (19%) were positive for *Blastocystis* by DLM. This high prevalence was in the same range as those observed in other neighboring countries such as Egypt and Iran.[18,19] It could roughly reflect the overall carriage rate of the parasite in the Lebanese population because most patients included in this study were followed up for various pathologies other than intestinal or were asymptomatic. However, the prevalence of *Blastocystis* in our Lebanese population was more likely underestimated because several authors[15,17] pointed out the poor sensitivity of DLM compared with either non-qPCR or qPCR assays. This strongly suggested that the actual prevalence of *Blastocystis* in Lebanon might be much higher making this parasite a potential major problem in public health.

Among the 42 positive samples by DLM, six were unsuccessfully amplified by either non-qPCR or qPCR due probably to the presence of known PCR inhibitors in fecal samples. The remaining 36 isolates were collected from 15 females and 21 males, ranging in age from 1 to 83 years (Table 1). The symptomatic group consisted of 19 patients presenting variously with diarrhea, abdominal pain, vomiting, constipation, some in association with fatigue and fever. As previously reported,[1,5] abdominal pain and diarrhea were the two major symptoms among *Blastocystis*-positive Lebanese patients. The asymptomatic group was composed of 17 individuals without any gastrointestinal symptoms. Each of the SSU rDNA gene sequences obtained from the 36 isolates showed 98–100% identity to representative sequences of *Blastocystis* STs reported so far, allowing the direct subtyping of these isolates (Table 1). For 8 of the 19 positive samples for which a 600 bp- or 520 bp-fragment of the SSU rRNA gene was cloned, the two sequenced clones were identical (Table 1). Clones showed one to four nucleotide differences for 10 of the remaining samples that could be explained by sequence variations between SSU rDNA genes copies within the same isolate[7,16,20]; for the last sample DS25 (ST3), there were 10 nucleotide differences between both clones suggesting a possible coinfection of the patient with two variants within the same ST. In this regard, substantial intra-ST diversity has been recently demonstrated in ST3[20]; the seven samples amplified by non-qPCR and qPCR yielded identical subtyping results.

All 36 samples genotyped in this study represented single infections. As shown in Table 1, ST3 (33.3%) and ST2 (33.3%) were the most common in our Lebanese population followed by ST1 (30.6%) and ST4 (2.8%). In most countries around the world, a majority of human *Blastocystis* infections were attributable to ST3 isolates[16]; this was also the case in the Lebanese population even if the frequencies of ST1 and ST2 were identical or roughly similar to that of ST3. The ST distribution in Lebanon can now be compared with those of other Middle Eastern countries such as Iran[19] and to neighboring countries

TABLE 1
Clinical data and *Blastocystis* subtypes among symptomatic and asymptomatic patients in Lebanon

