The prevalence and subtype distribution of Blastocystis sp. in humans and domestic animals in family units in Heilongjiang Province, China

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Research Article

Keywords: Blastocystis, Subtype, Allele, Human, Domestic animal, Zoonotic

Posted Date: May 5th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1618206/v1

Abstract

Background: *Blastocystis* is an enteric protozoan infecting humans and animals all over the world. Domestic animals play an important role in transmitting parasites to other domestic animals and humans. In the present study, a survey was conducted on *Blastocystis* among humans and domestic animals sharing habitats in northeastern China' s Heilongjiang Province, in order to investigate the prevalence, the subtype distribution, as well as to evaluate the risk of the zoonotic transmission of *Blastocystis* isolates.

Methods: A total of 314 (57 humans, 257 domestic animals) fecal samples were collected from 33 family households in three villages. The corresponding sequences of the barcode region of the *SSU rRNA* gene obtained in this study were subject to molecular analysis for subtype and allele identification of *Blastocystis* sp.

Results: The prevalence of *Blastocystis* was 14.0% (8/57) in humans and 17.9% (46/257) in domestic animals. Eight PCR-positive human samples, 100% (8/8) were successfully subtyped, allowing the identification of the ST3, followed by ST1 and ST2. The 46 PCR-positive animal samples, 65.2% (30/46) were successfully subtyped, ST5 followed by ST1 in pigs, ST5 in goats; ST10, ST14 followed by ST3 in cattle; ST7 in chickens. Phylogenetic analysis showed that *Blastocystis* ST3 sequences from humans in two geographical locations formed two distinct clades. Alleles were identified using the *Blastocystis* 18S database and a total of 10 different alleles were found in six STs.

Conclusions: The present study is the first description of the prevalence and subtype (allele) distribution of *Blastocystis* sp. by molecular analysis in humans and domestic animals in family units in China. *Blastocystis* ST2 in humans and ST5 in goats were reported in Heilongjiang Province for the first time. ST1 (a4) and ST3 (a34) overlaps were observed in humans and some domestic animal species (pig and cattle). The findings of potentially zoonotic subtypes in domestic animals suggest that these animals may serve as reservoirs of human *Blastocystis* sp. infections, thus help develop more efficient, targeted control strategies against human blastocystosis.

Background

Blastocystis sp. is a single-cell microorganism occurring in the gastrointestinal tract of humans and various animal species, with a worldwide distribution. This parasite was also the most frequently detected micro-eukaryote in some epidemiological surveys [1]. Concerning the pathogenicity of *Blastocystis* sp. remains controversial, mainly because of a high prevalence of asymptomatic carriers, the differences in host susceptibility, different pathogenic potential of different subtypes (STs) and intestinal microbiota. Recently, microbiome and metagenomics studies suggest that *Blastocystis* sp. colonization has an effect on or is usually linked to the constituents of human gut microbiota [2]. Moreover, *Blastocystis* sp. might be a potentially important factor in modulating the gut microbiota considered its positive association with higher bacterial richness and diversity [3]. However, in livestock, as in cattle, the high prevalence of *Blastocystis* sp. infection can lead to death [4]. Therefore, the pathogenicity of this organism is under strong debate.

In addition to humans, wildlife and zoo animals, *Blastocystis* is commonly found in domestic animals such as pigs, cows, goats, sheep, horses, chickens and geese [4, 5]. The mode of transmission of *Blastocystis* has not

been fully elucidated and the cyst form may be the infective stage of this protozoan. The oral-fecal route is the principal transmission pathway to infection, which occurs via direct or indirect between infected individuals and/or animals [6]. Recently, due to zoonotic and person-to-person contamination via the fecal-oral route and contaminated water as possible sources of *Blastocystis*, a high prevalence (80–100%) was reported in eight studies that identified poor hygiene and sanitation in small lower-income communities [6]. Infected persons can easily transmit the parasite to others if they are asymptomatic or they have poor personal hygiene [7]. The possibility of animal-to-human transmission of *Blastocystis* has been described in China and Thailand [7, 8]. The prevalence of animal handlers who worked in research institutions, zoos, and slaughterhouses are higher than those of people who are not in frequent contact with animals. Thus, it is necessary to consider the zoonotic potential of *Blastocystis* [9]. In addition, animal-to-animal transmission of *Blastocystis* could also be common, such as in zoological gardens and circus where grass and fodder is frequently contaminated by fecal [10].

Currently, multiple methods have been used for the detection of *Blastocystis*. *Blastocystis* diagnosis methods have relied traditionally on light microscopy and culture methods. Two methods do not seem to reflect the true prevalence of *Blastocystis* in fecal samples because of the low sensitivity and specificity of the diagnostic techniques [11]. Detection using molecular methods is more sensitive than traditional methods. The small subunit of ribosomal RNA (*SSU rRNA*) gene is currently popular gene marker used for accurate identification and subtyping of *Blastocystis* sp. [12]. For *SSU rRNA* sequence, the barcode region is a valid representation of the whole gene and the barcode sequences of the primers cover all polymorphic positions, ensuring that no phylogenetic signal information is lost [13, 14]. Moreover, the short barcode sequences can be used in publicly available sequence databases that can render consensus ST nomenclature and allele assignments per barcode sequence query much more conveniently than using full *SSU rRNA* sequences [14].

