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Intestinal Microbial Eukaryotes at the Human, Animal and Environment Interface in Rural Iraq

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Abstract

Intestinal microbial eukaryotic parasites represent a significant public and veterinary health burden, especially in low- and middle-income countries, yet their transmission dynamics at the human–animal–environment interface remain poorly characterized in certain countries. This study investigated the prevalence and genetic diversity of key microbial eukaryotes, including *Cryptosporidium* spp., *Giardia duodenalis*, *Blastocystis* spp., *Entamoeba histolytica*, and *Enterocytozoon bieneusi*, in a rural village in Iraq. Samples collected from humans (n = 50), livestock (sheep and goats, n = 50), water (n = 20), and soil (n = 20) were analysed using microscopy and molecular methods (qPCR and nested PCR). *Blastocystis* spp. (78% animals, 16% humans, 45% soil, 5% water) and *Cryptosporidium* spp. (26% animals, 12% humans, 5% soil, 15% water) were the most frequently found microeukaryotes using either microscopy and/or molecular detection. Molecular methods identified *Cryptosporidium parvum* in humans and sheep, hinting at zoonotic transmission potential. *Enterocytozoon bieneusi* and *Giardia* were also found. *Cryptosporidium ubiquitum* and *E. bieneusi* genotypes BEB6 and COS-I, respectively, were detected exclusively in sheep, suggesting roles as potential reservoirs. *Blastocystis* ST1 was detected in humans, while ST4 and ST10 occurred in sheep. Notably, molecular detection rates of *Blastocystis* were much lower than those of microscopy. *Entamoeba histolytica* was not detected. The detection of the same organisms in humans, animals and the environment suggest zoonotic and environmental transmission pathways, which warrant further investigation using the One Health approach.

Keywords: infectious diseases; zoonotic transmission; environmental contamination; protozoa; one health

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1. Introduction

Intestinal microbial eukaryotic (protozoan) parasites are globally significant pathogens, causing considerable morbidity in humans and livestock, particularly in low- and middle-income countries (LMICs) [1]. These organisms, including *Cryptosporidium* spp., *Giardia duodenalis*, *Entamoeba histolytica*, and *Enterocytozoon bieneusi*, contribute substantially to

diarrheal disease, malnutrition, and economic losses in affected communities [2,3]. Beyond their clinical and veterinary impact, these parasites also pose an economic burden due to livestock losses and reduced productivity [4,5].

Cryptosporidium spp. and *Giardia duodenalis* are known zoonotic agents, frequently transmitted between humans and animals via the faecal–oral route, often through contaminated water or food [6]. *Entamoeba histolytica* is one of the few invasive amoebae and remains a major cause of dysentery worldwide [7]. Meanwhile, *Blastocystis*, a genetically diverse organism whose pathogenicity remains controversial, has been gained increasing attention in recent years due to its high prevalence in both healthy and symptomatic individuals, and potential associations with a healthy gut microbiome [8]. Finally, *E. bieneusi* is an emerging microsporidian parasite found in various hosts, including humans, and is particularly problematic in immuno-compromised individuals [9].

While these eukaryotic microbes have been studied globally, investigations in Iraq remain limited, with most relying on microscopy-based methods [10]. Studies based on molecular methods are scarce, particularly for *Blastocystis* and *E. bieneusi*. Prior reports have documented these microbial eukaryotes in humans and animals in Iraq, but knowledge on their epidemiology and diversity remains limited [11,12].

To bridge these gaps, the current study employed both microscopic and molecular diagnostic methods to investigate the occurrence, genetic diversity, environmental contamination and potential zoonotic transmission of intestinal eukaryotic parasites between humans and small ruminants in a rural village in Iraq.

2. Materials and Methods

2.1. Ethics

The ethics committee (ACUC) at the University of Baghdad approved this study on 16 March 2022 under the project "One Health Approach—Iraq" (No.D.A.672).

2.2. Study Area

The study took place at Alissma village, which is located in the northeastern part of Iraq, near the border with Iran (Figure 1). Approximately 1000 individuals reside in this village. The nearest city (Mandli) is about 30 km away. The village is in a semi-desert area with nearly no tree cover, devoid of ponds and lakes. Oil River runs through the village in the winter, but its water is unfit for drinking and is used solely for irrigation purposes. Groundwater wells and underground well water are typically used for drinking after filtration and purification. The village latrines are basic and located outside of the household.

