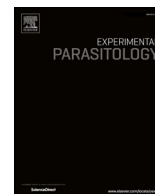




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# Establishing a protocol for water sample processing for the detection of *Blastocystis* sp.



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## ABSTRACT

This study was aimed at establishing a protocol for water sample processing for the detection of *Blastocystis* sp. using distilled water spiked with *Blastocystis* sp. cysts.

The study established a protocol involving eight technical aspects, namely, storage temperature, storage duration, minimum water sample volume, optimum relative centrifugal force, centrifugation duration, minimum number of cyst for inoculation in Jones' medium and turn-around-time for the detection of vacuolar forms of *Blastocystis* sp. Results showed a minimum of 1.0 L water sample should be collected and processed on the same day. Otherwise, it should be stored at 4 °C and processed within 3 days. Water sample should be centrifuged at 1400 × g for 10 min. For the isolation of *Blastocystis* sp. cysts, parasite pellet could be layered on top of Ficoll-Paque™ PLUS, centrifuged at 1400 × g for 20 min and washed twice using 0.9% saline with centrifugation at 1400 × g for 10 min. A minimum of 1 × 10<sup>5</sup> cysts could then be inoculated in Jones' medium supplement with 10% horse serum, incubated at 37 °C and examined for any presence of vacuolar forms of *Blastocystis* sp. after 3 days of inoculation. A protocol for water sample processing for the detection of *Blastocystis* sp. has successfully been established. The protocol was validated using 106 various water samples. This protocol will be very useful in determining the extent of *Blastocystis* sp. contamination in water sources in order to identify the seriousness of contamination.

## 1. Introduction

*Blastocystis* sp. is a commonly found intestinal parasite in any faecal survey. It exists as vacuolar, granular, multivacuolar, amoeboid and cyst forms (Tan, 2008a). Cyst is the transmissible stage of *Blastocystis* sp. in the environment (Tan, 2008b) and excyst to form vacuolar stages that are usually seen during *in vitro* cultures. Granular form of the parasite is usually detected in older culture (Tan, 2004). It is morphologically similar to vacuolar forms with an extra feature; the presence of granules in the central vacuole. Amoeboid form has one or more non-locomotive pseudopods. It has been reported in old or drug-treated cultures, colony growth (Zierdt, 1973; Tan et al., 1996) and in the culture of *Blastocystis* sp. isolates obtained from symptomatic patients (Tan and Suresh, 2006). This commonly found parasite in faecal samples has been detected using *in vitro* cultivation (Suresh and Smith, 2004; Termmathurapoj et al., 2004; Suresh et al., 2005), direct smear (Suresh and Smith, 2004; Termmathurapoj et al., 2004; Suresh et al., 2005), concentration technique (Suresh and Smith, 2004; Suresh et al., 2005) and or polymerase chain reaction (Termmathurapoj et al., 2004).

*Blastocystis* sp., one of the neglected waterborne parasitic protozoa, has been detected recently in water sources such as waste water (Suresh et al., 2005; Banaticla and Rivera, 2011), portable water tank from River Nile (Elshazly et al., 2007), drinking water (Leelayoova et al., 2008), tap water (Eroglu and Koltas, 2010), river (Mora et al., 2010; Ithoi et al., 2011), ponds (Khalifa et al., 2014) and drinking water treatment plant (Richard et al., 2016). However, these studies used different water processing methods as summarised by Plutzer and Karanis (2016) to obtain water concentrate that may contain *Blastocystis* sp. without a standardized protocol. Therefore, there is an absence of standard protocol for the detection of *Blastocystis* sp. in water sources unlike *Cryptosporidium* sp. whereby the standard detection protocol has been established (United States Environmental Protection Agency Method 1622; United States Environmental Protection Agency Method 1623). Hence, with the inclusion of *Blastocystis* sp. as a waterborne parasite by World Health Organization (WHO), as suggested by Suresh and Smith, in Waterborne Zoonoses - Identification, Causes, and Control, it recognises the possible threat that *Blastocystis* sp. may pose to the environment and humans. *Blastocystis* sp. has also been

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confirmed to exhibit low infectious dose; 10 cysts can potentially cause an infection (Yoshikawa et al., 2004). Therefore, there is an urgent need to establish a standard protocol for water sample processing for the detection of *Blastocystis* sp.