To date, based on polymorphism in *SSU rRNA* gene of *Blastocystis* sp., within the genus *Blastocystis*, 28 genetic groups named as subtypes have been proposed and designated as follows: ST1–ST17, ST21, ST23–ST29 and ST30–ST32 [6]. Among them, ST1 to ST4 are commonly identified in more than 90% of human cases of *Blastocystis*. The most prevalent subtypes, ST1, ST2 and ST4 are of high zoonotic subtypes, since they represented low host specificity [15]. ST3, an anthroponotic subtype, is recognized as the most common in humans, though it has been described from numerous animal hosts, such as cattle, chickens, pigs, monkeys, dogs, cats and so on [1, 15–18]. The other subtypes (ST5–ST9, ST10, ST12, ST14, ST16) are found both in humans and animals [6, 19], for example, ST5 in pigs [20], ST6 and ST7 in chickens [1], ST8 and ST9 in non-human primates [17, 19], ST10 and ST14 in cattle [15], ST12 in yaks, giraffes and kangaroos [21], ST16 in Kangaroos [22]. To sum up, *Blastocystis* has the ability to infect a wide range of hosts including humans and animals. Some researchers believed that the low host-specificity for the same subtypes in humans and animals increases the possibility of zoonotic or reverse zoonotic transmission [11]. Others also proposed zoonotic transmission is the origin of many human infections, when a large number of overlap of *Blastocystis* subtypes were reported in humans and other mammals and birds [5]. Subtypes and their respective alleles isolated from certain host groups have enabled a better understanding about *Blastocystis* transmission [23]. Furthermore, the comparison *SSU rDNA* alleles within the same ST can help determining the differences between the isolates in humans and animals, which may possibly contribute to the identification of the potential for zoonotic transmission and pathogenic isolates [24].

In China, *Blastocystis* infection has been reported in humans and animals distributed in at least 29 and 27 provinces or autonomous regions, respectively. The overall prevalence of *Blastocystis* in healthy humans have been reported, ranging from 0.007–43.3% in Xinjiang and Guangxi, respectively [25], while in domestic animals ranging from 0.4–100% in Anhui and Jiangxi, respectively [26, 27]. ST1–ST7 and ST12 were found in humans, and ST1–ST10, ST12–ST14, ST17–ST22 were found in mammal animals [28]. However, in China, few data of *Blastocystis* are available in humans and domestic animals in the family units. The aims of this study were to understand the prevalence and subtype (allele) distributions of *Blastocystis* in humans and domestic animals in northeastern China' s Heilongjiang Province, to elucidate the potential role of domestic animals as natural reservoirs of human *Blastocystis* infection, and to assess the risk of zoonotic transmission of this parasite among family members and domestic animals.

Methods

Sample collection

A total of 314 fresh fecal samples were collected between November 2020 and July 2021 from 57 villagers and 257 domestic animals from 33 households in three villages (Dongfanghong: DFH, Yuhe: YH and Shuangfa: SF) in northeastern China' s Heilongjiang Province. Among human samples, eight individuals who did not have close interactions with animals were collected in the same time period. Regarding the domestic animals, fecal samples were from livestock (n = 246) including pigs (n = 39), cattle (n = 20), goats (n = 14), rabbits (n = 2), chickens (n = 140), ducks (n = 9), geese (n = 22) and companion animals, such as dogs (n = 11) (Table 1). All the fecal samples were from fresh feces deposited on the ground after defecation by using disposable gloves and then placed into 50 ml sterile containers individually. Humans and animals were apparently healthy at the time of collection. All the samples were transported to the laboratory in a cooler with ice packs within 24 hours and stored in refrigerators at – 20°C prior to being used in molecular analysis.

Table 1
The prevalence and subtype distribution of *Blastocystis* in humans and domestic animals in three villages in Heilongjiang Province

Location	Host		No.of sample	No.of positive (%)[a]	Subtype (n)[b]
Yuhe	Humans	villager	10	1 (10.0)	ST2 (1)
	Animals	Pig	11	7 (63.6)	ST5 (6)
		Cattle	3	—	—
		Chicken	42	2 (4.8)	ST7 (1)
		Goose	9	—	—
		Dog	2	—	—
Dongfanghong	Humans	villager	26	5 (19.2)	ST1 (2); ST3 (3)
	Animals	Pig	28	21 (75)	ST1 (1); ST5 (13)
		Cattle	8	7 (87.5)	ST3 (1); ST10 (2); ST14 (2)
		Goat	12	5 (41.7)	ST5 (3)
		Dog	7	—	—
		Chicken	18	—	—
		Duck	1	—	—
Shuangfa	Humans	villager	21	2 (9.5)	ST3 (2)
	Animals	Cattle	9	1 (11.1)	—
		Goat	2	2 (100)	—
		Dog	2	—	—
		Rabbit	2	—	—
		Chicken	80	1 (1.3)	ST7 (1)
		Duck	8	—	—
		Goose	13	—	—
Total	Humans		57	8 (14.0)	ST1 (2); ST2 (1); ST3 (5)
	Animals		257	46 (17.9)	ST1 (1); ST3 (1); ST5 (22) ST7 (2); ST10 (2); ST14 (2)

[a] The prevalence of *Blastocystis* according to PCR amplification of approximately 260bp and 600bp (barcode region).

[b] Subtypes distribution of *Blastocystis* isolates by PCR amplification barcode region.

DNA extraction

Approximately 200 mg of frozen fecal samples were washed three times with distilled water by centrifugation at 1,500 g for 10 min at room temperature. Genomic DNA was extracted by using a commercially available a QIAamp DNA Mini Stool Kit (QIAgen, Hilden, Germany), following the manufacturer' s recommended procedures. The obtained DNA was stored at – 20°C until further use in polymerase chain reaction (PCR) analysis.

PCR amplification

To increase the detection rate of *Blastocystis* sp., all DNA preparations were screened for the presence of *Blastocystis* by PCR amplifying approximately 260 bp and 600 bp (the barcode region) of the *SSU rRNA* gene of *Blastocystis*. The 260 bp nucleotide fragments were only allowed detection of the parasite and the barcode region nucleotide fragments were used for detection, subtyping, allele identification and phylogenetic analysis of this parasite. The primers and the cycling parameters were used as described previously by Menounos and Scicluna [29, 30]. TaKaRa Taq DNA polymerase (TaKaRa Bio Inc., Tokyo, Japan) was used for all PCR amplification. A negative control (no DNA water control) and a positive control (DNA of a human-derived *Blastocystis* isolate) were used in all PCR tests. Each DNA preparation was analyzed at least twice by PCR. All PCR products were subjected to electrophoresis in a 1.5% agarose gel and were visualized under UV light after staining the gel with GelStrain (TransGen Biotech, Beijing, China).

