

***Blastocystis* spp. contaminated water sources in aboriginal settlements**

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Abstract. *Blastocystis* has been increasingly reported in water bodies. However, lack of studies to determine the presence of *Blastocystis* in water used by the aborigines in Malaysia has led to the birth of this research. This study was therefore aimed to determine the occurrence of *Blastocystis* in water samples in aboriginal settlements in Pahang, Malaysia. Water samples collected from seven sampling points of two rivers and other water sources in the villages were subjected to filtration and cultivation followed by trichrome staining. The trichrome stained slides were observed microscopically under 1000X magnification for the presence of *Blastocystis*. River samples were also measured for physicochemical parameters. From this study, 42.9% of the river water and 6.25% of other water samples were positive for *Blastocystis*. All river samples showed presence of *Escherichia coli* and *Enterobacter aerogenes*, indicating faecal contamination. Statistical analysis showed *Blastocystis* occurrence in the river were significantly correlated conductivity, turbidity, chemical oxygen demand (COD), total dissolved solid (TDS), concentration of sulfate and faecal coliforms. The river water used by the aborigines is a probable source for *Blastocystis* transmission in this community. Therefore, protection of the river from organic material and faecal contaminations are highly required in order to control the contamination by *Blastocystis*.

INTRODUCTION

Blastocystis spp., an anaerobic polymorphic parasite which causes gastrointestinal symptoms in humans is playing an increasingly important role in contaminating water sources since it has been detected in sewage treatment plants and drinking water. This has led to the inclusion of the detection of *Blastocystis* spp. in WHO Guidelines for Drinking-water Quality (WHO, 2011). New

Zealand for example has included *Blastocystis* spp. in its Guidelines for Drinking-water Quality Management where *Blastocystis* spp. should not be present in its drinking water (DWSNZ, 2013). The Australian Drinking Water Guidelines recommended and prioritize prevention of water contamination from humans and animals waste (NHMRC & NMRCC, 2011). Water treatment process may be effective in removing microorganisms, however, studies

on *Giardia* spp. and *Cryptosporidium* spp. showed that they successfully evade the filter barriers in the absence or deficiencies of water treatment (Karaniš *et al.*, 1998). However, the ability of *Blastocystis* spp. to survive during water treatment process has yet to be studied. The potential for environmental contamination with *Blastocystis* spp. depend upon a variety of factors; the burden of this infection in human and nonhuman hosts, human behaviour and activity, sanitation, seasonal influence and climate.

In Malaysia, *Blastocystis* spp. has been detected in recreational water from Sungai Batu and Sungai Congkak, with an average detection rate of 22.1% and 33.3%, respectively (Ithoi *et al.*, 2011). *Blastocystis* spp. cysts have also been isolated from rivers and lakes around Klang Valley, Malaysia (Suresh *et al.*, 2009). Based on a study in Malaysia, it was found that children with no access to piped water supply are more susceptible to *Blastocystis* spp. infection (Abdulsalam *et al.*, 2012). A similar finding was also reported in a study carried out among three different aboriginal tribes (the Proto-Malay, Negrito and Senoi); the finding indicated that drinking untreated water was a significant risk factor of blastocystosis (Anuar *et al.*, 2013). To the best of our knowledge, there was only single study on the occurrence of *Blastocystis* spp. in water from rivers and other sources used by the aboriginal community in Malaysia, therefore, this research was performed to provide more information on that. From our observations, the rivers in this study were also frequented by other communities from other villagers and places for bathing during shortage of water supply and fishing. Therefore, the chances of further spread of infection from the contaminated rivers to wider areas were likely to occur. It is hoped that the finding of this study will benefit the communities and public health authorities to initiate prevention and control program in the acquisition of *Blastocystis* spp. infection and other water-borne related infections.

