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ORIGINAL PAPER

Cross-sectional surveys and subtype classification of human *Blastocystis* isolates from four epidemiological settings in China

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Abstract The prevalence and geographical distribution of the intestinal protozoa *Blastocystis* in humans across China is unknown, and the relative importance of different subtypes has yet to be investigated. We assessed the community prevalence and relative frequencies of different *Blastocystis* subtypes in four epidemiological settings in China, i.e., Shanghai municipality, Yongjia county (Zhejiang province), Eryuan county, and Menghai county (both Yunnan province). *Blastocystis* infection was detected with the culture method, and the subtype was identified with polymerase chain reaction using a set of subtype-specific primers. The prevalence at the four study settings was 1.9, 5.9, 18.4, and 32.6%, respectively. People aged greater than or equal to 60 years had a higher prevalence in the former two settings, Shanghai and Yongjia, whereas the highest infection rate was found among individuals aged 10–17 years in the latter two settings, Eryuan and Menghai. A higher prevalence was found in men in the former two settings but in women in the latter two settings. Five different *Blastocystis* subtypes were identified from the 192 isolates. Subtype 3 was the predominant type, followed by subtype 1. In conclusion, the epidemiology of *Blastocystis* varies across China.

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Introduction

Human *Blastocystis* was first described in 1912 (Brumpt 1912). Numerous cross-sectional surveys have been carried out in different epidemiological settings, and hence *Blastocystis* is known to be one of the most frequently found protozoan parasites in human fecal samples. However, many aspects of this organism remain to be elucidated, including its taxonomy, speciation, pathogenetic potential, life cycle, and mode of transmission (Stenzel and Boreham 1996; Tan et al. 2002; Yoshikawa et al. 2004a). In addition, there is a lack of knowledge regarding the prevalence, incidence, and geographical distribution of human *Blastocystis* in many countries. Important underlying reasons are its small size and morphological diversity, which result in a low sensitivity of the widely employed diagnostic technique, i.e., parasite detection in stool samples by light microscopy of direct smears, fecal concentrates, or permanently stained smears (Stensvold et al. 2006). However, the culture technique is known to be more sensitive for detection of the parasite in fecal samples (Termmathurapoj et al. 2004). Short-term in vitro cultivation is useful for the detection and molecular study of *Blastocystis* in stool specimens.

Extensive genetic diversity was detected among *Blastocystis* populations, and several demes or subtypes were identified by different methods (Upcroft et al. 1989; Zierdt 1991; Bohm-Gloning et al. 1997; Clark 1997; Yoshikawa et al. 1998, 2000, 2003, 2004b; Noël et al. 2005; Scicluna et al. 2006; Stensvold et al. 2006, 2007). Recently, consensus terminology of different subtypes among *Blastocystis* isolates from mammals and avians was reported as subtypes 1 to 9 (Stensvold et al. 2007).

In China, *Blastocystis* has been largely neglected until the first national sampling survey of human parasites, which was carried out between 1988 and 1992, and reported a mean national prevalence of only 1.3% (Xu et al. 2000). Recently, screening of *Blastocystis* has been included in regional parasitological surveys, but the prevalence is considered to be underestimated, as most studies employed the direct fecal smear technique (Su et al. 1995; Wang and Li 2002; Jin et al. 2005). Moreover, little has been known about the genetic diversity of *Blastocystis* and the spatial distribution of different subtypes across China. Although there was only one report on diversity of *Blastocystis* subtypes isolated in Jiangxi province in China, a small sample size of the 35 isolates were employed for analysis in that study (Yan et al. 2006).

The objectives of the present study were (1) to determine the prevalence of human *Blastocystis* in four distinct epidemiological settings in China, stratified by age and sex, using a sensitive culture technique, and (2) to assess the relative frequency of different *Blastocystis* subtypes and the local heterogeneity therein.