At present, relative centrifugal force and its centrifugation duration for *Blastocystis* sp. and its isolation using Ficoll-Paque has been reported (Zaman, 1996). However, there is no information on other technical aspects. Therefore, this study was aimed at establishing a standard protocol for water sample processing for the detection of *Blastocystis* sp. focussing on eight technical aspects, namely, optimum relative centrifugal force, centrifugation duration, optimum storage temperature, storage duration, minimum water sample volume, relative centrifugal force and centrifugation duration for Ficoll-paque procedure, minimum number of cyst for inoculation in Jones' medium and turn-around-time for the detection of vacuolar forms of *Blastocystis* sp. All these were determined using distilled water spiked with *Blastocystis* sp. cysts.

## 2. Materials and methods

The same eight technical aspects were assessed using distilled water spiked with *Blastocystis* sp. cysts isolated from faecal materials from *Blastocystis* sp. infected monkeys. Each technical aspect was determined using a series of triplicates. Trypan blue dye exclusion count (Strober, 2001) was carried out to assess the recovery rate of viable *Blastocystis* sp. cysts.

### 2.1. Isolation of *Blastocystis* sp. from the faecal of infected monkeys

A pea size of faecal sample was cultured in Jones' medium and examined after 24 h for vacuolar forms of *Blastocystis* sp. The samples were then dissolved in 0.9% saline, filtered using gauze. Filtrate that contained *Blastocystis* sp. cysts was collected in a centrifuge tube and topped up to 14 ml using 0.9% saline. It was then centrifuged at  $1400 \times g$  for 10 min at room temperature. The supernatant was discarded; pellet containing *Blastocystis* sp. cysts was resuspended with five ml of 0.9% saline and layered on top of five ml of Ficoll-Paque™ PLUS (General Electric Healthcare Life Sciences, USA). It was centrifuged at  $1400 \times g$  for 20 min at room temperature. The top two layers that contained *Blastocystis* sp. cysts were transferred to a new centrifuge tube and topped up to 14 ml with 0.9% saline before centrifuging at  $1400 \times g$  for 10 min. This procedure was repeated twice before the supernatant was discarded and pellet was resuspended in one ml PBS. Fig. 1 showed *Blastocystis* sp. cysts isolated from rhesus monkeys.

An equal volume of cyst suspension and trypan blue were mixed for

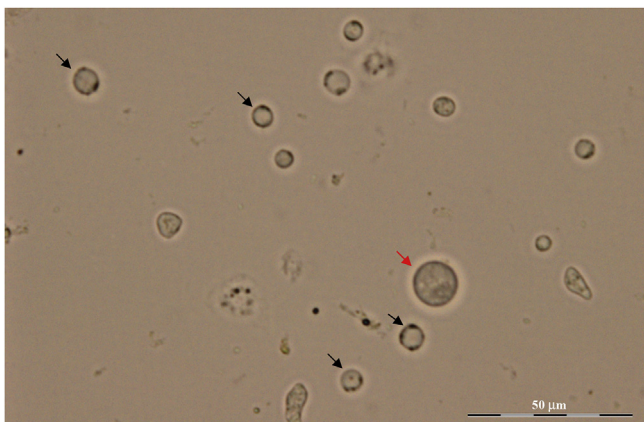


Fig. 1. Bright field image of isolated *Blastocystis* sp. cysts (black arrow) and vacuolar forms (red arrow) from rhesus monkey. Note that *Blastocystis* sp. cysts contained more than 1 nucleus and are smaller than  $10 \mu\text{m}$ . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

