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Molecular epidemiology of human *Blastocystis* in a village in Yunnan province, China

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Abstract

The purpose of this study was to improve our understanding of the molecular epidemiology of human *Blastocystis*, focusing on 239 randomly selected individuals in a single village in Yunnan province, China. Emphasis was placed on the relative frequency of different *Blastocystis* subtypes and underlying risk factors. We used a cross-sectional study design, by employing a pre-tested questionnaire to obtain demographic data and behavioural risk factors, and collected faecal samples for culture and subsequent identification of *Blastocystis*. DNA was extracted from *Blastocystis* isolates and the subtypes were identified using 7 subtype-specific sequenced-tagged site (STS) primers. Overall, 78 faecal samples were *Blastocystis* culture-positive (32.6%, 95% confidence interval: 26.7–38.6%). The majority ($n=73$, 93.6%) were single infections with one of the known subtypes, whereas 2 isolates consisted of 2 concurrent subtypes. The remaining 3 isolates could not be identified with the currently known STS primers. Risk factors for a *Blastocystis* infection were drinking unboiled water, consumption of raw water plants and pig ownership. The consumption of raw water plants was positively associated with subtype 1 infections, and drinking unboiled water with subtype 3 infections. In conclusion, human *Blastocystis* was common in this village in southwest China, and different subtypes were associated with distinct transmission routes or sources of infection, and hence *Blastocystis* subtypes might be linked to specific environmental compartments.

Keywords: *Blastocystis*; Molecular epidemiology; Subtype; Risk factor; China

1. Introduction

Blastocystis is a common protistan parasite infecting humans and many different animal species. It is found in both symptomatic and healthy individuals, and hence its pathogenic potential is still debated [1]. In addition, the taxonomy, life cycle, mode of transmission and zoonotic potential of *Blastocystis* warrant further clarification [2]. In view of the paucity of epidemiological studies pertaining to intestinal parasites, the true extent of *Blastocystis* infections among humans is unknown in many parts of the world. Moreover, diagnosis of *Blastocystis* is hampered by the relatively low sensitivity of widely employed diagnostic approaches, i.e. parasite detection in faecal samples by light microscopy of direct smears, faecal concentrates or permanently stained smears. The two main underlying reasons are the small size of *Blastocystis* and its large morphological diversity [1,3]. The use of culture methods have been suggested for more sensitive and more reliable *Blastocystis* detection [4,5].

In China, *Blastocystis* has not been investigated in a systematic manner, and the available data pertaining to human

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Blastocystis were derived from direct faecal smear examinations [6–8]. It follows that the epidemiology of human *Blastocystis* infections is not well understood in China and the overall prevalence might be considerably underestimated. The first national sampling survey on human parasites in China was conducted between 1988 and 1992 and estimated that the mean prevalence of *Blastocystis* was 1.3% [6].

The speciation of the genus *Blastocystis* is still controversial, but it is commonly assumed that all *Blastocystis*-like organisms isolated from human faeces belong to one species, i.e. *Blastocystis hominis*. Most isolates from non-human hosts are both morphologically and genetically similar to human *Blastocystis* [9]. The identification of protistan species by morphological characteristics is often challenging but recent advances with genetic analyses have opened up new avenues for the development of novel diagnostic tools. The highly conserved small-subunit ribosomal RNA (SSU rRNA) gene is frequently used as a genetic marker to identify species [10]. It is generally assumed that significant genetic variations in the SSU rRNA gene sequence occur between rather than within species [11,12]. The sequenced-tagged site (STS) primers developed by Yoshikawa and his colleagues can reliably distinguish 7 different subtypes within the morphologically identical *Blastocystis* isolates usually recovered from human faeces [13,14]. These subtypes or species could be associated with distinct symptoms, risk factors and zoonotic potential. Similar observations have been made for *Cryptosporidium* and *Entamoeba* [10,15,16]. In *Blastocystis*, the differences between subtypes have only been investigated with regard to the pathogenic potential of different subtypes [14,17–19]. In this connection, it is important to note that in experimental animals, the infection potential varies between different *Blastocystis* subtypes [20].

The purpose of this cross-sectional study carried out in a rural area of south-western China was to estimate the infection prevalence and subtype distribution of human *Blastocystis*, and to identify risk factors for infections in general and different subtypes in particular.