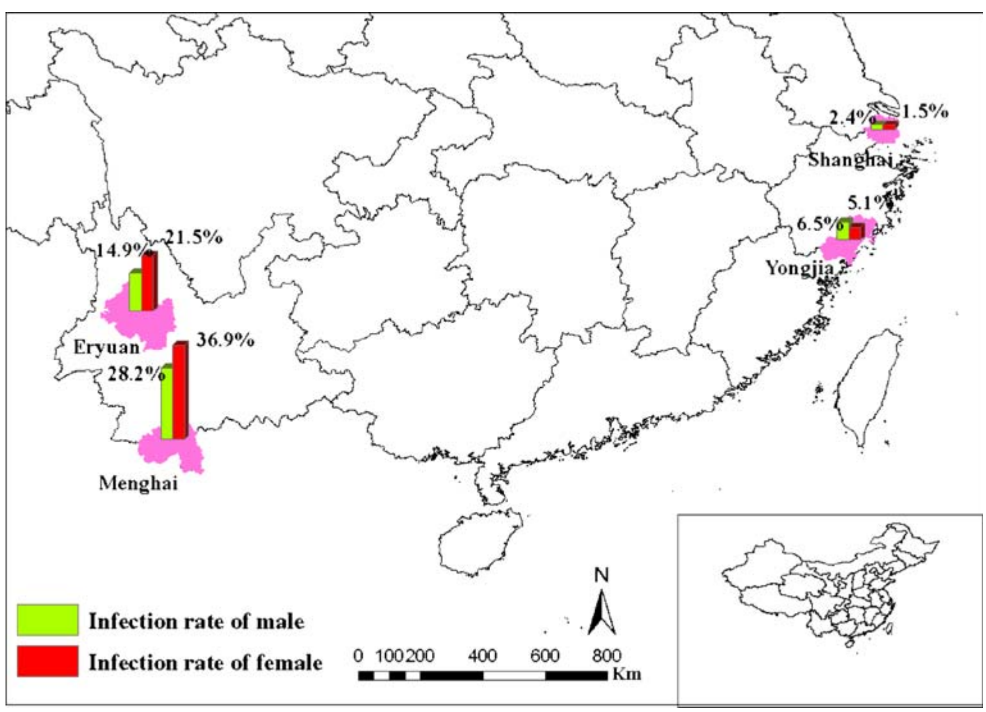

Fig. 1 Prevalence of human *Blastocystis*, stratified by sex, in four different epidemiological settings in China

Materials and methods

Study area and population surveyed

Figure 1 shows the location of the four study areas namely, (1) Shanghai municipality, (2) Yongjia county of Wenzhou city, Zhejiang province, (3) Eryuan county of Dali Bai autonomous prefecture, Yunnan province, and (4) Menghai county of Xishuangbanna Dai autonomous prefecture, Yunnan province. While Shanghai municipality and Yongjia county are located in the eastern and more developed part of China, the counties of Eryuan and Menghai are situated in the less industrialized and socio-economically underprivileged Yunnan province in southwestern China. Sanitation infrastructure in villages belonging to Eryuan and Menghai counties are generally poor or nonexistent, and the quality of water supply is inadequate, as water is neither filtered nor chlorinated. There is free forage of livestock (e.g., buffaloes, cattle, and pigs) and poultry in the villages. In contrast, residents in Shanghai municipality and Yongjia county have access to clean water and improved sanitation.

In Eryuan county, stool specimens were collected toward the end of 2005 from 407 people aged 5 to 79 years from 80 randomly selected families in four villages (20 families per village), as part of a large cross-sectional survey described in detail elsewhere (Steinmann et al. 2007a). In Shanghai municipality, stool specimens were collected in March 2006 from 1,505 people aged 2 to 96 years from 613 families living in the villages of Dianhu and Longhuaxin. In Menghai county, stool specimens were collected in June 2006 from 239 people aged 4 to 84 years from 71 families living in Nongyang village. Finally, in August 2006, stool specimens were obtained from 170 in-patients, aged 4 months to 90 years in the People's Hospital of Yongjia county.

Field and laboratory procedures

Stool specimens were collected in plastic containers, labeled with unique identification numbers, and transferred to nearby laboratories. From each study participant, demographic data, including age, sex, and ethnic background, was obtained using standardized questionnaires.

