

ORIGINAL ARTICLE

Prevalence and Molecular Identification of *Blastocystis* Subtypes in Sheep in Salah al-Din Province, Iraq

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ABSTRACT**Key words:***Blastocystis* sp., sheep, PCR, Sequencing, ST5, ST10***Corresponding Author:**Zuhair Mohammed Abed
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Background: *Blastocystis* sp. is among the most common and important pathogens identified in humans and various type of animals hosts including sheep, cattle, and goat, with a global distribution. **Objective:** determine the prevalence and molecular subtypes of *Blastocystis* in sheep from rural areas of Tikrit, Iraq. **Methodology:** 29 stool specimens (approximately 7-10 g per specimen) were taken from sheep suffering from diarrhea, as well as 154 specimens from sheep that did not show any clinical signs, samples were collected from different rural areas of Tikrit city, Iraq for the period from January to June 2025, DNA extraction from samples was performed by taking about 200 mg of fecal sample, by stool DNA kit. **Results:** The result of the PCR (Polymerase Chain Reaction) examination showed that the overall infection rate reached 16 (8.74%) out of 183 fecal samples were positive to *Blastocystis*, two subtype of *Blastocystis*, ST10 and ST5, were detected in this study, The sequencing results were recorded in the Gen Bank with the following numbers: PV876305.1, PV876306.1, PV876307.1, PV876308.1 PV876309.1. **Conclusion:** the identification of two *Blastocystis* subtypes in this study indicates the presence of genetic diversity among the detected isolates and this result confirms the importance of continuous molecular surveillance to understand the epidemiology and transmission routes.

INTRODUCTION

The genus *Blastocystis* is among the most common intestinal microorganisms in human and in numerous animals such as goat and sheep¹ *Blastocystis* is currently classified within kingdom: SAR and phylum: Stramenopiles².

Infection with *Blastocystis* in animals is often manifested by non-specific clinical signs, including abdominal pain, nausea and diarrhea³ in general, the different subtypes of *Blastocystis* influence the appearance of clinical symptoms and variation in their severity as well as in determining overall impact of the disease⁴ sanitation infrastructure, socioeconomic levels and geographical areas have an impact on the spread of *Blastocystis*, making its prevalence higher in developing countries than in industrialized countries⁵. The prevalence of this parasite in sheep and goats has been reported to range from 0.35 to 94.7% with subtype 10 being the most common⁶.

To date, several techniques have been applied to detect the presence of *Blastocystis* sp. including formalin-ether concentration method, direct smear examination, stained smear with iodine or trichrome, in culture medium, polymerase chain reaction (PCR) and sequencing.⁷ *Blastocystis* samples isolated from human and animals showed great genetic diversity according to evolutionary analysis conducted based on the sequence of the "SSU-rRNA" gene (this gene is widely used as a

reliable molecular marker in the taxonomic and evolutionary analysis of parasite, because it contains conserved region that facilitate PCR amplification)⁸. The data available so far have shown the presence of at least 42 subtypes of *Blastocystis* have been detected from different hosts². Among all these identified subtypes, ST1 to ST8 and ST12 were found in animals and humans, while ST9 was found only in humans⁹. The occurrence of zoonotic transmission from various animal populations is strongly supported, as the zoonotic potential of the parasite *Blastocystis* sp. was clearly demonstrated by molecular diagnosis which confirming the presence of identical genetic variants of ST1, ST5, ST6 or ST8, which was simultaneously revealed in captive primates and zookeepers within the same region¹⁰.

Therefore, the present study aimed to determine the prevalence and molecular subtypes of *Blastocystis* in sheep from rural areas of Tikrit, Iraq, using PCR and sequence based analysis of the "SSU rRNA".

METHODOLOGY

Samples collections: 29 stool specimens (approximately 7-10 g per specimen) were taken from sheep suffering from diarrhea, as well as 154 specimens from sheep that did not show any clinical signs. The samples were placed in plastic bottles with the information written on them (age, diarrhea and normal

stool), the fecal specimens were collected from different rural areas of Tikrit for the period from January to June 2025, the specimens were preserved in the Tikrit University Parasitology Laboratory at -20°C for use in molecular studies.