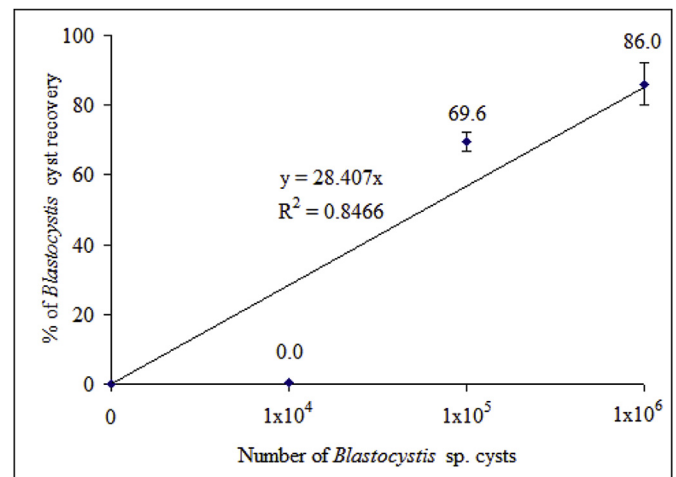


Fig. 2. A standard curve for the determination of the number of *Blastocystis* sp. cysts.

1 min. The mixture ( $10 \mu\text{l}$ ) was loaded on the chamber of a haemocytometer for counting at 400X magnification using an up-right microscope (CK40, Olympus, Japan).

### 2.2. Determination of number of *Blastocystis* sp. cysts

Number of *Blastocystis* sp. cysts was determined using haemocytometer with trypan blue dye exclusion method whereby  $1 \times 10^4$ ,  $1 \times 10^5$  and  $1 \times 10^6$  cysts were counted and seeded in 1 ml of distilled water, in a set of triplicates. The recovery rate was counted using haemocytometer. The preferred concentration was  $1 \times 10^5$  *Blastocystis* sp. cysts (Fig. 2). Therefore, eight technical aspects were assessed using distilled water spiked with  $1 \times 10^5$  *Blastocystis* sp. cysts. Distilled water is chosen as it has the most standard components while other water sources will have its array of contaminants that will potentially interfere with the results.

### 2.3. Determination of relative centrifugal force (RCF) to obtain parasite pellet

Spiked distilled water were centrifuged at various relative centrifugal forces (RCF) such as 500, 1000, 1400 and  $1900 \times g$  for 10 min and enumerated for number of cysts recovered and expressed as percentage of cyst recovery.

### 2.4. Determination of centrifugation duration

Spiked distilled water were centrifuged at  $1400 \times g$  for various centrifuge durations such as 5, 10, 15 min. The cysts obtained were enumerated for number of cysts recovered and expressed as percentage of cyst recovery.

### 2.5. Determination of storage duration and temperature ( $^{\circ}\text{C}$ )

Spiked distilled water was stored at room temperature ( $28\text{--}30^{\circ}\text{C}$ ) and  $4^{\circ}\text{C}$ . The samples incubated at room temperature and  $4^{\circ}\text{C}$ , each were centrifuged for 10 min at  $1400 \times g$  and enumerated for number of cysts recovered on day 0, 3, 5 and 7 for the percentage of cyst recovery.

### 2.6. Determination of minimum volume of water sample

Spiked distilled water with various volumes (0.5, 1.0, 5.0 and 10.0 L) were centrifuged for 10 min at  $1400 \times g$  and enumerated for number of cysts recovered and expressed as percentage of cyst recovery.

### 2.7. Determination of relative centrifugal force (RCF) for the isolation of purified *Blastocystis* sp. cysts

In the experiment that focus on the isolation of *Blastocystis* sp. cysts, one ml of  $1 \times 10^5$  *Blastocystis* sp. cysts were layered on five ml of Ficoll-Paque™ PLUS centrifuged at various RCFs such as 500, 1000, 1400 and  $1900 \times g$  for 20 min, washed twice with 0.9% saline and enumerated for number of cysts recovered and expressed as percentage of cyst recovery.

