# Comparison of Microscopy, Culture, and Conventional Polymerase Chain Reaction for Detection of *Blastocystis* sp. in Clinical Stool Samples

Tamalee Roberts,* Joel Barratt, John Harkness, John Ellis, and Damien Stark

*Department of Microbiology, SydPath, St. Vincent's Hospital, Sydney, Australia; Department of Medical and Molecular Biosciences, University of Technology, Sydney, Broadway, Australia*

*Abstract.* We tested 513 stool samples from patients in Sydney, Australia for *Blastocystis* by using five diagnostic techniques: microscopy of a permanently stained smear using a modified iron-hematoxylin stain, two xenic culture systems (a modified Boeck and Drbohlav's medium and tryptone, yeast extract, glucose, methionine-9 medium), and two published conventional polymerase chain reaction methods specific for the small subunit ribosomal DNA. Ninety-eight (19%) samples were positive for *Blastocystis* in one or more of the diagnostic techniques. The PCR 2 method was the most sensitive at detecting *Blastocystis* with a sensitivity of 94%, and the least sensitive was microscopy of the permanent stain (48%). Subtype 3 was the most predominant subtype (present in 43% of samples assigned to this group). This study highlights the low sensitivity of microscopy when used as the sole diagnostic modality for detection of *Blastocystis* sp.

## INTRODUCTION

*Blastocystis* is a single-celled enteric protozoan that has a world-wide distribution.[1] *Blastocystis* is the most common parasite isolated from human stool samples in developing and developed countries.[2,3] Rates of infection vary from 3.3% in developed countries[4] to 53.8% in developing countries.[5] *Blastocystis* is found in humans and animals. There has been some controversy over whether this protozoan is considered pathogenic in humans but many recent *in vivo* and *in vitro* studies strongly suggest that this organism is a pathogen.[6–8]

The wide range in prevalence of *Blastocystis* seen between countries can be attributed to several factors such as socioeconomic conditions, but also to the different diagnostic methods used for detection. The most common diagnostic technique used worldwide for identification of *Blastocystis* is the permanent stain. The use of xenic cultures, in which *Blastocystis* is grown *in vitro* with non-specific microorganisms, has been shown to be more sensitive in detecting *Blastocystis* but it is not commonly used in the diagnostic laboratory.[9–11] Molecular diagnosis by polymerase chain reaction (PCR) using the small subunit (SSU) ribosomal RNA gene is becoming more widely used for detection of enteric parasites. Although this technique is more costly, it is known to be more sensitive than the direct smear and xenic culture.[12] Because considerable diversity exists in the rDNA of *Blastocystis*, as the result of diversification of this species into a wide range of genotypes, the choice of primers is crucial from a diagnostic perspective. Some primers may amplify specific subtypes preferentially, which could result in some subtypes being missed in the analysis. Also it is preferable that the PCR primers are compatible with DNA extracted directly from stool.

There is a considerable degree of genetic heterogeneity shown within *Blastocystis*. Currently, human, mammalian, avian, and reptilian isolates can be assigned to one of 10 subtypes.[12–16] However, it is still not clear whether any of these subtypes are specific to human disease. Most human *Blastocystis* infections were shown to be attributed to subtype 3, having been reported in 40–92% of fecal samples.[17,18] Although, in some countries this is not the case as seen in Spain where subtype 4 was the most common subtype isolated from a human study group and no isolates were assigned to subtype 3.[19]

Most epidemiologic studies rely on molecular analysis from cultures containing *Blastocystis*. Such studies are limited by the fact they do not include *Blastocystis* that do not grow in culture. In addition, there have only been a few studies that compared the sensitivity of diagnostic techniques used for identification of *Blastocystis* sp.[20,21] Consequently, the aim of this study was to compare five diagnostic techniques (microscopy of a permanent stain using a modified iron-hematoxylin stain, two xenic culture systems (modified Boeck and Drbohlav's medium [MBD] and tryptose, yeast extract, glucose, methionine 9 [TYGM-9] medium), and two published conventional PCR methods specific for the SSU rDNA) for detection of *Blastocystis* sp. in stool samples.

## MATERIALS AND METHODS

**Stool specimens.** All stool specimens (n = 513) submitted to the microbiology department of St. Vincent's Hospital (Sydney, New South Wales, Australia) from patients during March 2008–December 2008 were included in the study. Samples were divided into three aliquots. A portion of the sample was mixed with sodium acetate acetic acid formalin (SAF) preservative and fixed overnight in preparation for permanent staining. A 10-mg sample was taken from the fresh fecal samples for culture and a portion was frozen to be kept for DNA extraction and further PCR analysis.

