Molecular analysis of *Blastocystis* sp. and its subtypes from treated wastewater routinely used for irrigation of vegetable farmlands in Iran

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ABSTRACT

Treated wastewater samples were collected, filtered using sterile 47-mm cellulose nitrate membrane and DNA extracted from the filtered materials. The presence of *Blastocystis* sp. was confirmed via polymerase chain reaction (PCR) targeting the SSU rRNA gene of *Blastocystis* sp. in 5/12 of samples. Based on the subtype analysis after sequencing, 2, 2 and 1 of ST2, ST6 and ST8 were detected among the isolates, respectively. Furthermore, both ST6s were allele 139, alleles 11 and 138 were identified in ST2 and the only ST8 was allele 95. The phylogenetic tree showed that one of ST2 was clustered together with those ST2 that were already reported from humans and animals. The presence of *Blastocystis* sp. in treated wastewater can indicate the potential role of this type of water for irrigation in the transmission of pathogenic microorganisms to downstream farmlands.

Key words | allele discrimination, *Blastocystis* sp., phylogenetic analysis, subtypes, treated wastewater

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INTRODUCTION

Waterborne diseases are considered as one of the most important public health problems, all over the world. Together with bacteria and viruses, protozoan parasites are frequently reported from waterborne outbreaks in both developing and developed countries (Karanis *et al.* 2007; Baldursson & Karanis 2011; Moss 2016; Efstratiou *et al.* 2017). Importantly, polluted water resources are a public health concern, which threaten millions of people, particularly those living in developing countries without access to safe water resources for drinking, bathing, and irrigation

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proposes (WHO/UNICEF 2014; Aldeyarbi *et al.* 2016; Plutzer & Karanis 2016; Mahmoudi *et al.* 2017; Rosado-Garcia *et al.* 2017; Ahmed *et al.* 2018). Therefore, people inhabiting areas without easy access to clean water are at risk for intestinal infections caused by waterborne parasites (Andrade *et al.* 2018). Although *Cryptosporidium* species and *Giardia duodenalis* (*G. intestinalis*, *G. lamblia*) are the most prevalent reported protozoan parasites, other protozoan parasites such as *Entamoeba histolytica, Cyclospora cayetanensis, Toxoplasma gondii*, *Isospora belli, Blastocystis* sp., *Acanthamoeba* spp., *Balantidium coli*, and *Naegleria fowleri* are also involved in waterborne outbreaks (Karanis *et al.* 2007; Baldursson & Karanis 2011; Plutzer & Karanis 2016; Efstratiou *et al.* 2017).

Blastocystis sp. has been found to be a highly prevalent intestinal parasite of humans that is mainly transmitted via contaminated food and water as well as close contact with animals (Parija & Jeremiah 2013; Wawrzyniak *et al.* 2013; Stensvold & Clark 2016). *Blastocystis* sp. is a non-invasive, unicellular, large intestinal protozoan parasite with multiple stages (Vassalos *et al.* 2010; Ahmed & Karanis 2018). According to results from clinical studies, *Blastocystis* sp. has been isolated from stool specimens of patients with diarrhea, constipation, vomiting, cutaneous rash, inflammatory bowel syndrome, and inflammatory bowel diseases (Carrascosa *et al.* 1996; Jimenez-Gonzalez *et al.* 2012; El Safadi *et al.* 2013; Casero *et al.* 2015; Mirjalali *et al.* 2017; Cifre *et al.* 2018; Rezaei Riabi *et al.* 2018). Wide distribution, high prevalence, and a broad spectrum of hosts have substantially increased the number of studies on this microorganism in recent years.

In recent decades, molecular tools have been applied to identify lineages of *Blastocystis* sp. Accordingly, based on the phylogenetic classification of discriminative fragment of the subunit ribosomal RNA (SSU rRNA) gene, 17 subtypes have so far been characterized (Stensvold *et al.* 2007; Alfellani *et al.* 2013a; Clark *et al.* 2013). Although correlation between clinical manifestations and certain subtypes is under question (Al-Fellani *et al.* 2007; Alinaghizade *et al.* 2017; Jalallou *et al.* 2017; Rezaei Riabi *et al.* 2017), subtype determination has mainly been applied for molecular epidemiology and source finding (Alfellani *et al.* 2013a; 2013b; Yoshikawa *et al.* 2016).

