SHORT COMMUNICATION

The microbial eukaryote *Blastocystis* is a prevalent and diverse member of the healthy human gut microbiota

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Abstract

To date, the majority of research into the human gut microbiota has focused on the bacterial fraction of the community. Inevitably, this has resulted in a poor understanding of the diversity and functionality of other intestinal microorganisms in the human gut. One such nonbacterial member is the microbial eukaryote *Blastocystis*, which has been implicated in the aetiology of a range of different intestinal and extra-intestinal diseases. However, prevalence data from different studies are conflicting, and crucially, there is limited information on its incidence and diversity in healthy individuals. Here, we survey the prevalence, genetic diversity and temporal stability of *Blastocystis* in a group of healthy adults (n = 105) using a sensitive PCR assay. *Blastocystis* was present in 56% of our sample set, which is much higher than previously reported from an industrialised county (Ireland). Moreover, a diversity of different subtypes (species) were detected, and *Blastocystis* was present in a subset of individuals sampled over a period of time between 6 and 10 years, indicating that it is capable of long-term host colonisation. These results show that *Blastocystis* is a common and diverse member of the healthy gut microbiota, thereby extending our knowledge of the microbial ecology of the healthy human intestine.

The human 'gut microbiota' is a collective terms used to refer to the microbial inhabitants of the human intestinal tract (IT). However, a thorough understanding of the composition of the gut microbiota and the microbial ecology of the healthy human gut is lacking due to a research focus on the bacterial fraction of the community. In addition to bacteria, a diverse collection of other microorganisms are present in the human gut (Scanlan & Marchesi, 2008; Hamad *et al.*, 2012) including members of the microbial eukaryotes. Such microorganisms are now the focus of increased interest (Parfrey *et al.*, 2011), especially following greater recognition of their relevance to host health (Iliev *et al.*, 2012). While some of the research that has taken place has focused solely on fungal diversity within the human gut (Chen *et al.*, 2011; Gouba *et al.*, 2013), a number of studies have taken a broader approach using universal primers to explore the diversity of all microbial eukaryotes present (Nam *et al.*, 2008; Scanlan & Marchesi, 2008; Hamad *et al.*, 2012; Pandey *et al.*, 2012). Even though the number of these universal studies is limited and sample sizes are small, one common trend emerging is the widespread prevalence of the intestinal protist *Blastocystis* (Nam *et al.*, 2008; Scanlan & Marchesi, 2008; Hamad *et al.*, 2012; Pandey *et al.*, 2012; Gouba *et al.*, 2013).

Blastocystis is a unicellular, nonflagellated member of the *Stramenopiles* (or Heterokonta) (Silberman *et al.*, 1996), which is a branch of the Eukarya that comprises a collection of uni- and multi-cellular organisms including diatoms, algae and oomycetes (Patterson, 1999). To date, seventeen different *Blastocystis* subtypes (arguably seventeen different species) have been described (Alfellani

FEMS MICROBIOLOGY ECOLOGY

et al., 2013) and in addition to colonising the human IT, *Blastocystis* is also found in a range of other hosts including other mammals, birds and reptiles (Ramirez *et al.*, 2013). With respect to its prevalence in human populations, *Blastocystis* has largely been investigated in the context of disease and some studies have implicated it in a number of different intestinal and extra-intestinal diseases including Inflammatory Bowel Disease, Irritable Bowel Syndrome, autism and urticaria (Boorom, 2007; Tan *et al.*, 2010; Poirier *et al.*, 2012). *Blastocystis* is also cited as the causative agent of an illness termed Blastocystosis (Tan *et al.*, 2010), which is characterised by a loose collection of nonspecific symptoms (*inter alia* abdominal pain, diarrhoea and bloating) that could in fact be attributed to and are associated with any number of other infectious microorganisms and/or intestinal disorders. Thus, the question of whether *Blastocystis* spp. play a role in certain human diseases or not remains both unclear and controversial. Indeed, although *in vitro* and genomic data that support the role of *Blastocystis* as an emerging pathogen have been published (Puthia *et al.*, 2006, 2008; Tan *et al.*, 2010; Mirza *et al.*, 2012), no links with human disease have been unequivocally demonstrated and its role as an aetiologic agent is still speculative (Tan *et al.*, 2010; Scanlan, 2012; Scanlan & Stensvold, 2013).

