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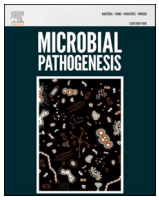

Blastocystis infection among diarrhea outpatients in Ningbo, Southeast China: A potential zoonotic health threat

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ABSTRACT

Background: Blastocystis is one of the important zoonotic parasites which can infect humans and various animals worldwide and has become a growing global public health concern. The study aims to obtain the data of *Blastocystis* infection and the information of the genetic characteristic.

Methods: In the present study, 489 fecal samples were collected from diarrhea outpatients in Ningbo, Zhejiang province, and were examined the presence of *Blastocystis* by polymerase chain reaction combined with sequencing.

Results: A total of 10 samples (2.04%, 10/489) were positive for *Blastocystis* with no significant difference among sex and age groups, respectively. Eight samples were successfully sequenced, and five zoonotic ST3 and three zoonotic ST1 with two new sequences were identified.

Conclusions: We first demonstrated the occurrence of *Blastocystis* infection in diarrhea outpatients in Ningbo, with two zoonotic subtypes (ST1 and ST3) and two new sequences being characterized. Meanwhile, mixed infection of *Blastocystis* and *E. bieneusi* was found which indicates the importance of investigation of multiple parasites. Finally, more extensive studies will be needed to better understand the transmission of *Blastocystis* at human-animal-environment interface and provide evidence for the development of one health strategies for the prevention and control of such diseases.

1. Introduction

Blastocystis is a common eukaryotic protist that can infect humans, domestic and wild mammals, birds, reptiles, amphibian, and even insects, which becomes a growing global public health concern [1,2]. The main transmission route of *Blastocystis* is fecal-oral pathway, and humans are commonly infected by ingesting food or water contaminated with *Blastocystis* cysts [3,4]. In addition, humans often have a great risk of infection through contacting with animals infected with *Blastocystis* [2]. The clinical feature is various, and is occupied by gastrointestinal symptoms such as diarrhea, nausea and abdominal pain [1,5]. In addition, several dermatological symptoms caused by *Blastocystis* such as acute or chronic urticaria, angioedema, and diffuse pruritus have also been reported recently [6–8].

Currently, molecular biological detection methods have been efficient ways and widely used for identification of *Blastocystis* infection in humans and animals. Now, *Blastocystis* has been classified into at least 28 subtypes (ST1-ST17, ST21, ST23-ST29 and ST30-ST32) based on molecular analyses of the small subunit ribosomal RNA (SSU rRNA) gene [9]. Of which, ST1 to ST8, ST10, ST12, ST14 and ST16 have been found in humans and animals, indicating its potentially zoonotic [10].

In previous studies, *Blastocystis* has been reported to be transmitted between human and animals, such as human-pigs or zookeepers-primates, indicating the significant health threats of the pathogen [11,

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12]. Meanwhile, *Blastocystis* has been listed as a waterborne zoonoses by the World Health Organization (WHO). In China, investigations related to *Blastocystis* infections has been conducted in several populations such as students in primary school and universities, with the infection rate varied from 1.10% to 14.93%. *Blastocystis* infection in residents in villages or HIV-positive/negative patients were also reported, while few studies about *Blastocystis* infection in diarrheal outpatients was conducted [13]. As we know, there were only two reports of *Blastocystis* infection in Shanghai, Zhejiang and Anhui province in Yangtze River Delta [14,15]. Ningbo, the fourth largest port city in the world, is located in eastern part of Zhejiang province and in the south wing of the Yangtze River Delta, China. Few research on investigation and characterization of intestinal parasites was reported in this area. In our previous study, *E. bieneusi* and *C. cayetanensis* were found in the molecular characterization of *E. bieneusi*, *C. cayetanensis* and *Cryptosporidium* spp. in fecal samples from diarrheal outpatients [16]. In the present study, we further investigated the *Blastocystis* infection and characterized the parasite in these diarrheal outpatients, thereby provide insights into the understanding of the epidemiology and management of *Blastocystis* infections.

2. Materials and methods

2.1. Ethics statement

Ethical clearance for the collection and examination of human fecal samples was obtained from the Ethics Committee of the National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention (Approval No. 2019003). The objectives, procedures and potential risks were orally explained to all participants. Parents/guardians provided consent on behalf of all infant participants.

2.2. Sample collection

The present study was conducted between January to December in 2019. A total of 489 samples were collected from patients that clinically diagnosed to be diarrhea with more than three events of diarrhea per day in outpatient clinics in Ningbo, Zhejiang province [16]. Each specimen were transported to the laboratory and stored at −20 °C until use.

