Comparison of Direct Examination and In Vitro Culture for the Detection of Blastocystis sp. in Orang Asli Stool Samples
(Perbandingan Pemeriksaan Langsung dan Kultur In Vitro untuk Pengesanan Blastocystis sp. dalam Spesimen Najis Orang Asli)

SAMSEH ABDULLAH, NORADILAH, NORDHAYATI MOKTAR, II LI LEE, FATMAH MD SALLEH & TENGGU SHAHRUL ANUAR

ABSTRACT
Microscopy-based technique has been widely used in the detection of Blastocystis sp. This study was conducted to compare the techniques used for screening of Blastocystis sp., namely in vitro cultivation of stool specimens in Jones’ medium (IVC) followed by Wheatley Trichrome staining and direct examination of stool samples preserved with polyvinyl alcohol (PVA) followed by Wheatley Trichrome staining with single-round polymerase chain reaction (PCR) as the reference technique. The study was performed on 466 stool samples obtained from the aboriginal community in Pahang, Malaysia. IVC showed higher detection rate of Blastocystis sp. (33.6%) than PVA (20.0%). Single-round PCR detected Blastocystis sp. in 41.0% of the stool specimens. The sensitivity and specificity of PVA and IVC in comparison to the reference technique were 75.3% (95% CI: 65.2-83.6) and 68.5% (CI: 63.7-73.3) and 88.6% (CI: 82.7-93.0) and 86.3% (CI: 81.9-90.0), respectively. The agreement between the reference technique and PVA showed statistically significant fair agreement by Cohen Kappa statistics of (K=0.318, p<0.001), meanwhile statistically significant substantial agreement was observed between PCR and IVC by Cohen Kappa (K=0.727, p<0.001). Therefore, in vitro cultivation in Jones’ medium followed by Wheatley Trichrome staining of stool specimens should be used as a screening technique in the detection of Blastocystis sp. infections.

Keywords: Blastocystis detection; in vitro cultivation; microscopy; polymerase chain reaction; polyvinyl alcohol

INTRODUCTION
Blastocystis sp. is a single-celled protist which has worldwide distribution with higher prevalence rates in developing countries (Graczyk et al. 2005). Detection of Blastocystis sp. in humans is usually based on microscopic examination of stool samples (Eida & Eida 2008). Direct microscopy is usually performed with either in wet mount preparations or stained stool specimens. Stains used in Blastocystis sp. detection include Lugol’s iodine for wet mount preparations and permanent stains including Wheatley Trichrome, Giemsa, acid-fast and Field’s. Among the stains used, Wheatley Trichrome has been the most practiced in many clinical parasitology laboratories as it is found to be more sensitive in the detection of Blastocystis sp. and many other intestinal protozoa than wet mount preparations stained with Lugol’s iodine. Detection rate of Blastocystis sp. improves greatly by microscopy with Wheatley Trichrome stain or when high quality optics are applied (Hazem 1993; Lee 1991; Markell & Udkow 1990). In Malaysia, almost all microscopic techniques in Blastocystis sp. detection used either direct smear of stool, formalin ethyl acetate concentration, staining or...
Blastocystis sp. portrays pleomorphic forms including vacuolar, multivacuolar, amoeboid, cyst and granular which vary in sizes. However, it may be difficult to distinguish Blastocystis sp. in different forms from leucocytes or trophozoites or cyst of other protozoa. The pleomorphic forms of Blastocystis sp. poses identification and diagnostic problems (Eida & Eida 2008). Therefore, several diagnostic approaches should be used in order to get higher detection of Blastocystis sp. in stool specimens (Tan et al. 2008). A reliable diagnosis is important in choosing appropriate microscopic technique (van Lieshout & Roestenberg 2015). The aim of this study was to compare fixed-polyvinyl alcohol stool specimens followed by Wheatley Trichrome staining and in vitro cultivation of stool specimens in Jones’ medium followed by Wheatley Trichrome staining in the detection of Blastocystis sp. in reference to polymerase chain reaction. To the best of our knowledge, this will be the first study to performed comparison on both techniques.