2. Materials and methods

2.1. Study area

Our study was carried out in Nongyang village, situated in Xishuangbanna prefecture, Yunnan province, China (geographical coordinates: 25.80° N latitude, 100.35° E longitude). The village is located in a hilly area, inhabited by members of the Bulang nationality, and primarily depends on farming and livestock breeding. Buffaloes, cattle, pigs and chickens roam freely in the village. Piped water, originating from a nearby river, is delivered to every household, though without previous filtering or chlorination. There is a lack of adequate sanitation; only 1 public latrine is available for the entire village, but is not used by all villagers.

2.2. Study population and informed consent

Approximately 150 families live in Nongyang village. Half of the households were randomly selected and informed about the purpose and procedures of the study. Written informed consent was sought from each head of the selected households or a designated literate substitute. In case a selected family was absent during our cross-sectional survey, a neighbouring family was invited to participate.

The study was approved by the institutional review boards of the National Institute of Parasitic Diseases (Shanghai, China) and the Swiss Tropical Institute (Basel, Switzerland).

2.3. Questionnaire survey and faecal collection

An individual questionnaire, previously tested and adapted to the current epidemiological setting, was administered to each participant. Data on demographic factors (e.g. age and sex), food consumption, hygiene behaviour and morbidity signs experienced during the preceding 2 weeks were obtained. Children below the age of 15 years answered the questionnaire with the help of their parents or a legal guardian. An additional questionnaire was addressed to all household heads to collect information on household characteristics and ownership of domestic animals.

After completion of the questionnaire surveys, pre-labelled collection containers were handed out and participants were asked to submit a fresh faecal sample in the early morning of the next day.

2.4. Laboratory procedures for diagnosis and subtype classification of Blastocystis

Faecal samples were collected in the morning and transferred to the laboratory where they were cultured at 37 °C in Ringer's solution, containing 0.05% asparagine and 10% horse serum [14], for 3 days. The cultures were screened microscopically for *Blastocystis* and positive samples were sub-cultured for another 3 days using fresh media. Subsequently, genomic DNA was extracted using DNAzol (Invitrogen Life Technologies, Carlsbad CA, USA), according to the manufacturer's instructions.

We adhered to the recently proposed terminology for the classification of *Blastocystis* subtypes [12]. Seven standardized subtype-specific STS primers were used, namely SB83 (351 bp) for subtype 1, SB340 (704 bp) for subtype 2, SB227 (526 bp) for subtype 3, SB337 (487 bp) for subtype 4, SB336 (317 bp)

Table 1
Prevalence of *Blastocystis* infection, as assessed by a culture method, and stratified by sex and age group among 239 inhabitants of Nongyang village, Yunnan province, China

Age group (years) [a]	Total		Male [b]		Female [b]	
	No.	No. (%) positive	No.	No. (%) positive	No.	No. (%) positive
<5	7	1 (14.3)	4	1 (25.0)	3	0 (0)
5–9	21	7 (33.3)	7	2 (28.6)	14	5 (35.7)
10–17	38	15 (39.5)	18	6 (33.3)	20	9 (45.0)
18–39	112	39 (34.8)	58	19 (32.8)	54	20 (37.0)
40–59	48	15 (31.3)	22	4 (18.2)	26	11 (42.3)
≥60	13	1 (7.7)	8	1 (12.5)	5	0 (0)
Total	239	78 (32.6)	117	33 (28.2)	122	45 (36.9)

[a] Difference between age groups: Fisher's exact test, $p=0.328$.
[b] Difference between males and females: $\chi^2=2.05$, $p=0.153$.

Table 2
Number (and percentage) of *Blastocystis* subtypes, isolated from faecal samples collected from humans living in Nongyang village, Yunnan province, China

Subtype [a]	No. (%)
Subtype 1	16 (20.5)
Subtype 2	1 (1.3)
Subtype 3	55 (70.5)
Subtype 4	1 (1.3)
Subtypes 1 and 2	1 (1.3)
Subtypes 1 and 3	1 (1.3)
Unknown	3 (3.9)
Total	78 (100)

[a] The new standard terminology for human *Blastocystis* subtypes has been used [12].

for subtype 5, SB332 (338 bp) for subtype 6 and SB155 (650 bp) for subtype 7 [13].