### 2.8. Determination of centrifugation duration for the isolation of *Blastocystis* sp. cysts

In the experiment that focus on the isolation of *Blastocystis* sp. cysts,  $1 \times 10^5$  *Blastocystis* sp. cysts were layered on five ml of Ficoll-Paque™ PLUS centrifuged at  $1400 \times g$  for various centrifuge durations such as 5, 10, 20 and 30 min, washed twice with 0.9% saline and enumerated for number of cysts recovered and expressed as percentage of cyst recovery.

### 2.9. Determination of inoculation number of *Blastocystis* sp. cysts

A total of 15 tubes containing various numbers of *Blastocystis* sp. cysts ( $1 \times 10^2$ ,  $1 \times 10^3$ ,  $1 \times 10^4$ ,  $1 \times 10^5$ ,  $1 \times 10^6$ ) in Jones' medium, supplemented with 10% horse serum were incubated at  $37^\circ\text{C}$  and examined for vacuolar forms of *Blastocystis* sp. for the next three consecutive days.

### 2.10. Determination of turn-around-time for the detection of vacuolar forms of *Blastocystis* sp.

Jones' medium spiked with *Blastocystis* sp., supplemented with 10% horse serum, incubated at  $37^\circ\text{C}$  and examined for vacuolar forms of *Blastocystis* sp. for five consecutive days.

### 2.11. Calculation and statistical analysis

Cyst recovery in samples spiked with *Blastocystis* sp. cysts were expressed in percentage of cyst recovery. For the determination of inoculation number of *Blastocystis* sp. cysts and turn-around-time for the examination of vacuolar forms of *Blastocystis* sp., the number of vacuolar forms of the parasite were enumerated and expressed as number of vacuolar forms of *Blastocystis* sp. per field. The results were presented as median with range and analyzed using one-way ANOVA test.

### 2.12. Validation of the established protocol

A total of 106 various water samples (31 river, 12 lake, 13 tap and 50 bottled drinking water), 1.0 L each, were collected and subjected to the established protocol for the determination of *Blastocystis* sp. The results were presented as the percentage of occurrence of vacuolar forms of *Blastocystis* sp.

## 3. Results

### 3.1. Determination of relative centrifugal force (RCF) to obtain parasite pellet

There is a linear relationship between the percentage of *Blastocystis* sp. cyst recovery and relative centrifugal force (RCF). As RCF increased, the percentage of cyst recovery increased (Fig. 3a). The highest *Blastocystis* sp. cyst recovery range was 62.4–74.0%. It was significant as compared to percentage of *Blastocystis* sp. cyst recovery range at  $500 \times g$  with  $p < 0.05$ . However, the percentage of *Blastocystis* sp. cyst recovery range decreased to 41.6–45.2% at  $1900 \times g$ . Therefore,  $1400 \times g$  is the optimum RCF to obtain parasite pellet for the detection of *Blastocystis* sp., which was used for the subsequent experiments.

### 3.2. Determination of centrifugation duration to obtain parasite pellet

The percentage of *Blastocystis* sp. cyst recovery increased from 48.0 to 57.6% at 5 min to 62.4–74.0% at 10 min (Fig. 3b). However, it decreased to 48.0–57.6% and 38.4–51.2% for 15 min and 20 min of centrifugation, respectively. The optimum RCF duration for the detection of *Blastocystis* sp. in water sample was determined as 10 min as it was significant ( $p < 0.05$ ) as compared to 20 min of centrifugation.