**Microscopy.** Samples fixed in SAF were stained by using a modified iron-hematoxylin stain (Fronine, Riverstone, New South Wales, Australia) according to the manufacturer's instructions and examined by oil-immersion microscopy. Diagnosis of *Blastocystis* was based on morphology of parasites observed in permanent stained smears.

**Culture.** All fresh fecal samples were inoculated into two culture systems upon arrival at the laboratory. Approximately 10 mg of the stool sample was put into monophasic xenic media TYGM-9 and a diphasic xenic system by using a dorset egg slope overlaid with approximately 5 mL of an in house made growth media containing 90% phosphate-buffered saline, 9% sterile horse serum, 1% of 20% [w/v] bacteriologic peptone, 1 mg of rice starch, and 500 μL of penicillin-streptomycin solution. Tubes were incubated at 35°C and a drop of sediment

*Address correspondence to Tamalee Roberts, Department of Microbiology, St. Vincent's Hospital, Darlinghurst 2010, New South Wales, Australia. E-mail: troberts@stvincents.com.au

was examined every two days for one week by phase-contrast microscopy for parasites. When parasites were seen in the sediment, a portion of it was fixed in SAF and stained with modified iron-hematoxylin stain for identification of the organism by oil-immersion microscopy.

**DNA extraction.** DNA was extracted from all frozen stool samples by using the QIAamp™ DNA Stool Minikit (Qiagen, Hilden, Germany) as per the manufacturer's instructions.

**Polymerase chain reaction for *Blastocystis* sp.** All DNA from the stool specimens was tested by PCR for *Blastocystis* using two described protocols.[12,21] Briefly, PCR 1 used primers b11400ForC (5′-GGA ATC CTC TTA GAG GGA CAC TAT ACA T-3′) and b11710RevC (5′-TTA CTA AAA TCC AAA GTG TTC ATC GGA C-3′), which amplify a 310-basepair fragment of SSU rDNA. The following reaction was used: 35 cycles with denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute, and extension at 72°C for 1 min. PCR 2 used primers F1 (5′GGA GGT AGT GAC AAT AAA TC-3′)[22] and BHCRseq3 (5′-TAA GAC TAC GAG GGT ATC TA-3′),[12] which are also specific for the SSU rDNA of *Blastocystis*. The PCR involved denaturation at 95°C for 7 minutes, 35 cycles at 94°C for 60 seconds, 56°C for 45 seconds, followed by a final extension step at 72°C for 7 minutes. Amplicons of 550–585 basepairs observed after electrophoresis on an agarose gel (Ready Agarose Gels; Bio-Rad, Marnes la Coquette, France) were considered a positive result.

The PCRs were conducted in a volume 25 μL and contained pure-Taq Ready-To-Go PCR beads (Amersham Pharmacia Biotech, Piscataway, NJ) (each containing 1.5 units of Taq DNA polymerase, 10 mM Tris-HCl, pH 9, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each dNTP, and stabilizers, including bovine serum albumin), 2 μL of genomic DNA extract and 0.5μm of each PCR primer.

Inhibition controls were run to exclude inhibition as a contributor to negative samples. Briefly, samples were spiked with an equal volume of genomic DNA from a *Blastocystis* control and run in parallel with an unspiked specimen.

**Sequence analysis.** DNA sequence analysis was performed on all PCR-positive samples. The PCR products were purified using the QIAquick™ PCR purification Kit (Qiagen) as per the manufacturer's instructions. The PCR products were then sequenced in both directions on an ABI prism 3700 automated sequencer (Applied Biosystems, Foster City, CA) at the SUPAMAC facility (Royal Prince Alfred Hospital, Sydney, New South Wales, Australia). The SSU rDNA sequences were then compared with those available in GenBank using the BLASTN program run on the National Center for Biotechnology Information server (http://www.ncbi.nlm.nih.gov/BLAST).

## RESULTS

A total of 513 samples from 462 patients were included in the study over a 10-month period. The specimens were obtained from 235 males and 227 females (age range = 3 months to 96 years, mean age = 46.2 years). Ninety-eight (19%) samples were positive for *Blastocystis* in one or more of the diagnostic methods, where a true positive result was defined as a sample identified as positive by use of one or more of the diagnostic techniques. When only a PCR result was positive, sequence analysis of the PCR product was used as a confirmatory test.