**MATERIALS AND METHODS**

**SAMPLES**

Fresh stool samples obtained from the Orang Asli community in Temerloh, Pahang Malaysia were divided into three aliquots. Approximately 1/3 of the collected stool was subjected to in vitro cultivation (IVC) in Jones’ medium, another 1/3 was placed in polyvinyl alcohol (PVA) and the remaining 1/3 stool was stored at 0°C for molecular characterization of Blastocystis sp. by DNA extraction and polymerase chain reaction.

**MICROSCOPY-BASED TECHNIQUES**

**PVA** Stool specimens fixed in polyvinyl alcohol with the followings; 70% alcohol with several drops of Tincture iodine (10-15 min), 70% alcohol (1 min), 70% alcohol (1 min), Trichrome stain (8-10 min), Acid alcohol (2-4 s), 95% alcohol (1 min), absolute alcohol (1 min) and Wintergreen oil (5 min) (Salleh et al. 2012). Positivity was cross-checked by experienced laboratory technician.

**IVC** A pea-sized of the second portion of the fresh stool samples were inoculated into 3 mL Jones’ medium supplemented with 10% horse serum and incubated at 37°C for 5 days (Farah et al. 2014). Approximately 100 μL of the cultured-products were smeared on a cover slip and air-dried for 24 h. The smeared cover slip was then subjected to Wheatley Trichrome staining as mentioned before.

**MOLECULAR-BASED TECNIQUE (REFERENCE TECHNIQUE)**

DNA of Blastocystis sp. from the stool of Orang Asli were extracted using QIAamp®DNA stool minikit (Qiagen, Germany) according to the manufacturer’s protocol. Screening of all samples were carried out using primer complementary to the SSU rDNA sequence from the samples using BhRDr and Rh5 primers (Seclunia et al. 2006). PCR amplification was performed using Eppendorf Pro-S thermal cycler (Hamburg, Germany) by 30 cycles of initial denaturation at 95°C for five min, followed by denaturation at 95°C for one min, annealing at 63.3°C for one min and 30 s, extension at 72°C for one min and an additional cycle of 10 min chain elongation at 72°C. The PCR products were separated in 1.5% agarose gel. Positive PCR products were sent for sequencing to obtain subtypes information based on comparisons with the data available in Genbank using BLAST program (http://www.ncbi.nlm.nih.gov/BLAST).

**RESULT AND DISCUSSION**

Positivity of Blastocystis sp. using PVA and IVC was dependent upon the presence of any of the four morphologies (vacuolar, granular, cyst and amoeboid) (Figures 1 & 2). Meanwhile, sample was considered positive when there was a band at ~600 bp on the agarose gel and confirmed by sequencing (Figure 3). The prevalence rates of Blastocystis sp. in all examined stool specimens using three different diagnostic techniques were 35.6% (IVC), 20.0% (PVA) and 41.0% (PCR) (Table 1). Hundred seven-teen (61.3%) and forty one (21.5%) stool specimens from Orang Asli were negative by PVA and IVC, respectively, proved to be positive by PCR assay. However, single-round PCR was unable to amplify forty two (15.3%) stool specimens among Orang Asli which was initially diagnosed by microscopic diagnostic methods (PVA and IVC). Sequencing of 191 positive PCR-product of Orang Asli stool specimens showed Blastocystis ST1, ST2, ST3 and ST4.

Sensitivity, specificity, PPV and NPV of microscopic techniques used in the detection of Blastocystis sp. in comparison to single-round PCR were presented in Table 2. The sensitivity and specificity of PVA and IVC compared to the reference technique were 75.3% (95% CI: 65.2-83.6) and 68.5% (CI: 63.7-73.3) and 86.6% (CI: 82.7-93.0) and 86.3% (CI: 81.9-90.0), respectively. The agreement between the reference technique and PVA showed fair statistically significant agreement by Cohen Kappa statistics of $K=0.318, p<0.001$, meanwhile substantial statistically significant agreement was observed between PCR and IVC by Cohen Kappa ($K=0.727, p<0.001$).