Molecular method

The genomic DNA was extracted to detect the presence of parasite cysts in sheep feces by taking 200 mg of stool sample. Extraction was carried out according to the manufacturer's (Bioneer Korea) protocol for the DNA stool kit. Positive samples were kept in a refrigerator at -20°C. Amplification of the "SSU- rRNA" gene was performed by Primers previously described by Khoshnood et al.,¹¹ and the sequences are as follows: forward 5'-GGAGGTAGTGACAATAAATC-3' and reverse 5'-TGCTTTCGCACTTGTTTCATC-3'. Conducted polymerase chain reaction in a total reaction volume of 25 µl. containing; 0.5 µl of each primer (12.5 pmol), 12.5 µl of 2Master Mix (which contains the *Taq* polymerase, dNTPs and MgCl₂) and 5 µl of template DNA. The thermal cycling (Gene Amp®, PCR System 9700) profile consisted of an initial step of denaturation at 95°C for 4 min, 35 cycles at 95°C for 30 s, 30 s at 54°C, 30 s at 72°C, and a final elongation at 72°C for 5 min. The amplified reaction products were separated by 1.5% agarose gel electrophoresis to identify the sample positive for *Blastocystis* sp.

Sequencing

Sequencing of some PCR products (on Sanger platform) to determine the subtype of *Blastocystis* by comparison of "SSU-rRNA" sequences with sequences stored in the NCBI was conducted by the BLAST. The phylogenetic tree was conducted in MEGA6¹².

Statistical Analysis

Statistical analysis was performed to compare the prevalence of *Blastocystis* sp. in sheep according to age (under 6 months vs. over 6 months) and stool condition (diarrhea vs. normal feces). Chi-square and Fisher's

exact test were used to determine the significance of differences, with P-value of $P < 0.05$ considered statistically significant. SPSS version 28.

RESULTS

The stool specimens were diagnosed by PCR was performed for amplification of a 500bp (Fig.1). The result of the PCR examination showed that the overall infection rate reached 16 (8.74%) out of 183 fecal samples contained parasite cysts. The occurrence of *Blastocystis* infection in sheep with diarrhea reached 4 (13.79 %) and normal fecal 12 (7.79 %), regarding age, the prevalence rate in sheep under 6 months was (9.21%), while in sheep over 6 months it was (8.41%), statistical analysis indicated that there were no significant differences between age groups (0.851), and there were also no significant differences in the prevalence of *Blastocystis* sp. in sheep with diarrhea and healthy sheep (0.375) (table1).

Table1. Prevalence of *Blastocystis* sp. in sheep according to age and stool condition.

	Collect	Positive sample	P. value
> 6 month	107	9 (8.41%)	0.851
< 6 month	76	7 (9.21%)	
Normal feces	154	12 (7.79%)	0.375
Diarrhea	29	4 (13.79%)	
Total	183	16 (8.74%)	

The subtype of *Blastocystis* was identified by performing sequence analysis of 5 PCR products. The sequencing results were recorded in the GenBank with the following numbers: PV876305.1, PV876306.1, PV876307.1, PV876308.1, PV876309.1, BLAST analysis revealed two subtypes ST10 and ST5 of *Blastocystis*.



Fig.1: lane1 and 9 are positive for *Blastocystis* sp.

