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Prevalence and subtyping of *Blastocystis* sp. in ruminants in Southwestern, Iran

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Blastocystis is the most common gastrointestinal protozoan parasite of humans and many vertebrates. This study was carried out to investigate the prevalence and determination subtype (ST) of _Blastocystis_ in domestic ruminants of Shahrekord County, southwestern Iran. In this descriptive cross-sectional study, 330 ruminant fecal samples (107 cows, 115 sheep, and 108 goats) were evaluated by parasitological methods (direct wet mount microscopic examination and formalin-ether concentration), Giemsa staining, In vitro xenic culture (The modified Dobell and Laidlaw culture method), polymerase chain reaction, and sequencing from 2018 to 2019, then data were analyzed using SPSS software version 21. The overall _Blastocystis_ positive in ruminants was 14.2% and the frequency of _Blastocystis_ sp. in cattle, sheep, and goats were 0.93%, 17.4%, and 24.1% respectively. Molecular diagnosis techniques revealed that ruminants were infected with four STs (genotypes) of _Blastocystis_ including ST5(21.3%), ST7(2.1%), ST10(17.1%) and ST14(57.4%). Also, the STs identified in cows were ST10, and the observed STs in sheep were ST5 (40%), ST7 (3%), ST10 (5%), ST14 (45%), and one unknown subspecies. Goats were infected by ST5 (7.7%), ST10 (23.1%), and ST14 (69.2%). In this study, ST14 was identified as the most common subtype of _Blastocystis_ sp. that was not common between humans and livestock, meanwhile, ST5 and ST7 are common between humans and animals accounted 21.3% and 2.1% of the positive cases, respectively, and reinforces the hypothesis that ruminants are reservoirs of blastocystosis in humans.

Keywords *Blastocystis*, Ruminants, Prevalance, Subtypes, Sequencing, Shahrekord, Iran

Blastocystis is a widespread strictly anaerobic unicellular zoonotic intestinal parasite belonging to the kingdom Stramenopiles, class Blastocystae, order Opalinata, and family Blastocystidae that infects a plethora of very diverse hosts including arthropods, reptiles, amphibians, birds and mammals such as human worldwide[1,2]. This microorganism is the most common intestinal parasite in humans with approximately 1 to 2 billion infected humans in various countries around the world, especially in tropical climates and developing countries with low sanitary and hygienic standards[3,4]. Some factors such as socioeconomic situation, climate conditions, the hygiene situation of the people including poor personal and environmental hygiene, inadequate health services and the consumption of contaminated water or food, travel to contaminated areas and closer contact with infected animals have important effects on the distribution of *Blastocystis* in different regions of developed and developing countries, therefore the prevalence of blastocystosis varies from up to 10% in developed countries to 50–60% in developing countries[5,6]. However epidemiological studies have indicated that the prevalence of blastocystosis in humans varies widely from 0.5 to 100% among different geographical areas of the world[6–9]. This parasite is a pleomorphic organism with six different forms with variable sizes and shapes in the life cycle including vacuolar, granular, amoeboid, avacuolar, multivacuolar, and cystic forms. Some of these forms (cyst, amoeboid, granular, and vacuolar) can be colonized in the large intestine such as the colon of different infected hosts for a long time, and seen in the stool of symptomatic and asymptomatic hosts[5,10]. The cysts are spherical to ovoid shape and smaller than the vacuolar and the granular forms, considered the most probable transmitted infectious form. The amoeboid form is possibly the pathogenesis stage of this microorganism. *Blastocystis* sp. is reproduced by binary fission, budding, and plasmotomy methods in vivo and in vitro culture[2,10,11]. Although the mode of

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transmission of *Blastocystis* has not been completely identified; however, different hosts such as humans and animals could be infected by the fecal-oral transmission, through ingestion of cyst-contaminated water (water-born transmission) and food (food-borne transmission) or direct contact with infected persons (anthroponotic transmission) or animals (zoonotic transmission)[5,12–14]. *Blastocystis* frequently detected and reported in both long-term asymptomatic healthy individuals and symptomatic infected humans, therefore its pathogenicity has not been completely proven and is controversial. So far relatively low studies have been carried out about the pathogenicity of this parasite, therefore the clinical significance of blastocystosis is not well known which may be because of the vague symptoms and non-occurrence clinical symptoms in asymptomatic infected individuals as carriers; hence, the definition of its pathogenicity is difficult. But blastocystosis can be considered as one of the causes of gastrointestinal complaints with symptoms such as watery diarrhea, nausea, abdominal pain, dyspepsia, constipation, vomiting, bloating, tenesmus, excessive gas (flatulence), loss of appetite, pruritus and fatigue[15–17]. Although the pathogenicity mechanism of *Blastocystis* is not completely understood, however, some investigations (in vitro and in vivo) have indicated the association between adhering to intestinal mucin and secreting cysteine proteases of *Blastocystis* that contribute to pathogenesis through degradation of secretory IgA, Rho/ROCK-mediated tight-junction compromise, NF-κB-mediated secretion of inflammatory cytokines, and enterocyte apoptosis which increased gut permeability[18] but other researchers believe that the colonization of *Blastocystis* sp. was associated with the presence of more diverse and nonpathogenic intestinal microbiota such as bacteria, fungi, archaea, and viruses, as well as single-celled eukaryotes than gut dysbiosis of hosts[19–21]. Furthermore, some researchers suggested the hypothesis of an association between blastocystosis and some chronic diseases such as inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), ulcerative colitis, Crohn's disease, and skin disorders such as itching, urticarial or allergic lesions[22–24]. Some studies also have suggested an association between the incidence of *Blastocystis* and the immunity status of the host as an opportunistic pathogenic microorganism, numerous epidemiologic surveys have reported the prevalence of *Blastocystis* sp. in the immunocompromised individuals such as AIDS patients and persons undergoing therapies for malignancies, transplants or lymphoproliferative disorders, Therefor this hypothesis can explain the high prevalence of blastocystosis in immunocompromised individuals than other healthy people with severe symptoms such as severe diarrhea and even death[25–27]. So far the molecular investigations of *Blastocystis* based on the small-subunit ribosomal RNA (SSU rRNA) gene have proven 44 STs (ST1-ST44) in many different host species that approximately 40 of them have been reported in mammalian and avian hosts but 38 STs (ST1–ST17, ST21, ST23–ST38, ST40 and ST42-ST44) have been identified as valid STs that indicate diverse biological features, such as host specificity, drug resistance, virulence or pathogenicity, and effects on the microbial flora of the gastrointestinal tract, while the validity of four STs (ST18, ST19, ST20 and ST22) remains contested according to the opinion of some researchers[28–31]. The molecular epidemiological studies showed that humans could be infected with 16 STs including ST1-ST10, ST12, ST14, ST16, ST23, ST35 and ST41 with varying prevalence in different geographical areas worldwide. The 14 STs of human infecting STs (ST1-ST10, ST12, ST14, ST16 and ST23) were detected in domestic and wild animals, therefore are classified as 'zoonotic STs' and indicating the possibility of zoonotic and reverse zoonotic transmission[32–36]. The ST1-ST4 are the most prevalent STs of humans with more than 90% of human infections due to human-to-human transmission, but ST3 is the most human-specific subtype in urban areas of many countries and is primarily transmitted among people. ST35 exclusively infects humans while the other STs were rarely privately detected in humans and do not have a strong host specificity for infecting humans and different animal groups but are frequently isolated from the gastrointestinal tract in various animal groups including primates, hoofed mammals and birds, therefore based on these findings have zoonotic potential that may infect human[36]. ST6 and ST7 infect poultry while ST8 infects non-human primates and dogs. ST5 mainly occurs in pigs, and ST10 and ST14 in livestock[37,38]. Therefore the potential significant occurrence of *Blastocystis* sp. in ruminants and carnivores such as dogs and cats could be a risk of zoonotic transmission for humans who are frequently in close contact with animals especially animal handlers such as those working in zoos, farms, and abattoirs, also shepherds and veterinarians[13,14,39]. Some molecular studies indicated that ruminants such as cattle, sheep, and goats can be predominant hosts of ST1-3, ST5-7, ST10 and ST12-15 of *Blastocystis* sp. that likely reflects ruminants as natural hosts for these STs in different geographic regions of the world[33,37,40–43]. Some persons such as veterinarians, shepherds, animal husbandry and slaughterhouse workers, zookeepers, butchers, and bird sellers through their jobs are in contact with animals, therefore these groups are at higher risk of being infected with potential zoonotic STs of *Blastocystis* sp. Identifying the subtyping of *Blastocystis* sp. in different hosts and environments as a source of *Blastocystis* sp. infections is an important key in preventing and controlling the spread of the parasite in human societies. In Iran, ruminants including cattle, sheep, and goats are bred as the main source of meat production. So the purpose of this study was to determine the prevalence and subtyping of *Blastocystis* sp. in domestic ruminants in Shahrekord County of Chaharmahal and Bakhtiari province, Southwest Iran using parasitological and molecular methods and evaluate the association between genetic diversity and the zoonotic potential of isolated *Blastocystis* sp. by phylogenetic comparison with STs of other investigations.