TABLE 1
Parasites found in conjunction with *Blastocystis* by using permanent stain

| Parasite | No. |
|---|---|
| *Blastocystis* sp. | 35 |
| *Blastocystis* and *Endolimax nana* | 4 |
| *Blastocystis* and *Giardia intestinalis* | 2 |
| *Blastocystis* and *Dientamoeba fragilis* | 1 |
| *Blastocystis* and *Entamoeba histolytica* complex* | 1 |
| *Blastocystis*, *Enteromonas hominis*, and *Chilomastix mesnili* | 1 |
| *Blastocystis*, *Iodamoeba butschlii*, *E. nana*, and *E. histolytica* complex* | 1 |
| *Blastocystis*, *E. nana*, *Entamoeba coli*, and *Entamoeba hartmanii* | 1 |
| *Blastocystis*, *Dientamoeba fragilis*, *E. coli*, *I. butschlii*, and *E. nana* | 1 |
| Total | 47 |

* *E. histolytica* complex = *E. histolytica*, *E. dispar*, or *E. moshkovskii*.

Seventy-eight samples were positive by the initial permanent modified iron-hematoxylin stain for any parasite and 47 (9%) of these were positive for *Blastocystis* (results are summarized in Table 1). Thirty-five samples were positive for *Blastocystis* and 12 showed co-infection with one or more other parasites. *Endolimax nana* was the most common protozoan parasite found in conjunction with *Blastocystis* (6), followed by *Giardia intestinalis* and the *Entamoeba histolytica/dispar/moshkovskii* complex (2 each). Parasites other than *Blastocystis* found in the samples are shown in Table 2. Thirty-eight (38.7%) of 98 patients with *Blastocystis* infection reported some form of gastrointestinal symptom and five had a history of travel. There were 69 patients in which *Blastocystis* was the only infectious agent detected.

Of all the diagnostic techniques used, PCR 2 was the most sensitive method for detecting *Blastocystis*; 92 of 98 samples were positive by any of the diagnostic techniques. The MBD media showed 81 positive results with stool samples. Eighty samples were positive when TYGM-9 medium was used. Sixty-five samples were positive by PCR1. Microscopy of permanent stains was least effective in detecting *Blastocystis*; only 44 samples showed positive results. Results are shown in Table 3. There was an almost equal specificity for all three diagnostic techniques: PCR and culture showed 100% specificity and microscopy showed 99.9% specificity. Less than 1% of samples were considered inhibitory when inhibition controls failed to produce an amplified product in 5 of 513 samples.

Although PCR 2 was the more sensitive method in detecting *Blastocystis*, PCR 1 showed positive results for samples in

TABLE 2
Number and prevalence of parasites other than *Blastocystis* in clinical specimens studied by using permanent stain

| Parasite | No. (%) |
|---|---|
| *Giardia intestinalis* | 11 (24.4) |
| *Endolimax nana* | 9 (20.5) |
| *Dientamoeba fragilis* | 7 (15.5) |
| *Entamoeba histolytica/E. dispar/E. moshkovski* | 4 (8.8) |
| *Entamoeba coli* | 3 (6.6) |
| *Cryptosporidium* sp. | 3 (6.6) |
| *Entamoeba hartmanii* | 2 (4.4) |
| *Iodamoeba butshlii* | 2 (4.4) |
| *Enteromonas hominis* | 2 (4.4) |
| *Chilomastix mesnili* | 1 |
| *Strongyloides* sp. | 1 |
| Total | 45 |

which PCR 2 showed negative results. These samples were not subtype specific. Four samples that were positive by microscopic analysis of samples treated with permanent stain and culture were initially negative by both PCRs. Repeated testing showed that these samples were positive by PCR 2. All of these false-negative samples were *Blastocystis* subtype 1.

Although there were almost equal numbers of positive samples for both culture techniques, more prolific growth occurred with MBD medium than TYGM-9 medium. Two samples were positive with permanent stain and PCR but negative with both culture techniques. These samples were stored at 4°C overnight before being cultured. Isolates in the MBD culture system lasted for more passages than those in the TYGM-9 medium, in which cultures only lasted 3–4 passages and lower numbers were seen after each passage. The MBD medium produced high numbers of cells after every passage and most cultures lasted for longer than 10 passages. Only a few cultures were maintained beyond this amount of time because of storage constraints. All positive cultures were confirmed by microscopy of wet preparations and permanent stains.