MATERIALS AND METHODS

Study areas

Water sampling was conducted in Sungai Krau, a river which flows along the Malay and aboriginal dwellings in Temerloh, Pahang. There are many villages situated along the river; Malay villages are the most upstream followed by aboriginal villages namely Kampung Terbol, Kampung Pian, Kampung Lubok Wong, Kampung Pasu and Kampung Penderas. Malay villages and Kampong Terbol are located near to the headwater area and the natural water sources from the hill. There are 245 people living in Kampung Terbol occupying 120 hectares of land. Kampung Lubok Wong is located at the midstream of Sungai Krau. It is populated with 268 people and living in a land area of 152.73 hectares. Both villages are not equipped with safe tap water supply; the villagers build up their own piping system direct from the hilly areas to their villages. Kampung Penderas is located at the downstream of Sungai Krau. Kampung Penderas is the most crowded area with 852 residents living in a land area of 255.15 hectares. Besides safe tap water supply, aborigines residing in Kampung Penderas also use the river for their daily activities. Aborigines used Sungai Krau for bathing, washing, and fishing as well as for open defecation. Besides Sungai Krau, Sungai Lompat which flows and meets Sungai Krau in Kampung Penderas was also included in this study.

Sampling sites and collection of water samples

The water samples were collected in three remote aboriginal settlements in Temerloh, Pahang, Malaysia commencing from October 2014 to November 2014. The aboriginal settlements were endemic for intestinal parasitic infection (Anuar *et al.*, 2013; Anuar *et al.*, 2012a, Anuar *et al.*, 2012b, Anuar *et al.*, 2014). Permission to conduct the sampling has been obtained from the Ministry of Rural and Regional Development Malaysia, reference number : JAKOA/PP.30.032Jld29(04).

Water from the river flowing along the three villages which is known as Sungai Krau were collected from the middle of the river. Six sampling points of the river were determined; K1 (1000 meters before Kampung Terbol, 3.83507°, 102.21404°), K2 (in the middle of Kampung Terbol, 3.81314°, 102.22804°), K3 (1000 meters before Kampung Lubok Wong, 3.78516°, 102.23596°), K4 (in the middle of Kampung Lubok Wong, 3.77014°, 102.23763°), K5 (1000 meters before Kampung Penderas, 3.74364°, 102.27091°) and K6 (in the middle of Kampung Penderas, 3.71301°, 102.28753°). Ten litres of water from each sampling points were collected. Water sampling was also carried out in one sampling point of Sungai Lompat (3.71259, 102.28839). One thousand and five hundred mL of water from each of the six sampling points and Sungai Lompat were collected for faecal coliforms count.

One thousand five hundred mL of water other than river were collected from all available sources within the aboriginal dwellings including untreated tap water and rain water storage tanks (Life Saver) in Kampung Terbol, untreated tap water, water stored in a moderately clean, uncovered container and fish pond in Kampung Lubok Wong and treated tap water, wells and stored water in a clean, uncovered container inside the houses and stored water left outside a house in an uncovered container in Kampung Penderas. No preservatives were added to the water samples and all were brought back to Community Laboratory in the Department of Parasitology and Medical Entomology, Faculty of Medicine, Universiti Kebangsaan Malaysia Medical Centre.

Physicochemical parameter of river water samples

Using a multiparameter (Hanna, USA, model HI 9829), data on pH, conductivity, temperature, salinity, dissolved oxygen, and total dissolved solid were recorded at each sampling point at Sungai Krau (K1-K6) and a point at Sungai Lompat. Turbidity was measured using microprocessor turbidity meter (Hanna, USA, model HI 93703). Chemical oxygen demand (COD) and sulfate concentrations were measured using a

colorimeter (Thermo Scientific, Singapore, model Orion AQ4000). Total chlorine was measured using a multiparameter bench photometer (Hanna, USA, model HI 83200). The results of all parameters were recorded for correlation analysis with the positively stained slides from in vitro cultivation of *Blastocystis* spp.

Examination of *Blastocystis* spp.

Ten liters of the water samples from each station of the river and one thousand five hundred mL of water samples collected from various other sources at the aboriginal settlements in the study area were filtered through a 1.2 µm pore size and 14 cm diameter mixed cellulose esters (MCE) membrane filter using flatbed membrane filtration system (Masterflex I/P, Millipore, model XX80EL230). The water concentrate from the filtration was resuspended and 100 µl was inoculated in 3 ml of Jones' medium supplemented with 10% horse serum and incubated at 37°C until day five. Three replicates of inoculation were carried out for each water sample. Positive and negative controls were repeated in triplicate by inoculation with *Blastocystis* spp. and PBS respectively.

