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Comparison of various diagnostic techniques for the detection of *Blastocystis* spp. and its molecular characterisation in farm animals in the United Arab Emirates

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Abstract

Introduction: *Blastocystis* spp. is a common anaerobic intestinal parasite infecting humans and a diverse range of animals. The aim of the study was to compare different diagnostic methods for the detection of *Blastocystis* and survey the occurrence of its subtypes in farm animals, namely sheep, cows and camels, in Al-Ain, United Arab Emirates. **Material and Methods:** Ninety-seven faecal samples comprised of 69 from sheep, 12 from cows and 16 from camels were submitted to DNA extraction, PCR and sequencing. *Blastocystis* was screened for microscopically in 65 samples using direct wet-mount, modified acid-fast staining, trichrome staining and *in vitro* culture techniques. **Results:** Fifteen (15.5%) samples were positive by PCR, twelve of which were confirmed by sequencing. Using PCR as a comparison standard, the sensitivity and specificity of the direct wet-mount, modified acid-fast staining, trichrome staining and *in vitro* culture methods were 40.0% and 78.3%, 40.0% and 83.3%, 80.0% and 80.0%, and 80.0% and 76.7% respectively. Only culture and trichrome tests were significantly associated with PCR (odds ratio (OR) = 13.14; 95% confidence interval (CI): 1.35–127.4; P = 0.007 and OR = 16; 95% CI: 1.63–156.5; P = 0.003, respectively) with trichrome detecting more positive cases than *in vitro* culture. The subtype (ST)10 was the only one found in all 12 sequenced sheep isolates. **Conclusion:** The study corroborated previous data indicating that sheep are the natural hosts for ST10. No zoonotic subtypes nor mixed-subtype colonisation were found. The report also confirmed the superiority of trichrome staining in detecting *Blastocystis* spp.

Keywords: *Blastocystis*, PCR, trichrome stain, sequencing, UAE.

Introduction

Blastocystis is a genus of common intestinal parasites of humans and a wide range of both wild and domestic animals (7, 26). A greater risk of *Blastocystis* infection was noted in people with close animal contact, suggesting its zoonotic potential (17, 25). Although infections are deemed to be associated with a variety of intestinal ailments such as irritable bowel syndrome, urticaria, and possibly gastrointestinal cancers (11, 27, 28), recent microbiome studies have shown that *Blastocystis* colonisation may be beneficial to human health (19, 21).

Molecular diagnostic tools based on small subunit ribosomal ribonucleic acid (SSU rRNA) subtyping have revealed the existence of extensive genetic diversity within *Blastocystis* (27). So far, 26 subtypes have been documented (13). Among them, nine (ST1 to ST9) have been identified in humans, all except the ninth being potentially zoonotic (7, 8).

Many studies conducted in different countries, including an earlier study performed by our group, revealed that subtype (ST)10 and ST14 were the most common subtypes detected in artiodactyls (sheep, cows and goats) (3). Unlike other researchers, we did not find any of the other subtypes such as ST1, ST3, ST4, ST5 or ST6 (4, 5). However, the artiodactyl sample size studied in our earlier report was limited and the information derived from it about the prevalence of *Blastocystis* infection would therefore have been inadequate to

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provide a detailed picture of the existence of potentially zoonotic subtypes in them and of their significance as carriers for human infections.

Microscopic investigation of wet-mount preparations and/or permanently stained slides is the main method of *Blastocystis* spp. detection (6). Nevertheless, the many different forms the parasite exhibits, the sporadicity of shedding of cysts and the parasite's similarity to normal structures typically found in a stool sample present challenges to correct identification and lead to underreporting (9, 15). Numerous studies have therefore suggested the necessity for dependable diagnostic techniques such as *in vitro* cultivation and polymerase chain reaction (PCR) (15, 20). Scicluna *et al.* (22) have developed a PCR method based on the analysis of the partial SSU rRNA gene. This method is routinely used in most diagnostic laboratories in developed nations for diagnosing *Blastocystis* infection. Among the many advantages of this approach are its abilities to determine the parasite's subtype and zoonotic potential (18, 24). However, because of financial constraints and the need for highly trained laboratory personnel if molecular detection is to be attempted, microscopy is often the only tool available for the screening of *Blastocystis* spp.