2.3. DNA extraction and PCR amplification

Genomic DNA was extracted using the QIAamp DNA stool Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. DNA was eluted in 200 μL of AE and stored at −30 °C until use for PCR amplification. A ~500 bp SSU rDNA gene fragment containing a variable region was amplified to subtype *Blastocystis* specimens, and the primer sequences, PCR volume and reaction conditions were described in the previous study [17]. One positive and negative control each was included in every PCR run, and the PCR products were analyzed through 1.5% agarose gel electrophoresis using Gel-Red staining. Each sample was amplified two times.

2.4. Sequence and phylogenetic analyses

The sequencing accuracy was confirmed with two-directional sequencing on an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, USA) with a Big Dye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems). And the PCR amplification primers were also used for sequencing [17]. The obtained sequences were assembled using the software ContigExpress. The sequence alignment was done used Clustal X 1.83 and compared with the GenBank sequence database by Basic Local Alignment Search Tool (BLAST). A neighbor-joining (NJ) tree was constructed with the Kimura 2-parameter using software MEGA 7 (http://www. megasoftware.net/), and a bootstrap analysis with 1000 replicates was used.

Table 1
The infection and subtype distribution of *Blastocystis* in diarrhea outpatients in Ningbo.

Sample number	Gender	Age	Subtype
NBH-13	Female	20	–
NBH-150	Male	56	ST3
NBH-162	Female	66	ST3
NBH-341	Male	80	ST1
NBH-367	Male	30	ST1
NBH-377	Male	32	ST3
NBH-412	Female	4	ST3
NBH-413	Female	40	–
NBH-416	Female	32	ST3
NBH-486	Male	26	ST1

Note: means the sample was not successfully sequenced.

Table 2
The variation of *Blastocystis* ST1 and ST3 sequences identified in the present study.

Sequences	Nucleotide position			
ST1	194	258	269	282
MK874789	G	G	T	G
486	G	G	T	G
367	T	G	C	C
341	G	C	C	C
ST3	284			
KU147402	G			
KU147332	T			
KU147400	C			
150	G			
377	G			
412	T			
162	C			
416	C			

2.5. Nucleotide sequence accession numbers

The obtained new nucleotide sequences from this study were deposited in GenBank under accession numbers OQ642266 and OQ642267.

3. Results

3.1. Infection of Blastocystis

A total of 489 fecal samples (268 were males and 221 were females) were tested in this study. A total of 10 samples (2.04%, 10/489) were positive for *Blastocystis* with the target fragment around 500 bp in the gel electrophoresis analysis. There was no significant difference among sex and age groups (Fisher's Exact Test, $P > 0.5$). However, only 8 samples were successfully sequenced, with three ST1 and five ST3 (Table 1).

3.2. Sequence analysis

Of the three *Blastocystis* ST1 sequences, sequences analysis indicated that one was identical with a reported human-derived sequence (GenBank NO. MK874789), and two new *Blastocystis* ST1 sequences were identified in this study (OQ642266 and OQ642267). Among the five ST3 sequences, two sequences (NBH-150 and NBH-377) showed 100% identity with the sequence (GenBank NO. KU147402) derived from humans from Mexico, two sequences (NBH-162 and NBH-416) showed 100% identity with the sequence (GenBank NO. KU147400) derived from pigs from Mexico, and the remaining one sequence was identical with human-derived sequence from Mexico (GenBank NO. KU147372) [18]. The nucleotide variations were displayed in Table 2.