Microscopy technique is the gold standard for the diagnosis of almost all intestinal protozoan infections. Microscopic techniques that usually used in parasitology laboratory for the identification of Blastocystis sp. include wet preparation and permanently stained of stool specimens. Among various known permanent stain used to aid in microscopic observation was Wheatley Trichrome. This stain is routinely used in many clinical microbiology laboratories and has shown to be more
FIGURE 1. Vacuolar form detected by PVA and stained with Trichrome in isolates from a diarrhoeic subject with large central vacuole (V), many nucleus (N) in the cytoplasm (C).

FIGURE 2. Amoeboid form of *Blastocystis* sp. detected using IVC and stained by Trichrome in isolates from the same diarrhoeic subject with many nucleuses at the rim of cytoplasm. P: pseudopod-like projection, N: nucleus, C: cytoplasm. This particular sample was positive for infection by *Blastocystis* sp. ST3 detected by PCR.

FIGURE 3. Results of the single-round PCR for DNA extracted from human stool samples.
sensitive in the detection of intestinal parasites than wet mount preparations stained with iodine (Gardner et al. 1980; Kellogg & Elder 1999).

However, despite of its wide usage in the diagnosis of Blastocystis sp., the microscopy technique has few disadvantageous; besides its low sensitivity, an unexperienced individual is unable to identify the great morphological diversity of this protozoa (vacuolar, granular, amoeboid and cyst) and to differentiate it with other protozoa, human cells and artefacts. The low numbers of this parasite in the stool specimens which reflected in low sensitivity of microscopy examination of direct stool smear can be improved by in vitro cultivation of stool specimens in culture medium. In vitro cultivation increases the detection rate and sensitivity of Blastocystis sp. in comparison to Wheatley Trichrome stained of stool smears (Roberts et al. 2011; Stensvold et al. 2007; Termmathurapoj et al. 2004). Of all the microscopy-related methods, xenic in vitro cultured-product (IVC) is considered the gold standard method for Blastocystis sp. detection (Poirier et al. 2011). IVC has been reported as more efficient in the detection of Blastocystis sp. than Wheatley Trichrome staining of direct stool smear although no significant difference between in vitro cultivation in Jones’ medium and Wheatley Trichrome staining were observed (Termmathurapoj et al. 2004). Culture medium of choice in this present study was Jones’ medium, a medium which has been widely used in many previous studies to cultivate Blastocystis sp. (Parkar et al. 2007; Stensvold et al. 2007).

In this present study, IVC in Jones’ medium followed by Wheatley Trichrome staining was not only descriptively able to detect more number of Blastocystis sp. infection, but this method also showed significantly higher detection rate of Blastocystis sp. infection in comparison to fixed-PVA stool specimens (PVA) followed by Wheatley Trichrome staining. The sensitivity of IVC and PVA followed by Wheatley Trichrome staining with PCR as reference method was 88.6% and 75.3%, respectively. The specificity of IVC was also higher (86.3%) than PVA (68.6%). Meanwhile, the negative predictive values of IVC suggested the detection of true blastocystosis-free was 93.2% in comparison to 91.8% with PVA. Meanwhile, the positive predictive value to determine the probability of having blastocystosis was 78.2% for IVC and 37.4% for PVA. Collectively, our present findings showed that IVC followed by Wheatley Trichrome stain was more superior in Blastocystis sp. detection in comparison to PVA followed by Wheatley Trichrome stain. However, no similar study has been performed before, therefore no confirmation can be warranted. Superiority of in vitro cultivation may have resulted from multiplication of Blastocystis sp. in the culture medium (Poirier et al. 2011). In addition to that, in this present study, Wheatley Trichrome stain was performed during the last day of