There are a number of factors that have hampered progress in establishing a definitive role for *Blastocystis* in human disease in the past. Central to this are methodological and experimental design issues (Tan, 2004; Tan *et al.*, 2010; Scanlan, 2012). Fortunately, these problems are now recognised and researchers have begun to switch to molecular-based screening approaches (Poirier *et al.*, 2011; Stensvold *et al.*, 2012a, b; Bart *et al.*, 2013; Clark *et al.*, 2013). Nonetheless, one of the outstanding issues is the lack of reliable epidemiological data (Tan *et al.*, 2010), and in particular, epidemiological data obtained from human populations of healthy, randomly sampled individuals using a standardised sensitive molecular-based approaches are lacking (Scanlan, 2012).

To date, reported prevalence rates are generally higher in developing countries and have been associated with sanitation levels, water source and contact with animals (Tan, 2008; Lee *et al.*, 2012; Wawrzyniak *et al.*, 2013). The highest prevalence reported for *Blastocystis* is 100% and is from a recent study of 93 children living in the Senegal River Basin (El Safadi *et al.*, 2014). In contrast to this, current estimates of *Blastocystis* prevalence in developed countries range from 0.5% to 30% depending on the methods used and the population sampled (Bart *et al.*, 2013; Scanlan & Stensvold, 2013; Wawrzyniak *et al.*, 2013). Here, we optimised a PCR assay using the existing *Blastocystis* primer set RD5 and BhRDr that targets the 18S rRNA gene (Scicluna *et al.*, 2006) to survey the prevalence of *Blastocystis* in our sample set which consisted of 100 healthy adults living in Cork, Ireland, that had participated in the Eldermet study (Claesson *et al.*, 2012), and five additional healthy adults from Ireland that had participated in an earlier study (Scanlan & Marchesi, 2008). These five additional individuals were also included in the longitudinal analysis, see Table 1; Supporting Information, Table S1.

Our assay had a limit of detection at *Blastocystis* cell densities of 10^3/cells per gram of faeces and at DNA concentrations of < 1 ng μL^{-1} per PCR. *Blastocystis* was detected in 59 individuals or 56% of the sample population ($n = 105$). Although this number is lower than an earlier report of 82% prevalence in an Irish population (Scanlan & Marchesi, 2008), the number of individuals assayed previously ($n = 17$) limited the scope of (statistical) analysis and conclusions. We found no significant difference in the prevalence of *Blastocystis* between elderly and nonelderly subjects ($\chi^2 = 0.645$, d.f. = 1, $P = 0.422$), or between female (61.8%) and male (46.7%) subjects ($\chi^2 = 1.861$, d.f. = 1, $P = 0.173$). *Blastocystis* transmission to humans is thought to occur via contact with other colonised humans or animals or through waterborne transmission (Tan, 2008; Lee *et al.*, 2012). Although it is difficult to trace the potential exposure of an individual

Table 1. Overview of participants, full details are provided in Table S1

Group (*n*)	Mean age and range (years)	Male to female ratio	Ethnicity	*Blastocystis* prevalence (%)	Sampling period
Eldermet* Control (< 65), ($n = 12$)	34.4 (28–46)	1 : 1	Caucasian	58	NA
Eldermet (> 65), ($n = 88$)	72.7 (64–93)	1 : 1.3	Caucasian	55	NA
Irish (prevalence and longitudinal analysis), ($n = 5$)	45.4† (27–67)	1 : 1.5	Caucasian	80	6–10 years
the Netherlands (longitudinal analysis), ($n = 5$)	31† (25–41)	1.5 : 1	Caucasian and Asian	0	7 years

*Please see Claesson *et al.* (2012) for further information on the Eldermet trial.