2.3. Sample Collection

The methodology used herein is graphically summarized (Figure 2). A total of 140 samples were collected between February 2022 and July 2022, each one comprising a singular sample for each participant in the study for humans and animals. Fifty of these were stool samples collected from humans. The participants did not have diarrhoea or other gastrointestinal symptoms and were not taking antibiotics and antiparasitic treatment at the time of sampling, with the exception of four participants, who had been diagnosed by a physician as having colitis. During sample collection, questionnaires were administered, and information about age, gender, type of drinking water, antibiotics, pets, career, and chronic disease was recorded.

Moreover, 50 samples were collected from animals, namely from goats (n = 15) and sheep (n = 35). These animals grazed near the village and drank unfiltered well water.

None of the animals had diarrhoea at the time, and the animal faecal samples were collected directly from the rectal ampulla.

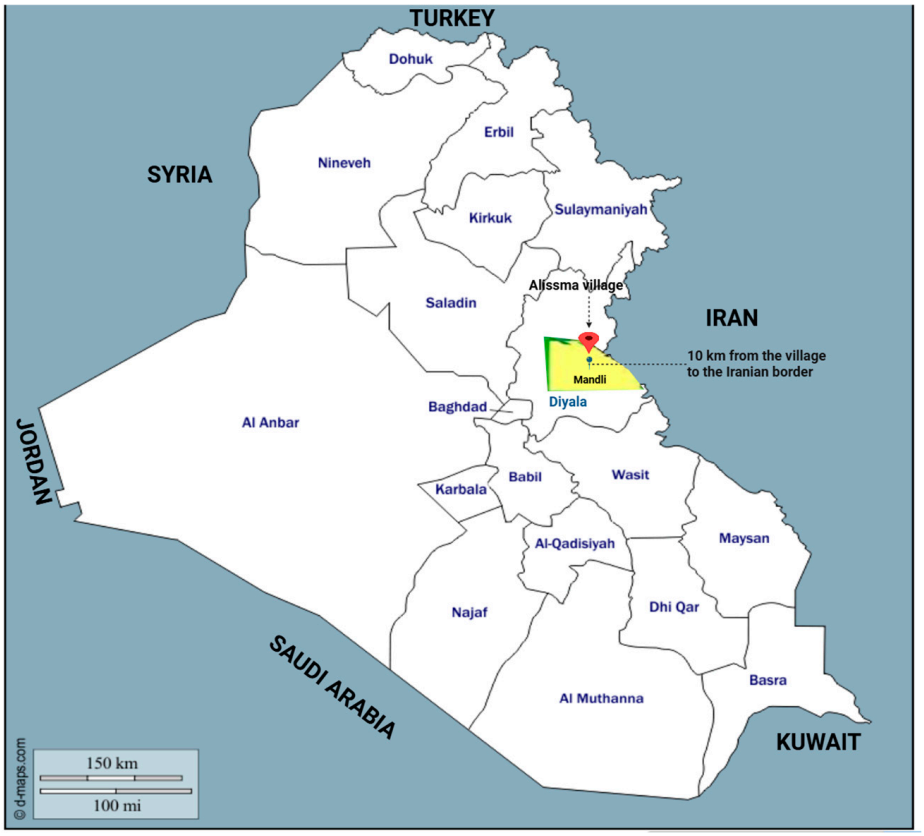

Figure 1. Map of Iraq and neighbouring countries. The study area is located in the eastern part of Iraq, within the Diyala Province. The village location is marked with the red pin and lies about 10 km from the Iranian border.