There were eight samples in which permanent stain and culture showed negative results but PCR showed positive results. There was no correlation between subtype and negative culture for four samples that had subtype 3; two had subtypes 1 and 4. There was no correlation between a negative diagnosis (by initial staining) and a positive PCR result for almost equal numbers of each subtype were found when there was a negative or positive staining result.

Eleven persons who had *Blastocystis*-positive results submitted more than one sample. Duplicate samples for four of these patients showed different results from the other sample. The first sample from the first patient was negative by PCR 1 and positive by PCR 2, and the second sample was positive by PCR 1 and PCR2. *Blastocystis* was found in cultures of both samples but this organism was only seen in the permanent stain for the second sample. The first sample from the second patient was positive by both PCR techniques but negative four days later. Culture and permanent stain were negative for this sample. The sample from the third patient had a negative result initially but 12 days later had a PCR-positive result; culture and permanent stain were negative. The first sample from the fourth patient initially had *Blastocystis* by permanent stain and culture, and both PCRs showed a positive result; sequencing showed that the isolate was subtype 1. The second sample from this person obtained one day later was negative by permanent stain but positive by culture and both PCRs; sequencing showed that the isolate was subtype 3.

Only vacuolar morphologic types were seen in initial permanent stain samples. Vacuolar and granular types were the main morphologic types seen during early stages of culture, and amoeboid cells were also present in older cultures.

Sequence analysis was conducted on all PCR-positive samples. Ninety-one readable sequences were obtained from 95 samples that were positive by either PCR. Six subtypes were identified. Subtype 3 was the most common subtype; 41 (43%) isolates belonged to this group. Subtype 1 was the second most common subtype; 28 (29%) isolates belonged to this group. Twelve (12%) isolates were subtype 4, 6 (6%) isolates were subtype 2, 3 (3%) isolates were subtype 6, and 1 (1%)isolate was subtype 8.

## DISCUSSION

We showed that PCR2 was the most effective method for detecting *Blastocystis* in clinical stool samples. Although PCR was the most sensitive method, its limitations are that it is expensive, time-consuming, and labor-intensive because of manual extraction of DNA and the need for specialized equipment. In a standard clinical diagnostic laboratory, permanent stains are the gold standard for diagnosis of enteric parasites. Although this method is effective for identification of a wide variety of parasites, we showed that more than 50% of the infections with *Blastocystis* sp. are missed. The low sensitivity of this method may result from the parasite being present in low numbers in the samples; thus, they are not detected by the staining procedure. The higher detection rates seen in cultures may be attributed to the organism needing more time to grow and replicate. The results from this study, with PCR being the most effective form of diagnosis, are consistent with those of studies that reported that molecular analysis is the most efficient method for detection of *Blastocystis*.[12,23] This finding is in contrast to those of another study,[20] which suggested that *in vitro* culture was superior to direct PCR for stool samples.

This study also demonstrated that in the Sydney population, there is a 19% incidence of *Blastocystis* in persons with gastrointestinal symptoms. This finding is similar to results reported from Germany, Thailand, and China, which reported incidences of 17.9%, 13.3%, and 32.6%, respectively.[17,24] These studies all used molecular techniques for diagnosis of *Blastocystis*. *Blastocystis* has been reported in much higher frequencies in studies in which molecular methods were not used and results obtained on the basis of microscopy alone (53.8% in Zambia,[5] 52.3% in Malaysia,[25] and 30% in travelers in Nepal[26]). These frequencies are extremely high and may be attributed to the fact that these studies were conducted in developing countries where there is usually a higher incidence of intestinal parasites caused by poor hygienic practices. The misidentification of *Blastocystis* as other organisms such as *Dientamoeba fragilis* or yeasts is a common problem and may confound studies limited to the use of microscopy alone.[27] Our study showed that for epidemiologic studies PCR should be the method of choice, but if this is not possible because of financial constraints, at least two diagnostic techniques should be used in conjunction with each other.

The sensitivity of the two PCRs varied greatly in this study. PCR 2 had a sensitivity of 94% and PCR 1 only had a sensitivity of 66%. These results show that it is important to choose the right set of primers and reaction conditions for detection of *Blastocystis* DNA by PCR.