### 3.3. Determination of storage duration and temperature

Results showed that *Blastocystis* sp. cysts were detected in all samples under different storage temperature and duration (Fig. 3c). There was a decline of *Blastocystis* sp. cysts in all samples (day 3, 5 and 7) incubated at room temperature and a significant difference was detected between day 0 and 7 with  $p < 0.05$ . Day 0 being sample collection day and therefore, containing the highest number of *Blastocystis* sp. cysts. Similar declining pattern was also observed in samples incubated at  $4^\circ\text{C}$  with a significant difference observed between day 0 and 7 with  $p < 0.05$ . The decrease of *Blastocystis* sp. cyst in the samples may be due to degradation of cyst over time regardless of temperature. As a result of this experiment, it is proposed that all water samples collected should be processed on the same day. However, whenever circumstances do not allow, it is suggested that all samples must be stored at  $4^\circ\text{C}$  and processed within 3 days to achieve more than 50.0% cyst recovery.

### 3.4. Determination of minimum volume of water sample

It is observed that the higher the volume of the sample led to the lower percentage of cyst recovery (Fig. 3d). But, there was not much difference observed in the percentage of *Blastocystis* sp. cyst recovery between 0.5 and 1.0 L. Therefore, either one of these volumes may be suitable as the minimum water volume for sample collection. Hence, it is recommended that 1.0 L of water sample should be collected as our working volume due to its shorter time spent for sample processing for the detection of *Blastocystis* sp.

### 3.5. Determination of relative centrifugal force (RCF) for the isolation of purified *Blastocystis* sp. cysts

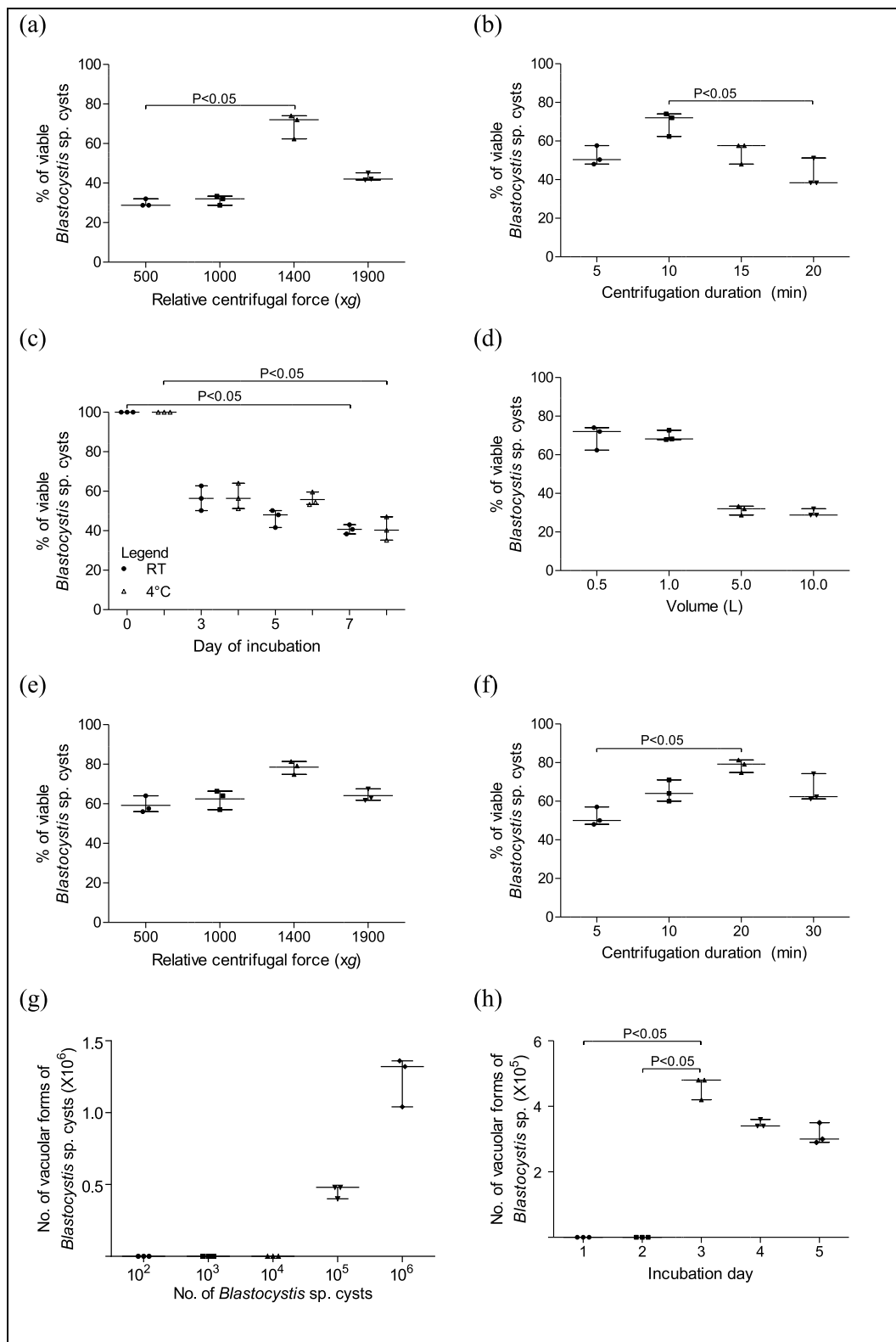
There is a mild bell shape graph observe for percentage of *Blastocystis* sp. cyst recovery and relative centrifugal force (RCF). As RCF increased, the percentage of cyst recovery increased but it decreased after  $1400 \times g$  (Fig. 3e). The optimum RCF was determined to be as  $1400 \times g$  for the maximum recovery of *Blastocystis* sp. cyst at 74.9–81.4%. Therefore,  $1400 \times g$  is the optimum RCF the isolation of *Blastocystis* sp. cysts, which was used for the subsequent experiments.