2.5. Data management and statistical analysis

Questionnaire and parasitological data were double-entered and validated in EpiData version 3.1 (EpiData Association, Odense, Denmark). Statistical analyses were conducted using SAS version 8.0 (SAS Institute Inc., Cary, USA). Only participants with complete data records were considered for the final analysis. The likelihood-ratio chi-square test and Fisher's exact test were used, as appropriate, to explore associations between the infection status with *Blastocystis* or different subtypes and demographic variables, as well as associations between independent variables.

Bivariate logistic regression was used to test for an association between an infection with *Blastocystis* and different food consumption habits (e.g. drinking unboiled water, consumption of raw food dishes) and self-reported morbidity indicators. The association between the infection status and ownership of domestic animals was analyzed using a multi-level logistic regression model, because animal ownership differs at the family level. Finally, multiple logistic regression models were employed to further explore the association between an infection with *Blastocystis* or specific subtypes and the drinking of unboiled water or consumption of raw food items, as well as livestock ownership. Variables were included at a level of $p=0.1$, using a stepwise forward selection approach.

3. Results

Overall, 283 individuals from 71 families were enrolled. Complete data records (i.e. filled-in questionnaires and cultured faecal samples) were obtained from 239 participants (84.5%). The study cohort consisted of 122 females (51.1%), and the age of the participants ranged from 4 to 84 years. The 44 individuals without complete questionnaire data or the lack of a faecal sample showed similar age and sex profiles as the final study cohort. *Blastocystis* could be cultured from the faeces of 78 individuals, corresponding to a prevalence of 32.6% (95% confidence interval: 26.7–38.6%).

Table 1 shows the infection status with *Blastocystis*, stratified by sex and age group. The prevalence among males and females was 28.2% and 36.9%, respectively ($\chi^2=2.05$, $p=0.153$). The infection rate by age group increased from 14.3% in children <5 years of age to 39.5% (10–17 years), 34.8% (18–39 years) and was 7.7% in those ≥60 years (Fisher's exact test, $p=0.328$).

Table 2 presents the frequency of the different *Blastocystis* subtypes. The 78 successfully cultured and morphologically identified *Blastocystis* isolates belonged to 4 different subtypes, with the large majority being single infections with known subtypes ($n=73$; 93.6%). Subtype 3 was the dominant type (70.5%). Subtype 1 accounted for 20.5% of the single subtypes found in the village. Two isolates were mixed infections consisting of 2 different subtypes. The remaining 3 isolates could not be assigned to any of the known subtypes, and hence might represent new subtypes.

The results of the bivariate logistic regression analysis are shown in Table 3. An infection with *Blastocystis* was positively associated with drinking unboiled water (odds ratio (OR)=

Table 3
Association between *Blastocystis* infection and raw food consumption and domestic animal ownership among 239 study participants from Nongyang village, Yunnan province, China

Risk factor	*Blastocystis*		Subtype 1		Subtype 3	
	OR (95% CI)	*p*	OR (95% CI)	*p*	OR (95% CI)	*p*
Food and drinking habits [a]						
Drinking unboiled water	8.56 (1.11, 65.80)	0.039	1.27 (0.16, 10.18)	0.824	–	0.015 [b]
Consumption of raw water plants	0.89 (0.50, 1.59)	0.694	8.28 (1.08, 63.58)	0.044	0.59 (0.32, 1.10)	0.099
Consumption of raw pork meat	0.55 (0.31, 0.97)	0.038	1.07 (0.39, 2.99)	0.892	0.64 (0.34, 1.19)	0.156
Consumption of raw fish	0.56 (0.32, 1.01)	0.053	0.85 (0.30, 2.39)	0.763	0.69 (0.36, 1.30)	0.248
Consumption of raw crab	0.45 (0.13, 1.64)	0.227	1.94 (0.40, 9.35)	0.408	0.20 (0.03, 1.54)	0.122
Ownership of domestic animals [c]						
Pigs	2.82 (0.99, 7.99)	0.052	1.42 (0.18, 11.31)	0.743	2.59 (0.59, 11.27)	0.204
Buffaloes	0.91 (0.48, 1.71)	0.764	0.48 (0.13, 1.73)	0.261	0.89 (0.45, 1.74)	0.725
Dogs	0.64 (0.33, 1.22)	0.171	1.27 (0.28, 5.18)	0.964	1.97 (0.74, 5.27)	0.174

CI: confidence interval; OR: odds ratio.
The new standard terminology for human *Blastocystis* subtypes has been used [12].
[a] Univariate logistic regression.
[b] Fisher's exact test.
[c] Multi-level logistic regression.