Many studies used Jones medium successfully as the medium of choice for xenic culture of *Blastocystis*.[10–12,23] In our study, two media, MBD and TYGM-9, were used for comparison of growth of *Blastocystis* in culture. These culture media were used instead of Jones medium because it was known that *Blastocystis* grows successfully in Jones medium. These other two media were used to investigate which other media could

be used for cultivation of *Blastocystis*. Other media may be more suitable for diagnosis of *Blastocystis* than the MBD and TYGM-9 used in this study (giving a higher growth yield). This possibility should be considered in future studies.

No mixed subtype infections were found. One patient who submitted two samples in two days had a different subtype isolated from each sample: subtype 1 from the initial sample and subtype 3 from the second sample. This finding could indicate a mixed infection in both samples. However, the PCR primers were only able to detect one subtype in each sample. Different subtypes may have different shedding rates, which could explain why two subtypes were detected in one person. Three other persons who submitted more than one sample had different results. The initial sample of the first patient was PCR 1 negative and PCR 2 positive. However, the second sample showed positive results in both PCRs. The low sensitivity of PCR 1 can be attributed to the fact that it did not detect *Blastocystis* in the first sample. The first sample of the second patient was PCR2 positive but the second sample, which was submitted four days later, was negative for *Blastocystis* by all diagnostic techniques used. This finding could have been caused by the self-limiting effect of *Blastocystis* and also because the patient may have been treated for another form of disease, which may have removed the *Blastocystis*. The first sample of the third patient was initially negative by all diagnostic techniques for *Blastocystis* but the second sample obtained 12 days later was positive by both PCRs. Because this person was admitted to a hospital, the patient may have acquired the disease from contaminated food or water brought into the hospital. It is also possible that the patient had intermittent shedding of *Blastocystis*. These findings show the importance of submitting more then one sample over several days to ensure that an accurate diagnosis is made.

There was no correlation between permanent stain and a negative culture or PCR-positive results. This finding suggests that the culture systems used in this study do not discriminate between subtypes of *Blastocystis*. However, these results show that it is necessary to perform PCR on all culture-negative and microscopy-negative samples to acquire a true perspective of the prevalence of *Blastocystis* sp. because isolates may be missed if molecular techniques are used to test only positive culture samples. Many epidemiologic studies reported only further use of molecular analysis if there are samples positive by culture.[4,17,24,28–30] An obvious conclusion is that these studies did not provide a true representation of the prevalence of *Blastocystis* infections in human population studies where some culture systems used may not enable certain strains of *Blastocystis* to grow.

Sequence analysis of all PCR-positive samples showed that subtype 3 was the most common subtype in the population in Sydney. Our study is the first molecular epidemiologic study to identify subtype distribution of *Blastocystis* sp. in a population in Australia. Results were similar to those of other studies, in which subtype 3 was also the most common subtype.[31–35]

In conclusion, this is the first large-scale study to compare five diagnostic techniques used in a clinical laboratory for diagnosis of *Blastocystis* sp. The PCR was the most efficient diagnostic technique used. Microscopy detected only 48% of the positive samples. We showed that persons in Sydney, Australia had a *Blastocystis* prevalence of 19% and that subtype 3 was the most predominant subtype (43%). If the use of PCR is not feasible for a diagnostic laboratory, it is recommended that at least two different diagnostic techniques be used for detection of *Blastocystis*. These results show the need to be cautious when interpreting prevalence reports, especially for studies that rely solely on microscopy.

Authors' addresses: Tamalee Roberts, Joel Barratt, and Damien Stark, Department of Microbiology, St. Vincent's Hospital, Darlinghurst, New South Wales, Australia, and Department of Medical and Molecular Biosciences, University of Technology, Sydney, Broadway, New South Wales, Australia, E-mails: troberts@stvincents.com.au, Joel.Barratt-1@uts.edu.au, and dstark@stvincents.com.au. John Harkness, Department of Microbiology, St. Vincent's Hospital, Darlinghurst, New South Wales, Australia, E-mail: jharkness@stvincents.com.au. John Ellis, Department of Medical and Molecular Biosciences, University of Technology, Sydney, Broadway, New South Wales, Australia, E-mail: john.ellis@uts.edu.au.