One hundred µl of the samples in culture were fixed with 100 µl of polyvinyl alcohol (PVA) and smeared on a cover slip and air-dried for 24 hours. The smeared slips were then subjected to Wheatley's trichrome staining, mounted on the glass slide and examined under compound microscope under 1000X magnification (Nikon eclipse E100). Slides with *Blastocystis* spp. in any forms (vacuolar, amoeboid, granular and cyst) were recorded as positive.

Assessment of faecal coliforms

River water samples from all sampling points were filtered through 0.45 µm, 5cm diameter nitrocellulose membrane filter. The membrane filter was then transferred onto membrane lactose glucuronide agar (MLGA) and incubated at 37°C for 24-36 hours. Agar were observed daily for three days for the presence of faecal coliforms. Colonies were counted and indicated by colours: green for *Escherichia coli* and yellow for *Enterobacter*

aerogenes. The results were expressed in number of colonies in every 100 ml water sample.

Statistical analysis

Spearman's rho analysis was carried out using a statistical software package (SPSS version 22). Correlations were considered statistically significant at *p*-value of 0.05.

RESULTS

Physicochemical parameters of river water sample

Physicochemical parameters of the river water were measured to determine any association of the occurrence of *Blastocystis* sp. with the water parameters. The mean pH was 6.75±0.08 and the lowest pH was recorded in water sample collected in K4. The conductivity reading ranged from 32.00-42.00 µS/cm. The lowest reading was recorded in K6. Temperature ranged from 24.24-26.55°C with the lowest in K2. Salinity ranged from 0.001-0.002% and turbidity reading ranged from 3.00-75.00 NTU with highest recorded in K1 while dissolved oxygen (DO) reading ranged from 5.85-14.9 mg/L. Chemical oxygen demand (COD) reading ranged from 214.75-531.89 mg/L. High COD was recorded in K1. Total dissolved solid (TDS) ranged from 16.00-21.00 NTU with the highest recorded in K1. The total chlorine reading ranged from 0.03-0.29 mg/L

and the concentration of sulfate ranged from 2.40-34.20 mg/L with the highest measured in K1.

Examination of *Blastocystis* spp. in Jones' medium and assessment of faecal coliforms

Culture tubes of water samples taken in K1, K5 and K6 were positive for *Blastocystis* spp. (Table 1). Faecal coliforms were significantly high in K1, K5 and K6 as compared to water samples in K2, K3 and K4. Mean ± SE of the faecal coliforms in the water from all sampling points was 0.55 ± 0.28 x 10⁶. Faecal coliforms ranged from 0.01 to 2.02 x 10⁶ CFU per 100 mL of water.

Water stored in an open aluminium container outside the house of the village chief of Kampung Lubok Wong was positive for the presence of *Blastocystis* spp. However, water samples collected from wells, tap waters, water from tank and aquarium and water stored in a container inside the house were negative for *Blastocystis* spp. (Table 2).

Correlation of *Blastocystis* spp. with physicochemical parameters, faecal coliforms and monthly total rainfall volume

The occurrence of *Blastocystis* spp. showed significant correlations with conductivity ($r_s = 0.756$, $p < 0.05$), turbidity ($r_s = 0.866$, $5C < 0.05$), chemical oxygen demand (COD) ($r_s = 0.866$, $p < 0.05$), total dissolved solid

Table 1. Examination of *Blastocystis* spp. isolated from samples of river water and faecal coliforms

Point of sampling	Occurrence of <i>Blastocystis</i> spp.	Faecal coliforms		
		Green colonies	Yellow colonies	(CFU X 10 ⁶ /100 ml water)
Sungai Krau K1	+	1	10	2.02
Sungai Krau K2	-	2	11	0.06
Sungai Krau K3	-	1	9	0.13
Sungai Krau K4	-	2	4	0.01
Sungai Krau K5	+	1	12	0.92
Sungai Krau K6	+	1	13	0.58
Sungai Lompat	-	0	5	0.12
Total (%)	3/7 positive (42.9)	8	64	0.55 (Mean)

+ : positive for any forms of *Blastocystis* spp.