Sequencing analysis and subtype (allele) identification

All positive PCR products were directly sequenced with two sets of primers for approximately 260 bp and 600 bp nucleotide fragments [29, 30] on an ABI PRISMTM 3730 DNA Analyzer (Applied Biosystems, USA), using a BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster, CA, USA). Sequence accuracy was confirmed by two-directional sequencing. Nucleotide sequences obtained in the present study were subjected to BLAST searches (http://www.ncbi.nlm.nih. gov/blast/) and then aligned with each other and reference sequences downloaded from GenBank database using the program Clustal X 1.83 (http://www.clustal.org/) to determine *Blastocystis* subtypes. 18S allele analysis offering higher resolution than subtyping alone, sequences obtained in the present study were submitted to the *Blastocystis* 18S Sequence Typing (MLST) databases (http://pubmlst.org/blastocystis/) for subtype confirmation and allele identification [31]. In the end, the nucleotide sequences generated have been deposited in GenBank under accession numbers OM218638–OM218649.

Phylogenetic analysis

To confirm the *Blastocystis* sp. subtype results and to explore the genetic and geographical relationship of *Blastocystis* isolates obtained in our study and previously reports, phylogenetic analysis of barcoding region of the *SSU rRNA* gene of *Blastocystis* isolated from humans and domestic animals was performed. The phylogenetic tree was constructed based on the neighbor-joining (NJ) method and the Kimura-2-parameter model using the program MEGA v6.0 (http://www.megasoftware.net). A bootstrap analysis with 1000 replicates was used to assess the reliability of the tree. Reference sequences were downloaded from the GenBank, and the sequences were labeled by NCBI accession number, country and the host origin. *Proteromonas lacertae* (U37108, AY224080) was selected as outgroup.

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics 25. Associations were assessed by Chi-square test or Fisher' s exact test between the prevalence of *Blastocystis* and demographic characteristics, personal hygiene habits and other possible risk factors for *Blastocystis* carriage. A value of $P < 0.05$ was considered statistically significant.

Results

The prevalence of Blastocystis

A total of 72 DNA preparations were successfully amplified and sequenced in either the 260 bp or barcode regions of the *SSU rRNA* gene (Additional file 1). Based on sequence analysis, 54 DNA preparations were confirmed to be positive for *Blastocystis* (Additional file 2) The prevalence of *Blastocystis* sp. was 14.0% (8/57) in humans and 17.9% (46/257) in animals: pigs 71.8% (28/39), cattle 40% (8/20), goats 50% (7/14) and chickens 2.1% (3/140) from three villages. However, there were absence of *Blastocystis* in rabbits, geese, ducks, and companion animal dogs (Table 1). In the present study, 57 participants from 33 households were asked to fill out questionnaires. All 57 completed questionnaires were used for evaluation of possible risk factors for *Blastocystis* carriage. The prevalence of *Blastocystis* between contacting animals and non-contacting animal villager were statistically significant ($\chi 2 = 6.38$, $P < 0.05$). However, without significant differences were found in gender, aged, seasons and hygiene habits (Table 2).

Table 2
Analysis of possible risk factors for *Blastocystis* infection in humans from three villages in Heilongjiang Province

Variable	Examined no.	No.of positive (%)	OR (95% CI)[a]	χ2/P-value
Gender				
Male	34	7 (20.6)	0.18 (0.02–1.54)	3.00/0.08
Female	23	1 (4.3)		
Age				
< 45	11	2 (18.2)	ref	
46–69	38	5 (13.2)	0.682(0.11–4.12)	0.18/0.68
> 69	8	1 (12.5)	0.64 (0.05–8.62)	0.11/0.74
Seasons				
Winter	7	1 (14.3)	0.98 (0.10–9.39)	0.00/0.98
Summer	50	7 (14.0)		
Eating unwashed vegetables and fruits				
Yes	12	1 (8.3)	2.02 (0.22–18.29)	0.41/0.52
No	45	7 (15.6)		
Contacting animals				
Very closely	9	4 (44.4)	ref	
Generally close	40	4 (10.0)	0.14 (0.05–0.74)	**6.38/0.01**[b]
No contact with animals	8	0	0.56 (0.31–1.00)	4.65/0.08

[a] CI, confidence interval.

[b] Bold type for values indicates statistical significance.

Distribution of subtype and allele

Out of the 54 samples of humans and animals origin that tested positive for *Blastocystis* sp. by PCR amplification, 70.4% (38/54) were successfully subtyped by sequence analyses barcode region of the *SSU rRNA* gene. Seven subtypes (ST1–ST3, ST5, ST7, ST10, ST14) were identified. *Blastocystis* ST3 (n = 5), ST1 (n = 2) and ST2 (n = 1), were found in humans in this study. In the case of the livestock, ST5 (n = 19) and ST1 (n = 1) were found in pigs, with the majority being ST5 (95%, 19/20); ST5 (n = 3) in goats; ST10 (n = 2), ST14 (n = 2) and ST3 (n = 1) in cattle. For the poultry, ST7 (n = 2) in chickens (Table 1).

Using the *Blastocystis* 18S database, a total of 10 different alleles were identified in six subtypes, namely ST1 (a4), ST2 (a9), ST3 (a34), ST5 (a115, a118, a153), ST7 (a96), and ST10 (a43). A few of the sequences showed similarity to ST3 (a122), ST7 (a99), and ST14 (a157), although not entirely identical, and therefore potentially new alleles. To date, there are no reports available on allele 118 in pigs globally. Detailed information on subtype (allele) distributions was summarized in Fig. 1.