Materials and methods

Study area and sample collection

This cross-sectional descriptive study was carried out in Shahrekord County of Chaharmahal and Bakhtiari Province, Southwest Iran, from December 2018 to May 2019, one of the mountainous parts of Iran's central plateau which is situated between 32° 20′ and 33° 31′ north latitude, as well as between 49° 22′ and 50° 49′ east longitude, with a population of about 315,980 persons. The jobs of many people in this province are animal husbandry, farming, and gardening therefore, this province is one of the centers of heavy and light livestock breeding in Iran. A total of 330 fecal samples (20–50 gr) of ruminants were randomly collected in the lottery method according to

the number of livestock animals in Shahrekord County from 107 cattle, 115 sheep, and 108 goats that were bred in traditional farming (n = 173), industrial farming (n = 20) or were referred to slaughterhouse (n = 122 samples) and veterinary clinics (n = 15) (Fig. 1). Traditional breeding is carried out predominantly forage-based and healthy water systems in the houses or grazing in the environment and pastures with unhealthy water, and industrial breeding in farms is carried out mainly forage-based and healthy water systems, with an on maintaining animal health through improved welfare and a reduction in the use of routine, conventional veterinary treatments. All animal owners were informed about the study's aim and their permission was obtained before collecting the fecal samples. The age and sex of the animals and sampling location were recorded with the sample collection dates. The animals did not have any specific gastrointestinal symptoms; specifically, they did not have diarrhea. Fecal samples were directly collected from the rectum of the animals (91 cattle, 86 sheep, and 82 goats) using sterile gloves or immediately after defecation on the ground if the animal was observed defecating (16 cattle, 29 sheep, and 26 goats), Samples contaminated by soil or other environmental matters were avoided by picking the inner part of the feces, then were stored in sterile pre-labelled plastic containers and transported to the laboratory short-term for microscopic examination, in vitro culture, and further analyses (molecular testing).

Microscopic examination and culture

At first, all fecal samples were directly examined within 2–4 h after collection using direct saline wet-mount and lugol's iodine staining and observed microscopically at 100X and 400X magnifications followed with formalin-ether concentration technique and Giemsa staining to detection and identification of *Blastocystis*[44]. Fresh fecal samples (2–5 g) were transported into 10 ml of 10% formalin in a 15-ml sterile screw-capped tube, the stool and formalin were thoroughly mixed, and the mixture was stood for a minimum of 30 min for fixation. Then, a sufficient quantity was filtered through two layers of wet gauze into a conical 15-ml centrifuge tube. 4–5 ml of ethyl acetate was added to the mixture; the contents were mixed thoroughly and centrifuged at 500 g for 10 min. After centrifugation and decanting the supernatant fluid of the obtained concentrate, the resulting pellet was thoroughly mixed with an applicator stick. Then a drop of the pellet (25 µl) was spilled onto dry, grease-free microscope slides in duplicate, then 1 drop (25 µl) each of iodine solution and normal saline was added to the sediment, mixed, and examined under an optical microscope (Olympus CX52) with low power (The magnifications of 100 × and 400 ×). Also, Giemsa staining was carried out by prepared smears using 25 µl of concentrated sample which spread over a glass slide in an area of approximately 1.5 to 5 cm wide and 2 to 7 cm long and fixed by absolute methanol for 5 min. Then smears were stained for 20 min with a 1:20 dilution of Giemsa stock, washed under gently running tap water, and dried in the air of the house. Finally examined under the microscope by × 400 magnification[45]. Furthermore, All samples short-term after collection were xenical cultured in-vitro culture according to Dobell and Laidlaw with a slight modification of the HSr + S medium[46] [10% heat-inactivated sterile horse serum (Razi Serum Institute, Iran), ringer with starch rice]. In this medium egg-white was not used in the liquid phase, to avoid fungal contamination of the medium[47]. This process was carried out by inoculating approximately 50 mg of each fresh fecal sample into a 15-ml sterile screw-capped tube containing 2-ml slant of coagulated horse serum overlying with 1 ml of autoclaved ringer and powdered starch rice together with 10% penicillin–streptomycin (1000 IU/ml and 500 µg/ml)[48]. Following, all inoculated tubes were tightly closed, incubated at 37 °C for 5–7 days, and were examined every 24–48 h to observe any of the four morphologies forms of *Blastocystis* by placing aliquots of culture sediments (20 µl) onto a glass slide, coated coverslip and assayed by using an optical microscope with magnifications of 100 × and 400 × .