The aims of the present study were to determine by PCR the occurrence, subtype distribution and zoonotic potential of *Blastocystis* spp., particularly in farm animals of the Artiodactyla order, and to compare and evaluate the sensitivity and specificity of different diagnostic techniques in the detection of *Blastocystis* spp. in faecal samples compared with the PCR assay as the gold standard.

Material and Methods

Sample collection and storage. Ninety-seven fresh faecal samples were obtained from sheep (69), cows (12) and camels (16) in Al-Ain, United Arab Emirates (UAE) and kept in leak-proof containers without preservatives. Samples were transported immediately to the University of Sharjah and submitted for DNA extraction and PCR. Of the 97 samples, 65 were separated into two containers (one aliquot stored at −20°C and the other used for microscopy and *in vitro* culture analysis). All of these 65 samples were screened using wet-mount, trichrome stain, acid-fast stain and *in vitro* culture techniques as described below. The remaining 32 samples were not screened using these four techniques as they were discarded prior to the microscopic examination and following the extraction of DNA.

Iodine stain. Lugol's iodine was applied to non-fixed smears and the smears were examined microscopically using 10× and 40× objective lenses.

Trichrome stain. Smeared slides were allowed to air dry, stained with trichrome stain and mounted with distrene, plasticizer, and xylene (Sigma Aldrich; St. Louis, MO, USA) before they were examined microscopically under 100× magnification for the presence of *Blastocystis* spp. Slides were cross-checked by other members of the group.

Modified acid-fast stain. Smears were fixed with absolute methanol for 30 s, stained with Kinyoun's carbolfuchsin for 1 min, decolourised using acid alcohol for 2 min and then counterstained using malachite green for 2 min. The stained slides were examined microscopically using 40× and 100× oil immersion objective lenses to confirm internal morphology.

***In vitro* culture.** Fresh faecal samples were subjected to short-term xenic *in vitro* culture using modified Tanabe-Chiba medium with 10% horse serum at 37°C (14). Following three days of culture, sediments were examined microscopically for the presence of any of the four known morphologies. Samples negative for *Blastocystis* were re-examined after two more days of culture and marked negative when no parasites were observed on either of these occasions (2). A sample was reported as positive if any of the four morphologies of *Blastocystis* spp. was observed.

DNA extraction, PCR, and analysis of DNA sequencing. In this stage, first DNA was extracted from all stool samples using the Bioline stool DNA Kit (London, UK) as per the manufacturer's recommendations and stored at −20°C until it was analysed. The 600-bp barcoding region of the SSU rRNA gene of *Blastocystis* was amplified using BhRDr and RD5 primers (22, 23). Reaction products were separated in 1.2% agarose gels stained with ethidium bromide and the size confirmed using a 100-bp ladder. The PCR products were gel-purified using a PureLink Quick PCR Purification Kit from Invitrogen (Carlsbad, CA, USA) according to the manufacturer's instructions, and sent for sequencing in a commercial sequencing facility (McLab; South San Francisco, CA, U.S.A).

Subtyping and phylogenetic analysis of *Blastocystis* isolates. Products of the PCR were sequenced on both strands, and the resulting sequences assembled online using CAP3 Sequence Assembly Program online tool (available at https://doua. prabi.fr/software/cap3/). The CLC Main Workbench 6 package (Qiagen, Redwood City, CA, USA) was also used for sequence end trimming and editing, in addition to contig assembly. Sequences were aligned with sequences covering the "barcoding region" of the SSU rRNA gene retrieved from GenBank to provide representatives of the known subtypes of *Blastocystis*. For phylogenetic analysis, SSU rRNA gene sequences were analysed together with an outgroup sequence (*Proteromonas lacertae*, GenBank accession no. U37108), and the evolutionary relationship between taxa was inferred using MEGA X software. To perform DNA sequence alignment, we used the Clustal W algorithm in MEGA X, with default parameters. Phylogenetic analysis was performed using the neighbour-joining method. Selected reference subtype sequences and *Blastocystis* sequences available from GenBank were included in the construction of the phylogenetic tree. We also used the public *Blastocystis* database

at https://pubmlst.org/ blastocystis/ to check the STs and the alleles.