Distribution of Blastocystis **sp. within family members and domestic animals**

All 314 samples from 57 members and 257 domestic animals were obtained. In total, we obtained samples from 33 families and at least one *Blastocystis* positive person or animal was observed in 13 families. In DFH, a couple (H36, H37) raising numerous pigs shared ST1 (a4), which was found in one pig (P20) from another family. Besides this, in the same village, we obtained ST5 (a153) in two goats (S2, S3) and one pig (P12) in one family, but the presence of this subtype was not confirmed in their owners. Furthermore, *Blastocystis* ST3 (a34) was found in one cattle (B4) from one family and three humans (H6, H39, H40) in three families (Table 3).

Table 3
Data of *Blastocystis* positive villagers and domestic animals examined in family units by PCR amplification of barcode region

Region	Family ID	Host	Sample ID	ST (n)	Allele (n)	Genbank ID number
Yuhe	YH-4	Owner	H44	ST2 (1)	a9 (1)	OM218645
	YH-7	Pig	P29, P30, P31, P33, P36	ST5 (5)	a153 (5)	OM218647
			P38	ST5 (1)	a115 (1)	OM218648
		Chicken	C137	ST7 (1)	a96 (1)	OM218641
Dongfanghong	DFH-1	Owner	H40	ST3 (1)	a34 (1)	OM218642
	DFH-2	Cattle	B2, B3	ST14 (2)	a157 (2)	OM218638
			B4	ST3 (1)	a34 (1)	OM218642
	DFH-3	Owner	H36, H37	ST1 (2)	a4 (2)	OM218644
		Pig	P3	ST5 (1)	a115 (1)	OM218648
			P5	ST5 (1)	a153 (1)	OM218647
	DFH-4	Goat	S1	ST5 (1)	a115 (1)	OM218648
	DFH-5	Owner	H39	ST3 (1)	a34 (1)	OM218642
		Cattle	B5, B6	ST10 (2)	a43 (2)	OM218639
	DFH-6	Pig	P12	ST5 (1)	a153 (1)	OM218647
		Goat	S2, S3	ST5 (2)	a153 (2)	OM218647
	DFH-8	Pig	P14	ST5 (1)	a115 (1)	OM218648
	DFH-9	Owner	H6	ST3 (1)	a34 (1)	OM218642

a: allele.

Region	Family ID	Host	Sample ID	ST (n)	Allele (n)	Genbank ID number
		Pig	P15, P17, P19, P22, P23, P24, P27	ST5 (7)	a153 (7)	OM218647
			P20	ST1 (1)	a4 (1)	OM218646
			P21	ST5 (1)	a115 (1)	OM218648
			P28	ST5 (1)	a118 (1)	OM218649
Shuangfa	SF-9	Chicken	C84	ST7 (1)	a99 (1)	OM218640
	SF-11	Owner	H54	ST3 (1)	a122 (1)	OM218643
	SF-12	Owner	H52	ST3 (1)	a122 (1)	OM218643

a: allele.

Phylogenetic analysis

A phylogenetic analysis indicated the seven subtypes obtained in the present study belonged to two groups. Sequences of STs1, 2, 3, 5, 10 and 14 were clustered into group 1. In group 1, the ST1 sequences of one pig and two humans were clustered into one clade with a high bootstrap value. Sequences of ST5 from DFH and YH pigs were in one clade. However, sequences of ST3 were further subdivided into two clades: ST3 from SF humans were in one clade and ST3 from DFH humans and cattle were in another clade. Sequences of ST7 from SF and YH chickens were clustered into group 2 (Fig. 2).

Genetic diversity analysis

The twelve haplotypes obtained in this study were compared with each other. By the nucleotide sequences analyzed, a high degree of genetic variation within *Blastocystis* sp. was observed at the barcoding region of the *SSU rRNA* gene. The degree of intra-subtype and inter-subtype variations was high, up to 24.9% and 25.8%, respectively (Table 4).

Table 4
Values of nucleotide variation in the *SSU rRNA* gene detected between pairs of *Blastocystis* sp. sequences, expressed as percentages

Sample ID.	P20-ST1	H36-ST1	H44-ST2	H6-ST3	H52-ST3	P14-ST5	P28-ST5	P12-ST5	C137-ST7	C84-ST7	B5-ST10	B2-ST14
P20-ST1	—											
H36-ST1	0.2	—										
H44-ST2	3.8	4.0	—									
H6-ST3	8.2	8.4	7.0	—								
H52-ST3	24.6	24.8	25.8	24.9	—							
P14-ST5	6.6	6.8	7.3	7.5	23.4	—						
P28-ST5	7.8	8.0	8.4	8.7	23.9	1.4	—					
P12-ST5	6.6	6.9	7.0	7.8	23.7	0.6	2.0	—				
C137-ST7	12.4	12.7	13.0	11.3	25.0	11.1	11.8	10.9	—			
C84-ST7	12.4	12.7	13.2	10.8	24.9	11.4	12.4	11.2	1.2	—		
B5-ST10	7.7	7.9	9.1	6.2	23.1	8.0	8.6	8.2	11.9	11.6	—	
B2-ST14	6.6	6.9	7.5	8.2	23.6	2.6	4.0	2.6	10.7	10.9	7.1	—