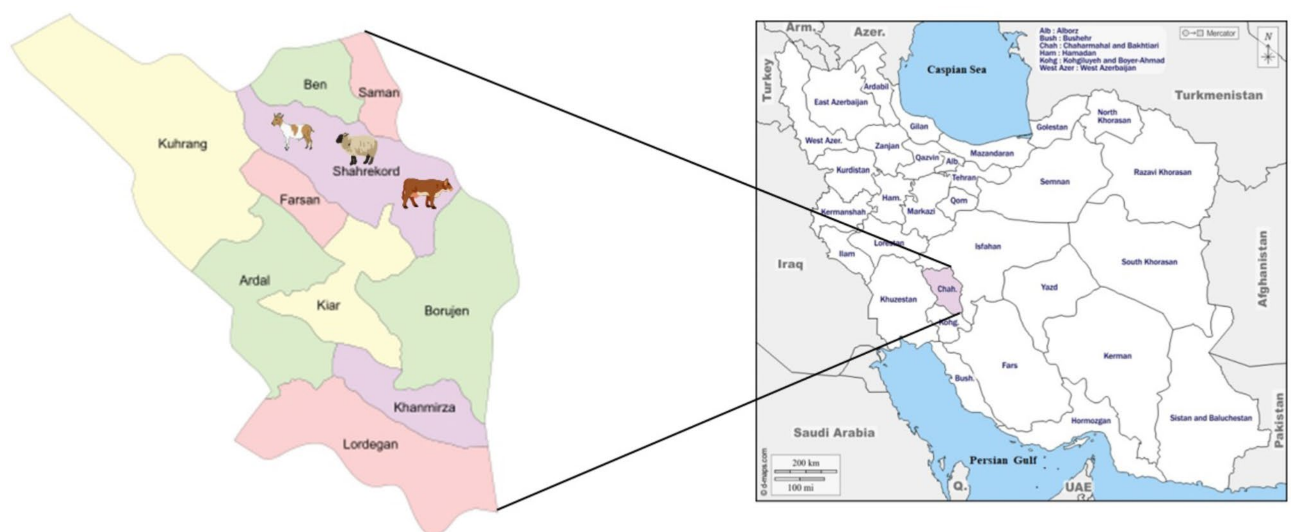

Fig. 1. Map of the study area. The sampling sites were located in the Shahrekord County, which is situated in the Chaharmahal and Bakhtiari province. The origin of the animals (picture) is indicated.

DNA extraction, PCR, and sequencing

Total genomic DNA of each *Blastocystis* isolate was extracted from 200 µl of the positive cultures after 5–7 days of cultivation that washed three times with sterile phosphate-buffered saline (PBS) by using a commercial DNA extraction kit (DNG-Plus™ (DN8118C) Cinnaclon Co., Iran) according to the manufacturer's instructions. Furthermore, DNA of some negative cultures based on indiscernible growth of the parasite after 10 days was extracted to investigate the slow or hard culture *Blastocystis* isolates. Then, the concentration and quality of extracted DNA were determined using an ND-1000 spectrophotometer (Nanodrop Technologies, USA) and were stored at – 20 °C until further molecular examinations. The PCR assay was carried out on extracted DNA to amplify a ~600 bp fragment of the SSUr DNA gene of the *Blastocystis* using the primers RD5 (5′-ATCTGG TTGATCCTGCCAGTA-3′), BhRDr (5′-GAGCTTTTTAACTGCAACAACG-3′)[49]. PCR was performed in a reaction mixture (25 µL) containing 12.5 µl of the master mix (Amplicon, Denmark), 5.5 µl of nuclease-free water, 2 µl DNA template (50–100 ng/µL), and 2.5 µl of each primer (4 pmol concentration). The PCR was carried out in conditions including a pre-denaturation at 95 °C for 5 min, followed by 35 cycles including denaturation at 94 °C for 60 s, annealing at 58 °C for 60 s, and extension at 72 °C for 60 s and at the end, final extension of 10 min at 72 °C. The PCR product was mixed with power load and loading buffer loaded on 2% agarose gel, then electrophoresed and visualized on a UV transilluminator by Gel Doc, which is a ~600 bp fragment. The PCR products of positive samples were sequenced using the BhRDr primer by the service of Bioneer Laboratories (Bioneer, Daejeon, Korea). The obtained generated nucleotide sequences of isolated *Blastocystis* sp. were identified by manually edited and evaluated by the Chromas v. 2.6.6 software (http://www.technelysium.com.au/Chroma.sPro.html) and were determined by investigation the exact match or closest similarity against all known *Blastocystis* sp. ST homologous sequences available in the National Centre for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST) program (http:// www. ncbi. nlm. nih. gov) then Phylogenetic tree was constructed using forty-six nucleotide sequences representative of the current study and GenBank database reference sequences (KC922151.1, MW767072.1, MK801414.1, MK801418.1, KY610168.1, MW682191.1, MK240457.1, MK240481.1, MK930352.1, MW078483.1, MF186696.1, MF072954.1, MF541107.1, LT594969.1, KC148206.1, MF974619.1, MW078491.1, MF186707.1, MF541102.1, ON796562.1, KY488610.1, KC148205.1, MT898459.1, MG000950.1, MW648987.1, MW648927.1, MF186668.1, MK240467.1, MT645670.1, KT438702.1, and KF447173.1) by the neighborjoining and maximum composite likelihood methods with the Tamura-3 parameter substitution model in Molecular and Evolution Genetic Analysis software version 6.0 (MEGA) [Pennsylvania State University, State College, PA, USA, (https://www.megas.oftware.net/)]. The gene sequence of *Proteromonas lacerate* LA (U37108.1) was used as an outgroup. The bootstrap consensus tree was inferred from 1000 replicates.

Statistical analysis

Data were presented using frequency and percentage. Compare between groups were done using Fisher's exact or Chi-square test (X^2) where appropriate. The comparison between diagnostic methods was performed using Cochran's Q test with Bonferroni for pairwise comparison. Statistical significance was defined as $P < 0.05$ in all tests and analysis was performed by SPSS version 21 software (SPSS Inc., Chicago, IL, USA).

Ethical approval

The study was approved by the Research Ethics Committee of Shahrekord University of Medical Sciences (IR.SKUMS.REC.1397.272).

Results

In this study, a total of 330 fecal samples of different domestic ruminants including cattle (107), sheep (115), and goats (108) were investigated for the presence of *Blastocystis* sp. with considered factors such as animal type, sex, and sampling location (Table 1) using direct smear, formalin-ether concentration technique, Giemsa staining, and culture methods (Fig. 2). The results of the *Blastocystis* screened in ruminants indicated that 27(8.2%), 38(11.5%),

Characteristic	No. of cattle (%)	No. of sheep (%)	No. of goats (%)
Gender			
Male	68 (63.5)	78 (67.8)	44 (40.7)
Female	39 (36.5)	37 (32.2)	64 (59.3)
Age group			
Puberty	86 (80.4)	93 (80.9)	92 (85.2)
Unpuberty	21 (19.6)	22 (19.1)	16 (14.8)
Sampling location			
Traditional animal husbandry	49 (45.8)	43 (37.4)	81 (73.2)
Industrial animal husbandry	20 (18.7)	0 (0)	0 (0)
Slaughterhouse	29 (20.8)	66 (57.4)	27 (26.8)
Veterinary clinic	9 (8.4)	6 (5.2)	0 (0)
Total	107 (100%)	115 (100%)	108 (100%)

Table 1. The socio-demographic characteristics of ruminants in this study.