†For longitudinal analysis, between three and eight samples were analysed per person. Each of the four positive Irish samples were sampled at year 0, 1, 2 and 6, and the remaining Irish individual (*Blastocystis* negative) was sampled at year 0, 1, 2, 3, 4, 6, 8 and 10 years. Each of the Dutch samples was taken at year 0, 3 and 7. The mean of the averaged age for the time period analysed is given, and the range is the highest and lowest ages for all individuals in that group over the time period analysed. Due to potential biases arising from geographical variation in *Blastocystis* prevalence, only Irish samples were analysed and reported in the overall prevalence figures (i.e. 59/105 or 56% prevalence).

to *Blastocystis* over their lifetime, we looked for a relationship between their current water source (urban or rural) and the incidence of *Blastocystis*. However, no significant relationship was evident ($P = 0.754$) with equivalent prevalence in urban and rural groups.

We also analysed the temporal stability of *Blastocystis* in ten individuals to provide some insight into long-term colonisation trends. This sample set consisted of a subset of our Irish population ($n = 5$) and an additional five individuals from the Netherlands ($n = 5$) (Rajilic-Stojanovic *et al.*, 2012). These individuals were sampled over different time periods, ranging from 6 to 10 years, see Table 1; Table S1 (Scanlan & Marchesi, 2008; Rajilic-Stojanovic *et al.*, 2012). Four of these individuals were consistently positive for the same *Blastocystis* strain (determined at allele level) over the time period sampled indicating that, once established, *Blastocystis* is a stable component of the healthy human gut microbiota. Of the four individuals that were positive, all were Irish; that is, none of the samples from the Netherlands study testing positive. These five Dutch individuals plus one of the Irish subjects tested negative for *Blastocystis* and, similar to the consistency in the temporal trend observed for positive samples, these individuals were negative at all time-points sampled up to a period of 10 years.

To date, nine different *Blastocystis* subtypes (arguably separate species) have been found in humans (Clark *et al.*, 2013). All 59 positive PCR products provided single-trace reads indicating the absence of mixed infections. At the subtype (ST) level, all reads could be assigned to one of five STs using the online site http://pubmlst.org/blastocystis/ (Jolley & Maiden, 2010; Stensvold *et al.*, 2012a, b); however, the relative frequency of STs within the dataset was significantly different ($\chi^2 = 27.02$, d.f. = 4, $P < 0.0001$). ST3 was the most common ST accounting for 42% of positive PCR products, followed by ST2 (25%), ST4 (19%), ST1 (12%) and ST8 (2%), see Fig. 1a. *Blastocystis* ST distribution within the positive subpopulation was independent of host age or gender ($P = 0.772$ and 0.883, respectively). Although there are differences in the geographic distribution of different subtypes (for example, ST4 is much less common in African populations compared with European populations), of the nine STs associated with humans, recent analysis has shown that STs 1–4 are the most common and account for *c.* 90% of all STs identified in *Blastocystis* subtyping surveys of human samples (Alfellani *et al.*, 2013b, Alfellani *et al.*, 2013c). Our data are consistent with this pattern of ST1-ST4 predominance in human populations. These subtypes were further delineated to 10 different alleles (intra-subtype variants). Similar to the analysis of ST frequencies, the relative frequencies of different alleles within the sample population differed significantly ($\chi^2 = 76.92$, d.f. = 10, $P < 0.0001$). ST3-34 accounted for 37% of alleles in the dataset. The greatest within subtype diversity was observed for ST2 with five different alleles detected, two allele variants were detected for ST3, and only one allele type was identified for ST1, ST4 and ST8, see Fig. 1b.