Table 4
Multiple logistic regression analysis of *Blastocystis* infection and subtype 1 infection among 239 study participants from Nongyang village, Yunnan province, China

Infection and risk factors	OR (95% CI)	p
Blastocystis, all subtypes [a, b]		
Drinking unboiled water	9.45 (1.28, 69.93)	0.028
Consumption of raw pork meat	0.54 (0.31, 0.95)	0.031
Ownership of pigs	2.78 (0.84, 9.21)	0.096
Blastocystis, subtype 1 [a, c]		
Consumption of raw water plant	8.28 (1.08, 63.58)	0.044

A stepwise forward selection procedure was used and variables at the level of $p=0.1$ were eliminated.
CI: confidence interval; OR: odds ratio.
[a] The new standard terminology for human *Blastocystis* subtypes is used [12].
[b] Multi-level logistic regression.
[c] Bivariate logistic regression.

8.56, $p=0.039$) and negatively associated with the consumption of raw pork meat (OR=0.55, $p=0.038$). The stratification by subtype showed that subtype 3 was only found in participants who reported to have drank unboiled water (Fisher's exact test, $p=0.015$). The consumption of raw water plants was positively associated with subtype 1 infections (OR=8.28, $p=0.044$). No significant associations were found between an infection with any of the observed *Blastocystis* subtype and sex, age group or self-reported signs and symptoms (data not shown).

The results of the multivariate logistic regression between an infection with *Blastocystis* in general and an infection with subtype 1 in particular, and the risk factors identified in the bivariate analysis, are summarised in Table 4. Drinking unboiled water was a risk factor for an infection of any *Blastocystis* subtype (OR=9.45, $p=0.028$). Pig ownership resulted in an OR of 2.78, but showed no statistical significance ($p=0.096$). The consumption of raw pork meat appeared to be a protective factor (OR=0.54, $p=0.031$), but this might be a chance finding. The consumption of raw water plants was significantly associated with a subtype 1 infection (OR=8.28, $p=0.044$).

4. Discussion

To our knowledge, this is the first investigation into the molecular epidemiology of human *Blastocystis* in a typical rural area of the Yunnan province in south-western China. Using pre-tested questionnaires and a sensitive faecal culture method, we found a *Blastocystis* prevalence of 32.6% among a random population sample from a single village, and identified underlying risk factors. It is conceivable that the 'true' prevalence of *Blastocystis* in this setting is even higher, since we only collected 1 stool specimen per person for subsequent culturing in the laboratory, and hence some infections might have been missed. The genetic classification of the isolates revealed the presence of 4 different known subtypes of variable prevalence. Three of the cultured *Blastocystis* isolates, however, could not be assigned to any of the known subtypes. Specific risk factors could be linked to positive cultures and infections with different subtypes, respectively.

The sanitary infrastructure (or rather the lack thereof) in the village studied here, and people's living conditions, and local food and water consumption habits are consistent with known risk factors for the faecal–oral transmission of parasitic diseases, including *Blastocystis* [21–23]. The first national sampling survey on the distribution of human parasites in China, carried out between 1988 and 1992, reported a *Blastocystis* prevalence of 8.6% for the southern part of the Yunnan province [24]. However, the diagnostic method used at the time, i.e. direct smear and iodine staining, only has a low sensitivity compared to the culture method used in the current investigation [4]. Hence, the 'true' prevalence of *Blastocystis* in the Yunnan province and across China in general, might have been significantly underestimated during the first national sampling survey.