Although the interest in this parasite has rapidly increased in recent years, few studies have indicated the importance of water transmission of *Blastocystis* sp. to human communities (Lee *et al.* 2012; Anuar *et al.* 2013; Angelici *et al.* 2018). Accordingly, *Blastocystis* sp. was detected in drinking water (Leelayoova *et al.* 2004, 2008), water catchment (Noradilah *et al.* 2016), recreational water (Ithoi *et al.* 2011) as well as river and sea waters (Koloren *et al.* 2018). More recently, a protocol was established and explained by Lee *et al.* (2019) for detection of *Blastocystis* sp. from water samples.

In the current study, we investigated the presence of *Blastocystis* sp. and its subtypes and alleles in treated wastewater (TW) that was routinely used for irrigation of vegetable farmlands.

METHODS

Sampling

In the current study, we performed investigations on 12 treated wastewater samples that were collected in our previous study by Javanmard *et al.* (2018a) from a one-year follow-up study on a wastewater treatment plant (WTP) located south of Tehran, Tehran province, Iran. This WTP is the biggest sewage treatment complex and collects a huge proportion of wastewaters in Tehran. Coagulation, flocculation, sedimentation, activated sludge, and disinfectant were used during the treatment process. The WTP collects approximately more than 70% of the sewage of Tehran. This study was a one-year follow-up of this WTP, thus, only 12 samples were investigated. All samples were collected from the WTP during monthly microbial quality control from a depth of 50 cm below the surface. Gathered samples were aseptically transported to the Parasitology and Water Research Laboratory of Foodborne and Waterborne Diseases Research Center in Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran for further processing.

Processing samples

In the laboratory, samples were processed in the same way as our previous study by Javanmard *et al.* (2018a). Briefly,

samples were filtered using a six-branch filtration system (Sartorius, Gottingen, Germany) and sterile 47-mm cellulose nitrate membrane with pore size 0.4 μm together with a sterile water as the negative control. Then, the filtered membranes were washed in 30 mL of sterile PBS and kept at 4–8 °C for 24 h. Finally, samples were centrifuged at 2,500 × g for 20 min at 4 °C, supernatant was discarded and the remaining pellet was stored at −20 °C until DNA extraction.

DNA extraction and PCR amplification

DNA of samples were extracted using a YTA DNA extraction kit (Yekta Tajhiz, Tehran, Iran) according to the manufacturer's instructions with some modifications. Briefly, 250 μL of pellet sample was washed three times with sterile PBS (pH = 7.5), and after final centrifugation, supernatant was discarded and the pellet was added to 2 mL sterile tubes containing glass beads. After 2 h incubation of samples at 60 °C together with 40 μg/mL of proteinase K, DNA was extracted and purified. Isolated DNA was stored at −20 °C until use.

In order to amplify the discriminative fragment for subtyping, the primer set BhRDr (GAGCTTTTTAACTGCAACAACG) and the broad-specificity eukaryote-specific primer RD5 (ATCTGGTTGATCCTGCCAGT), described elsewhere (Scicluna *et al.* 2006), were employed to target ~620-bp fragment of the SSU rRNA gene of *Blastocystis* sp. according to the following conditions: an initial denaturing step of 95 °C for 5 min and 35 cycles consisting of 94 °C for 30 s, 60 °C for 30 s, and 30 s at 72 °C. A final extension at 72 °C was performed for 5 min. Subsequently, 10 μL of each polymerase chain reaction (PCR) product was electrophoresed on a 1.5% agarose gel in TBE (0.09 M Tris, 0.09 M boric acid, 2 mM EDTA) stained with 0.5 μg/mL ethidium bromide and visualized using a UV Transilluminator. Finally, an ABI (3130) sequencer (California, USA) sequenced 20 μL of all PCR products. The generated sequences were edited and trimmed by Chromas software and the results were compared with those available sequences in GenBank database (BLAST; http://blast.ncbi.nlm.nih.gov/). Furthermore, in order to characterize alleles, edited sequences were compared (http://pubmlst.org/blastocystis/). All sequences were submitted in the GenBank database with accession numbers MH915564–MH915567.