The laboratory procedures for *Blastocystis* culture and identification have been described previously (Li et al. 2007). In brief, stool samples were cultured in Ringer's solution containing 10% horse serum and 0.05% asparagine at 37°C (Yoshikawa et al. 2004b). After 3–4 days, cultures were examined under a light microscope for *Blastocystis*. Positive samples were subcultured in the same medium for another 3–4 days.

Subtyping of *Blastocystis* genotypes

The subtyping procedures have also been detailed elsewhere (Li et al. 2007). In brief, genomic deoxyribonucleic acid (DNA) was extracted using DNAzol (Invitrogen, Carlsbad CA) and subjected to polymerase chain reaction (PCR) amplification using subtype-specific sequence-tagged site (STS) primer developed by Yoshikawa et al. (1998, 2000, 2003) with positive control DNAs for each primer.

Sequencing and phylogenetic analysis of the SSU rRNA genes

Small subunit (SSU) ribosomal ribonucleic acid (rRNA) gene of an isolate negative with all the primers was amplified with a pair of conserved primers targeting at the entire SSU rRNA gene (SSU ribosomal DNA [rDNA]; Yoshikawa et al. 2000). The PCR product was purified with a PCR rapid purification kit (Beijing BioDev-Tech, Beijing) and ligated into pGEM T-Easy Vector (Promega, Madison WI) and sequenced. Primers synthesis and SSU rDNA sequencing were finished by Invitrogen. The sequences of the SSU rDNA were aligned manually by using the maximum likelihood method with Mega software 4.0.

Data management and analysis

Double-data entry and internal consistency checks of the database were done in EpiData version 3.1 (EpiData Association, Odense, Denmark). Statistical analyses were carried out in SAS version 8.0 (SAS Institute, Cary, NC). The likelihood-ratio chi-square test (χ^2) and Fisher's exact test as appropriate were used to assess the relationship of the *Blastocystis* infection rate between study areas and according to demographic factors (i.e., sex and age, the latter stratified into five age groups: infants and young children [less than 10 years], adolescents [10–17 years], young adults [18–39 years], middle-aged adults [40–59 years], and old and retired adults [greater than or equal to 60 years]). Finally, the frequency of individual subtypes of *Blastocystis* was compared between the different settings.

Results

Overall, 2,321 stool specimens were collected and cultured for subsequent examination of *Blastocystis*: 1,505 from Shanghai municipality, 407 from Eryuan county, 239 from Menghai county, and 170 from Yongjia county. All participants in the Shanghai and Yongjia cross-sectional surveys were Han Chinese, whereas in Menghai county, people belonged to the Bulang nationality. In Eryuan county, 79% were Bai, and the remaining 21% were Han, with more than 90% of the latter ethnic group living in the same village.

Blastocystis prevalence

The highest prevalence of *Blastocystis* was observed in Menghai county namely, 32.6% (95% confidence interval [CI]=26.7–38.6%), followed by Eryuan, where a prevalence of 18.4% was found (95% CI=14.7–22.2%). Much lower prevalence was observed in Yongjia county (5.9%, 95% CI=2.4–9.5%) and Shanghai municipality (1.9%, 95% CI=1.2–2.6%). There was a highly statistically significant difference in the prevalence of *Blastocystis* in the four study areas (χ^2=323.42, degree of freedom [df]=3, P<0.001).

Figure 1 shows the prevalence of *Blastocystis*, stratified by sex, in the different epidemiological settings. The overall prevalence of males and females was similar. Locally, however, the prevalence of *Blastocystis* showed considerable difference between males and females, although not significant at the 5% level. In Eryuan and Menghai counties, females were more likely to be infected with *Blastocystis* than males (Eryuan=21.5 vs. 14.9%, χ^2=2.90, df=1, P=0.088; Menghai=36.9 vs. 28.2%, χ^2=2.05, df=1, P=0.153). The opposite was found in Yongjia county and Shanghai municipality, where the prevalence among females was lower than in males (Yongjia=5.1 vs. 6.5%, χ^2=0.15, df=1, P=0.700; Shanghai=1.5 vs. 2.4%, χ^2=1.41, df=1, P=0.235).

