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Prevalence and Subtype Identification of *Blastocystis* sp. in Healthy Individuals in the Tunis Area, Tunisia

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Abstract. *Blastocystis* sp. is currently the most common eukaryotic parasite found in humans. Despite its potential public health impact, epidemiological data regarding its prevalence and molecular subtype (ST) distribution in Maghreb are rarely reported. Therefore, the aim of this study was to determine the prevalence of the parasite in a cohort of healthy food handler Tunisian individuals and to acquire the first molecular data regarding the distribution of *Blastocystis* sp. STs in this country. Therefore, 524 fecal samples were collected, and 68 of them (13%) were identified as positive for the parasite by direct-light microscopy of smears. Seventeen samples of 100 negative by microscopy were also shown to be positive by real-time quantitative polymerase chain reaction. Among all the positive samples, 61 *Blastocystis* isolates were subtyped using partial small subunit ribosomal RNA gene analysis. ST3 was the most abundant (51%) followed by ST1 (30%), ST2 (16%), and ST4 and ST7 (both 1.6%).

Blastocystis sp. is a parasite inhabiting the gastrointestinal tract of humans and a wide range of animals. This single-celled eukaryote is currently the most prevalent protozoa found in human fecal samples with a worldwide distribution.^{1,2} Its prevalence considerably varies between geographic areas, reaching 100% in developing countries and 56% in industrialized ones.^{3,4} Poor health care and hygiene can explain these differences between countries since its main transmission mode is the fecal–oral route through the consumption of contaminated food or water.¹ It has also been shown that prevalence data may depend on detection methods, especially direct-light microscopy (DLM) of fecal smears versus polymerase chain reaction (PCR) assays.⁵ For many years, *Blastocystis* sp. was considered with no clinical relevance mainly because asymptomatic colonization is very common.⁶ However, recent *in vivo* and *in vitro* studies have led to the identification of plausible models of pathogenesis.⁷ Clinical features of *Blastocystis* sp. infection were reported to include many nonspecific symptoms including diarrhea and abdominal pain, as well as urticarial lesions.^{1,8} In addition, *Blastocystis* sp. was also recently associated with irritable bowel syndrome.⁹ At the molecular level, the genus *Blastocystis* exhibits a large genetic diversity since at least 17 different ribosomal lineages so-called subtypes (STs) were identified.¹⁰ Nine of them (ST1–ST9) were found in humans with varying prevalence.¹¹ In most countries worldwide, ST3 is globally predominant followed by ST1 and ST2, whereas ST4 is rarely found outside Europe. Interestingly, almost all of these nine human STs are also shared by various nonhuman hosts, highlighting both the low host specificity of the parasite and its zoonotic potential.¹⁰ In spite of the potential impact of *Blastocystis* sp. in public health, few reports are available on the prevalence and ST distribution of the parasite in some geographical regions including Maghreb. Therefore, the aim of this study was to clarify the prevalence of the parasite and

to acquire first molecular data, assessed directly from stool samples, regarding the distribution of *Blastocystis* sp. STs in the Tunisian population.

To conduct this study, a total of 524 stool samples were collected from healthy food handlers working in the Tunis area, in the setting of the routine activity of the Department of Parasitology at the Pasteur Institute of Tunis, Tunisia, during the period of October–November 2014. Following formalin ether concentration technique, stool samples were examined by DLM of smears for the presence of intestinal parasites. Specimens positive for *Blastocystis* sp. by DLM as well as 100 negative ones were subjected for DNA extraction and molecular analysis. Total genomic DNA was extracted from fecal samples using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. DNA was stored at –20°C until use. To detect and subtype *Blastocystis* sp., each DNA sample was amplified by real-time quantitative PCR (qPCR) targeting the small subunit (SSU) rRNA coding region of the parasite as previously described.⁹ Each sample was tested in duplicate, and each run included both negative (DNA matrix replaced by water) and positive (DNA obtained from a *Blastocystis* ST4 culture) controls. Negative samples by qPCR were checked for inhibition by spiking these samples with 1 µL of the positive control. The expected 320-bp qPCR product from each positive sample was directly sequenced for subtyping. The SSU rRNA gene sequences obtained in this study were deposited in GenBank under accession numbers KX378175–KX378235. These new sequences were compared with all *Blastocystis* sp. homologous sequences available from the National Center for Biotechnology Information using the Basic Local Alignment Search Tool program. STs were identified by determining the exact match or closest similarity against all known *Blastocystis* STs.