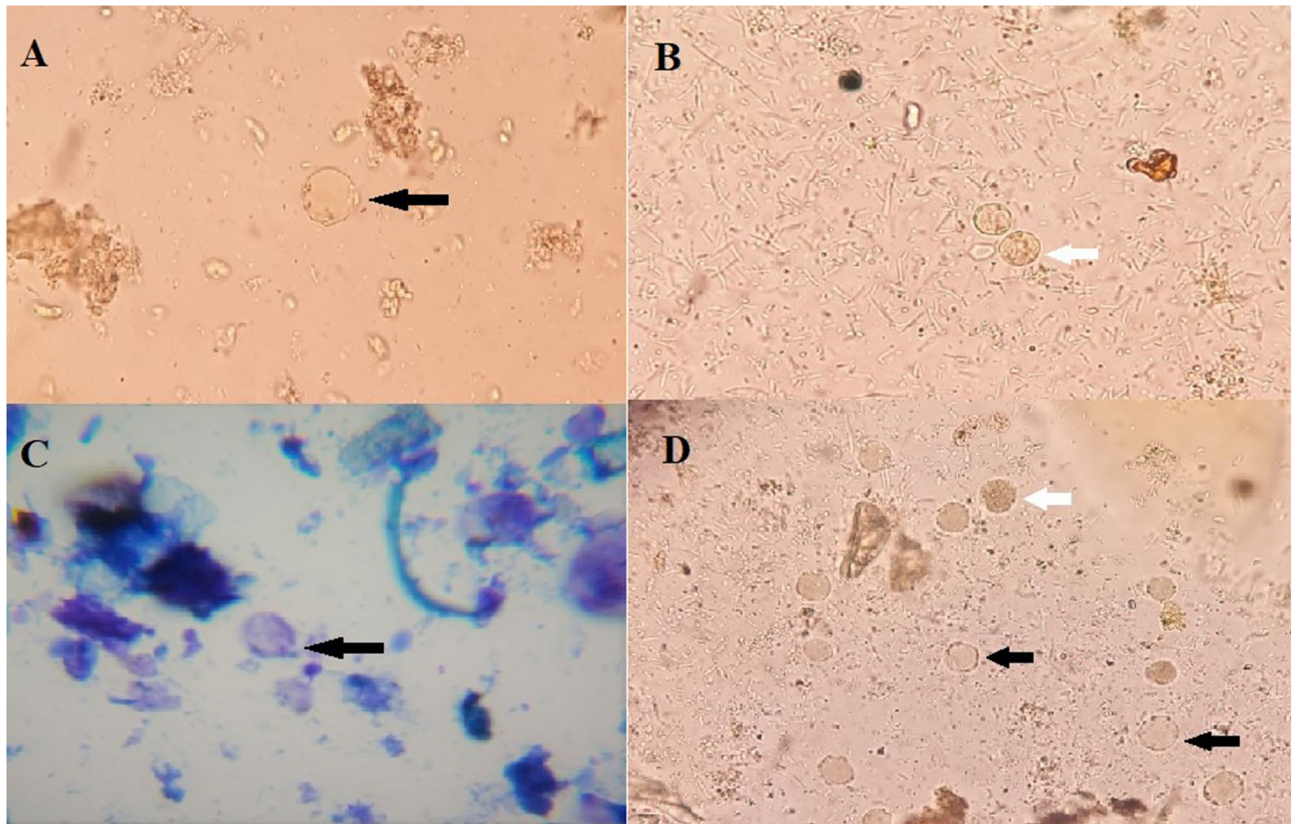

Fig. 2. *Blastocystis* sp. in the stool of ruminants, (**A**) direct smear, (**B**) formalin-ether concentration technique, (**C**) Giemsa staining, and (**D**) culture methods (400X magnification) (Black arrow: vacuolar forms, White arrow: granular forms).

35(10.6%), and 47(14.2%) of animals were found to be *Blastocystis*-positive using direct smear, formalin-ether concentration technique, Giemsa staining, and culture methods respectively. The 47 positive *Blastocystis* in the culture of stool samples were confirmed by the PCR-based molecular method that indicated success DNA-amplified with an approximately ~600 bp fragment of SSU rRNA in PCR products (Fig. 3). Furthermore, the molecular investigation of negative cultures after 10 days did not indicate the infection of *Blastocystis* sp. The comparison of the results of different diagnostic methods for *Blastocystis* shows that culture and PCR methods

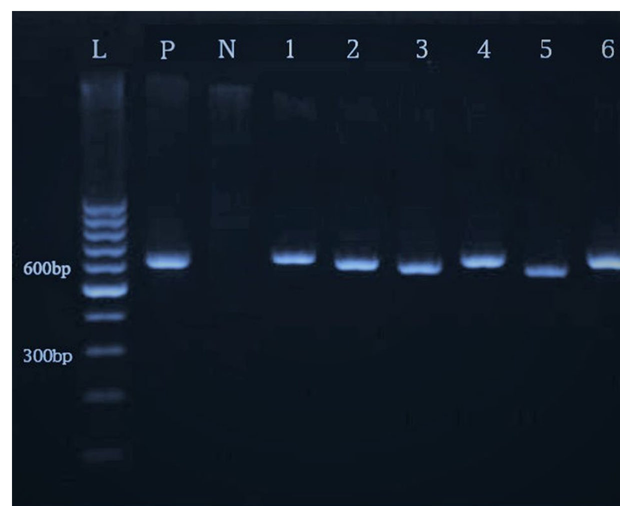

Fig. 3. The PCR amplification of Blastocystis sp. Lanes: L, molecular marker of a 100-bp ladder; P Positive control, N Negative control, Lines: 1–6: Blastocystis sp. in different ruminants.

have the highest sensitivity for diagnosis of blastocystosis (Tables 2 and 3). Also, the culture of samples showed that the prevalence of *Blastocystis* sp. in cattle, sheep, and goats were 0.93%, 17.4%, and 24.1% respectively. The vacuolar form with a central-body was the most common form of the protozoan which was detected in the direct smear, formalin-ether concentration technique, and Giemsa staining method. The investigation of in vitro culture of samples in HSr + S medium indicated vacuolar with central-body and granular forms were shown as the most common forms of *Blastocystis* sp. Of the 330 stool samples examined, 190 and 140 samples belonged to male and female animals, of which 18 male (9.5%) and 29 female (20.7%) animals were infected with *Blastocystis* sp. by the culture and PCR methods. The successful consensus sequences of partial SSU rDNA of 47 *Blastocystis* isolated in this study were manually edited by Chromas (version 2.6.6) to remove regions of ambiguity and analyzed using the Basic Local Alignment Search Tool (BLAST). The identified nucleotide sequences were deposited in the genetic sequence database at the National Center for Biotechnical Information (NCBI) using Bankit program (https://www.ncbi.nlm.nih.gov/WebSub/) under the following accession numbers MN315558 to MN317333, then were compared with sequences in the GenBank database with high similarity genetic identity of 97% to 100% for determining the STs of them (Table 4). These findings revealed that ruminants were infected with four STs of *Blastocystis* including ST5, ST7, ST10, and ST14 with a prevalence of 21.3%, 2.1%, 17.1%, and 57.4% respectively. Furthermore, obtained results demonstrated cattle were affected with ST10 only, but sheep and goats were infected with ST5, ST7, and ST14 (Table 5). The phylogenetic relationships of the Nucleotide sequences of *Blastocystis* small subunit ribosomal RNA (SSU rRNA) gene of isolates in this study were compared and evaluated with identified sequence of databases in GenBank using the ML phylogram method that showed similarity with other published isolates in GenBank (Fig. 4). The analysis of different identified STs of *Blastocystis* sp. and the demographic characteristics of animals revealed that a significant relationship was not seen between different STs with gender, sampling location, and age groups of ruminants (Table 6). In the end, the statistical analysis of the findings demonstrated a significant relationship between the prevalence of *Blastocystis* sp. and variables including ruminant type, sex of the animals, and sampling location (Table 7).