| TABLE 1. Comparison of different diagnostic techniques in the detection of Blastocystis sp. infections |
|-----------------------------------------------|
| Diagnostic techniques                           | Positive (%) | Negative (%) |
| Wheatley Trichrome staining of fixed PVA stool samples | 93 (20.0)   | 373 (80.0) |
| Wheatley Trichrome staining of IVC in Jones’ medium | 166 (35.6)  | 300 (64.4) |
| Single-round PCR followed by sequencing *       | 191 (41.0)  | 275 (59.0) |

*Specific for Blastocystis sp. subtypes: ST1, ST2, ST3, ST4

| TABLE 2. Sensitivity and specificity of PVA followed by Wheatley Trichrome staining and IVC followed by Wheatley Trichrome staining against the reference technique (single-round PCR) |
|-----------------------------------------------|
|                                           | PVA followed by staining | IVC followed by staining |
|                                           | Positive | Negative | Positive | Negative |
| Positive by PCR (n= 191)                     | 70      | 117      | 147      | 41      |
| Negative by PCR (n= 275)                     | 23      | 256      | 19       | 259     |
| Total                                        | 93      | 373      | 166      | 300     |

|                                           | Sensitivity (%) | Specificity (%) |
|                                           | 75.3            | 68.6            |
| 95% CI                                     | 65.2 – 83.6     | 82.7 – 93.0     |
|                                           | 95% CI          | 86.3            |
|                                           | 63.7 – 73.3     | 81.9 – 90.0     |
| PPV (%)                                    | 37.4            | 78.2            |
| NPV (%)                                    | 91.8            | 93.2            |
| Accuracy (%)                               | 70.0            | 87.1            |
| Kappa                                      | K=0.318         | K=0.727         |
| (p-value)                                  | p<0.001         | p<0.001         |
incubation of the cultured samples which may have aided in the detection of small size cysts or small vacuolar forms. Cysts of Blastocystis sp. are common in stool samples of infected patients, however due to their small size; this form is often missed during microscopy, hence leads to significant underestimation of Blastocystis sp. detection rate (Ree et al. 2009; Suresh & Smith 2004).

In the attempt to determine the epidemiology and dynamic transmission of Blastocystis sp. infection in a community, a highly sensitive detection method is required (Poirier et al. 2011). Polymerase chain reaction (PCR) has been identified as the most effective method in the detection of Blastocystis sp. infection (Roberts et al. 2011). In this present study PCR assay and PCR-product sequencing was used as a reference technique to detect and differentiate various Blastocystis sp. subtypes. The result showed 41.0% (191/466) of the specimens were positive for Blastocystis sp.; 70 specimens detected by PVA and 147 specimens detected by IVC were amplified by single round PCR and later differentiated into subtypes. In line with that, this present study concurred with the previous study findings since we detected the highest number of Blastocystis sp. infection using PCR technique (Parkar et al. 2007; Roberts et al. 2011). PCR was used as reference technique in this present study due to its high sensitivity; besides that the primary advantages of using this technique is the ability of this technique to discriminate different Blastocystis sp. subtypes. These findings are useful in the understanding of epidemiology and dynamic transmission of Blastocystis sp. in a community. However, despite of its sensitivity, several limitations including the needs of high-tech laboratory, expensive reagents and equipment, as well as labour-intensive of the DNA extraction tend to make other cheaper, conventional method such as microscopy and permanent stain still being practised (Meurs et al. 2017; Roberts et al. 2011).

In conclusion, this present study reaffirmed the need to perform IVC followed by Trichrome staining as the screening tools for the detection of Blastocystis sp. especially in community laboratories that are usually not equipped with the more sensitive but costly method, which is molecular technique (PCR). High specificity of IVC followed by Trichrome stain indicated that this technique is able to discriminate individuals infected with Blastocystis sp. and free-Blastocystis sp.. Implementation of a method with relatively high sensitivity and specificity at a low cost and easy to be performed makes this technique a reliable technique for screening of Blastocystis sp.. PCR showed high sensitivity and specificity in the identification of Blastocystis sp.; therefore, it potentially will become the 'gold standard' by which other diagnostic techniques are measured.