A total of 68 (13.0%) were DLM positive for *Blastocystis* sp. Its prevalence ranked the highest followed by *Dientamoeba fragilis* (4.2%), *Entamoeba coli*, and *Endolimax* (1.3% each). Despite the confirmed absence of PCR inhibitors, five of the 68 DLM-positive samples were negative by qPCR, suggesting low parasite load or misidentification of the parasite by microscopy. The prevalence of the parasite in this study

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was significantly higher than those observed using DLM in two previous Tunisian surveys reporting a prevalence of 7.3% and 1.4% in two cohorts of 3,257 and 8,502 patients, respectively.^{12,13} By comparison, the prevalence reported in other Maghreb countries was 11.2% in Algeria ($N = 1,042$) and 13.5% and 15.0% in Morocco ($N = 4,000$ and $N = 300$, respectively) using the same detection method.^{14–16} Another study reported a prevalence of 39.4% by DLM in a population of 673 schoolchildren in the area of Tetouan, Morocco.¹⁷ In Libya, the overall prevalence of *Blastocystis* sp. infection ranged from 22.1% to 28% by xenic in vitro culture followed by DLM in three different cohorts of patients attending the Central Laboratory in Sebha.^{11,18,19} From all these data, the average prevalence of *Blastocystis* sp. in the Maghreb would be roughly comprised between 15% and 30%, depending on the country and the studied population. However, the real prevalence of the parasite in this geographic area could greatly be underestimated because nonmolecular methods have a low diagnostic sensitivity compared with qPCR.⁵ The present study confirms this observation, since 17 of 100 DLM-negative samples were subsequently shown to be positive for *Blastocystis* sp. by qPCR.

Among the 80 qPCR-positive samples, sequence analysis was contributive for only 61 (46 DLM-positive and 15 DLM-negative samples) since DNA sequencing chromatograms of 19 qPCR positive samples had been difficult to interpret. SSU rRNA gene sequences obtained from the 61 remaining isolates showed 96–100% identity to representative sequences of *Blastocystis* sp. STs reported so far, allowing the direct subtyping of these isolates and all represented single infection. ST3 was the predominant ST present in our Tunisian cohort (51%), followed by ST1 (30%) and ST2 (16%). ST4 and ST7 were both identified in only one individual (1.6%). No others STs were identified, which may be due to either the low number of subtyped isolates or the lack of occurrence of additional STs in Tunisia. The *Blastocystis* ST distribution in Tunisia was quite similar to that globally observed in most countries around the world, with the predominance of ST3 followed by ST1 and ST2.¹¹

These three STs represented about 97% of the subtyped isolates in our study. The ST distribution in Tunisia can be compared with those obtained in the few studies conducted in other Maghreb countries such as Libya^{11,19} and in neighboring countries including Egypt.^{20–22} It is worth noting that those molecular data were obtained after *Blastocystis* culturing in liquid medium, which could result in a selection bias of some STs. In Libya, ST1 showed the highest frequency (more than 50% of the isolates), followed by either ST3¹¹ or ST2.¹⁹ On the other hand, ST3 was largely predominant in the three studies conducted in different Egyptian areas.^{20–22} Since ST3 is rarely identified in animals that may be in contact with humans, a large-scale interhuman transmission likely explains the predominance of this ST worldwide.^{1,2,11} As stated above, ST1 represented 29.6% of the subtyped isolates colonizing Tunisian food handlers. Interestingly, ST1 is predominantly found in bovidae,¹⁰ suggesting that an as yet undetermined proportion of ST1 human infections in our Tunisian cohort might be of zoonotic origin. In that case, the importance of widespread practice of cattle and sheep rearing in Tunisia could be a risk factor for the dissemination of *Blastocystis* sp. ST1. Regarding ST4, it was detected in only one individual in our Tunisian cohort and still unidentified in

Egypt and Libya, thus confirming the virtual absence of ST4 in north African countries.^{19–22} More globally, ST4 is much less frequently detected or absent in Africa, Middle East, Asia, and North America while it is commonly found in Europe.^{5,11} The recently proposed ST4 emergence in the European population might explain such heterogeneous geographical distribution of this ST.² Finally, ST7 was identified in only one case in our Tunisian cohort. ST7 together with ST6 are usually hosted by birds (so-called avian STs) and rarely colonize mammalian groups including humans.^{10,11} A single isolate belonging to ST7 was also identified in Libya, whereas ST6 and ST7 were unexpectedly commonly found in two Egyptian studies.^{20,22} Regarding their host specificity, human infections by both avian STs are likely of zoonotic origin. Interestingly, if we focused on the potential zoonotic reservoirs identified in this study, they strikingly were the same as those suspected in the transmission of *Cryptosporidium* spp. isolates in Tunisia, insofar as the cattle species *Cryptosporidium parvum* and the avian species *Cryptosporidium meleagridis* were identified in human cryptosporidiosis.²³

In conclusion, this study first complements the limited data available on the prevalence of *Blastocystis* sp. in Maghreb countries. From our report, *Blastocystis* sp. is the most common protozoa colonizing the digestive tract of our Tunisian individuals and thus appears as an appropriate indicator of the overall level of intestinal parasitism. Our survey also represents the first investigation of ST distribution of the parasite in Tunisia, directly assessed from stool samples, with the identification of five STs, ST3 being largely predominant. This distribution should be confirmed in further studies, including a larger number of patients. However, our survey fills a geographic gap in our knowledge of *Blastocystis* ST distribution and contributes to a better understanding of the molecular epidemiology of the parasite.

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