Results

Ninety-seven stool samples were collected and screened for the presence of *Blastocystis* by PCR. Fifteen (15.5%) were determined positive by PCR (1 from a camel, 2 from cows and 12 from sheep). Twelve of the fifteen samples were confirmed by sequencing to be *Blastocystis* and were deposited in GenBank (accession numbers MT895705–MT895716). These were from sheep. The remaining three samples were not sequenced due to financial constraints. Subtype 10 (allele 152) was the only *Blastocystis* subtype detected. No zoonotic subtypes were detected, no mixed infections involving different STs were identified, and no intra-subtype genetic variation was observed within ST10. The isolates in the present study clustered in two areas within the clade comprising ST10 (Fig. 1). Isolates 4 (MT895715) and 5A (MT895716) clustered in a different sub-clade from the other isolates in the study. Isolates 2, 5B, 7 and 10 (MT895706, MT895708, MT895710 and MT895713) shared 80.72%, 84.74%, 78.36% and 80.53% identity with isolates from cattle in Malaysia (MG831493, MG831498 and MG831501), respectively. Isolate 6 (MT895709) shared 90.29% similarity with an isolate from deer in China (MK357785) and isolate 4 (MT895715) shared 97.03% identity with an isolate from an elk in the UK (MF186696). Interestingly, isolate 5A (MT895716) shared 99.11% similarity with one from a camel in Libya (KC148207). All twelve sequences were found to match *Blastocystis* spp. ST10 isolate CA (KC148207) when compared against the https://pubmlst.org/organisms/blastocystis-spp database. However, it was surprising that a few isolates (5 in total) did not return any matches when compared with Blastocystis spp. ST10 isolate CA (KC148207) using BLAST. These isolates had no score tags in Table 1. Table 1 shows the BLAST scores of our sequences compared to the previously mentioned KC148207 isolate in this database, wherein that isolate was the only match identified.

The performance of the direct wet-mount modified acid-fast stain, trichrome stain, and *in vitro* culture methods in terms of sensitivity and specificity were compared to the PCR assay as the gold standard technique (Table 2). Calculations of sensitivity and specificity were made from the data of 65 samples. The sensitivity and specificity of the direct wet-mount and modified acid-fast stain techniques compared to PCR were 40% and 78.3% and 40% and 83.3%, respectively. However, the sensitivity and specificity of the trichrome stain and *in vitro* culture compared to PCR were 80% and 80% and 80% and 76.7%, respectively. The sensitivity of iodine stain (direct wet-mount), modified acid fast stain, trichrome stain and *in vitro* culture varied widely with 40%, 40%, 80% and 80% being seen, respectively, while the specificity of the four techniques was almost the same at 78.3%, 83.3%, 80.0%, and 76.7%, respectively. Table 2 shows the association of the iodine, acid-fast, trichrome, and culture tests with the PCR. Only the trichrome and culture tests were significantly associated with PCR (odds ratio (OR) = 16.00; 95% CI: 1.63–156.5; P = 0.007 and OR = 13.14; 95% CI: 1.35–127.4; P = 0.003, respectively) and it seems that the trichrome method was able to detect more positive cases than the culture method.

Table 1. GenBank DNA BLAST sequence of *Blastocystis* in sheep (present study) compared to *Blastocystis* ST10 isolate CA (KC148207)

Isolate	GenBank ID	Maximal score	Total score	Query cover (%)	Identity (%)
1	MT895705	571	571	94	85.41
2	MT895706	No score	No score	No score	No score
3	MT895707	407	407	87	81.01
5B	MT895708	No score	No score	No score	No score
6	MT895709	No score	No score	No score	No score
7	MT895710	No score	No score	No score	No score
8	MT895711	540	540	94	84.20
9	MT895712	512	512	95	87.33
10	MT895713	No score	No score	No score	No score
12	MT895714	560	560	99	86.15
4	MT895715	968	968	97	97.03
5A	MT895716	1016	1016	97	99.11