ACKNOWLEDGEMENTS
We gratefully acknowledge the Ministry of Rural and Regional Development Malaysia to permit this research to be conducted and Meteorological Department of Pahang for their technical support. This study was supported partly by the UKMMC Fundamental Research Grant (FF-2014-219) and UKM Publication Enhancement Grant (DLP-2014-013).

REFERENCES
Abdulsalam, A.M., Ithoi, I., Al-Mekhlafi, H.M., Khan, A.H., Ahmed, A., Surin, J. & Mak, J.W. 2012. Prevalence, predictors and clinical significance of Blastocystis sp. in Sebha, Libya. Parasites & Vectors 6: 86.
Eida, A.M. & Eida, M.M. 2008. Identification of Blastocystis hominis in patients with irritable bowel syndrome using microscopy and culture compared to PCR. Parasitology United Journal 1: 87-92.
Farah, H.M.T., Chandrawathani, P., Mohd Zain, S.N., Suresh, K., Hemalatha, C. & Premalaath, B. 2014. A preliminary study of Blastocystis sp. isolated from chicken in Perak and Selangor, Malaysia. Malaysian Journal of Veterinary Research 5: 21-25.
Gardner, B.B., Del Junco, D.J., Fenn, J. & Hengesbaugh, J.H., 1980. Comparison of direct wet mount and trichrome staining techniques for detecting Entamoeba species trophozoites in stools. Journal of Clinical Microbiology 12: 656-658.
Ghani, M.K.A. & Yusof, H. 2011. Blastocystis hominis: Kedahirannya di dalam sampel leses kanak-kanak Orang Asli di Pos Lenjang, Pahang, Malaysia. Sains Malaysia 40(10): 1123-1127.
Graczyk, T.K., Shiff, C.K., Tamang, L., Munsaka, F., Beitin, A.M. & Moss, W.J. 2005. The association of Blastocystis hominis and Endolimax nana with diarrheal stools in Zambian school-age children. Parasitology Research 98: 38.
Hanapian, Y.L., Mak, J.W. & Chen, P.C.Y. 2014. An intestinal parasitological survey among the Jehai Orang Aslis (aborigines) of the Temenggor forest, Perak state, Malaysia. International e-Journal of Science, Medicine & Education 8: 18-23.
Hazen, K.C. 1993. Controversial fungal and protozoan gastrointestinal infections. Current Opinion in Infectious Diseases 6: 77-82.
Ishak, S.A., Othman, H. & Sahani, M. 2008. A preliminary study of Blastocystis hominis in some development areas in Alor Gajah district, Melaka. Malaysian Journal of Health Sciences 6: 109-115.
Kellogg, J.A. & Elder, C.J. 1999. Justification for use of a single trichrome stain as the sole means for routine detection of intestinal parasites in concentrated stool specimens. Journal of Clinical Microbiology 37: 835-837.
Lee, M.J. 1991. Pathogenicity of Blastocystis hominis. Journal of Clinical Microbiology 29: 2089.
Markell, E.K. & Udkow, M.P. 1990. Association of Blastocystis hominis with human disease?. Journal of Clinical Microbiology 28: 1085.
Meurs, L., Polderman, A.M., Melchers, N.V.V., Brienlen, E.A., Verweij, J.J., Grooshojan, B., Mendes, F., Mechendura, M., Hepp, D.H., Langenberg, M.C. & Edelenbosch, R. 2017. Diagnosing polyparasitism in a high-prevalence setting in beira, Mozambique: Detection of intestinal parasites in faecal samples by microscopy and real-time PCR. PLOS Neglected Tropical Diseases 11: e0005310.
Parkar, U., Traub, R.J., Kumar, S., Munghin, M., Vitali, S., Leelayoova, S., Morris, K. & Thompson, R.C. 2007. Direct characterization of Blastocystis from faeces by PCR and evidence of zoonotic potential. Parasitology 134: 359-367.
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. *Journal of Clinical Microbiology* 49: 975-983.