Discussion

Although it has been over a hundred years since Alexeieff described *Blastocystis* and many researchers have carried out studies on various aspects of this microorganism such as classification, life cycle, pathogenicity, diagnosis, status of epidemiology in different areas, determined of STs and importance of animal reservoirs, but there are still some controversial and unknown aspects about it such as pathogenicity that need to scrutiny investigations[34,50]. The absence of unique morphological characteristics, a wide variety of hosts from invertebrates to vertebrates, relatively widespread distribution globally, a high degree of diverse genotypes, and diagnosis of protozoa in the stool of healthy individuals as well as in patients with gastrointestinal disorders are important causes why this protist has remained a mysterious microorganism[1,2,5]. Therefore, it has been an interesting subject for researchers, in recent years[33]. The molecular epidemiologic studies about the subtyping of *Blastocystis* sp. in different hosts such as mammalian and avian hosts indicated different STs in nature[1,13,37,39]. The successful

Methods	Positive N (%)	Negative N (%)	Overall p-value*
Direct smear and Lugol's iodine staining	27 (8.2)	303 (91.8)	<0.001
Formalin-ether concentration	38 (11.5)	292 (88.5)	
Giemsa staining	35 (10.6)	295 (89.4)	
Culture	47 (14.2)	283 (85.8)	
PCR	47 (14.2)	283 (85.8)	

Table 2. The prevalence of blastocystosis based on different diagnostic methods of *Blastocystis* sp. in the ruminants, Shahrekord County, Iran. *Based on Cochran,s Q test.

Methods	Compare with	Adjusted significance
Direct smear	Formalin-ether concentration	0.006
Direct smear	Giemsa staining	0.131
Direct smear	Culture	<0.001
Direct smear	PCR	<0.001
Formalin-ether concentration	Giemsa staining	1
Formalin-ether concentration	Culture	0.053
Formalin-ether concentration	PCR	0.053
Giemsa staining	Culture	0.002
Giemsa staining	PCR	0.002
Culture	PCR	1

Table 3. Pairwise comparison of different diagnostic methods of *Blastocystis* sp. in the ruminants, Shahrekord County, Iran.

Subtypes	Source	Age groups	Gender	Sampling location	Accession numbers
ST5	Sheep	Puberty	Male	Traditional animal husbandry	MN315558
ST5	Sheep	Puberty	Female	Slaughterhouse	MN315561
ST5	Goat	Puberty	Male	Traditional animal husbandry	MN315565
ST5	Sheep	Unpuberty	Female	Slaughterhouse	MN315567
ST5	Sheep	Puberty	Male	Slaughterhouse	MN315647
ST5	Sheep	Puberty	Female	Traditional animal husbandry	MN316527
ST5	Goat	Puberty	Male	Slaughterhouse	MN316539
ST5	Sheep	Puberty	Female	Traditional animal husbandry	MN316540
ST5	Sheep	Puberty	Female	Traditional	MN317314
ST5	Sheep	Puberty	Female	Traditional animal husbandry	MN317330
ST7	Sheep	Puberty	Male	Slaughterhouse	MN315568
ST10	Sheep	Puberty	Female	Traditional animal husbandry	MN315563
ST10	Goat	Puberty	Male	Traditional animal husbandry	MN316600
ST10	Goat	Puberty	Female	Traditional animal husbandry	MN316628
ST10	Goat	Puberty	Female	Traditional animal husbandry	MN316633
ST10	Goat	Puberty	Female	Traditional animal husbandry	MN316645
ST10	Goat	Puberty	Female	Traditional animal husbandry	MN316666
ST10	Goat	Puberty	Female	Traditional animal husbandry	MN316700
ST10	Cattle	Puberty	Female	Traditional animal husbandry	MN317318
ST14	Sheep	Puberty	Male	Traditional animal husbandry	MN315564
ST14	Sheep	Puberty	Male	Slaughterhouse	MN315566
ST14	Sheep	Puberty	Female	Slaughterhouse	MN316528
ST14	Sheep	Puberty	Female	Traditional animal husbandry	MN316531
ST14	Sheep	Puberty	Male	Slaughterhouse	MN316532
ST14	Sheep	Puberty	Female	Traditional animal husbandry	MN316533
ST14	Sheep	Unpuberty	Female	Traditional animal husbandry	MN316541
ST14	Goat	Puberty	Female	Traditional animal husbandry	MN316587
ST14	Goat	Puberty	Female	Slaughterhouse	MN316596
ST14	Goat	Puberty	Female	Traditional animal husbandry	MN316597
ST14	Goat	Puberty	Male	Traditional animal husbandry	MN316598
ST14	Goat	Puberty	Female	Slaughterhouse	MN316599
ST14	Goat	Puberty	Male	Traditional animal husbandry	MN316613
ST14	Goat	Puberty	Female	Traditional animal husbandry	MN316634
ST14	Goat	Puberty	Female	Traditional animal husbandry	MN316657
ST14	Goat	Puberty	Male	Traditional animal husbandry	MN316658
ST14	Goat	Puberty	Male	Slaughterhouse	MN316663
ST14	Goat	Puberty	Female	Traditional animal husbandry	MN316665
ST14	Goat	Puberty	Male	Traditional animal husbandry	MN316667
ST14	Goat	Puberty	Male	Traditional animal husbandry	MN316674
ST14	Goat	Puberty	Male	Slaughterhouse	MN316675
ST14	Goat	Puberty	Female	Traditional animal husbandry	MN316676
ST14	Sheep	Puberty	Female	Traditional animal husbandry	MN317319
ST14	Goat	Puberty	Male	Slaughterhouse	MN317320
ST14	Sheep	Puberty	Female	Traditional animal husbandry	MN317331
ST14	Goat	Puberty	Male	Traditional animal husbandry	MN317332
ST14	Goat	Puberty	Female	Slaughterhouse	MN317333

Table 4. Nucleotides sequence data from *Blastocystis* sp. in the ruminants, Shahrekord County, Iran.

experimental transmission of some STs of *Blastocystis* of humans to animals such as chickens and rats demonstrates the possibility of zoonotic transmission of this protist[51]. Additionally, there is a higher occurrence of blastocystosis in some occupational groups who had more contact with animals because of their jobs such as workers of animal husbandry, research institutions, zookeepers, slaughterhouse workers, shepherds, and veterinarians in comparison with other persons who are not in frequent contact with animals is conclusive evidence that strongly indicates the assumption of the zoonotic potential of some *Blastocystis* sp. and reservoir of animals for human blastocystosis[37,40,52]. Milk, meat, and other products of animals are important food sources for humans that cause the breeding of ruminants, pigs, and poultry in all countries worldwide. Therefore, if these animals are infected with zoonotic pathogens microorganisms such as parasites can be transmitted to animal handlers

Subtypes	Ruminants			
	No. of infected cattle (%)	No. of infected sheep (%)	No. of infected goat (%)	Total
ST5	0 (0)	8 (40)	2 (7.7)	10 (21.3%)
ST7	0 (0)	1 (5)	0 (0)	1 (2.1%)
ST10	1 (100)	1 (5)	6 (23.1)	8 (17.1%)
ST14	0 (0)	9 (45)	18 (69.2)	27 (57.4%
Unknown	0 (0)	1 (5)	0 (0)	1 (2.1%)

Table 5. Frequency (%) of *Blastocystis* STs in ruminants in Shahrekord County, Iran.