## REFERENCES

1. Stenzel DJ, Boreham PF, 1996. *Blastocystis hominis* revisited. *Clin Microbiol Rev 9:* 563–584.
2. Tan KS, Singh M, Yap EH, 2002. Recent advances in *Blastocystis hominis* research: hot spots in terra incognita. *Int J Parasitol 32:* 789–804.
3. Tan KS, 2004. *Blastocystis* in humans and animals: new insights using modern methodologies. *Vet Parasitol 126:* 121–144.
4. Wong KH, Ng GC, Lin RT, Yoshikawa H, Taylor MB, Tan KS, 2008. Predominance of subtype 3 among *Blastocystis* isolates from a major hospital in Singapore. *Parasitol Res 102:* 663–670.
5. Graczyk TK, Shiff CK, Tamang L, Munsaka F, Beitin AM, Moss WJ, 2005. The association of *Blastocystis hominis* and *Endolimax nana* with diarrheal stools in Zambian school-age children. *Parasitol Res 98:* 38–43.
6. Stark D, van Hal S, Marriott D, Ellis J, Harkness J, 2007. Irritable bowel syndrome: a review on the role of intestinal protozoa and the importance of their detection and diagnosis. *Int J Parasitol 37:* 11–20.
7. Boorom KF, Smith H, Nimri L, Viscogliosi E, Spanakos G, Parkar U, Li LH, Zhou XN, Ok UZ, Leelayoova S, Jones MS, 2008. Oh my aching gut: irritable bowel syndrome, *Blastocystis*, and asymptomatic infection. *Parasit Vectors 1:* 40.
8. Tan KS, 2008. New insights on classification, identification, and clinical relevance of *Blastocystis* spp. *Clin Microbiol Rev 21:* 639–665.
9. Zman V, Khan KZ, 1994. A comparison of direct microscopy with culture for the diagnosis of *Blastocystis hominis*. *Southeast Asian J Trop Med Public Health 25:* 792–793.
10. Leelayoova S, Taamasri P, Rangsin R, Naaglor T, Thathaisong U, Mungthin M, 2002. *In-vitro* cultivation: a sensitive method for detecting *Blastocystis hominis*. *Ann Trop Med Parasitol 96:* 803–807.
11. Suresh K, Smith H, 2004. Comparison of methods for detecting *Blastocystis hominis*. *Eur J Clin Microbiol Infect Dis 23:* 509–511.
12. Stensvold CR, Arendrup MC, Jespersgaard C, Molbak K, Nielsen HV, 2007. Detecting *Blastocystis* using parasitologic and DNA-based methods: a comparative study. *Diagn Microbiol Infect Dis 59:* 303–307.
13. Noel C, Peyronnet C, Gerbod D, Edgcomb VP, Delgado-Viscogliosi P, Sogin ML, Capron M, Viscogliosi E, Zenner L, 2003. Phylogenetic analysis of *Blastocystis* isolates from different hosts based on the comparison of small-subunit rRNA gene sequences. *Mol Biochem Parasitol 126:* 119–123.
14. Yoshikawa H, Morimoto K, Wu Z, Singh M, Hashimoto T, 2004. Problems in speciation in the genus *Blastocystis*. *Trends Parasitol 20:* 251–255.
15. Noel C, Dufernez F, Gerbod D, Edgcomb VP, Delgado-Viscogliosi P, Ho LC, Singh M, Wintjens R, Sogin ML, Capron M, Pierce R, Zenner L, Viscogliosi E, 2005. Molecular phylogenies of *Blastocystis* isolates from different hosts: implications for

genetic diversity, identification of species, and zoonosis. *J Clin Microbiol 43:* 348–355.

16. Parkar U, Traub RJ, Vitali S, Elliot A, Levecke B, Robertson I, Geurden T, Steele J, Drake B, Thompson RC, 2010. Molecular characterization of *Blastocystis* isolates from zoo animals and their animal-keepers. *Vet Parasitol 169:* 8–17.

17. Yoshikawa H, Wu Z, Kimata I, Iseki M, Ali IK, Hossain MB, Zaman V, Haque R, Takahashi Y, 2004. Polymerase chain reaction-based genotype classification among human *Blastocystis hominis* populations isolated from different countries. *Parasitol Res 92:* 22–29.

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

19. Dominguez-Marquez MV, Guna R, Munoz C, Gomez-Munoz MT, Borras R, 2009. High prevalence of subtype 4 among isolates of *Blastocystis hominis* from symptomatic patients of a health district of Valencia (Spain). *Parasitol Res 105:* 949–955.