Discussion

Blastocystis has been reported in humans worldwide. The prevalence of *Blastocystis* usually varies based on geographical regions and studied population [31, 32]. The prevalence of *Blastocystis* has been reported in humans ranged from 0.5% in Thailand to 100% in Senegal [6]. However, in a study, *Blastocystis* as a common member of the healthy human gut microbiota is present in 56% of sample-sets which is much higher than previously reported from an industrialized country (Ireland) [33, 34]. In the present study, the overall prevalence of *Blastocystis* in healthy humans reached 14.0% (Table 1), which corresponds well with prevalence data from a previous study ranged from 0.007–43.3% in China [25]. The differences in prevalence may be related to the following factors such as age, contacting animals, traveling, diet, hygienic standard or drinking habits [3]. In the present study, the prevalence was observed in humans in contact with domestic animals, which were considered statistically significant compared to those who did not have contact with animals (Table 2). This result was consistent with previous studies, and suggested that humans were close contact with livestock or

birds could increase the risk of *Blastocystis* infection [3, 35]. The prevalence of *Blastocystis* varies in mammals between and within countries worldwide and is as high as 100% in some studies, such as in pigs from China and cattle from Indonesia [27, 36]. Nevertheless, Wang et al. (2018) described the prevalence as low as 9.5% in cattle, 8.8% in pigs, with an absence in goats in China's Heilongjiang Province [37]. In the present study, *Blastocystis* sp. was detected in three mammal species (pigs, goats and cattle) and one bird species (chickens), with the prevalence in domestic animals ranged from 2.1% in chickens to 71.8% in pigs. Goats infected with *Blastocyst* sp. were reported for the first time of Heilongjiang Province and the prevalence was 50% (7/14) (Table 1). However, domestic animals infection with *Blastocystis* sp. were present in 17.9% (46/257) of our sample set compared to other studies where prevalence ranged from 6.3–81.4% [4, 7, 38–40]. The fact of low prevalence could be explained by that a large proportion of domestic animal samples were from poultry, in which an absence of *Blastocystis* were found in geese and ducks and *Blastocystis* was only found in three chickens. Similar to our finding, *Blastocystis* sp. infection was absence in examined geese and ducks in UK [5], in Côte d' Ivoire [41], in Malaysia [38], in Algeria [42], in Italy [43], in Mauritius [5]. Additionally, the stool samples from free-roaming poultry were collected directly from the ground in November and in February in Northeast China. The low temperature and the lower stocking density may be decrease the chance of transmission of *Blastocystis* sp. in poultry [44, 45]. Furthermore, most of the poultry were vaccinated. Previous studies showed that vaccine may protect against bacterial, fungal, parasitic or viral infections in animals [46].

Molecular epidemiological studies have revealed a significant difference in the distribution of subtypes across host species and geographical regions [31]. ST3 is the most common subtype in humans. The second most common is ST1 in humans except the studies from continental Europe and the UK, where ST4 is common [6]. In China, ST3 and ST1 in humans are common subtypes in eight provinces, excluding the study from Hebei province, where ST2 is predominant [28]. In the present study, the most common subtype in the human sample set was ST3 (62.5%; 5/8), followed by ST1 (25%; 2/8) and ST2 (12.5%; 1/8) (Table 1). Our result was consistent with most studies, although the difference in constitutions and ratios of *Blastocystis* subtypes might vary in different populations studied [3, 47]. *Balstocystis* ST2 (allele 9) was only detected in an elderly man from YH and this is the first report of *Blastocystis* ST2 in humans in Heilongjiang Province. However, ST2 as common subtype was found in children of China' s Hebei Province and Senegalese [28, 48]. According to a previous study, ST2 was commonly identified in fecal samples of cynomolgus monkeys [49], and was also identified in domestic animals [11]. Humans were in frequent contact with monkeys and domestic animals will increase the risk of infection with *Blastocystis* [11, 50]. In the present study, because of the absence of *Blastocystis* ST2 in domestic animals in the investigated areas, we cannot give a satisfactory explanation for zoonotic transmission of *Blastocystis.*

Interestingly, the present study allowed us to see if *Blastocystis* sp. and its subtypes could circulate within family members or domestic animals. We obtained samples from 33 families and detected at least one *Blastocystis* sp. subtype in 13 families (Table 3). However, in one DFH family that raised pigs, we discovered two members infection with *Blastocystis* ST1 (a4). The result might suggest that *Blastocystis* sp. circulates between two members or they could have been colonized from the same source [3] (Fig. 1). If humans are asymptomatic or they have poor personal hygiene within a family, infected person can easily spread the parasite to others [7]. Meanwhile, in the present study, ST1 (a4) was identified not only in two humans in one

family, but also in one pig from another family in DFH. The *Blastocystis* ST1 sequences of pig (P20) and two humans (H36, H37) were clustered into one branch with a high bootstrap value, which may further be suggested potential transmission of *Blastocystis* infection between humans and pigs in DFH (Fig. 2). *Blastocystis* ST1 are considered be zoonotic and observed in some other animals, such as livestock, companion animals, birds, non-human primates, wildlife [11]. In the present study, besides ST1, ST5 are the most predominant subtypes in pigs in DFH and YH. Because of a low prevalence of *Blastocystis* ST1 (7.1% 1/14) in DFH pigs (Table 1), it seems unlikely that pigs are a zoonotic source of *Blastocystis* ST1 infection in this area. Our results are consistent with result of previous study in pigs in Thailand [7]. Therefore, the role of pigs to transmit *Blastocystis* ST1 should be further evaluated in the future studies.