who are in frequent close contact with animals[13,14,39,47]. Ruminants, particularly cattle and sheep can be natural reservoirs of human *Blastocystis* infection that notifies the public health point of view of this parasitic protist in humans who have close contact with animals. Thus, determining the prevalence and subtyping of *Blastocystis* sp. in these animals is necessary to consider[14,41,47]. Many epidemiological investigations demonstrate the widespread distribution of *Blastocystis* in humans of different areas of Iran with a highly variable prevalence from 0.08 to 45% that is affected by some factors such as sample sizes, diagnostic methods, the sanitation conditions, socioeconomic situation, quality and expectancy of the life and education, and climate conditions of the place of the study population[53–56]. Furthermore, a few studies indicated the prevalence and subtyping of this parasite in animals in Iran. Badparva et al. reported that 9.6% of cattle in Iran were infected with *Blastocystis*[57]. Also in another study, Asghari et al. demonstrated that *Blastocystis* was detected in 43.7% of birds (42.9% and 44.4% of pigeons and crows respectively) in Iran[58]. The study carried out by Mohammadpour et al. indicated 29 (18.8%) dogs, 21 (17.7%) cats, and 20 (15.8%) rats in Iran were infected by *Blastocystis* sp.[43]. In another study, Rostami et al. reported that 35%, 19.4%, and 15.9% of cattle, sheep, and poultry in the northwest of Iran were infected by *Blastocystis* sp.[59]. However, the studies in this field are very limited and the information about the prevalence and genotypes of *Blastocystis* sp. in various animals in different areas of Iran is still unknown, particularly in the southwest region. The present study was carried out to determine the prevalence and subtyping of *Blastocystis* sp. in ruminants by screening 330 fecal samples of cattle, sheep, and goats using parasitological and molecular methods. The results of the current study revealed that *Blastocystis* was detected in 27(8.2%), 38(11.5%), 35(10.6%) and 47(14.2%) animals using microscopic, formalin-ether concentration technique, Giemsa staining, and culture method respectively which were confirmed by PCR-based molecular diagnostic method. The comparison of diagnostic methods used in this study indicated that the culture method was more sensitive to other parasitological methods. Salehi et al. reported that the stool cultivation method such as using HSr + S medium enhances the chance of detection of *Blastocystis* from fresh stool samples compared to the microscopy method[47]. Furthermore, Maryanti et al. indicated that the detection of *Blastocystis* by the culture method using modified Jones medium with sheep serum is superior and recommended compared to direct microscopic examination because it can cause the immediate growth of parasites and will increase their numbers[60]. On the other hand, based on the literature the sensitivity and specificity of the culture method are 90% and 100%, respectively. However, the culture method needs more time compared to direct microscopic examination which can be directly examined when getting a sample but microscopic examination needs an expert because it is difficult to distinguish different forms of *Blastocystis* from yeast cells or fecal debris[5,61]. Whereas, other researchers claimed that DNA extraction of cultivated stool samples in comparison to direct extraction of stool samples is more confident because of the elimination of the PCR inhibitors increasing the chance of detection of the parasite by molecular methods[62,63]. On the other hand, molecular techniques have a high specificity and sensitivity compared to other diagnostic methods. In the present study, the prevalence of *Blastocystis* detected in cattle, sheep, and goats were 0.93%, 17.4%, and 24.1% respectively. The higher prevalence of *Blastocystis* sp. in sheep and goats than in cattle may be due to access to infected forage and water in the free environment in the surroundings for consuming water, grazing, and feeding. The results of the current study showed that the prevalence of blastocystosis in adult goats was higher than in other animals. Moreover, the blastocystosis rate was higher in female than in male animals. Previous epidemiological studies conducted in some countries in different regions of the world reported prevalence rates of *Blastocystis* sp. infection in cattle is very different from 1.8% in Spain[64] to 100% in Indonesia[65]. Meanwhile, investigations carried out in other countries to assess blastocystosis in cattle reported that the prevalence of *Blastocystis* were 9.6% and 50.6% in Iran[47,57], 9.6% in China[66], 11.25% in Türkiye[67], 16.7% in Nepal[12], 2.9% and 19.15% in the United States of America[68,69], 22.7% in United Arab Emirates[52], 34.5% in Malaysia, 41.7% in Libya[50], 54.1% in Japan[70], 63.4% in Lebanon[71] and 80% in Colombia[72]. Although the prevalence rate in our study is low and almost similar to Spain but is lower than in other countries which may be due to sample size, using different diagnostic methods in various studies, or quality hygiene and sanitary conditions on farms. Regarding the infection in sheep and goats, our results revealed that 17.4% and 24.1% of sheep and goats were infected with *Blastocystis* sp., respectively. The epidemiological studies reported very different prevalence rates of *Blastocystis* sp. infection in small ruminants with an average estimated 25.3% and 20.5% in sheep and goats, respectively. However, the prevalence rates of blastocystosis in ruminants varied from 6% in China to 100% in Colombia in sheep and from 0.3% in China to 100% in Colombia in goats in different regions across the globe[73]. Thereby, epidemiological investigation of blastocystosis in sheep shows that 19.3–42.9% in different areas in Iran, 23.5% in the UK, 33.3% in Brazil, 38.2 in Turkey, 50% in Poland, 57.9% in Malaysia and 81.8% in Italy were infected with *Blastocystis* sp.[73]. The other surveys carried out in goats revealed that the prevalence of *Blastocystis* sp. varies in different countries such as Nepal (3.4%), Indonesia (5%) Libya (10.5%), Egypt (28.6%), France (50%)