- : negative for any forms of *Blastocystis* spp.

Table 2. Occurrence of *Blastocystis* spp. in water from other sampling sources

Point of sampling	Total number of samples	Occurrence (%)
Kampung Terbol		
Tap water	3	-
Water in tank (LifeSaver)	1	-
Kampung Lubok Wong		
Water stored outside a house	1	+
Tap water	1	-
Fish pond	1	-
Kampung Penderas		
Water stored in the house	2	-
Water stored outside the house	1	-
Tap water	4	-
Well	2	-
Total	16	1/16 positive (6.25)

+ : positive for any forms of *Blastocystis* spp.
 - : negative for any forms of *Blastocystis* spp.

(TDS) ($r_s = 0.780, p < 0.05$), concentration of sulfate ($r_s = 0.866, p < 0.05$) and faecal coliforms ($r_s = 0.866, p < 0.05$). There were no significant correlations of *Blastocystis* spp. occurrence with pH, temperature, salinity, dissolved oxygen and total chlorine.

DISCUSSION

Blastocystis spp. is a widely distributed parasite which can be isolated from the environmental samples (Puthia *et al.*, 2008). Being one of the most common enteric parasites infects aboriginal communities (Abdulsalam *et al.*, 2012; Anuar *et al.*, 2013); the presence of *Blastocystis* spp. in the environment should be determined to examine the possibility of environmental source such as river water being the source of *Blastocystis* spp. infection. To date, there is no published data on this. Therefore, in this present study we examined the occurrence of *Blastocystis* spp. in Sungai Krau and Sungai Lompat, Temerloh Pahang, as the river water are frequently used by the aboriginal communities for bathing, washing clothes, fishing and open defecation. Human settlements in these villages are mostly located along the two rivers and most of the houses are not equipped with safe water supply and proper sewage disposal.

The physical parameters analysed were pH, conductivity, temperature, salinity and turbidity. The mean of pH, conductivity and salinity of the rivers were within the standard National Water Quality Standards, Malaysia (NWQS) for Malaysian rivers (DOE, 2006) and classified under Class I while temperature and turbidity were classified under Class IIA. With the exception of mean of chlorine concentration, COD and total coliform count, the chemical parameters (mean of dissolved oxygen, sulphate concentration and TDS) were classified under Class I. High and above permissible limits of COD values were recorded in all sampling points that were positive for *Blastocystis* spp. which suggested that *Blastocystis* spp. was present at the rivers with high organic material contamination. Higher value of conductivity, salinity, turbidity, COD, TDS, sulphate and total chlorine concentration were recorded in K1 as compared to other sampling points were possibly because of lumbering activities in the more upstream area within the time of sampling which may have affected the physical and biochemical properties of water in K1.

Faecal coliforms count also exceeded the permissible limit by NWQS and belongs to Class III (DOE, 2006). Coliforms were detected in all sampling points with high count detected in K1, followed by K6 and K5.

This study highlighted that the coliforms were faecal coliform of *E. coli* and *E. aerogenes* as shown by the presence of green and yellow colonies respectively on the agar plates. Therefore, Sungai Krau was contaminated with faeces, either from humans, domesticated or wild animals. It can be postulated that open defecation into the river could be the main source of faecal coliform in Sungai Krau as it was a common practice seen in this aboriginal community. According to Dufour (1977) and Allen and Edberg (1995), about 94% to 97% and 2% to 6% of coliforms in human's stool were *E. coli* and *Enterobacter*, respectively. In animal stool, 93% to 94% of the coliforms were *E. coli* and 4% to 7% were *Enterobacter* (Dufour 1977; Allen & Edberg, 1995).