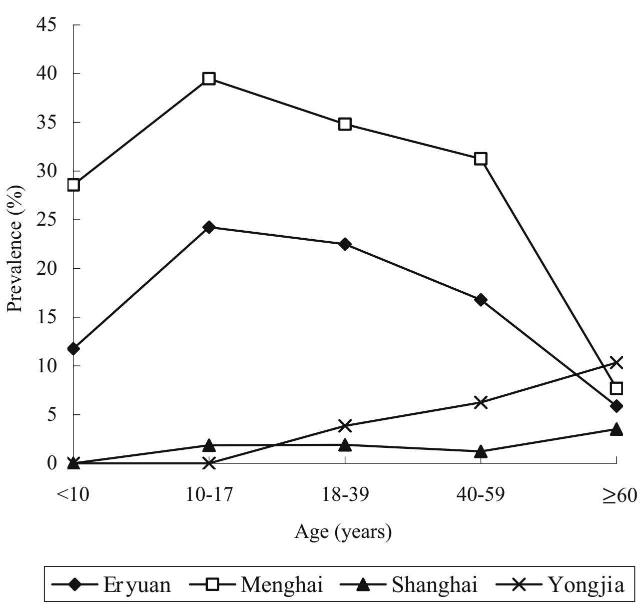

Fig. 2 Age–prevalence curves of human *Blastocystis* in four different epidemiological settings in China

Figure 2 depicts age–prevalence curves of *Blastocystis* for the different study settings. While the prevalence significantly differed between age groups when all the data were pooled (χ^2=66.68, df=4, P<0.001), no statistically significant differences were seen for the individual study sites. Similar *Blastocystis* age–prevalence curves were observed in Eryuan and Menghai counties with the peak prevalence recorded among the 10–17-year-olds. On the other hand, in Shanghai municipality and Yongjia county, participants aged greater than or equal to 60 years had the highest infection rates.

The relationship between prevalence and ethnic group living in the same area could only be studied for Eryuan county. In this county, a significantly higher prevalence of *Blastocystis* was found among ethnic Bai (20.5%) when compared to Han (10.6%; χ^2=4.39, df=1, P=0.036).

Frequency of *Blastocystis* subtypes

The relative subtype frequencies of the *Blastocystis* isolates are summarized in Table 1, according to the nomenclature established by Stensvold et al. (2007). Single-subtype infections were found in 174 of the 192 isolates (90.6%), and two different subtypes from the same patients were concurrently identified in ten cultures (5.2%). We failed to amplify the remaining eight isolates (4.2%) with the standard primers utilized in our study. There was no statistically significant difference in the relative frequencies of the different *Blastocystis* subtypes between study sites, age groups, and sex. In 39 families, two or more family members were infected with *Blastocystis*. Among them, all members of 21 (53.8%) families were infected with only one subtype.

The SSU rRNA gene of only one of the eight isolates that could not be amplified with the standard STS primers could be amplified with the conserved primers targeting the entire SSU rRNA gene and sequenced. Phylogenetic analysis showed that this isolate and two other reported China isolates (DQ366343 and EF079872 in GenBank) were clustered into an additional monophyletic clade (clade X), which branched from a clade of the subtype 7 in the phylogenetic tree with 100% bootstrap value (Fig. 3).

Discussion

Although the public health significance of *Blastocystis* is still debated, common intestinal parasites are indicators for the fecal contamination of the environment (Muller et al. 1989; Schulz and Kroeger 1992). It is also suspected that *Blastocystis* is associated with lower anthropometric indices in children (Ertug et al. 2006) and water-associated outbreaks of parasitic protozoan disease (Karanis et al.