Previous studies reported an association between *Blastocystis* infection rates and age [21,25]. In our study, we did not observe such a relationship, but this might be explained by the relatively small sample size ($n=239$). The prevalence of *Blastocystis* among females (36.9%) was considerably higher than that in males (28.2%), but this difference showed no statistical significance ($p=0.153$). Our findings corroborate results from other studies although it is generally assumed that sex is not a risk factor for an infection with *Blastocystis* [1]. Drinking unboiled water has been identified before as a risk factor for infection [22,23]. This might indicate that *Blastocystis* is mainly transmitted through contaminated drinking water [1,2]. However, work-related exposure to animals is another known risk factor for infection [26]. Recently, it could be shown that certain *Blastocystis* isolates have a zoonotic potential [20], and isolates from humans are now commonly classified into the same subtypes as isolates obtained from animals that were in close contact with humans [27]. Note that previous studies found high prevalence of *Blastocystis* in domestic animals [28,29]. Future studies should investigate the susceptibility of humans and domestic animals to different *Blastocystis* subtypes to elucidate the role of domestic animals in the transmission of different subtypes of *Blastocystis*. Pig ownership was a risk factor for a *Blastocystis* infection in our study, suggesting that pigs might play a more prominent role in the local epidemiology of *Blastocystis* than other domestic animals in the present village. Similar observations have also been reported by Abe and co-workers in Japan [29].

New research is needed to shed light on the role of *Blastocystis* and its different subtypes on morbidity [1]. In the current study, no significant relationship was found between self-reported morbidity and an infection with *Blastocystis*. However, the evaluation of parasite-specific morbidity was complicated by the very high prevalence of soil-transmitted helminth infections and multiple species parasitic infections (data not shown).

Still today, men have a higher social status than women in certain parts of China [30]. This inequity is also mirrored in the diet; males consume more expensive, protein-rich food such as chicken, eggs, fish and meat, which are traditionally regarded as superior to typical carbohydrate diets. In our study, the consumption of raw pork dishes, fish and crab was more common among males than females ($p<0.001$), whereas no gender difference was found regarding the consumption of raw water plants (mainly wild cress).

Consequently, many women are "passive" (= non self-elected) vegetarians, while males consume mixed diets. The plasma zinc concentration is lower in vegetarians than in non-vegetarians [31,32]. Zinc is an essential trace element and zinc deficiency has detrimental effects on the immune system [33]. *Blastocystis*-infected males have lower zinc concentrations than their non-infected female counterparts [34]. Moreover, there is evidence that infections with *Blastocystis* are more common and more severe in immunocompromised patients than in healthy controls [35,36]. These observations might offer an explanation for the negative association between *Blastocystis* infection and the consumption of raw pork, and the considerably lower prevalence among males than females.

Recently, Yan and colleagues [15] had investigated the genetic variability among human *Blastocystis* isolates from Jiangxi province, eastern China. The authors reported 3 different subtypes and found a higher prevalence of subtype 1 (37.1%) than Yoshikawa and his group [14]. We found 4 different subtypes in a single village, suggesting that different *Blastocystis* strains can co-exist in the same location in China. In our study, subtypes 1 and 3 were the most common strains, corroborating previous reports by Yan et al. [15] and Yoshikawa et al. [14]. However, we found a lower prevalence of subtype 1 than the previous report from China [15], which might be due to the different sample size and location. Studies in animals showed that different subtypes isolated from human faecal samples have different infection potentials [20]. However, we are not aware of previous attempts to identify specific risk factors for infection with different *Blastocystis* subtypes. In our study, the consumption of raw water plants was a risk factor for subtype 1 infections. Drinking unboiled water, on the other hand, was significantly associated with infections involving subtype 3. It is noteworthy that subtypes 1 and 3 are the most widely separated classes if the SSU rRNA gene is considered [11]. In Nongyang village, water plants are grown in ponds while tap water used at home originated from a small river. This suggests that different *Blastocystis* subtypes prevail in specific environmental compartments or have different reservoirs.

In conclusion, our study indicates important differences in the epidemiology of various *Blastocystis* subtypes at a small spatial unit. This calls for additional molecular epidemiological investigations pertaining to *Blastocystis* in humans and animals to reveal the distribution and relative importance of the various subtypes, and to identify their respective reservoirs and ways of transmission. This knowledge-base, in turn, will be crucial to tailor setting-specific control measures to lower the transmission potential of *Blastocystis*.

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