Patients	Sex/age	Symptoms	*Blastocystis* ST by non-qPCR*	Nucleotide differences†	*Blastocystis* ST by qPCR*	Accession no.
DS1	M/71	Diarrhea			1	KC294143
DS2	M/65				3	KC294144
DS3	F/10	Abdominal pain, vomiting			2	KC294145
DS4	M/4	Diarrhea, vomiting, fever			1	KC294146
DS5	M/69	Diarrhea, abdominal pain			1	KC294147
DS6	M/33				2	KC294148
DS7	M/44				1	KC294149
DS8	M/21	Diarrhea, abdominal pain, vomiting			1	KC294150
DS9	M/12	Diarrhea, vomiting, fever	2 (Sc)	4	2	KC294151-3
DS10	M/13	Diarrhea, abdominal pain, fever, fatigue	1 (Sc)	2	1	KC294154-6
DS11	F/27	Diarrhea	2 (Sc)	0		KC294157
DS12	F/23		3 (Sc)	0	3	KC294158-9
DS13	M/60		3 (Sc)	0	3	KC294160-1
DS14	F/30	Abdominal pain	3 (Sc)	0	3	KC294162-3
DS15	M/34	Abdominal pain			3	KC294164
DS16	M/40				2	KC294165
DS17	M/29	Diarrhea, abdominal pain			1	KC294166
DS18	F/6				2	KC294167
DS19	F/31	Abdominal pain	2 (Sc)	0		KC294168
DS20	F/5		3 (St)	1		KC294169-70
DS21	M/51				2	KC294171
DS22	F/83				2	KC294172
DS23	F/20	Abdominal pain, fatigue, constipation	1 (St)	3	1	KC294173-5
DS24	M/3	Diarrhea, abdominal pain, vomiting			1	KC294176
DS25	M/24		3 (Sc)	10		KC294177-8
DS26	F/11	Diarrhea, abdominal pain	3 (Sc)	1		KC294179-80
DS27	F/5	Abdominal pain, vomiting, fatigue	1 (St)	1		KC294181-82
DS28	F/8		4 (Sc)	0		KC294183
DS29	M/8		2 (Sc)	0		KC294184
DS30	M/5	Abdominal pain			2	KC294185
DS31	M/16	Abdominal pain	1 (Sc)	2		KC294186-7
DS32	F/23		3 (St)	1		KC294188-9
DS33	F/22				2	KC294190
DS34	F/40		3 (Sc)	2		KC294191-2
DS35	M/35		3 (St)	0		KC294193
DS36	M/1	Diarrhea, abdominal pain	3 (St)	2	3	KC294194-6

*According to the new standard terminology[8]; (St) and (Sc): non-qPCR using the primer pair described by Stensvold and others[15] and Scicluna and others,[14] respectively.
†Determined in the common region of two clones sequenced for each sample.

like Turkey[21,22] and Egypt[23,24]; in these countries, ST1 was the second most common variant after ST3 while it follows at third position in Lebanon but still has a high frequency. The ST2 was globally poorly represented in Iran and Egypt, whereas it was commonly found in Turkey and Lebanon. In addition, in our Lebanese population only 1 of 36 isolates has been genotyped as ST4. This ST has not been found in Egypt and Iran and was only identified in a single patient in Turkey. Overall, ST4 is common in Europe[25] and much less frequent or absent in other geographical regions. In summary, our data showed a prevalence of ST1, ST2, and ST3 and a virtual absence of ST4 in the Middle Eastern and neighboring countries as well as some geographic variation in the frequency of ST2 that might reflect different exposure to animal and/or environmental infection sources.

To evaluate the pathogenic potential of the different *Blastocystis* STs in our Lebanese population, the phylogenetic distribution of the 36 genotyped isolates from symptomatic and asymptomatic individuals was examined. The ST3 (8 of 12 isolates) and ST2 (7 of 12) were dominant in the asymptomatic group reinforcing the hypothesis that most isolates of these subtypes are likely to be non-pathogenic.[5] The single ST4 isolate of our study was asymptomatic, whereas ST4 has been shown to be common in patients with acute diarrhea.[26] Strikingly, 10 of 11 ST1 isolates composed the symptomatic group and a statistical analysis done with GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA) using the Fisher's exact test showed a significant association between ST1 and gastrointestinal symptoms ($P = 0.0113$). Recently, epidemiological surveys have reported the frequency of STs from symptomatic and asymptomatic individuals in China,[27] Turkey,[28] and Iran[19] and showed that ST1 was over-represented in groups of symptomatic patients. Moreover, ST1 was the most prevalent ST of *Blastocystis* in patients with IBS[24,29] and human ST1 isolates were associated with elevated pathogenicity in experimentally infected rats.[30] However, patient symptomatic status was uncorrelated with *Blastocystis* ST and symptoms in the context of several other epidemiological studies.[16,21,31,32]

To our knowledge, this is the first investigation of prevalence and molecular epidemiology of *Blastocystis* in Lebanon. In this country, the prevalence of this parasite would be high with predominance of ST3, ST2, and ST1 isolates. A consistent link between ST1 and gastrointestinal symptoms was identified and should be confirmed in further studies, including a larger number of patients.

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