Phylogenetic analysis

All *Blastocystis* sp. sequences obtained from the new isolates were multi-aligned, with ClustalW software, with those of humans and animals, which were previously deposited in GenBank. A phylogenetic tree was drawn using Molecular and Evolution Genetic Analysis Software (MEGA v.7) based on the maximum-likelihood model and Tamura-3-parameter option (Tamura *et al.* 2013). In order to calculate statistical significance, bootstrap 1,000 was employed.

RESULTS

PCR amplification, subtyping and allele discrimination

The targeted fragment of the SSU rRNA gene was successfully amplified in 5/12 of samples. All five *Blastocystis* sp.-positive samples were sequenced such that the result showed the presence of 2, 2, and 1 of ST2, ST6, and ST8 among treated water samples, respectively. The findings showed that *Blastocystis* sp. was present once in the investigated treated waters in all four seasons, while two *Blastocystis* sp.-positive isolates were identified in the summer period. Furthermore, allele discrimination showed that both ST6s were allele 139, while alleles 11 and 138 were identified in ST2 and the only ST8 was allele 95 (Table 1).

Phylogenetic analysis

A phylogenetic tree was drawn that showed all obtained subtypes were clearly separated. Accordingly, ST2 obtained from the current study was placed together with other subtypes 2, which were isolated from humans and cattle, while it was molecularly closer to human isolates. Both subtypes 6 were grouped together and far from other ST6, which were isolated from chickens and humans even though molecular diversity was seen between them. Indeed, the only ST8 was separated from other subtypes and placed in a clade closer to ST6 than those ST8 that were characterized from non-human primates (Figure 1).

Table 1 | *Blastocystis* sp. and its subtypes and alleles isolated from treated wastewater using amplification and sequencing of 'barcoding region' of SSU rRNA gene, regarding season

Sampling time		Treated wastewater (positive samples)		
		Subtype	Allele	Accession number
Autumn	September	**ST6**	**139**	MH915567
	October	–	–	–
	November	–	–	–
Winter	December	–	–	–
	January	**ST2**	**138**	NP
	February	–	–	–
Spring	March	–	–	–
	April	–	–	–
	May	**ST2**	**11**	MH915566
Summer	June	–	–	–
	July	**ST6**	**139**	MH915565
	August	**ST8**	**95**	MH915564

Note: NP: not provided. These isolates did not provide enough length of nucleotide sequence to be submitted in GenBank database.

DISCUSSION

Blastocystis sp. is a prevalent eukaryote that was recently included in check lists of WHO guidelines for drinking water quality control (WHO 2017). Importantly, despite the importance of human to human transmission of this protist, evidence of epidemiologic studies highlights the crucial role of zoonotic and/or anthroponotic transmission and water resources in the distribution of *Blastocystis* sp. to human communities. *Blastocystis* sp. is a prevalent protozoan in humans and animals in Iran. According to the published studies in the past decade, prevalence of this protozoan in humans was up to 25% (Javanmard *et al.* 2018b).

Microscopic morphotypes of *Blastocystis* sp. have been widely described in the literature (Vassalos *et al.* 2010; Ahmed & Karanis 2018). Pairwise evolutionary differences and nucleotide percentage similarities between (SSU) rDNA sequences in water samples and reference sequences of *Blastocystis* sp. from GenBank and a phylogenetic analysis summary have been provided in a recent paper by Koloren *et al.* (2018). However, the aim of the present study was to identify the subtypes of *Blastocystis* sp. in treated wastewater (TW) samples used for irrigation of vegetable farmlands. Although almost all of the studies in Iran using