20. Termmathurapoj S, Leelayoova S, Aimpun P, Thathaisong U, Nimmanon T, Taamasri P, Mungthin M, 2004. The usefulness of short-term *in vitro* cultivation for the detection and molecular study of *Blastocystis hominis* in stool specimens. *Parasitol Res 93:* 445–447.

21. Stensvold R, Brillowska-Dabrowska A, Nielsen HV, Arendrup MC, 2006. Detection of *Blastocystis hominis* in unpreserved stool specimens by using polymerase chain reaction. *J Parasitol 92:* 1081–1087.

22. Bohm-Gloning B, Knobloch J, Walderich B, 1997. Five subgroups of *Blastocystis hominis* from symptomatic and asymptomatic patients revealed by restriction site analysis of PCR-amplified 16S-like rDNA. *Trop Med Int Health 2:* 771–778.

23. Parkar U, Traub RJ, Kumar S, Mungthin M, Vitali S, Leelayoova S, Morris K, Thompson RC, 2007. Direct characterization of *Blastocystis* from faeces by PCR and evidence of zoonotic potential. *Parasitology 134:* 359–367.

24. Li L-H, Zhou X-N, Du Z-W, Wang X-Z, Wang L-B, Jiang J-Y, Yoshikawa H, Steinmann P, Utzinger J, Wu Z, Chen J-X, Chen S-H, Zhang L, 2007. Molecular epidemiology of human *Blastocystis* in a village in Yunnan Province, China. *Parasitol Int 56:* 281–286.

25. Noor Azian MY, San YM, Gan CC, Yusri MY, Nurulsyamzawaty Y, Zuhaizam AH, Maslawaty MN, Norparina I, Vythilingam I, 2007. Prevalence of intestinal protozoa in an aborigine community in Pahang, Malaysia. *Trop Biomed 24:* 55–62.

26. Sohail MR, Fischer PR, 2005. *Blastocystis hominis* and travelers. *Travel Med Infect Dis 3:* 33–38.

27. Stark DJ, Beebe N, Marriott D, Ellis JT, Harkness J, 2006. Dientamoebiasis: clinical importance and recent advances. *Trends Parasitol 22:* 92–96.

28. Hoevers J, Holman P, Logan K, Hommel M, Ashford R, Snowden K, 2000. Restriction-fragment-length polymorphism analysis of small-subunit rRNA genes of *Blastocystis hominis* isolates from geographically diverse human hosts. *Parasitol Res 86:* 57–61.

29. Kaneda Y, Horiki N, Cheng XJ, Fujita Y, Maruyama M, Tachibana H, 2001. Ribodemes of *Blastocystis hominis* isolated in Japan. *Am J Trop Med Hyg 65:* 393–396.

30. Rivera WL, 2008. Phylogenetic analysis of *Blastocystis* isolates from animal and human hosts in the Philippines. *Vet Parasitol 156:* 178–182.

31. Li LH, Zhang XP, Lv S, Zhang L, Yoshikawa H, Wu Z, Steinmann P, Utzinger J, Tong XM, Chen SH, Zhou XN, 2007. Cross-sectional surveys and subtype classification of human *Blastocystis* isolates from four epidemiological settings in China. *Parasitol Res 102:* 83–90.

32. Ozyurt M, Kurt O, Molbak K, Nielsen HV, Haznedaroglu T, Stensvold CR, 2008. Molecular epidemiology of *Blastocystis* infections in Turkey. *Parasitol Int 57:* 300–306.

33. Yoshikawa H, Wu Z, Pandey K, Pandey BD, Sherchand JB, Yanagi T, Kanbara H, 2009. Molecular characterization of *Blastocystis* isolates from children and rhesus monkeys in Kathmandu, Nepal. *Vet Parasitol 160:* 295–300.

34. Souppart L, Sanciu G, Cian A, Wawrzyniak I, Delbac F, Capron M, Dei-Cas E, Boorom K, Delhaes L, Viscogliosi E, 2009. Molecular epidemiology of human *Blastocystis* isolates in France. *Parasitol Res 105:* 413–421.

35. Souppart L, Moussa H, Cian A, Sanciu G, Poirier P, El Alaoui H, Delbac F, Boorom K, Delhaes L, Dei-Cas E, Viscogliosi E, 2010. Subtype analysis of *Blastocystis* isolates from symptomatic patients in Egypt. *Parasitol Res 106:* 505–511.