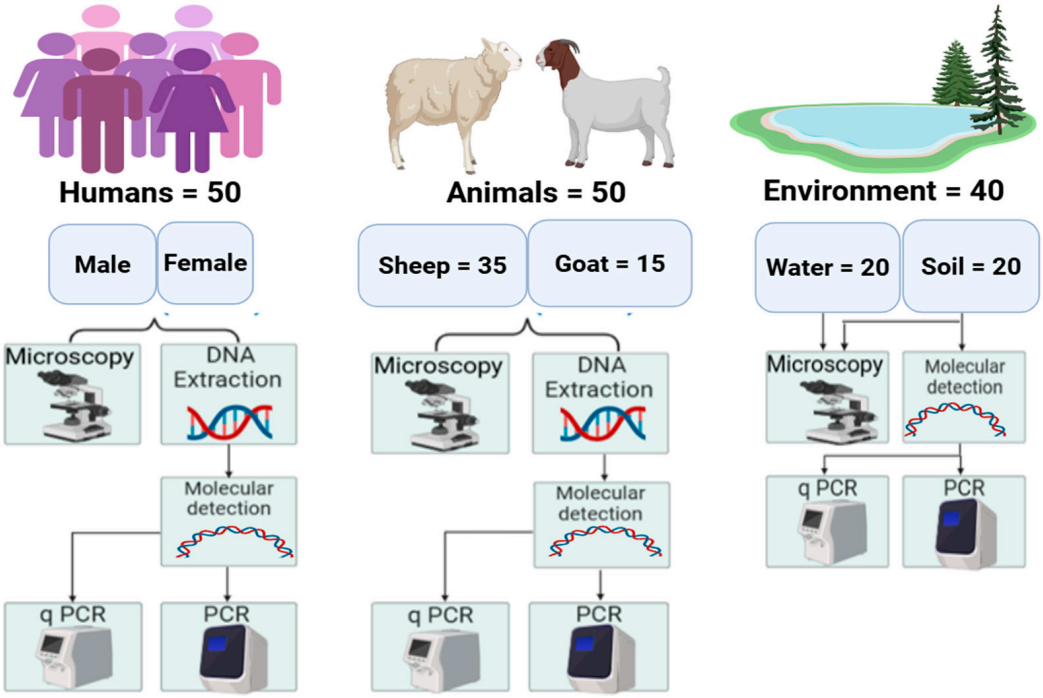

Figure 2. Methodology summary for the samples and approaches used in the study.

Additionally, 20 water samples were collected from sources used by both humans and animals in 5 mL plastic tubes. These included filtered drinking water (n = 5), tap water (n = 5), well water (n = 5), and river water (n = 5). Twenty soil samples were also collected. These included home garden soil (n = 5), field soil (n = 5), animal grazing field soil (n = 5), and river edge soil (n = 5). Five grams of soil were collected in plastic tubes after scraping five centimetres of soil surface.

2.4. Microscopic Examination

All samples were examined microscopically using wet mount smears. Iodine staining was used for the human and animal faecal samples. The flotation method was also used in this study as follows: 5 g of stool was diluted in 20 mL of distilled water and filtered using clean gauze to remove the faecal debris. 5 mL of the mixture was combined with 10 mL of saturated NaCl (40 g of salt in 100 mL of water), and placed into a 15 mL centrifuge tube until a convex meniscus was obtained, in which the liquid curved upwards. The meniscus was then covered with a coverslip. After standing for 15 to 20 min to allow lighter parasitic cysts or oocysts to float, the coverslip was gently removed and examined using a microscope (Olympus Corporation, Tokyo, Japan) with the 10×, 40×, and 100× objective lenses [13].

2.5. DNA Extraction and Molecular Detection

For DNA extraction, 200 mg of each faecal and soil sample was used using the PureLink™ Microbiome Genomic DNA Purification Kit (Invitrogen, Thermo Fisher Scientific, Waltham, CA, USA) according to the manufacturer's protocol. The extracted DNA was used for qPCR and nested PCR (Table 1).

Herein, we screened samples for *Blastocystis* sp., *Cryptosporidium* spp., *Entamoeba histolytica*, *Giardia duodenalis* and *E. bieneusi*. The SSU rRNA gene was used to identify *Cryptosporidium* spp. and *gp60* was used for *Cryptosporidium parvum* subtyping. For *Blastocystis* spp., SSU rRNA was used; beta-giardin (*bg*) and triosephosphate isomerase (*tpi*) for *Giardia*; and internal transcribed spacer (*ITS*) for *E. bieneusi*. A positive and negative control were included in each PCR run. The reaction conditions differed according to parasite and genetic marker (Table 1). Probe-based qPCR was used to amplify a fragment of the SSU rRNA of *G. duodenalis* and *E. histolytica*. The positive PCR products were purified using a Thermo Scientific GeneJET Gel Extraction Kit (Thermo Fisher Scientific, Waltham, CA, USA) according to the manufacturer's protocol. The methodology used in this study was undertaken according to previous investigations [14,15].

2.6. Sequencing Analysis

The purified positive PCR amplicons were sequenced unidirectionally at Eurofins genomics (Cologne, Germany). The obtained raw reads were trimmed manually at both ends to remove ambiguous bases using SnapGeneViewer v.6.0.2. The acquired sequences were used as queries to perform BLAST version 2.14.1 against the NCBI database. The newly generated nucleotide sequences were submitted to GenBank under accession numbers PV521084, PV521085, PV521086, PV521087, PV504621, PV504622, PV504623, and PV504624.