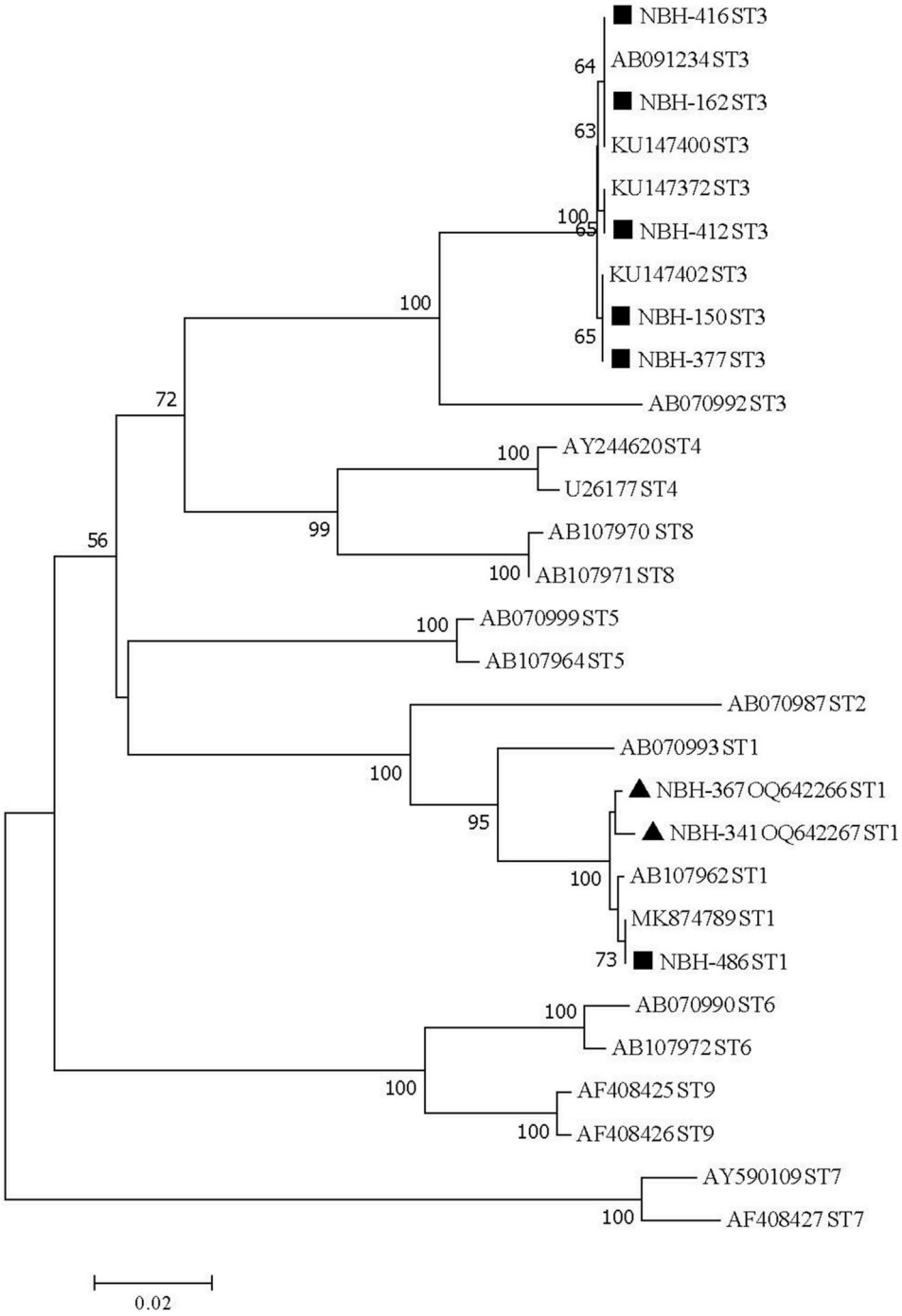

Fig. 1. Phylogenetic relationships of the SSU rDNA gene sequences of *Blastocystis* identified in the present study inferred by a neighbor-joining analysis. The squares and triangles filled in black indicate known and new sequences identified in this study, respectively.

3.3. Phylogenetic analysis of Blastocystis SSU rDNA gene sequences

Based on the phylogenetic analysis of the neighbor-joining (NJ) tree of *Blastocystis* SSU rDNA gene sequences, the sequences identified in the study were clustered in to ST1 and ST3 groups (Fig. 1), which was consistent with sequence alignment results.

4. Discussion

Blastocystis has been recognized as a public health concern due to its global distribution in humans and diverse animals, which is probably the most common intestinal protozoa in parasitological surveys in the world, with prevalence ranging from 0.8% to 100% in different countries [19,20]. Recently, PCR-based molecular techniques with high sensitivity and specificity were used in more and more investigations on intestinal protozoa including *Blastocystis*.

In the present study, 8 of 10 PCR-positive samples were successfully sequenced and analyzed, with three ST1 and five ST3, which were all zoonotic subtypes. First, there is a low infection rate (2.04%, 10/489) of *Blastocystis* in the diarrheal individuals with no significant difference among sex and age groups being found. The infection rate was lower than a study on *Blastocystis* in diarrheal patients in urban region in China [18]. To our knowledge, at least 12 provinces have reported the prevalence of *Blastocystis* in humans in China, with the highest infection rate