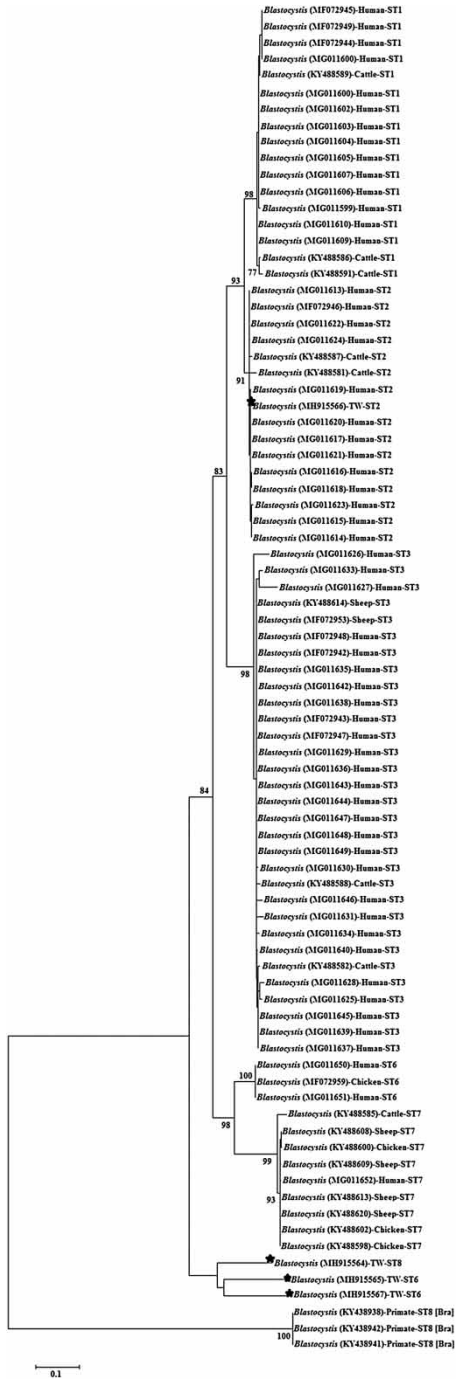

Figure 1 | Phylogenetic analysis of 'barcoding region' of SSU rRNA gene of *Blastocystis* sp. isolated from the current study and those that were previously reported from humans and animals. The tree was drawn by using the maximum likelihood method based on the Tamura-3-parameter model. The number above the branch indicates bootstrap percentage. The branches without numbers have bootstrap lower than 75%. TW, treated wastewater; Bra, Brazil. All *Blastocystis* sp. isolated from the current study are indicated with black-filled asterisks. *Note:* One of the isolates did not provide enough length of nucleotide sequence to be included in phylogenetic analysis.

sequencing of the 'barcoding region' showed the majority of ST1–3 in both human and animals, some other less prevalent subtypes were recently reported from human cases. In a study conducted by Rezaei Riabi et al. (2018), ST6 and ST7 were isolated from human cases. However, they concluded that probably animal sources were involved in the transmission cycle of *Blastocystis* sp. Indeed, based on the non-host specificity feature of most human-infecting subtypes of *Blastocystis* sp. (Stensvold 2013; Stensvold & Clark 2016), it seems that not only close contact with animals, but also water resources that are polluted with feces of animals can play an important role in the transmission of *Blastocystis* sp. to human communities. Angelici *et al.* (2018) reported the case of a symptomatic patient who carried *Blastocystis* sp. subtype 1 and based on the history of the infected subject, they strongly suggested that drinking from an unpotable water source was the source of transmission.

The important role of water resources, particularly surface water in transmission of *Blastocystis* sp. to humans, was previously indicated (Table 2). Leelayoova *et al.* (2008) in a study on the prevalence of *Blastocystis* sp. in schoolchildren of a rural community in Thailand proposed that the water supply within a school was most probably the source of infection. Researchers in Malaysia investigated the presence of *Blastocystis* sp. among primary schoolchildren and claimed that, based on the epidemiologic evidence, the source of drinking water most probably was infected with *Blastocystis* sp. (Abdulsalam *et al.* 2012). At the same time, another study in Nepal by Lee *et al.* (2012), which was performed on animals and river water, showed the majority of *Blastocystis* sp. ST4 in both animals and river waters and suggested high probability of zoonotic transmission of *Blastocystis* sp. via water resources.

The presence of *Blastocystis* in wastewater has also been reported. Suresh *et al.* (2005) investigated the presence of *Blastocystis* sp. in influent and effluent of a WTP and showed the presence of viable cysts of *Blastocystis* sp. in both types of sewage. In another study conducted by Banaticla & Rivera (2011), viable cysts of *Blastocystis* sp. were detected from both influent and effluent of a WTP. Although the number of positive samples in the discharge water was significantly lower than that observed in raw sewage, the authors concluded that the presence of viable cysts in effluent may indicate inefficiency of the usual wastewater treatment process. Therefore, it seems that *Blastocystis* sp. can not only be resistant to the water treatment process and be distributed in water environments,