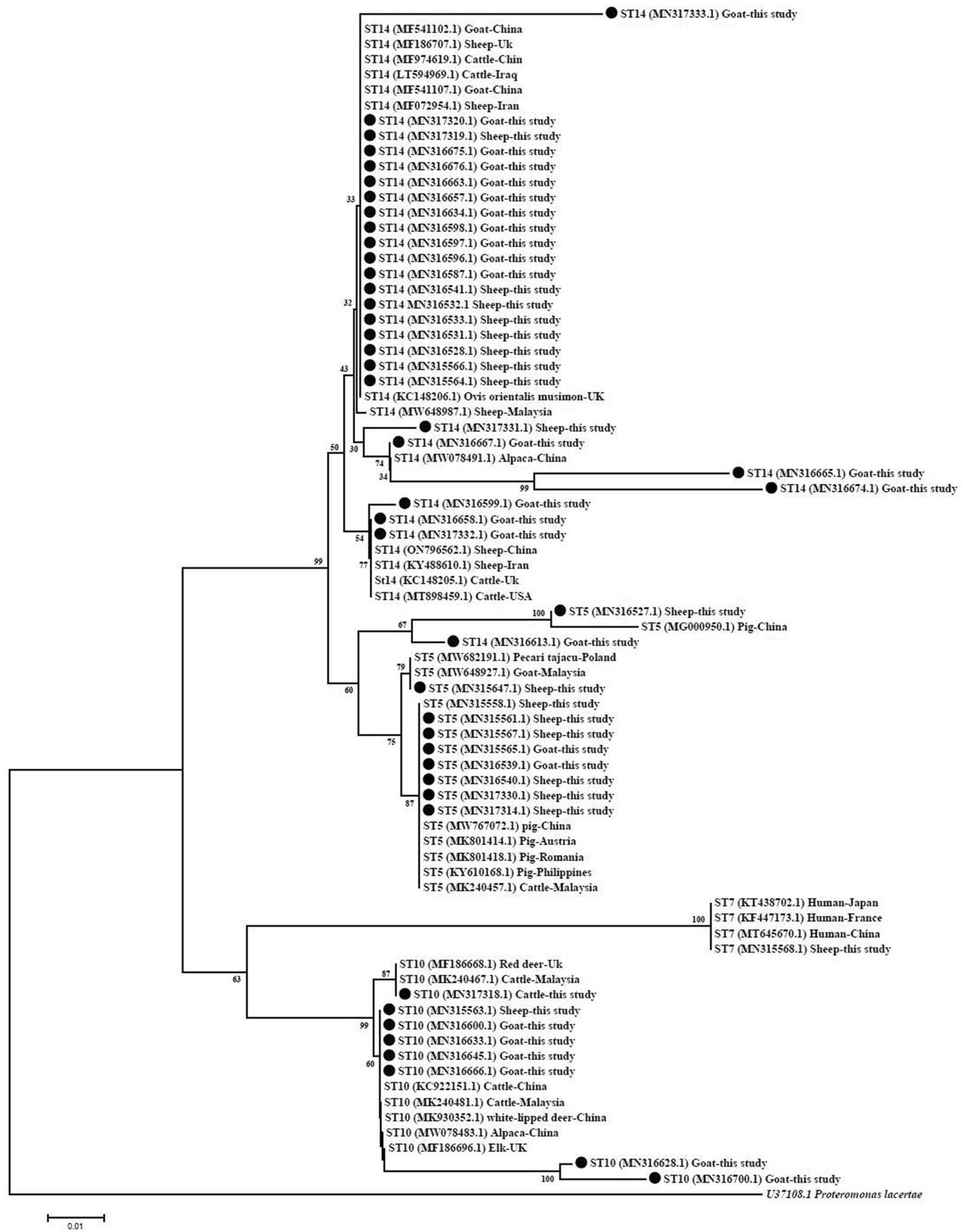

Fig. 4. Phylogenetic relationships among nucleotide sequences of barcode regions of small subunit ribosomal RNA (SSU rRNA) of *Blastocystis* sp. in the ruminants, Shahrekord County, Iran. The neighbor-joining method was used to construct the trees using the Kimura-3-parameter model. The numbers on the branches are percent bootstrapping values from 1000 replicates. Each sequence is identified by its STs, accession number, hosts, and country. *Blastocystis* STs identified in the present study are indicated by solid cycle.

and Poland (87.5%)[73]. Based on nucleotide differences sequences of the small-subunit rRNA (SSU rRNA) gene *of Blastocystis* a total of 38 STs (ST1–ST17, ST21, ST23–ST38, ST40 and ST42-ST44) were reported in mammalian and avian hosts[28]. In Iran, eight STs (ST1–ST7, and ST9) of sixteen *Blastocystis* STs recorded in humans were identified in different areas, of which ST3 was the most common subtype (Table 8)[74]. Additionally, 11 STs of

Risk factor	ST5	ST7	ST10	ST14	Unkown ST	P value*
Gender						
Male	5 (27.8)	1 (5.6)	1 (5.6)	11 (61.1)	0 (0)	0.203
Female	6 (20.7)	0 (0)	7 (24.1)	15 (51.7)	1 (3.4)	
Sampling location						
Traditional animal husbandry	6 (18.8)	0 (0)	8 (25)	18 (56.3)	0 (0)	0.058
Industrial animal husbandry	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	
Slaughterhouse	5 (33.3)	1 (6.7)	0 (0)	8 (53.3)	1 (6.7)	
Veterinary clinic	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	
Age groups of ruminants						
Cattle	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)	0.07
Calf	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	
Sheep	8 (44.4)	1 (5.6)	1 (5.6)	7 (38.9)	1 (5.6)	
Lamb	1 (50)	0 (0)	0 (0)	1 (50)	0 (0)	
Goat	2 (7.7)	0 (0)	6 (23.1)	18 (69.2)	0 (0)	
Kid	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	

Table 6. Prevalence of different STs of *Blastocystis* sp. in ruminants according to demographic characteristics, Shahrekord County, Iran. *Based on Fisher exact test.

Risk factor	Infected with *Blastocystis* sp.		P value*
	No of positive (%)	No of negative (%)	
Gender			
Male	18 (9.5)	172 (90.5)	0.006
Female	29 (20.7)	111 (79.3)	
Sampling location			
Traditional animal husbandry	32 (18.5)	141 (81.5)	0.029
Industrial animal husbandry	0 (0)	20 (100)	
Slaughterhouse	15 (12.3)	107 (87.7)	
Veterinary Clinic	0 (0)	15 (100)	
Age groups of ruminants			
Cattle	1 (1.2)	85 (98.8)	0.001
Calf	0 (0)	21 (100)	
Sheep	18 (19.4)	75 (80.6)	
Lamb	2 (9.1)	20 (90.9)	
Goat	26 (28.3)	66 (71.7)	
Kid	0 (0)	16 (100)	

Table 7. Prevalence of *Blastocystis* sp. in ruminants according to demographic characteristics, Shahrekord County, Iran. *Based on Fisher exact test.

Blastocystis (ST1–ST8, ST10, ST13- ST14) have been reported in non-human hosts in different areas of Iran, including cattle (ST1, ST3, ST5, ST7, ST10 and ST14)[47,59,75,76], sheep (ST3, ST5, ST7, ST10 and ST14)[47,59,75,76], equus animals [(horses, donkeys, and mules) (ST1-ST4, ST6-ST7, ST10 and ST14)][77], birds (ST6, ST7, ST10, ST13 and ST14)[47,58,59], rodent (ST1, ST3 and ST4)[43] and domestic carnivorous [(dog and cat (ST1-ST5, ST7-ST8, ST10)][43,78]. In a second step of the current study, 47 detected isolates of *Blastocystis* sp. were sequenced by BhRDr primer and compared with available sequences in databases that indicated four genotypes of *Blastocystis* including ST5 (21.3%), ST7 (2.1%), ST10 (17.1%), ST14 (57.4%) and 1 (2.1%) unknown subtype. Furthermore, the only subtype identified in cattle was ST10, and the observed STs in sheep were ST5 (40%), ST7 (5%), ST10 (5%), ST14 (45%) and one unknown subspecies. The goats were infected with ST5 (7.7%), ST10 (23.1%) and ST14 (69.2%). In this study, the potentially zoonotic ST5 was detected among the positive samples of sheep and goats which can be a source of infection for people who have close contact with these animals. The investigation of the prevalence and characterization of *Blastocystis* sp. in humans in the area of our study (Southwest of Iran) show that 6.4% of individuals who were referred to Shahrekord's clinics for their recent illness or periodic checkups were infected with four STs of *Blastocystis* including ST1 (29.1%), ST2 (27.3%), ST3 (36.4%) and ST7 (7.3%)[79]. These findings and our results indicate that ST7 may be transmitted from animals to humans. Recent-year investigations conducted in geographic regions of different continents, from Asia to Oceania worldwide, indicated 23 different STs of *Blastocystis* including ST1-ST7, ST10-ST14, ST15, ST17, ST21, ST23-ST26, ST30, ST32, ST40,