In conclusion, the high percentage of individuals positive for *Blastocystis* and the temporal stability of *Blastocystis* (when present) show that *Blastocystis* is a common member of the healthy human intestinal microbial ecosystem and, where present, appears to be a stable feature. Moreover, the range of different *Blastocystis* subtypes and genotypes present in individuals indicate that a diversity of different *Blastocystis* spp. can colonise the healthy human gut without resulting in symptomatic carriage. Nonetheless, the

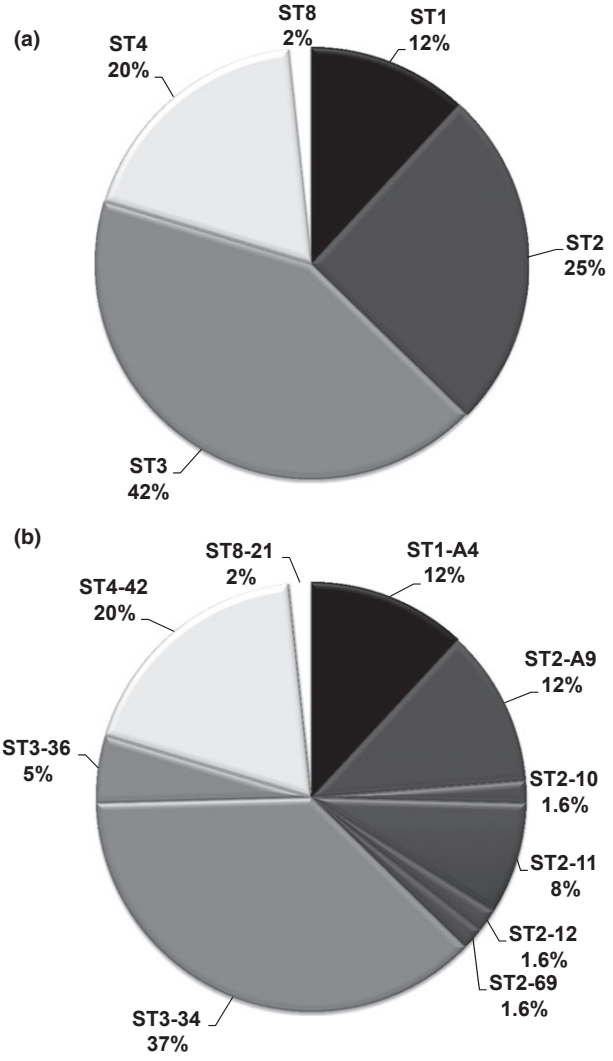

Fig. 1. Overview of subtype (a) and intra-subtype/allele (b) percentage distributions within the *Blastocystis* positive subpopulation.

question of what role *Blastocystis* plays in intestinal illness and disease remains outstanding. If a specific *Blastocystis* sp. is found to be virulent or disease causing, this would be analogous to what is now known about *Entamoeba* spp. distribution and pathogenicity in human populations. A diversity of different *Entamoeba* species have been detected in humans to date; however, only some species cause illness and the majority of individuals that are host to *Entamoeba* spp. are colonised by apparently nonpathogenic species (Stensvold *et al.*, 2011). Similarly, the majority of *Blastocystis* spp. may be harmless and the virulence of *Blastocystis* spp. might be limited a subset of genetic variants or intra-subtypes (Tan *et al.*, 2010; Scanlan, 2012). An additional scenario is that different variants might be opportunistic in different host backgrounds (Genotype × Genotype interaction) or where an environmental component(s) that, for example, compromises the immune status of the host facilitates disease initiation and progression (Genotype × Genotype × Environment interaction). As more data emerge from carefully controlled studies that use appropriate methods, this will hopefully facilitate a better understanding of the relationship between *Blastocystis* and disease, determine what factors govern host colonisation and move towards understanding the ecological role (if any) of this common and diverse resident of the human gut.

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References

Alfellani MA, Taner-Mulla D, Jacob AS, Imeede CA, Yoshikawa H, Stensvold CR & Clark CG (2013a) Genetic diversity of *Blastocystis* in livestock and zoo animals. *Protist* **164**: 497–509.