of 100% in Guangdong province and the lowest infection rate of 0.8% in Fujian province [21]. The prevalence of *Blastocystis* infection may be related to many factors, such as diagnostic methods, geographic locations, the immune status of hosts, eating and drinking habits, etc. It has been confirmed that PCR detection methods were more effective to detect *Blastocystis* for its high sensitivity, and widely used in identify *Blastocystis* [22]. The conventional microscopic examination method was very time-consuming and heavily dependent on the professional and technical experiences. Second, 99.79% of sequence identity was found among the two new ST1 in this study with the previous reported pig-derived sequence (GenBank NO. EU445486) [23] and horse-derived sequence (GenBank NO. ON932511) [24]. So, our results of sequence analysis also indicated the potential zoonotic of *Blastocystis* found in patients in Ningbo city. Third, it was reported that ST1 is likely related to clinical symptoms such as diarrhea, while ST3 is common in asymptomatic *Blastocystis* infection [13,25]. Interestingly, all the positive participants found in the present study had gastrointestinal symptoms of diarrhea at least 3 times a day. In addition, of the 489 fecal samples examined in this study, a total of 35 (7.2%, 35/489) and three (0.6%, 3/489) samples were positive for *E. bieneusi* and *C. cayetanensis* respectively [16]. More importantly, one fecal sample was mixed infected with *Blastocystis* and *E. bieneusi* (Type IV), indicating the importance of investigation of multiple parasites in humans.

In the present study, ST3 was the predominant subtype and sequence analysis have also indicated the potential zoonotic transmission [18]. In fact, there have been lots of reports on the transmission mode of *Blastocystis*, confirming the occurrence of human-animal-environment transmission. In Japan, three patients have been reported to be infected with *Blastocystis* ST3 from two long-term health care facilities and provided the first molecular evidence of possible human-to-human transmission [26]. In Malaysia, *Blastocystis* was commonly found among the animal handlers indicating the presence of human-animal transmission, and animals may pose a significant zoonotic source of *Blastocystis* for humans [27].Meanwhile, more and more studies have provided molecular evidence of zoonotic transmission between animal and animal handlers, for example, four zoo-keeper *Blastocystis* isolates were 100% similarity with the isolates from the southern hairy nosed wombat and five primate species in Australia [28,29]. Moreover, previous studies also demonstrated high prevalence of *Blastocystis* in sewage in Malaysia and Scotland [30]. In addition, three *Blastocystis* ST1 isolates were identified in patients in the present study, which is more likely to be transmitted by water. A study focused on a possible source of *Blastocystis* ST1 infection in school children proved the possibility of drinking water as waterborne transmission [31]. *Blastocystis* has also been reported to be transmitted through contaminated water, and the irrigation, surface, and sewage water were also the source of infection [32,33]. Furthermore, it has also been reported in fresh produce and shellfish [34,35]. Therefore, the survey of *Blastocystis* in humans, animals and environments is of great importance, especially for a seaside city in the south wing of the Yangtze River Delta.

Recently, to better deal with the serious public health challenges of the twenty-first century, One Health approach is widely recommended that aims to prevent the spread of disease transmission at human-animal-environment interface. Effective One Health approaches are essential to reduce the zoonotic parasitic diseases [36]. First, an integrated and sensitive surveillance system including humans, animals and environment is critical in the control of transmission of parasitic diseases. It can provide timely and effective epidemic information to support for policy makers. Secondly, the research and development of monitoring tools should be accelerated, especially a user-friendly, rapid and sensitive multi-pathogens detection tool, which can be promoted for use in the clinic or in the field, thereby improve our understanding the real epidemiology of these pathogens, particularly the neglected intestinal protozoa such as *Blastocystis*. Thirdly, health education and promotion on the control and prevention of these diseases should be aimed not only at the general public, but also at the large number of health workers. In fact, poor sanitary conditions, keeping pets, not washing hands after going to the toilet, and many others have been reported to be the risk factors of *Blastocystis* infection or other pathogens [37,38]. Additionally, the realization of the above-mentioned efforts is certainly inseparable from multi-sectoral cooperation to jointly promote the coordinated management of human-animal-environment, effectively respond to various infectious diseases such as parasitic diseases including *Blastocystis* infection, and protect people's health.

5. Conclusions

In conclusion, we demonstrated the occurrence of *Blastocystis* infection in diarrhea outpatients in Ningbo. Two zoonotic subtypes of ST1 and ST3 with two new sequences were characterized. In addition, the mixed infection of *Blastocystis* and *E. bieneusi* was found which indicates the importance of investigation of multiple parasites. In view of the transmission routes of *Blastocystis* (feces, water or food), it is recommended that more extensive studies should be carried out to better understand the epidemiology of intestinal protozoa such as *Blastocystis* at human-animal-environment interface, thereby provides evidence for the development of one health strategies for the prevention and control of such diseases.

CRediT authorship contribution statement

Hua Liu: Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation. **Hongxia Ni:** Resources, Investigation, Data curation. **Na Zhu:** Resources, Investigation. **Shike Liu:** Resources, Investigation. **Rong Wang:** Investigation. **Jianping Cao:** Supervision, Funding acquisition. **Yujuan Shen:** Writing – review & editing, Supervision, Funding acquisition. **Jianhai Yin:** Writing – review & editing, Validation, Supervision, Resources, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declared that they have no conflicts of interest.

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