Rene, B.A., Stensvold, C.R., Badsberg, J.H. & Nielsen, H.V. 2009. Subtype analysis of Blastocystis isolates from Blastocystis cyst excreting patients. *The American Journal of Tropical Medicine and Hygiene* 80: 588-592.

Roberts, T., Barratt, J., Harkness, J., Ellis, J. & Stark, D. 2011. Comparison of microscopy, culture, and conventional polymerase chain reaction for detection of Blastocystis sp. in clinical stool samples. *The American Journal of Tropical Medicine and Hygiene* 84: 308-312.

Salleh, F.M., Anuar, T.S., Yasin, A.M. & Moktar, N. 2012. Wintergreen oil: A novel method in Wheatley’s trichrome staining technique. *Journal of Microbiological Methods* 91: 174-178.

Scicluna, S.M., Tawari, B. & Clark, C.G. 2006. DNA barcoding of Blastocystis. *Protist* 157: 77-85.

Stensvold, C.R., Suresh, G.K., Tan, K.S.W., Thompson, R.C.A., Traub, R.J., Viscogliosi, E., Yoshikawa, H. & Clark, C.G. 2007. Terminology for Blastocystis subtypes - a consensus. *Trends in Parasitology* 23: 93-96.

Suresh, K. & Smith, H. 2004. Comparison of methods for detecting Blastocystis hominis. *European Journal of Clinical Microbiology & Infectious Diseases* 23: 509-511.

Tan, T.C., Suresh, K.G. & Smith, H.V. 2008. Phenotypic and genotypic characterisation of Blastocystis hominis isolates implicates subtype 3 as a subtype with pathogenic potential. *Parasitology Research* 104: 85-93.

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. *Parasitology Research* 93: 445-447.

van Lieshout, L. & Roestenberg, M. 2015. Clinical consequences of new diagnostic tools for intestinal parasites. *Clinical Microbiology and Infection* 21: 520-528.

Samseh Abdullah Noradilah*
Department of Medical Sciences II
Faculty of Medicine and Health Sciences
Universiti Sains Islam Malaysia
55100, Pandan Indah, Kuala Lumpur, Federal Territory
Malaysia

Norhayati Moktar
Department of Pre-Clinical Sciences
Faculty of Medicine and Health Sciences
Universiti Tunku Abdul Rahman, Sungai Long Campus
43000 Kuala Kertajaya, Penang
Malaysia

Li Lee
Kulliyyah of Medicine and Health Sciences
Sultan Abdul Halim Mu’adzam Shah International Islamic University
09300 Kuala Ketil, Kedah Darul Aman
Malaysia

Fatmah Md Salleh
Department of Parasitology and Medical Entomology
Faculty of Medicine
Universiti Kebangsaan Malaysia Medical Centre
Jalan Yaacob Latif, Bandar Tun Razak
56000 Cheras, Kuala Lumpur, Federal Territory
Malaysia

Tengku Shahrul Anuar
Centre of Medical Laboratory Technology
Faculty of Health Sciences
Universiti Teknologi MARA, Puncak Alam Campus
42300 Bandar Puncak Alam, Selangor Darul Ehsan
Malaysia

Tengku Shahrul Anuar
Integrative Pharmacogenomics Institute
Universiti Teknologi MARA, Puncak Alam Campus
42300 Bandar Puncak Alam, Selangor Darul Ehsan
Malaysia

*Corresponding author; email:noradilah82@gmail.com

Received: 8 February 2018
Accepted: 14 May 2018