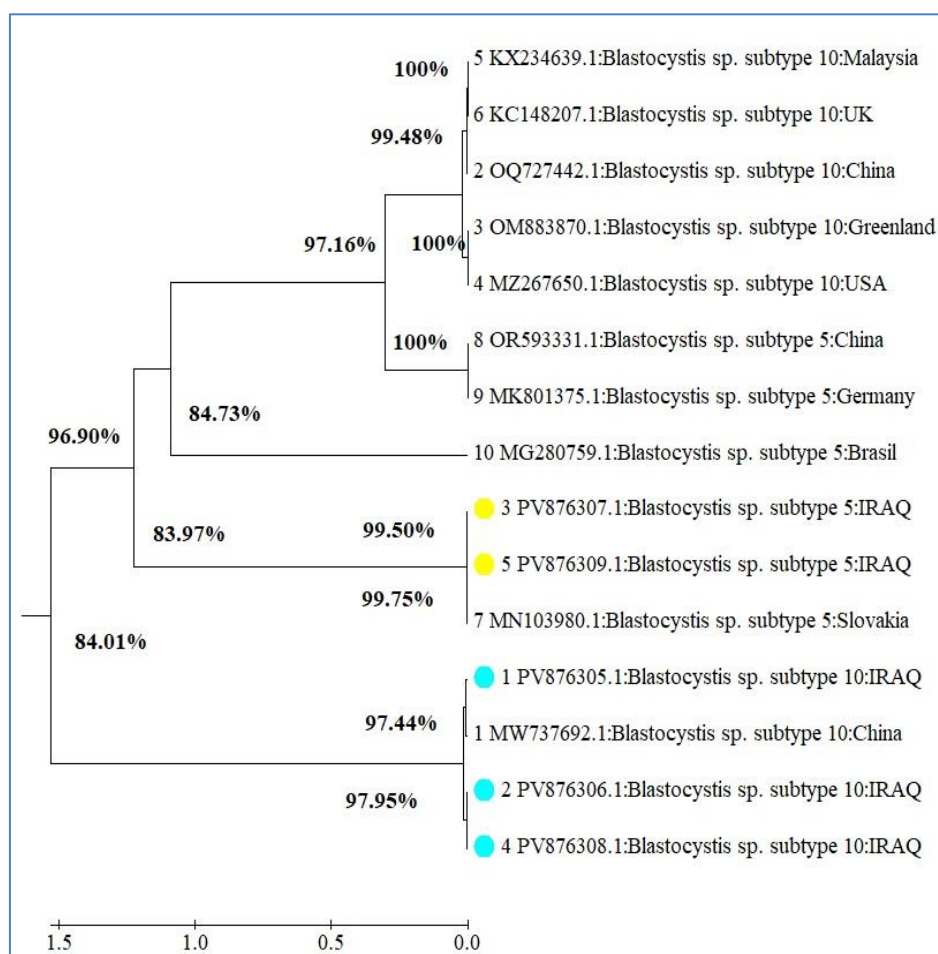


Fig. 2: Phylogenetic tree of *Blastocystis* sp isolated from sheep. and reference sequence from Gen Bank

Table 2: Genetic rapprochement of "SSU- rRNA" gene for the isolate of *Blastocystis* sp.

	18S ribosomal RNA gene					
	Accession	Country	Isolation Source	Source	date of registration	Compatibility
1.	ID: MW737692.1	China	Cattle	<i>Blastocystis</i> sp. subtype 10	2021	99%
2.	ID: OQ727442.1	China	Goat	<i>Blastocystis</i> sp. subtype 10	2023	98%
3.	ID: OM883870.1	Greenland	Muskox	<i>Blastocystis</i> sp. subtype 10	2022	99%
4.	ID: MZ267650.1	USA	Deer	<i>Blastocystis</i> sp. subtype 10	2021	99%
5.	ID: KX234639.1	Malaysia	Bird	<i>Blastocystis</i> sp. subtype 10	2016	97%
6.	ID: KC148207.1	UK	Camelus dromedaries	<i>Blastocystis</i> sp. subtype 10	2012	97%
7.	ID: MN103980.1	Slovakia	Sow	<i>Blastocystis</i> sp. subtype 5	2019	99%
8.	ID: OR593331.1	China	masked palm civets	<i>Blastocystis</i> sp. subtype 5	2023	99%
9.	ID: MK801375.1	Germany	Sow	<i>Blastocystis</i> sp. subtype 5	2019	99%
10.	ID: MG280759.1	Brasil	-----	<i>Blastocystis</i> sp. subtype 5	2017	98%

DISCUSSION

The genus *Blastocystis* is among the most frequently identified pathogen in animals and humans¹³. Cattle and sheep may be the most important ruminants that act as natural reservoirs of human *Blastocystis* infection that raising public health concerns about the presence of this parasite in human who have being in close proximity to animals. Therefore, it is important to know the prevalence and subtypes of *Blastocystis* sp. in these animals¹⁴.