Blastocystis spp. was detected in water collected from sampling points K1, K5 and K6. Their occurrence in three sampling points was significantly correlated with faecal coliforms and COD. At the most upstream located few Malay villages followed by Kampung Terbol. These villages were located near to the headwater area and the natural water sources from the hill. *Blastocystis* spp. was detected 1000 meters from Kampung Terbol; therefore, we suspected that the source of this parasite was probably from infected humans from the Malay villages who defaecated within this area of river. The possibility of faeces from animals as the source of *Blastocystis* spp. cannot be ruled out as this parasite is a common parasite in animals (Ramirez *et al.*, 2014). There was an absence of *Blastocystis* spp. in river water collected from Kampong Terbol and 1000 meters from Kampong Lubok Wong and in Kampung Lubok Wong; faecal coliforms and COD values were also low as compared with water samples taken from the upstream and downstream of Sungai Krau. This suggested that river water in these areas were less exposed to contamination with *Blastocystis* spp. possibly due to low population density with minimal water-related activities and low burden of *Blastocystis* spp. in human and non-human hosts.

On the other hand, river water sample collected from downstream, 1000 meters

from Kampung Penderas and Kampung Penderas were examined positive for *Blastocystis* spp. with high faecal coliforms and COD value. High population density, high burden of population infected with *Blastocystis* spp., open defecation practice among the villagers and high water-related activities in Kampung Penderas could be the possible reasons that lead to contamination of the river by *Blastocystis* spp.. Abraham (2010) reported growth of human population increases the rate of domestic sewage discharge which contributes to deterioration of water quality. In addition, dislodgement of *Blastocystis* spp. cysts that was sedimented at the river bed and through more water-related activities by population in this area causing them to float downstream. Hence, reared animals including dogs, cats, chickens, swans, ducks and goats in these areas might also contribute to the contamination. In Denmark, a waterborne outbreak related with gastrointestinal symptoms stated 8.6% of the affected patients were positive for *Blastocystis* spp.. Massive stool contamination of the water distribution system with coliforms and *E. coli* were reported as the cause of this outbreak (Vestergaard *et al.*, 2007). Another study on the surface water in Malaysia reported a significant correlation of *Blastocystis* spp. and faecal coliforms (Ithoi *et al.*, 2011).

Water sampled from other sources in Kampung Penderas was negative for *Blastocystis* spp., which means there was no contamination of water stored in the house, tap waters, water tank and wells. In Kampung Terbol, water samples collected from tap water in the house were negative for *Blastocystis* spp. Water from the tank provided by the government namely Life Saver nearby the community hall was also negative for *Blastocystis* spp. However, rain water collected from a roof of a house and stored in an aluminium container outside the house in Kampung Lubok Wong was positive for *Blastocystis* spp. This raised a question on the source of this contamination: was the rainwater contaminated by bird droppings on the roof or was it contaminated while stored in the aluminium container, as we observed a dead dragon fly and two nematodes in the

water. Few studies have reported on the isolation of *Blastocystis* spp. from insects including cockroaches and house flies [Zaman *et al.*, 1993; Yoshikawa *et al.*, 2007, Altimonelli, 1940). However, there was no documented study on the isolation of *Blastocystis* spp. from dragon fly as well as in free-living or parasitic nematodes.

This study also highlighted a significant correlation of the occurrence of *Blastocystis* spp. with conductivity, turbidity, total dissolved solid and sulphate. In this present study, the mean conductivity reading of the river water was within the permissible limit; significant association between conductivity and presence of *Blastocystis* spp. suggests that *Blastocystis* spp. is only able to survive in permissible limit of water conductivity. Significant correlation of high or above permissible limit COD and the present of *Blastocystis* spp. may be related to lower dissolved oxygen leading to anaerobic conditions. *Blastocystis* spp. is an anaerobes; hence, this might explain the occurrence of *Blastocystis* spp. in high or above permissible limit COD of sampling water point especially in Kl.

CONCLUSION

This present study comprehensively provides essential information on the occurrence of *Blastocystis* spp. in water and water quality of Sungai Krau and Sungai Lompat used by the aboriginal community. The river water sample was highly contaminated with organic materials and faeces of humans and animals. The occurrence of *Blastocystis* spp. were significantly correlated with COD and faecal coliforms, indicating this parasite was excreted in faeces of humans and animals into the rivers. Long term strategies incorporating health education regarding good personal hygiene practices, the provision of safe water supply, proper sewage disposal and the importance of their usage need to be adopted by the aborigines in each villages in order to control the spread of *Blastocystis* spp. and other water-borne or faecal oral route microorganisms.

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