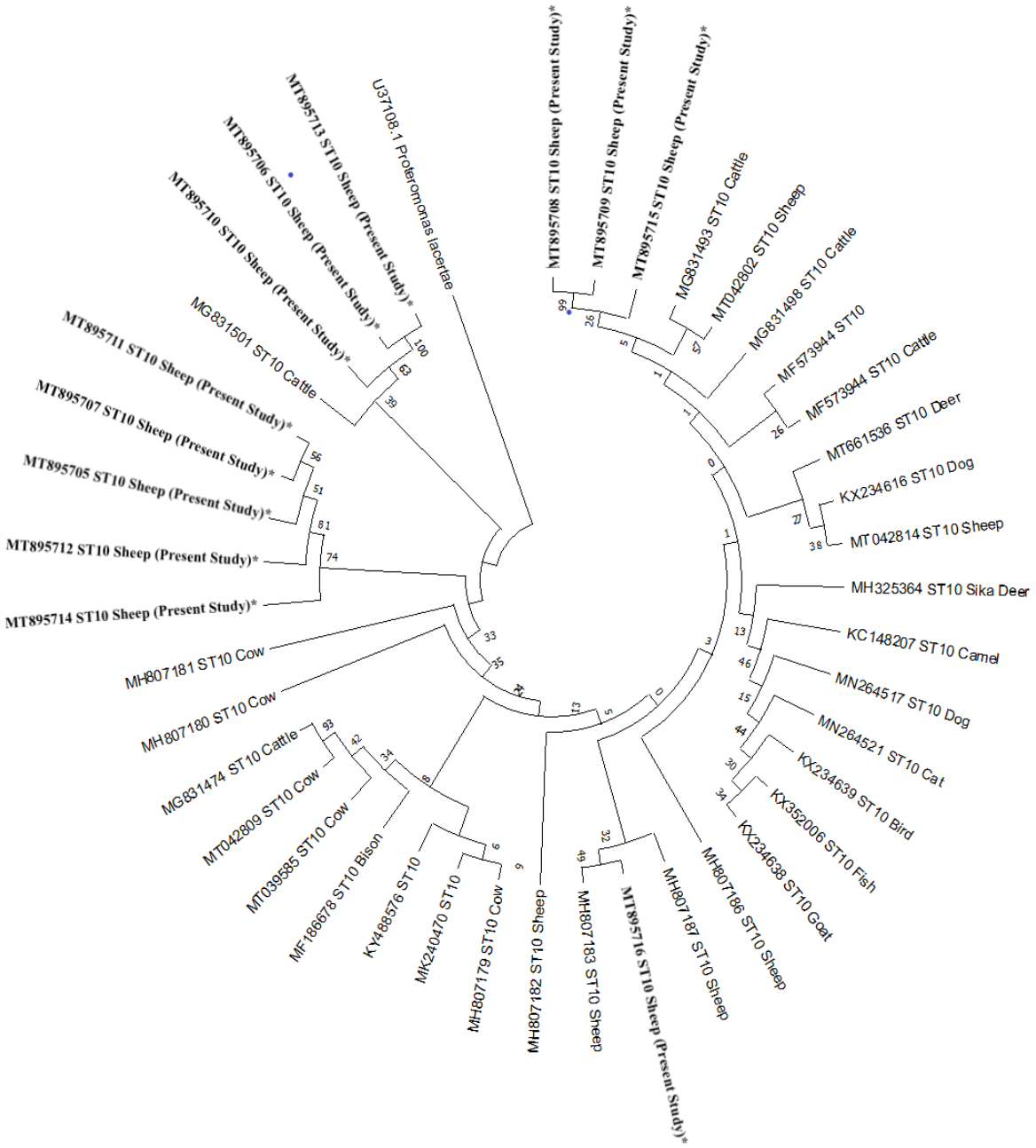


Fig. 1. Evolutionary relationships of *Blastocystis* SSU rRNA sequences inferred using the neighbour-joining method
* – Sequences from the present study, shown in bold

Table 2. Sensitivity and specificity of iodine, acid-fast stain, trichrome and culture techniques

		Polymerase chain reaction (gold standard)		Sensitivity	Specificity	Odds ratio	95% Confidence interval		P-value
		Positive (n = 5)	Negative (n = 60)				Lower	Upper	
Iodine	Positive	13.3 (2)	86.7 (13)	0.4	0.783	2.41	0.36	15.98	0.35
	Negative	6 (3)	94 (47)						
Acid-fast stain	Positive	16.7 (2)	83.3 (10)	0.4	0.833	3.33	0.49	22.59	0.19
	Negative	5.7 (3)	94.3 (50)						
Trichrome	Positive	**25 (4)**	75 (12)	0.8	0.800	16	1.63	156.5	**0.003**
	Negative	2 (1)	**98 (48)**						
Culture	Positive	**22.2 (4)**	77.8 (14)	0.8	0.767	13.14	1.35	127.4	**0.007**
	Negative	2.1 (1)	**97.9 (46)**						