Alfellani MA, Stensvold CR, Vidal-Lapiedra A, Onuoha ES, Fagbenro-Beyioku AF & Clark CG (2013b) Variable geographic distribution of *Blastocystis* subtypes and its potential implications. *Acta Trop* **126**: 11–18.

Alfellani MA, Jacob AS, Perea NO, Krecek RC, Taner-Mulla D, Verweij JJ, Levecke B, Tannich E, Clark CG & Stensvold CR (2013c) Diversity and distribution of *Blastocystis* sp. subtypes in non-human primates. *Parasitology* **140**: 966–971.

Bart A, Wentink-Bonnema EM, Gilis H, Verhaar N, Wassenaar CJ, van Vugt M, Goorhuis A & van Gool T (2013) Diagnosis and subtype analysis of *Blastocystis* sp. in 442 patients in a hospital setting in the Netherlands. *BMC Infect Dis* **13**: 389.

Boorom KF (2007) Is this recently characterized gastrointestinal pathogen responsible for rising rates of inflammatory bowel disease (IBD) and IBD associated autism in Europe and the United States in the 1990s? *Med Hypotheses* **69**: 652–659.

Chen Y, Chen Z, Guo R, Chen N, Lu H, Huang S, Wang J & Li L (2011) Correlation between gastrointestinal fungi and varying degrees of chronic hepatitis B virus infection. *Diagn Microbiol Infect Dis* **70**: 492–498.

Claesson MJ, Jeffery IB, Conde S *et al.* (2012) Gut microbiota composition correlates with diet and health in the elderly. *Nature* **488**: 178–184.

Clark CG, van der Giezen M, Alfellani MA & Stensvold CR (2013) Recent developments in *Blastocystis* research. *Adv Parasitol* **82**: 1–32.

El Safadi D, Gaayeb L, Meloni D *et al.* (2014) Children of Senegal River Basin show the highest prevalence of *Blastocystis* sp. ever observed worldwide. *BMC Infect Dis* **14**: 164.

Gouba N, Raoult D & Drancourt M (2013) Plant and fungal diversity in gut microbiota as revealed by molecular and culture investigations. *PLoS One* **8**: e59474.

Hamad I, Sokhna C, Raoult D & Bittar F (2012) Molecular detection of eukaryotes in a single human stool sample from Senegal. *PLoS One* **7**: e40888.

Iliev ID, Funari VA, Taylor KD *et al.* (2012) Interactions between commensal fungi and the C-type lectin receptor Dectin-1 influence colitis. *Science* **336**: 1314–1317.

Jolley KA & Maiden MC (2010) BIGSdb: scalable analysis of bacterial genome variation at the population level. *BMC Bioinformatics* **11**: 595.

Lee LI, Chye TT, Karmacharya BM & Govind SK (2012) *Blastocystis* sp.: waterborne zoonotic organism, a possibility? *Parasit Vectors* **5**: 130.

Mirza H, Wu Z, Teo JD & Tan KS (2012) Statin pleiotropy prevents rho kinase-mediated intestinal epithelial barrier compromise induced by *Blastocystis* cysteine proteases. *Cell Microbiol* **14**: 1474–1484.

Nam YD, Chang HW, Kim KH, Roh SW, Kim MS, Jung MJ, Lee SW, Kim JY, Yoon JH & Bae JW (2008) Bacterial, archaeal, and eukaryal diversity in the intestines of Korean people. *J Microbiol* **46**: 491–501.

Pandey PK, Siddharth J, Verma P, Bavdekar A, Patole MS & Shouche YS (2012) Molecular typing of fecal eukaryotic microbiota of human infants and their respective mothers. *J Biosci* **37**: 221–226.

Parfrey LW, Walters WA & Knight R (2011) Microbial eukaryotes in the human microbiome: ecology, evolution, and future directions. *Front Microbiol* **2**: 153.

Patterson DJ (1999) The diversity of *Eukaryotes*. *Am Nat* **154**: S96–S124.