As for *Blastocystis* ST3, which has been classified as more anthroponotic subtype and was also reported from animals closely contacted with humans, such as cattle, pigs, sheep, racoon dogs, chickens, non-human primates, dogs and cats [11, 51]. In the present study, *Blastocystis*-infected three humans and one cattle with the same ST3 (a34) were found from different families in DFH (Table 3). The result can likely be explained by reverse zoonosis from human handlers to animals, as suggested in previous studies focused on various animal hosts (pigs, cattle, cows, sheep, goats, dogs, cats, chickens and ducks) [11, 52]. In the present study, apart from ST3, ST10 and ST14 were predominant in cattle (Table 1). *Blastocystis* ST10 and ST14 were also observed in other hosts, such as ST10 was observed in dogs, cats, pigs, sheep, goats, rodents, ostriches, monkeys, wild and zoo hoofed animals and ST14 was found in sheep, goats, rabbits, birds, wild cat, wild and zoo hoofed animals [11]. Meanwhile, some studies have reported that cattle may be assigned as specific host for ST10 and ST14, which globally represented the most widely distributed subtypes, such as in Korea, Lebanon, Turkey, China and United States [11, 13, 15, 53–55]. However, in a recent study, ST10 and ST14 have also been identified in Senegalese school children, who were infected with *Blastocystis* sp. may be through either direct contact with livestock or water-borne transmission [48]. In the present study, ST10 and ST14 were predominant in cattle, followed by ST3 (Table 1). Thus, *Blastocystis* ST10 and ST14 in cattle may play a prominent role in the transmission of zoonotic *Blastocystis* subtypes to humans, which should be considered for implementation of future preventive measures [15].

According to the results of previous studies, pigs are natural host for *Blastocystis* ST5, due to most of *Blastocystis*-positive pigs harbored ST5 regardless of geographical setting [7, 20, 56]. Besides pigs, ST5 has also been detected in some domestic animals (cattle, sheep, goats) and wildlife (blesbok, oryx, grey brocket, wild boar, black rhinoceros, wild cat) [11] Although ST5 is considered rare in humans, it has been reported to be a potential zoonosis in rural China [27]. In the present study, *Blastocystis* ST5 (a153) was not only identified in one pig, but also in two goats in one family. While, *Blastocystis* ST5 (a115) was also found in three pigs and one goat from different families in DFH. *Blastocystis* ST5 was not detected in any human samples from the studied areas. All ST5 sequences had 99–100% similarity with ST5 isolated from animal-derived sequences in GenBank (Table 5). It suggested that this parasite could be transmitted from pigs to goats or goats to pigs in DFH [7]. In addition, fecal contamination of grass and fodder was common, which may increase the risk of *Blastocystis* infection in animals [10]. Interestingly, a118 (ST5) in pig is a novel finding: there are no reports of the allele in pig globally.

Table 5
Homology analysis of nucleotide sequences of *Blastocystis* subtypes

Host	Isolate ID	ST/allele (n)	GenBank ID (host) [b]	Homology (%)
Human	DFH (H36, H37)	ST1/a4 (n = 2)	EU445486 (Pig)	100
	DFH (H6, H39, H40)	ST3/a34 (n = 3)	MN338083 (Monkey); MK934333 (Human) MW888497 (Racoon dog); AB107963 (Pig); AB107965 (Cattle)	100
	YH (H44)	ST2/a9 (n = 1)	MN326606 (Human); OL623671 (Human); MN836828 (Human)	100
	SF (H52, H54)[a]	ST3/a122 (n = 2)	MN339604 (Dog); MK782518 (Human)	99.65
Pig	DFH (P20)	ST1/a4 (n = 1)	EU445486 (Pig); MN585811 (Human); MK782495 (Human)	99.83
	DFH (P3, P14, P21); YH (P38)	ST5/a115 (n = 4)	MK801386 (Pig); MN493729 (Wild boar); MF991106 (Sheep)	100
	DFH (P5, P12, P15, P17, P19, P22, P23, P24, P27); YH (P29, P30, P31, P33, P36)	ST5/a153 (n = 14)	MK801419 (Pig); MF186709 (Wild boar); MW850525 (Sheep)	100
	DFH (P28)	ST5/a118 (n = 1)	MW242641 (Squirrel); KY989561 (Pig)	100
Cattle	DFH (B2, B3) [a]	ST14/a157 (n = 2)	MW426241 (Cattle)	100
	DFH (B4)	ST3/a34 (n = 1)	MN338083 (Monkey); MK934333 (Human) MW888497 (Racoon dog); AB107963 (Pig); AB107965 (Cattle)	100
	DFH (B5, B6)	ST10/a43 (n = 2)	MF1866689 (Deer)	100

a: allele.

[a] Closest match: 18S rRNA full length: a122; a157; a99.

[b] Accession no. indicating the sequences downloaded from GenBank, which have 99–100% homology with the sequences obtained in the present study.

Host	Isolate ID	ST/allele (n)	GenBank ID (host) [b]	Homology (%)
Goat	DFH (S1)	ST5/a115 (n = 1)	MK801421 (Pig); MK085081 (Cattle); MN493729 (Wild boar); MW888498 (Racoon dog); MF991106 (Sheep)	100
	DFH (S2, S3)	ST5/a153 (n = 2)	MK801419 (Pig); MF186709 (Wild boar); MW850525 (Sheep)	100
Chicken	YH (C137)	ST7/a96 (n = 1)	MK010975(Chicken); MW867033 (Quail); FJ809939 (Human)	100
	SF (C84)[a]	ST7/a99 (n = 1)	MF326200 (Chicken)	100

a: allele.

[a] Closest match: 18S rRNA full length: a122; a157; a99.

[b] Accession no. indicating the sequences downloaded from GenBank, which have 99–100% homology with the sequences obtained in the present study.

ST6 and ST7 were considered “avian STs”, because of their relative predominance in birds [1]. In Brazil, Poland, Czech Republic and Lebanon, there were several reports of ST6 or ST7 in humans [1, 3, 23]. Indeed, ST6 isolates were found to transmit from chickens to two staff members of a poultry slaughter house in Lebanon, probably because of repeated exposure to a large amount of chicken feces [1]. Moreover, in Brazil, ST7 was detected from two children and a household member, as well as an asymptomatic individual and a transplant candidate [23, 57, 58]. In the present investigated areas, only ST7 (a99), ST7 (a96) were found in chickens in SF and YH, respectively. However, one *Balstocystis* ST7 (a96) sequence from chicken in YH was identical to that from human (FJ809939) in China, suggesting potential zoonotic transmission of *Blastocystis* infection (Table 5).