Table 1 Number of study participants and *Blastocystis* isolates, stratified by subtypes, as obtained during cross-sectional surveys carried out in four different epidemiological settings in China

Study area	Number of participants	Number of *Blastocystis* isolates	Number (%) of subtypes										
			1	2	3	4	5	6	7	1+3[a]	1+2[a]	2+3[a]	Unknown
Eryuan county	407	75	22 (29.3)	6 (8.0)	38 (50.7)	0	0	0	0	5 (6.7)	0	1 (1.3)	3 (4.0)
Menghai county	239	78	16 (20.5)	1 (1.3)	55 (70.5)	1 (1.3)	0	0	0	1 (1.3)	1 (1.3)	0	3 (3.8)
Shanghai municipality	1,505	29	6 (20.7)	1 (3.4)	17 (58.6)	0	0	1 (3.4)	0	2 (6.9)	0	0	2 (6.9)
Yongjia county	170	10	3 (30.0)	1 (10.0)	6 (60.0)	0	0	0	0	0	0	0	0
Total	2,321	192	47 (24.5)	9 (4.7)	116 (60.4)	1 (0.5)	0	1 (0.5)	0	8 (4.2)	1 (0.5)	1 (0.5)	8 (4.2)

[a] Mixed infection

2007). We assessed the prevalence of *Blastocystis* and relative frequency of *Blastocystis* subtypes in four distinctively different epidemiological settings in China. We found extensive genetic diversity of *Blastocystis* irrespective of the study location but noted marked differences in the prevalence and age-prevalence curves of *Blastocystis* between eastern and western China. In fact, the prevalence of *Blastocystis* in the two counties in Yunnan province, western China, was several-fold higher when compared to Shanghai municipality and Yongjia county in eastern China. In the highly endemic settings, the peak prevalence occurred in younger age groups, whereas in the low-endemic settings, the peak prevalence was observed in the oldest age group. This phenomenon, the so-called peak shift, is a well-known feature in parasite epidemiology (Woolhouse 1998).