Poirier P, Wawrzyniak I, Albert A, El Alaoui H, Delbac F & Livrelli V (2011) Development and evaluation of a real-time PCR assay for detection and quantification of *Blastocystis* parasites in human stool samples: prospective study of patients with hematological malignancies. *J Clin Microbiol* **49**: 975–983.

Poirier P, Wawrzyniak I, Vivares CP, Delbac F & El Alaoui H (2012) New insights into *Blastocystis* spp.: a potential link with irritable bowel syndrome. *PLoS Pathog* **8**: e1002545.

Puthia MK, Sio SW, Lu J & Tan KS (2006) *Blastocystis ratti* induces contact-independent apoptosis, F-actin rearrangement, and barrier function disruption in IEC-6 cells. *Infect Immun* **74**: 4114–4123.

Puthia MK, Lu J & Tan KS (2008) *Blastocystis ratti* contains cysteine proteases that mediate interleukin-8 response from human intestinal epithelial cells in an NF-kappaB-dependent manner. *Eukaryot Cell* **7**: 435–443.

Rajilic-Stojanovic M, Heilig HG, Tims S, Zoetendal EG & de Vos WM (2012) Long-term monitoring of the human intestinal microbiota composition. *Environ Microbiol* **15**: 1146–1159.

Ramirez JD, Sanchez LV, Bautista DC, Corredor AF, Florez AC & Stensvold CR (2013) *Blastocystis* subtypes detected in humans and animals from Colombia. *Infect Genet Evol* **22**: 223–228.

Scanlan PD (2012) *Blastocystis*: past pitfalls and future perspectives. *Trends in parasitology* **28**: 327–334.

Scanlan PD & Marchesi JR (2008) Micro-eukaryotic diversity of the human distal gut microbiota: qualitative assessment using culture-dependent and -independent analysis of faeces. *ISME J* **2**: 1183–1193.

Scanlan PD & Stensvold CR (2013) *Blastocystis*: getting to grips with our guileful guest. *Trends Parasitol* **29**: 523–529.

Scicluna SM, Tawari B & Clark CG (2006) DNA barcoding of *Blastocystis*. *Protist* **157**: 77–85.

Silberman JD, Sogin ML, Leipe DD & Clark CG (1996) Human parasite finds taxonomic home. *Nature* **380**: 398.

Stensvold CR, Alfellani M & Clark CG (2012a) Levels of genetic diversity vary dramatically between *Blastocystis* subtypes. *Infect Genet Evol* **12**: 263–273.

Stensvold CR, Ahmed UN, Andersen LO & Nielsen HV (2012b) Development and evaluation of a genus-specific, probe-based, internal-process-controlled real-time PCR assay for sensitive and specific detection of *Blastocystis* spp. *J Clin Microbiol* **50**: 1847–1851.

Stensvold CR, Lebbad M, Victory EL, Verweij JJ, Tannich E, Alfellani M, Legarraga P & Clark CG (2011) Increased sampling reveals novel lineages of *Entamoeba*: consequences of genetic diversity and host specificity for taxonomy and molecular detection. *Protist* **162**: 525–541.

Tan KS (2004) *Blastocystis* in humans and animals: new insights using modern methodologies. *Vet Parasitol* **126**: 121–144.

Tan KS (2008) New insights on classification, identification, and clinical relevance of *Blastocystis* spp. *Clin Microbiol Rev* **21**: 639–665.

Tan KS, Mirza H, Teo JD, Wu B & Macary PA (2010) Current views on the clinical relevance of *Blastocystis* spp. *Curr Infect Dis Rep* **12**: 28–35.

Wawrzyniak I, Poirier P, Viscogliosi E, Dionigia M, Texier C, Delbac F & El Alaoui H (2013) *Blastocystis*, an unrecognized parasite: an overview of pathogenesis and diagnosis. *Ther Adv Infect Dis* **1**: 167–178.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Summary data of all study participants.