Table 2 | All reports of *Blastocystis* sp. and its subtypes isolated from different types of water regarding method of detection and characterization, worldwide

No.	Country	Sample	Subtype	Prevalence of *Blastocystis* sp.	Method of detection	References
1	Iran (Asia)	Treated wastewater	ST2, ST6, ST8	5/12	PCR sequencing	Current study
2	Malaysia (Asia)	River water	ST1, ST2, ST3, ST4, ST8, ST10	27.7%	PCR sequencing	Ithoi *et al.* (2011)
3	Malaysia (Asia)	Wastewater	–	Influent 50%, effluent 28%	Microscopy	Suresh *et al.* (2005)
3	Thailand (Asia)	Drinking water	ST1, ST2	–	PCR-RFLP	Leelayoova *et al.* (2008)
4	Philippines (Asia)	Wastewater treatment plants	ST1, ST2	Influent 23%, effluent 7%	PCR sequencing	Banaticla & Rivera (2011)
5	Turkey (Europe)	River water, drinking water	ST1, ST3	10%	PCR sequencing	Koloren *et al.* (2018)
6	Italy (Europe)	Non-potable drinking water	ST1	Positive	PCR-RFLP	Angelici *et al.* (2018)
7	Egypt (Africa)	Ponds and canal water	–	15.87%	Microscopy	Khalifa *et al.* (2014)
8	Egypt (Africa)	Potable water tanks, river	–	1%	Microscopy	Elshazly *et al.* (2007)
9	Venezuela (South America)	River water	–	–	Microscopy	Mora *et al.* (2010)

but also remain viable during the common processes that are employed for treatment of drinking water and sewage.

In the current study, three subtypes, ST2, ST6, and ST8, were characterized among the TW samples. Apart from ST2, which is usually reported from human subjects, ST6 and ST8 were mainly described from birds and non-human primates, respectively. Regarding the fact that Iran is not the habitat of non-human primates, it seems that together with ST2, ST8 came from a human source. In the case of ST6, it is necessary to mention that the WTP was not covered, and thus, most probably, the source of this subtype was free-range birds with access to the WTP.

Interestingly, the results of phylogenetic analysis confirmed the probable sources of each subtype. In the phylogenetic analysis, ST2 was placed together with other subtypes 2, which were previously obtained from human subjects, while ST8 was located in a separate branch from those ST8 that were reported from non-human primates. This observation indicated that, most probably, the presence of ST8 in TW was correlated with human sources. Inversely, both ST6 obtained from the current study were grouped together but separated from those subtypes 6 that were previously isolated from human subjects in Iran. The current finding strengthens the hypothesis that the source of ST6 in TW was probably birds.

Surprisingly, allele analysis of *Blastocystis* sp. showed that apart from one of the ST2 isolates (allele 11), all other subtypes revealed alleles that were not previously reported in humans and animals. Accordingly, ST2 allele 11 was previously characterized by Ramirez *et al.* (2016, 2017) in South America and Rezaei Riabi *et al.* (2008) in Iran. Presumably, the presence of this allele in TW indicated the insufficiency of the usual method for elimination of *Blastocystis* sp. cysts from raw wastewater. On the other hand, in the current study, we identified ST2 allele 138 together with ST6 allele 139 and ST8 allele 95 and there are no data about their distribution in humans. However, detection of these alleles may suggest secondary contamination of treated wastewater with non-human isolates.

CONCLUSION

Epidemiological frequency of *Blastocystis* sp. subtypes in symptomatic and asymptomatic patients has been reported in different countries and their prevalence is variable according to geographical distribution. Although knowledge on the prevalence and distribution of *Blastocystis* sp. and its subtypes has increased to a great extent, it seems that this parasite is largely underestimated and there are not enough data about the role of water resources in transmission of this protozoan parasite. Furthermore, there is no comprehensive study on the sufficiency of wastewater treatment processes in elimination of *Blastocystis* sp. from sewage. The current study showed that treated water can remain either contaminated with *Blastocystis* sp. after wastewater treatment or polluted with this protozoan via access of free-range animals. However, evaluation of small sample size was the limitation of the current research, therefore, more intensive studies on large water samples, axenization and/or genetic analyses of new subtypes will be beneficial to improve the knowledge about *Blastocystis* sp. species and its subtypes in water resources.

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