Based on the currently known subtypes, all subtypes were clearly separated by the phylogenetic analysis. However, *Blastocystis* ST3 sequences originating from DFH and SF humans formed two distinct clades (Fig. 2). Therefore, the distribution of *Blastocystis* ST3 is clearly subject to a variation in the geographic locations [32]. In pairwise distance comparisons, between ST3 were found to have large variations (Table 4).

Additionally, the fact that ST3 (a34) was identical in humans and animal (cattle) in DFH, which was differed from ST3 (a122) found in humans in SF. This result showed the possible potential for between humans and animals transmission of *Blastocystis* subtype in the same area.

Conclusions

The present study describes prevalence, subtype (allele) distribution and genetic diversity of *Blastocystis* for the first time in humans and domestic animals (pigs, cattle, goats, rabbits, dogs, chickens, ducks and geese) in family units in northeastern China' s Heilongjiang Province. The prevalence of *Blastocystis* was 14.0% in humans and 17.9% in domestic animals. Seven subtypes were identified, with ST1 (a4) and ST3 (a34) overlaps being observed in humans and some domestic species (pig and cattle). These results might suggest that *Blastocystis* sp. circulates between family members or family members and domestic animals. By phylogenetic analysis, the distribution of *Blastocystis* ST3 in humans from DFH and SF is clearly subject to high variations in the geographic locations, which will stimulate interest in the field of parasite evolution (including host specificity and parasite phylogenography). Although *Blastocystis* is of uncertain pathogenicity in humans, health education to reduce zoonotic risk among farmers, breeders and veterinarians who have close contact with these animals is necessary. Therefore, larger studies with sampling from humans and domestic animals sharing habitats should further elucidate transmission dynamics of *Blastocystis* in the investigated areas.

Abbreviations

ST: Subtype; DFH: Dongfanghong village; YH: Yuhe village; SF: Shuangfa village; A: Allele;*SU rRNA*: Small subunit of ribosomal RNA; NJ: Neighbour-joining; PCR: Polymerase chain reaction.

Declarations

Acknowledgements

The authors would like to thank the owners for cooperation during the collection of human and animal fecal samples.

Funding

The study was supported by the Natural Science Foundation of Heilongjiang Province of China (No. H2017006) and was supported by Basic Medical Science Research Program for Youth, Harbin Medical University (No.31041210065). The funder had no role in study design, data collection and analysis, and preparation of the manuscript.

Availability of data and materials

All data generated or analyzed during this study are included in this published article. The nucleotide sequences were deposited in GenBank database under the accession numbers OM218638-OM218649.

Authors' contributions

Experiments were conceived and designed by FY. HC, YH and YL collected human and animal samples and demographic data. HC, YH and MX performed molecular experiments and HC, YH and HL analyzed the PCR and sequence data. The manuscript was drafted by HC, and revised by FY, HL, AL and WZ. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This project was a minimum risk investigation for the participants and was approved by the Research Ethics Committee and the Animal Ethical Committee of Harbin Medical University. Oral and written informed consents were obtained from participants prior to sample collection. In the case of domestic animal samples, the owners provided a written consent for sample collection.

