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Variable geographic distribution of *Blastocystis* subtypes and its potential implications

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ABSTRACT

Blastocystis is a common intestinal micro-eukaryote found in both humans and non-human hosts and known to be genetically very diverse. It has been divided into numerous subtypes (STs), nine of which have been identified in humans to date. Surveys of ST prevalence have started to emerge over the past few years but to date no data are available for any African country except Egypt and Tanzania. In this study, we determined the prevalence of *Blastocystis* STs in populations from Libya, Liberia and Nigeria, as well as expanding the dataset available for the UK. A total of 356 *Blastocystis* STs were identified in this study, 271 from the UK, 38 from Libya, 25 from Liberia and 22 from Nigeria. SSU rRNA gene sequences revealed the presence of eight of the nine STs known from humans but at varying frequencies between countries. ST1 was the most common ST in Libya and Nigeria whereas ST3 showed the highest frequency in the other two countries, as indeed is the case in most populations around the world. ST4 was absent in Libya and ST2 in Nigeria, while no ST5, ST6, ST8 or ST9 infections were detected in any of the three African populations. The picture emerging from this and other surveys suggests that there is significant variation in ST prevalence between populations. Some of the possible reasons for and implications of this diversity are discussed.

1. Introduction

Blastocystis is a common intestinal parasitic micro-eukaryote with a worldwide distribution and is often the most frequently detected parasite in epidemiological surveys (Clark et al., in press). Its prevalence varies from country to country and among various communities within the same country. Generally, however, the prevalence in developing countries is higher than in developed countries, which has been linked to standards of hygiene, waste disposal, exposure to animals, and consumption of contaminated food or water (Tan, 2008), although direct evidence for some of these is lacking.

DNA-based methods have been developed that are able to identify genetic variation between *Blastocystis* organisms that are morphologically indistinguishable under the microscope. Molecular studies have focused mostly on determining the prevalence of *Blastocystis* subtypes (STs) in asymptomatic and symptomatic individuals (Böhm-Gloning et al., 1997; Kaneda et al., 2001; Souppart et al., 2009; Stensvold et al., 2009, 2011a; Yan et al., 2006; Yoshikawa et al., 2004). In spite of recent methodological advances, the molecular epidemiology of *Blastocystis* infections is still unknown in many parts of the world. Recent studies are starting to provide more information on the distribution of STs among human populations (Tan, 2008; Clark et al., in press) but most have been carried out in temperate regions. To date there is some indication of geographic variation in the prevalence of STs (e.g. Forsell et al., 2012a,b) as well as reports of associations between specific STs and disease, although with conflicting conclusions (e.g. Domínguez-Márquez et al., 2009; Jones et al., 2009; Stensvold et al., 2011a; Tan et al., 2008).

In this study our aim is to investigate the distribution of *Blastocystis* STs from unselected individuals in North and West Africa, where climates, cultures and ecological conditions are likely to be quite different from most regions surveyed to date. This will fill a geographic gap in our knowledge of *Blastocystis* diversity around the world and also provide an insight into whether such variables influence ST distribution. In addition, we greatly expand the data available on ST prevalence for the UK.

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2. Materials and methods

2.1. Source of specimens

UK samples. A total of 271 new *Blastocystis* isolates from samples submitted for routine ova and parasite analysis were studied; 136 came from Irritable Bowel Syndrome (IBS) clinics in England, while the other 135 were submitted by the patient's physician, usually because the patient was symptomatic but the cause was unknown. Both sample groups were received through the Diagnostic Parasitology Laboratory of the London School of Hygiene and Tropical Medicine. The final patient diagnosis is not known and it is likely that some IBS clinic samples were from patients later diagnosed with non-IBS causes of their symptoms and, equally, some of the physician-submitted samples were likely from patients with IBS. Ethical approval for work on the UK samples was obtained (LSHTM reference no.5026).

Libyan samples. 150 faecal samples submitted for ova and parasite examination by Libyan patients attending the Sebha Central Medical Laboratory were studied. The samples were submitted in order for the patient to get a health certificate, join the army, or attend university, rather than because the patient was symptomatic. 42 (28%) of these samples were positive by direct smear and 38 of these grew in culture. The Ethics Committee of Sebha University concluded that no specific approval was needed for the further analysis reported here. Sebha is a city in southwestern Libya (27°2'0" North, 14°26'0" East) with a population of 130,000. It was historically the capital of the Fezzan region and is now capital of the Sebha District. The weather is hot and dry in summer and cold and dry in winter.

Liberian samples. 43 faecal samples from children aged 6–18 from Bomi, Bong and Margibi counties, Liberia, were included. These three countries are to the Northwest, Northeast and East of the capital Monrovia (6°18′48″ North, 10°48′5″ West), respectively 30. (70%) of the samples were positive for *Blastocystis* by PCR. Informed consent was obtained from the parents or school director for analysis of samples from under-age children. Liberia has a hot equatorial climate with the rainy season between May to October.