### 3.6. Determination of centrifugation duration for the isolation of purified *Blastocystis* sp. cysts

The percentage of *Blastocystis* sp. cyst recovery for the isolation of purified *Blastocystis* sp. cysts was peak at 20 min (Fig. 3f). However, it decreased to 61.2–74.3% at 30 min of centrifugation. There was a significant difference between the percentage of *Blastocystis* sp. cyst recovery at 5 min and 20 min ( $p < 0.005$ ). Therefore, centrifugation at  $1400 \times g$  for 20 min is the optimum duration for the isolation of purified *Blastocystis* sp. cysts in water sample.

### 3.7. Determination of inoculation number of *Blastocystis* sp. cysts

After various numbers of *Blastocystis* sp. cysts were inoculated in Jones' medium, vacuolar forms of *Blastocystis* sp. were counted after 24 h. It was reported that there was absence of vacuolar forms of *Blastocystis* sp. in culture inoculated with  $1 \times 10^2$ ,  $1 \times 10^3$  and  $1 \times 10^4$



**Fig. 3.** Determination of eight technical aspects in water sample processing for the detection of *Blastocystis* sp. in spiked distilled water. Determination of (a) optimum relative centrifugal force (RCF) for parasite pellet, (b) centrifugation duration for parasite pellet, (c) storage temperature and duration, (d) minimum volume, (e) optimum relative centrifugal force (RCF) for isolating purified cysts, (f) centrifugation duration for isolating purified cysts, (g) inoculation number of *Blastocystis* sp. cysts, (h) turn-around-time for the detection of vacuolar forms of *Blastocystis* sp. Data represents the median with range of three replicates. Data was analysed using one-way ANOVA test.