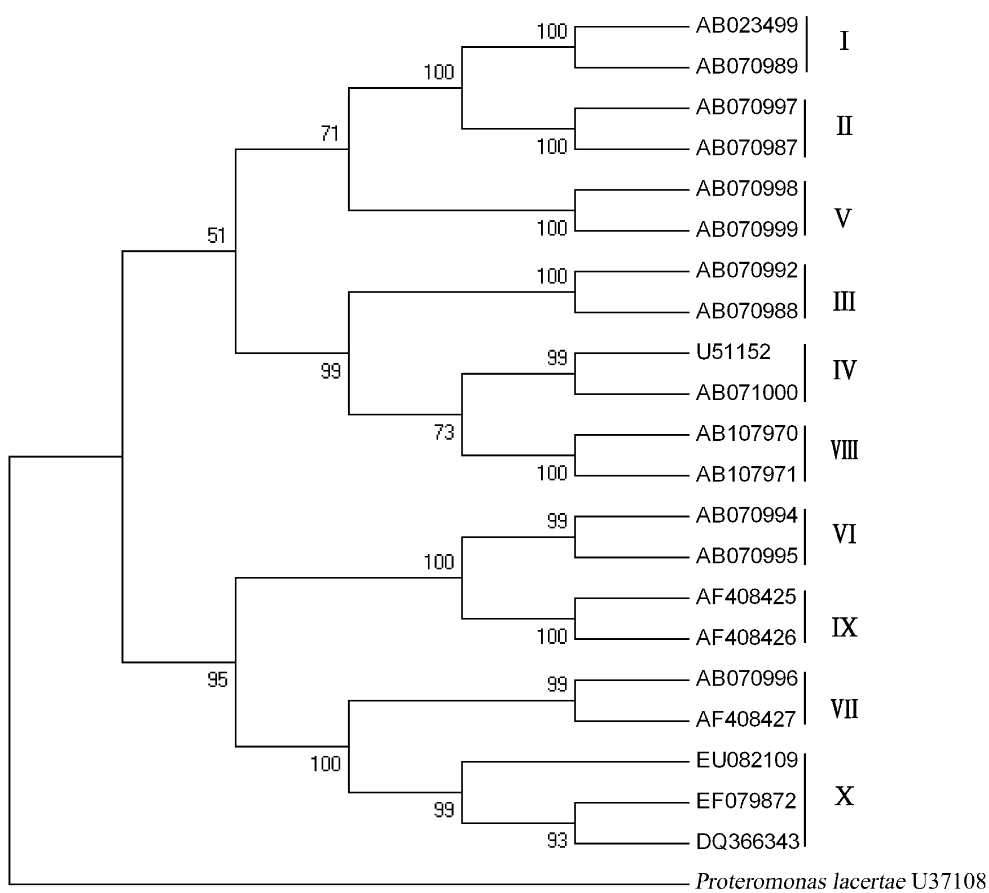

Fig. 3 Subtype classification and phylogenetic tree of the SSU rDNA sequence of three isolates including one from our study (EU082109) and other two China isolates (EF079872, DQ366343) combined with the known sequence data of 18 *Blastocystis* isolates with *Proteromonas lacertae* (U37108) as the outgroup. All *Blastocystis* isolates from mammals and birds were listed by using accession numbers in the GenBank. The subtype classification from I to IX was followed to the nomenclature by Stensvold et al. (2007). Note a new clade of the subtype X comprised of our isolate (EU082109) and two reported China isolates (EF079872 and DQ366343) was branched from the subtype VII supported with 100% bootstrap value

Blastocystis infections are common in tropical and subtropical environments, particularly in poor rural communities among lower socio-economic strata and in temperate zones where hygiene standards are low (Stenzel and Boreham 1996; Tan et al. 2002). We found higher prevalence in the southern study settings compared to the northern areas, both in East and West China, and higher prevalence in the less developed inland province of Yunnan than in the coastal provinces of Shanghai and Zhejiang. The economic development of China started in the coastal provinces and is only about to spread to more remote areas. The uneven economic development has impacted on the epidemiology and distribution of various parasitic diseases (Banister and Zhang 2005).

The *Blastocystis* prevalence recorded in Shanghai municipality, i.e., 1.9%, is higher than the prevalence reported in the first national sampling survey of human parasites in China, when the prevalence of *Blastocystis* in Shanghai was below 0.1%. In Yongjia county and the two settings in Yunnan province, we found several-fold higher prevalence than those reported in the national sampling survey for Zhejiang province (0.8%) and Yunnan province (4.5%; Xu et al. 2000). The most likely explanation for these differences arises from the lack of sensitivity of direct microscopic examination of stool specimens (Termmathurapoj et al. 2004). The national sampling survey relied on direct fecal smears and an iodine stain technique, which was reported to only have 16.7% sensitivity compared with the culture, the technique employed in the current study (Termmathurapoj et al. 2004). Therefore, by adapting a more sensitive detection method, the present investigation for the first time revealed a more accurate situation of the human *Blastocystis* prevalence in China.

Previous investigations have reported differences in the prevalence of *Blastocystis* between various ethnic groups in China (Wang et al. 1994; Liu et al. 1997). In Eryuan county, we found a significantly higher prevalence among members of the Bai than Han, and the highest point prevalence was found in a village inhabited by the Bulang minority in Menghai county. However, we hypothesize that these differences are due to prevailing local living conditions and customs, rather than differences in the susceptibility to infection (Lai 1992). In Eryuan county, most Han lived in the fertile and economically more advanced plain areas, whereas the Bai inhabited more remote mountainous areas. Additional differences could be found in dietary customs and hygiene conditions (Steinmann et al. 2007a). Nongyang village in Menghai county, which was inhabited by ethnic Bulang, was located in the subtropical climate zone. From the same village, exceptionally high prevalences of intestinal helminths (i.e., *Ascaris lumbricoides*, hookworm, and *Trichuris trichiura*) have been reported (Steinmann et al. 2007b).

A previous review concluded that the age and sex distribution of *Blastocystis* infections shows spatial heterogeneity (Stenzel and Boreham 1996). Regarding age, we already noted that in the highly endemic areas (i.e., Eryuan and Menghai county), the peak prevalence occurred at a younger age namely, among those aged 10–17 years. Similarly, a study by El-Shazly et al. (2005) in Egypt reported a peak prevalence in participants aged between 10 and 20 years. People aged 60 years and above had the highest prevalence of *Blastocystis* in Shanghai municipality and Yongjia county, which is similar to findings reported from Glasgow, UK. There, an overall prevalence of *Blastocystis* of 3.9% was found with the highest infection rates among the elderly (Suresh and Smith 2004).