Nigerian samples. 47 faecal samples were obtained from Nigerian patients (median age 32 y, inter-quartile range: 24–41 y) attending a clinic in Lagos. The Ethics and Experimentation Committee of the College of Medicine, University of Lagos PEPFAR/APIN and Lagos University Teaching Hospital, concluded that no ethical permission was needed for this work. 23 (49%) of the samples were positive for *Blastocystis* by PCR. Lagos is a metropolitan city located in the southwest region of Nigeria (6°27′11″ North, 3°23′45″ East). A typical tropical rainforest climate exists in the city. The population of Lagos is estimated at over 10 million.

2.2. Sample processing

Approximately 50 mg of each Libyan and UK faecal sample was inoculated into Robinson's medium (Clark and Diamond, 2002) or (in Libya only) a modification of Jones' medium (Leelayoova et al., 2002) using a Luria agar slant and supplemented with 10% human serum. The culture was incubated at 37 °C and examined every 2 days. Positive cultures were passaged into fresh medium for another 3–4 days, then *Blastocystis* was harvested by centrifugation and the pellet resuspended in lysis buffer for DNA extraction using the Puregene core kit A (Qiagen, Crawley, UK) according to the manufacturer's protocol. This rapid DNA extraction prevents the possibility of differential ST growth affecting subsequent analyses in our experience. DNA of samples from Nigeria and Liberia was extracted directly from faeces using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations.

2.3. PCR amplification and sequencing

PCR amplification of partial *Blastocystis* small subunit ribosomal RNA genes (SSU-rDNA) for subtyping of samples from the UK, Liberia and Libya was performed (at LSHTM) in a 40 μl volume reaction using Biomix (Bioline, London, UK) and the primers BhRDr (5′-GAG CTT TTT AAC TGC AAC AAC G-3′) and RD5 (5′-ATC TGG TTG ATC CTG CCA GTA-3′) at 2 μM concentration. These primers amplify a 600 bp region of the SSU-rDNA that contains sufficient information for unambiguous assignment of STs to samples (Scicluna et al., 2006). The following amplification profile was used: 30 cycles of denaturation at 94 °C for 30 s, annealing at 59 °C for 30 s and extension at 72 °C for 30 s. PCR products were purified using the QiaQuick gel extraction kit (Qiagen, Hilden, Germany) or the GeneJet PCR Purification kit (Fermentas, Epsom, UK) and sequenced using the BhRDr primer as described previously (Scicluna et al., 2006).

Nigerian samples were screened for *Blastocystis* by PCR (at the SSI) using the primers bl1400ForC and bl1710RevC, which amplify a 310 bp SSU-rDNA product, or the primers F1 and BHCRseq3, amplifying a 550 bp product, and Extract-N-Amp PCR ReadyMix (Sigma–Aldrich Denmark, Brøndby, Denmark). PCR products were sequenced as described previously (Stensvold et al., 2006, 2007).

STs were assigned based on the results of BLAST analysis against the databases at NCBI and/or www.pubmlst.org/blastocystis. Nucleotide sequences of samples from which unambiguous barcode sequences were obtained have been submitted to the isolate database at www.pubmlst.org/blastocystis.

3. Results and discussion

3.1. UK samples

The samples from the UK had two distinct origins and could therefore have had distinct ST characteristics: half of the samples were submitted by IBS clinics. The ST profiles of the two sets of samples were compared using the chi-square test. Although there was a clear excess of ST4 in IBS samples, the distribution of STs in the two sample groups did not show any significant difference overall ($p = 0.18$; Table 1), so we have combined the two sets of data for geographic comparisons.

The distribution of STs in the UK samples is generally similar to those reported previously in the UK and elsewhere in Europe (Table 2), where ST3 is the most frequently detected ST, followed by ST4, with STs 1 and 2 each having a prevalence of about 10%.

3.2. African samples and comparison with UK samples

In Libya the prevalence of *Blastocystis* has been reported previously to be 26.6% (Alfellani et al., 2007), which is very similar to the prevalence in the current samples (28%). The prevalence of *Blastocystis* in Liberia has not been reported to date and the only report from Nigeria is a prevalence of 2.5% (in Ogun State) using microscopy (Reinthaler et al., 1988).

The only previous reports of ST prevalence in Africa are from three studies conducted in Egypt and a small one in Tanzania (Table 2). Libya, Nigeria, Liberia and Egypt cover a range of distinct climate conditions. Despite this, ST3 was highly prevalent in all countries (ca. 30–60%; Table 3), although ST1 was detected at the highest frequency in both Libya and Nigeria, with 50% and 45.5% respectively. In neither the Nigerian samples nor in two of the studies from Egypt (Egypt1 and Egypt3; Table 3) was ST2 detected, in contrast to most previous surveys and the other three locations sampled in this study.

Despite ST4 being the second most common ST detected in the UK and being quite common in both Liberia and Nigeria, in Libya and