*Blastocystis* sp. cysts (Fig. 3g). However, vacuolar forms of *Blastocystis* sp. were detected in cultures inoculated with  $1 \times 10^5$  and  $1 \times 10^6$  of *Blastocystis* sp. cysts with the number of vacuolar forms of *Blastocystis* sp. determined as  $4.0\text{--}4.8 \times 10^5$  and  $10.4\text{--}13.6 \times 10^5$  respectively. Therefore, it indicated that  $1 \times 10^5$  is the minimum number of *Blastocystis* sp. cysts must be present to initiate a parasite growth in the culture for positive detection of vacuolar forms of *Blastocystis* sp. This amount of *Blastocystis* sp. cysts was used in the subsequent experiment for the determination of turn-around-time.

3.8. Determination of turn-around-time for the detection of vacuolar forms of *Blastocystis* sp.

Vacuolar forms of *Blastocystis* sp. were not detected on day 1 and 2 but on day 3 onwards whereby the parasite number started to decrease on day 4 and 5 from  $4.2\text{--}4.8 \times 10^5$  to  $3.4\text{--}3.6 \times 10^5$  and  $2.9\text{--}3.6 \times 10^5$ , respectively (Fig. 3h). There were similar significant differences observed between day 1 and 3, and day 2 and 3 ( $p < 0.05$ ). Since the first detected vacuolar forms of *Blastocystis* sp. were on day 3 onwards, it is determined as the day for examination of sample for *Blastocystis* sp., also known as the turn-around-time for the detection of vacuolar forms of *Blastocystis* sp.

3.9. Validation of the established protocol

Amongst the four types of water samples, vacuolar forms of *Blastocystis* sp. were only detected in river water samples (Fig. 4) with an occurrence of 45.2% (14/31) as shown in Table 1.

4. Discussion

Many attempts were carried out to collect faecal materials from patients infected with *Blastocystis* sp., however, the supply of positive *Blastocystis* faecal samples from infected patients were not constant for us to mount studies on cysts. Moreover, the positive faecal samples collected were sporadically available and yielded low numbers of *Blastocystis* cysts. Therefore, faecal samples were collected from the same long-tailed macaque (*Macaca fascicularis*) infected with *Blastocystis* sp. with unknown subtype, which were housed near our institution for a regular and constant high supply of cysts. A study had indicated that the cytology (which includes structure, function and chemistry) and ultrastructure of *Blastocystis* sp. isolated from monkeys are comparable to that found in the faecal samples of humans (McClure et al., 1980). Another study reported that cysts of *Blastocystis* sp. isolated from humans and monkeys are similar except variations in size and contents of vacuoles for vacuolar forms of the parasite (Yamada et al., 1987). Therefore, studies using cysts collected from monkeys' faecal materials at best for practical reasons could be a substitute for

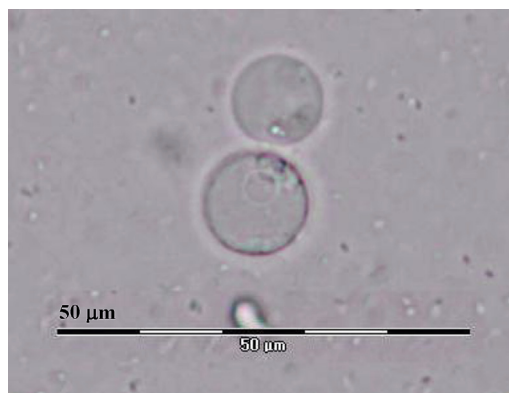


Fig. 4. Vacuolar forms of *Blastocystis* sp. detected in river water samples at 400X magnification using bright field microscopy, BX51 Olympus, Japan.

Table 1

The percentage of occurrence of vacuolar forms of *Blastocystis* sp. in various water samples.

Water source	n/N, (% of occurrence)
River	14/31 (45.2)
Lake	0/12 (0.0)
Tap	0/13 (0.0)
Bottled drinking water	0/50 (0.0)
	14/106 (13.2)

n = number of samples examined positive for *Blastocystis* sp.  
N = number of samples collected.