Bolded numbers represent statistically significant results

Discussion

As in our previous study, all twelve sequenced sheep samples were confirmed to be the ST10 subtype (3). Some of the sequences obtained in this survey shared high identity with the *Blastocystis* spp. sequences in GenBank. Only allele 152 was detected in all of these cases. Similar ST10 alleles were detected in neighbouring Iran in dogs and cats (16). On the other hand, allele 43 of this subtype was detected in sheep and cows in a study in the Czech Republic, possibly evidencing intra-subtype variability in ST10 due to different geographical regions and diets (12).

Some *Blastocystis* subtypes have zoonotic potential; in a recent study Wang *et al.* (26) detected four subtypes in sheep (ST1, ST5, ST10 and ST14) with ST1, ST5 and ST14 identified in this animal species for the first time in Heilongjiang Province in northeastern China. *Blastocystis* ST10 was the only subtype found, with all isolates from the present study clustering in two sub-clades. None of the potentially zoonotic subtypes was detected. Thus, non-zoonotic subtypes seem to be more frequent in the UAE.

We have previously indicated the presence of two non-zoonotic subtypes (ST10 and ST14) in two sheep among individuals with mixed subtypes (3). Unlike our earlier study the present investigation did not find any mixed-subtype infections. The isolates in our previous study were cloned, allowing for the detection of mixed subtypes (3). The current report identified *Blastocystis* isolates using a fragment of the SSU-rDNA gene as a unique marker by PCR sequencing. This technical approach is not apt to identify co-infections with different STs in the same specimen because of the inherent nature of PCR, which preferentially amplifies the predominant subtype(s). This is a limitation of the investigation in respect of the variety of STs possible to detect per sample. A more accurate measure of the detection rate of particular subtypes, and therefore of potentially zoonotic types, in the Artiodactyla order in this country would be provided by screening large numbers of different member species.

The study also compared the efficacy of iodine stain, acid-fast stain, trichrome stain, *in vitro* culture and PCR for the detection of *Blastocystis* in 65 samples and indicated that *in vitro* culture and trichrome stain had comparable sensitivities, returning the same number of positive cases: 4/5 (80%) by culture and 4/5 (80%) by trichrome stain. Both tests detected positive cases that were false negative by iodine or modified acid-fast stain. Since the trichrome stain is rapid, provided a clear image of the organism and is less expensive than *in vitro* culture, it may be a better option to use in the examination of samples for *Blastocystis* spp. As it was in samples from other experimental groups, the vacuolar form was the most observed form in the samples from the sheep comprising this experimental group (1, 10, 15). The present study showed that when both microscopic techniques (trichrome and *in vitro* culture) were used together, the overall sensitivity and specificity of *Blastocystis* spp. detection was markedly improved (Table 2).

This study confirmed that trichrome staining has the highest sensitivity, is suitable for use in hospitals and clinics where *Blastocystis* testing is requested and is also more feasible to use in laboratories in developed and less developed countries because it is cheap, rapid, easy to perform and renders the definitive morphological details of the parasite clearly visible. Although comparable in sensitivity to the trichrome stain, the *in vitro* culture method is cumbersome, tedious and time-consuming, making it more suitable for use in research institutes. The results of this study concurred with what Mohammad *et al.* (15) reported on the relative utility of these techniques. Moreover, the finding of ST10 for the second time in artiodactyls corroborates previous studies which contended that the members of this order are natural hosts for these subtypes. No potentially zoonotic subtypes were detected in either this or our previous investigation. Further studies are required to ascertain the variety of subtypes present in farm animals and the zoonotic potential of the subtypes identified. Whether *Blastocystis* ST circulation is also linked to geographical areas remains to be elucidated. Therefore, sampling more hosts for *Blastocystis* from various geographical regions and analysing alleles within identified subtypes may offer an answer.

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