Sex is generally not considered as a risk factor for *Blastocystis* infection, although different studies reported slightly higher prevalence among females than males (Stenzel and Boreham 1996). We found setting-specific sex differences with higher infection rates among women in the high-prevalence sites in Eryuan and Menghai counties and higher prevalence among males in the low-prevalence settings of Shanghai municipality and Yongjia county, although none of the differences showed statistical significance ($P>0.05$). It is conceivable that the marked differences in the prevalence of *Blastocystis* from one setting to another and within-setting differences in age and sex patterns indicate distinct ways of transmission or different exposition to the sources of transmission. The underlying reasons for these patterns remain to be evaluated.

Using the PCR-based subtype classification with standardized STS primers developed by Yoshikawa et al. (1998, 2000), we identified five *Blastocystis* subtypes among the 192 cultured isolates from the four study sites. To our knowledge, this is the first attempt to study and compare the genetic diversity of *Blastocystis* in different parts of China. Subtype 3 was the predominant subtype, followed by subtype 1, corroborating recent results (Yan et al. 2006). In addition, we also repeatedly identified subtype 2. Subtypes 4 and 6 were each isolated only once. Subtypes 5 and 7 could not be detected in our study. It is interesting to note that in only one isolate among eight unidentified isolates could the entire SSU rDNA be amplified. Phylogenetic analysis including subtypes 1 to 9 (Stensvold et al. 2007) showed that the isolate was positioned as an additional monophyletic clades X with other two China isolates (DQ366343 and EF079872 in GenBank) supported by a 100% bootstrap value (Fig. 3). Based on the SSU rDNA phylogeny, it is evident that our isolate and the other two isolates are a new subtype. Our study also demonstrated that the PCR analysis using the known STS primers is a practical tool to identify unknown subtypes.

So far, the correlation of different subtypes with geographic origin, prevalence, and pathology is still unknown.

We summarized the subtype proportion in China, Japan, Germany, and UK (Bohm-Gloning et al. 1997; Clark 1997; Yoshikawa et al. 2000, 2003, 2004b; Scicluna et al. 2006; Yan et al. 2006) and revealed that subtype 3 is the most dominant subtype in humans. The proportions of other subtypes differ between locations. Subtype 1 is the second most common variant in China and Germany, while subtype 6 follows at position two in Japan. Subtype 4 is second in the UK and also common in Germany but rarely observed in China and Japan. Subtype 7 is common in Japan but not in the other countries, and subtype 2 is common in the UK and China. The relative proportions of the different subtypes might indicate different epidemiological characteristics, i.e., reservoirs and ways of transmission (Hunter et al. 2004). However, the direct public health relevance of these subtypes is debated because none of the subtypes could be associated with particular intestinal symptoms (Bohm-Gloning et al. 1997; Yoshikawa et al. 2004b; Yan et al. 2006). A particular challenge to any conclusion about the significance of the different subtypes is the limited number of relevant studies and their poor comparability resulting from the use of different techniques and terminologies (Ho et al. 1994; Init et al. 1999; Stensvold et al. 2007).

Direct transmission of *Blastocystis* between humans has been reported (Yoshikawa et al. 2000), and it is suspected that different parasite subtypes have different reservoirs (Hunter et al. 2004). We found the same subtype in more than one member of 21 out of 39 families with at least two infected individuals. This indicates human-to-human transmission between family members or shared exposure to a common source of infection. The presence of multiple subtypes in the same families and communities indicates the concurrent existence of different sources of infection or infection at different locations.

We conclude that heterogeneity is an important feature in the epidemiology of *Blastocystis* in China, governed by locality, age, and sex. Stool culture is the recommended method for the study of this intestinal protozoan parasite owing to its superior sensitivity over other diagnostic approaches. Genotyping using standardized methods will reveal new insights into this common but research-neglected parasite. Additional studies are warranted to identify specific risk factors for infection, which could in turn explain the different prevalence encountered among members of different nationalities living in the same area. Finally, new research should aim at animal and environmental isolates to identify different *Blastocystis* reservoirs and transmission routes.

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Conflict of interest The authors declare that they have no conflict of interest.

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