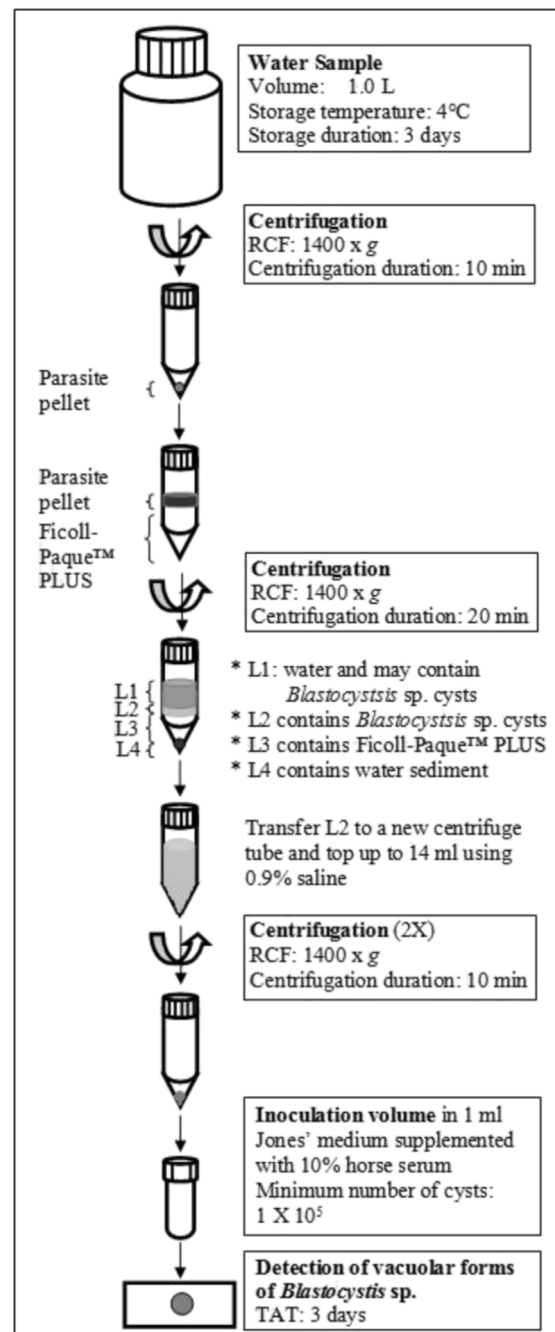


Fig. 5. A summary of the standard protocol for the detection of *Blastocystis* sp. from water sample. Note: RCF is relative centrifugal force; TAT is turn-around-time for the detection of vacuolar forms of *Blastocystis* sp.

*Blastocystis* sp. cysts from humans.

## 5. Conclusion

This is the first study to comprehensively optimize the technical aspects for water sample processing using centrifugation for the detection of *Blastocystis* sp. in water sources as outline in Fig. 5. In short, 1.0 L water sample should be collected and processed on the same day. Otherwise, it should be stored at 4 °C and processed within 3 days. Water sample should be centrifuged at 1400 × g for 10 min. For the isolation of *Blastocystis* sp. cysts, parasite pellet is layered on top of Ficoll-Paque™ PLUS, centrifuged at 1400 × g for 20 min and washed twice using 0.9% saline with centrifugation at 1400 × g for 10 min. A minimum of  $1 \times 10^5$  *Blastocystis* sp. cysts could then be inoculated in Jones' medium supplement with 10% horse serum, incubated at 37 °C and examined for any presence of vacuolar forms of *Blastocystis* sp. after 3 days of inoculation.

The method has been validated when it was applied to 106 water samples. It is a very cost-effective method to process the minimum volume of 1.0 L of water sample which can easily be carried out in most laboratories especially in developing nations using a simple centrifuge. The present proposal of a qualitative detection protocol, provides a framework for future studies to further adapt, modify and optimize the detection protocol for *Blastocystis* sp. in water sources such as drinking and recreational water.

## Conflicts of interest

There is no conflict of interest declared.

## Funding

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.exppara.2019.01.007>.

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