The result obtained revealed that the overall prevalence of *Blastocystis* among sheep was, 16(8.74%) out of 183. This result is almost similar to studies conducted in China where the prevalence rate was 8.55%¹⁵, 10.12%¹⁶, 7.5%¹, but were low compared to the prevalence rate in Sulaymaniyah province of Iraq, which reached 24.4% in ruminant¹⁷ United Arab Emirates 63.64%¹⁸, Iran 17.4%¹⁹, China 16.26%²⁰, Brazil 33.3%²¹ and Italy 81.8%²². The variation in the infection rate of *Blastocystis* be due to several factors, including animal age, feeding condition, climate, farm management and geographic location²³. In a study, although the prevalence rate was higher in sheep with diarrhea, but it was not significant and no significant differences were found among age groups.

The sequencing analysis of the "SSU-rRNA" gene indicated the detection of two subtypes ST10 and ST5 in sheep samples, with these data being documented in the NCBI and the numbers were taken PV876305.1, PV876306.1, PV876307.1, PV876308.1 PV876309.1. are in concordance of 97-99% as shown in table 2. and Fig2. of the phylogenetic tree of *Blastocystis* sp.

In the investigated areas, two subtypes (ST5 and ST10) were found in sheep. Previous studies indicated the presence of more than one subtypes of *Blastocystis* in sheep (ST1 to ST5, ST7, ST10, ST12, ST14, ST15, ST21, ST23, ST24 and ST26)²⁴ ST10 was found more frequently than other subtype, followed by ST14. among the three subtypes identified in the study (ST10, ST12 and ST14)¹⁶ as well as, the sequences analyses showed the presence of four subtypes of *Blastocystis* (TS5, ST10, ST14 and ST30) in sheep¹⁹ in contrast one subtype ST10 has been identified in sheep in a study in Italy²¹. Ten subtypes (ST1 to ST3, ST5, ST10, ST14, ST21, ST24, ST26 and ST40) have been identified in sheep in Egypt with a different infection rate for each subtypes¹⁴. Molecular studies have shown that host species and geographical regions have a significant influence on the distribution of subtypes²⁵. In a study, six subtypes (ST1, ST2, ST3, ST5, ST7, and ST14) were detected in human and other animals, ST1-ST3 subtypes were detected in domestic animals²⁶, in addition, ST10 and ST14 were identified in apparently healthy school children²⁷, as in studies in which subtype10 was identified in human. The zoonotic significance of this subtype has been demonstrated²⁸

Subtype5 commonly found worldwide in ungulates animals (pigs and sheep), and it was also occasionally identified in people who had close contact with animals, therefore subtype5 is considered potential zoonotic⁹, it was observed to be the most prevalent in sheep and pig²⁰ Subtype10 was more hesitant in both Tibetan sheep and Tibetan goats¹⁵. *Blastocystis* is a microorganism of great importance, frequently identified in the feces of animals and humans and with global distribution, and the STs of *Blastocystis* shared between human and animals lead to the risk of transmission from an animal source to human²⁹.

CONCLUSION

This study investigated the occurrence of *Blastocystis* in sheep from Salah al-Din Province. The overall prevalence rate was 8.74%. There were no significant differences in infection rates between diarrhea and healthy sheep or among different age groups. Two subtypes, ST10 and ST5, were identified in examined samples. It is recommended that further molecular epidemiological studies be conducted on a larger number of livestock species and across different regions to better understand the transmission dynamics and potential zoonotic significance of *Blastocystis* in Iraq.

Declarations:

Consent for publication: Not applicable

Availability of data and material: Data are available upon request.

Competing interests: The author(s) declare no potential conflicts of interest with respect to the research, authorship and/or publication of this article. This manuscript has not been previously published and is not under consideration in another journal.

Funding: Authors did not receive any grants from funding agencies.

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