City/province	Positive number/sample size	STs (number)	Participant	Method
Shiraz	45/100	ST1(20), ST2(4), ST3(16)	Persons who were referred to health centers	RFLP
Tehran	100/420	ST1(21), ST3(25), ST6(21), Mix 1, 3(14), Mix 3,5(2), Mix 3,6(4), M 1,5(1)/M 1,6(4)/M 1,5,3(4)/M 1,3,6(4)	persons who were referred to health centers	STS primers
Hamadan	41/250	41/250 ST1(23), ST5(3), ST3(9), Mix 1, 3(6)	persons who were referred to health centers	STS primers
West Azerbaijan	57/900	ST1(23), ST2(5), ST3(29)	persons who were referred to health centers	STS primers
Khorramabad	30/511	ST3(17), ST5(4), ST6(6), Mix 3,5(2), Mix 3,6 (1)	persons who were referred to health centers	STS primers
Baghmalek	17/1410	ST3(3), ST4(9), ST5(2), ST7(3)	persons who were referred to health centers	PCR/sequencing
Tehran	58/400	ST1(18), ST2(21), ST3(19)	diarrheic and non-diarrheic patients	PCR/sequencing
Ahvaz	50/481	ST1(11), ST2(3), ST3(20), ST4(1), ST5(4), Mix 1, 3(3), M 1,4(3) and M 3,4(5)	persons who were referred to health centers	STS primers/sequencing
Ahvaz	51/268	ST1(11), ST2(6), ST3(29), ST6(2), Mix 1, 3(3)	HIV patients	STS primers/sequencing
Tehran	13/161	ST1(7), ST2(5), ST3(1)	Tuberculosis patients	PCR/sequencing
Qazvin	25/864	ST1(14), ST2(7), ST3(4)	children (referred to hospital)	PCR/sequencing
Mazandaran	7/420	ST3(7)	persons who were referred to health centers	PCR/sequencing
Urmia, Tabriz, Maragheh	16/300	ST1(3), ST2(3), ST3(10)	persons who were referred to health centers	PCR/sequencing
Sanandaj	24/1383	ST1(2), ST2(6), ST3(16)	persons who were referred to health centers	PCR/sequencing
Shiraz	37/802	ST1(12), ST2(9), ST3(13), ST7(3)	persons who were referred to health centers	PCR/sequencing
Tehran	32/58	ST1(1), ST3(28), ST9(3)	Schizophrenia patients	STS primers/sequencing
Kerman	66/210	ST1(5), ST2(1), ST3(37), ST4(7), ST5(6), ST7(3), Mix 3, 4(3), Mix 3, 5(1), Mix 1,2, 3(3), Mix 4, 5(1), Mix 1, 3(3), Mix 3, 4,7(1),	persons who were referred to health centers	Real-time PCR molecular method
Northeast of Iran	22/1878	ST1(4), ST2(7), ST3(10), ST4(1)	persons who were referred to health centers	PCR/sequencing
Shahrekord	55/864	ST1(16), ST2(15), ST3(20), ST7(4)	Persons who were referred to health centers	PCR/sequencing
Kashan	51/1118	ST1(20), ST1(10), ST3(21)	Persons who were referred to different medical diagnostic laboratories	PCR/sequencing
Kermanshah	33/950	ST1(5), ST3(15), ST5(4), Mix 1, 3(2), Mix 1, 5(1), Mix 1, 6(1), Mix 3, 4(1), Mix 2, 5(1), Mix 3, 5(1), Mix 1, 5,6(1), Mix 3, 6(1)	Persons who were referred to health centers	STS primers
Bandar Abbas	8/378	ST1(4), ST2(1), ST3(3)	Persons who were referred to hospitals and health centers	PCR/sequencing

Table 8. The prevalence of different STs of *Blastocystis* sp. in humans in Iran[74,84,85].

ST42 and ST44 can infect large and small ruminants that among them, eleven STs are zoonotic (ST1-ST7, ST10, ST12, ST14 and ST23). According to these studies, the detected STs of this protozoa that were isolated from cattle are ST1-ST7, ST10-ST14, ST17, ST21, ST23-26, ST32, ST42 and ST44 (Twenty-one STs) which the ST10 is the most common of them[73,80–83]. The STs of *Blastocystis* that isolated from sheep were ST1-ST5, ST7, ST10, ST12, ST14, ST15, ST21, ST23-ST26, ST30, ST40, ST43 and ST44 whereas, goats can be infected with nineteen STs such as ST1, ST3-ST7, ST10, ST12-ST14, ST21, ST23-ST26, ST30, ST32, ST43 and ST44 that the most frequency of STs in small ruminants was related to ST10 in many countries of the world[73,81,83]. The studies carried out in Iran indicate that ST3, ST5, ST7, ST10 and ST14 were isolated from sheep, furthermore the STs of ST1, ST3, ST5, ST6, ST7, ST10 and ST14 were identified in infected cattle[47,73,80].

Conclusion

Some previous studies have predicted the risk of zoonotic transmission of some STs of *Blastocystis*. So, the main goal of the current study was to determine the frequency and genotype of *Blastocystis* in ruminants. The findings of this survey indicated that in this region, blastocystosis in goats was reported for the first time in Iran. Additionally, ST14 was identified as the most common subtype of *Blastocystis* in this study that was not common between humans and livestock, but ST5 and ST7 are common between humans and animals accounting for 23.4% and 2.1% of the positive cases, respectively and the hypothesis is reinforced that ruminants are reservoirs of human infection of *Blastocystis* parasite. Therefore, local government and health authorities should take measures to

reduce the contamination of water and food sources by this parasite and thus, protect the health of animals and humans who have contact with animals, particularly those with immune system defects in the context of the One Health concept. So, further studies should be carried out on the other hosts such as rodents and birds to determine the distribution pattern of *Blastocystis* sp. and can control infection in this area of the world. The limited sample size and failure to check people who in contact with animals were limitations of the present study. Therefore, more studies with large sampling from different domestic animals and their in-contact humans would further indicate the zoonotic transmission potency of *Blastocystis* sp., and which animals pose a risk of human infection and to what extent in the investigated areas.

Data availability

Sequence data that support the findings of this study have been deposited in the National Center for Biotechnology Information (NCBI) with the primary accession codes MN315558, MN315561, MN315565, MN315567, MN315647, MN316527, MN316539, MN316540, MN317314, MN317330, MN315568, MN315563, MN316600, MN316628, MN316633, MN316645, MN316666, MN316700, MN317318, MN315564, MN315566, MN316528, MN316531, MN316532, MN316533, MN316541, MN316658, MN316663, MN316665, MN316667, MN316674, MN316675, MN316676, MN317319, MN317320, MN317331, MN317332, MN317333, MN316587, MN316596, MN316597, MN316598, MN316599, MN316613, MN316634 and MN316657.

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R.A. conception of the study, study design, methodology, formal analysis and investigation, interpretation of the results, writing original draft of manuscript; M.H., K.M.N., S.K. methodology, formal analysis and investigation, interpretation of the results, editing manuscript; R.A., K.M.N. and S.K resources of the experiment. R.A. and K.M.N language profiling. R.A. mentoring, manuscript editing-revision, supervision. All authors have read and agreed to the published version of the manuscript.

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Competing interests

The authors declare no competing interests.

Additional information

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