Consent for publication

No applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

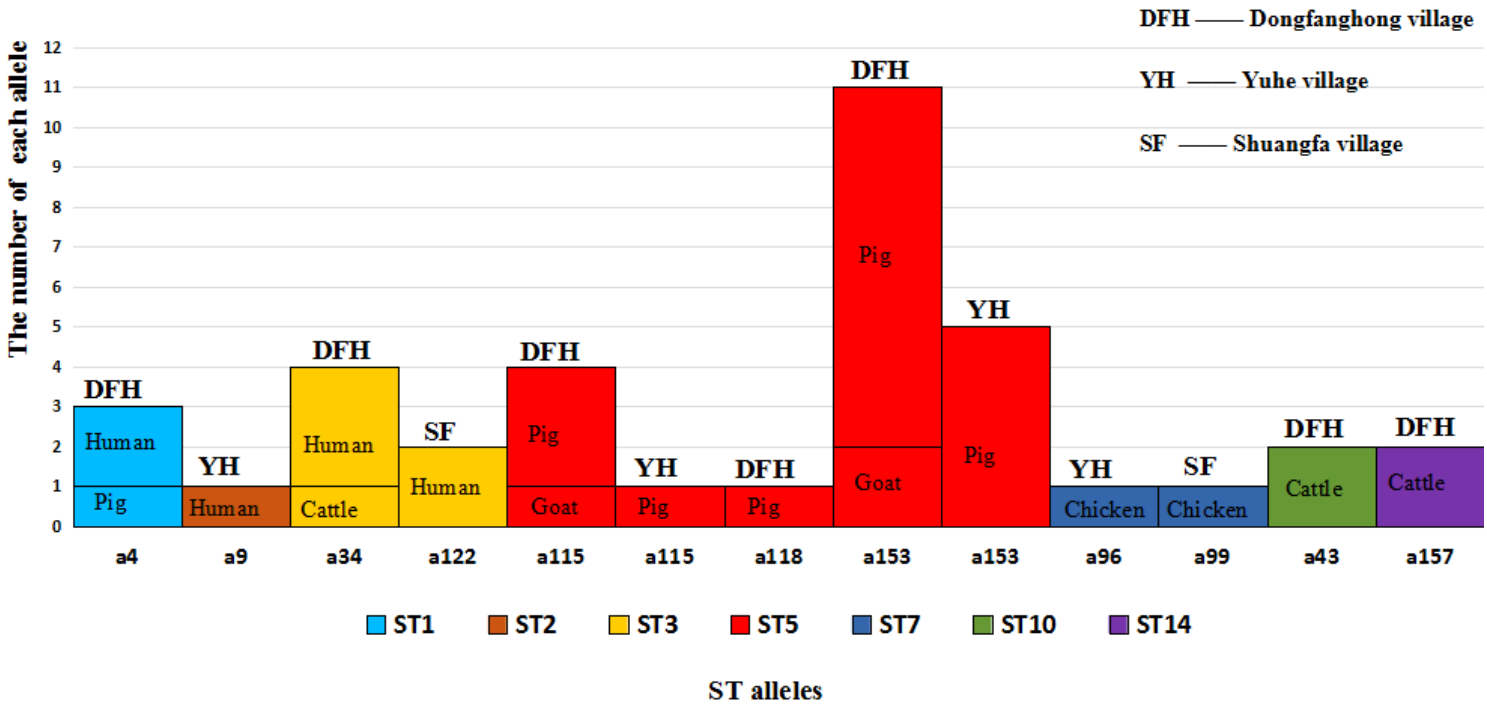

Figure 1

Distribution of *Blastocystis* 18S alleles based on the positive samples for each subtype in humans, pigs, cattle, goats, and chickens in three villages

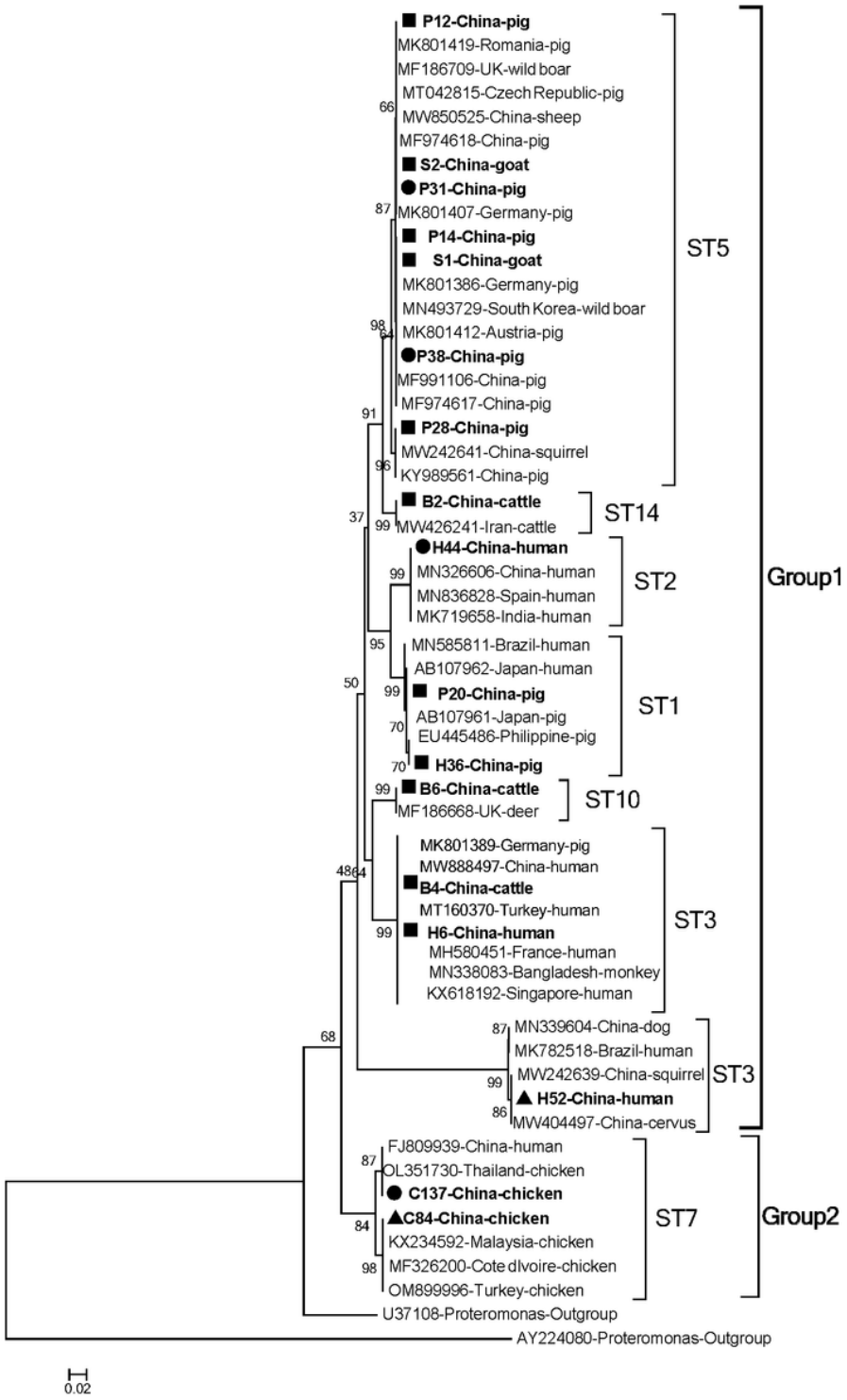

Figure 2

Molecular phylogenetic relationships between various *Blastocystis* sp. samples isolated from humans and domestic animals as inferred by the NJ tree based on nucleotide sequences of barcode regions of the 18S rRNA gene. The NJ method was used to construct the tree by the Kimura-2-parameter model. The numbers on the branches are percent bootstrapping values from 1000 replicates. Each reference sequence is identified by its accession number, country, and hosts. *Blastocystis* subtypes identified in the present study are indicated in bold type. The squares, circles and triangles filled in black represent Dongfanghong (DFH), Yuhe (YH) and Shuangfa (SF), respectively. *Proteromonas lacertae* (AY224080, U37108) were used as outgroup

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