Table 1. Parasites, genes, primer sequences, amplification procedures, and the expected fragment size (bp), which were used in the study.

Parasite of Interest	Target Gene	Detection Method	Primer Sequences (5′-3′)	Amplification Condition	Amplicon Size (bp)	References
Cryptosporidium spp.	SSU	nPCR	CRY-SSU-F1: GATTAAGCCATGCATGTCTAA CRY-SSU-R1: CTTGAATACTCCAGCATGGAA	95 °C: 2 min; 24 cycles: 94 °C: 50 s 53 °C: 50 s 72 °C: 1 min 72 °C: 10 min	723 bp	[14,16]
			CRY-SSU- F2: CAGTTATAGTTTACTTGATAATC CRY-SSU- R2: GAAAATTAGAGT-GCTTAAAGCAGG	94 °C: 2 min; 30 cycles: 94 °C: 50 s 56 °C: 30 s 72 °C: 1 min 72 °C: 10 min	631 bp	
	GP60	nPCR	F1: AL3531: ATAGTCTCCGCTGTATTC R1: AL3535: GCAAGGAACGATGTATCT	94 °C: 3 min; 35 cycles: 94 °C: 45 s 50 °C: 45 s 72 °C: 1 min 72 °C: 7 min	1000 bp	[16,17]
			F2 AL3532: TCCGCTGTATTCTCAGCC R2 AL3534: GCAGAGGAACCAGCATC	94 °C: 3 min; 35 cycles: 94 °C: 45 s 50 °C: 45 s 72 °C: 1 min 72 °C: 7 min	850 bp	
Giardia duodenalis	SSU	qPCR	GIARDIA-80-F: GACGGCTCAGGACAACGGTT GIARDIA-127-R: TTGCCAGCGGTGTCCG Probe: FAM: CCCGCGGCGGTCCCTGCTAG	95 °C: 2 min; 50 cycles: 95 °C: 15 s 58 °C: 30 s 72 °C: 30 s	62 bp	[14,18,19]
	Bg beta-giardin	nPCR	F1(G7F): AAGCCCGACCTCACC-CGCAGTGC	94 °C: 5 min; 35 cycles: 94 °C: 30 s 66 °C: 30 s 72 °C: 1 min 72 °C: 7 min	753 bp	[20]
			F2(G376): CATAAGGACGC-CATCGCGGCTCTGAGG R (G759R): GAGGCCGCCCTG-GATCTTCGAGACGAC	94 °C: 3 min; 30 cycles: 94 °C: 30 s 65 °C: 15 s 72 °C: 30 s 72 °C: 7 min	292 bp	
	Tpi triosephos-phate isomerase	nPCR	Tpi_AL3543_F1: AAAT/IDEOXYL/ATGCCTGGTCG Tpi_AL3546_R1: CAAAC-CTT/IDEOXYL/TCCGCAAACC	94 °C: 3 min; 35 cycles: 94 °C: 45 s, 50 °C: 35 s 72 °C: 30 s 72 °C: 10 min	530 bp	[21]
			Tpi_AL3544_F2: CC-CTTGATCGG/IDEXYL/GGTAACTT Tpi_AL3545_R2: GTGGCCAC-CAC/IDEOXYL/CCCGTGCC	94 °C: 3 min; 35 cycles: 94 °C: 35 s 47 °C: 35 s 72 °C: 30 s 72 °C: 10 min	332 bp	
Blastocystis	*SSU*	nPCR	RD3—F1: GGGATCCTGA TCCTTCCGCAGGTTCACCTAC RD5—R1: GGAAGC TTATCTGGTTGATCCTGCCAGTA	94 °C: 3 min; 35 cycles: 94 °C: 1 min 60 °C: 1 min 72 °C: 100 s 72 °C: 7 min.	600 bp	[15,22]
			BsRD5F—F2: ATCTGGTTGATCCTGCCAGT BhRDr—R2: GAGCTTTTTAACTGCAACAACG	94 °C: 3 min; 35 cycle: 94 °C: 1 min 60 °C: 1 min 72 °C: 100 s 72 °C: 10 min	650 bp	
Entamoeba histolytica	SSU	qPCR	End-239F: ATTGTCGTGGCATCCTAACTCA End-88R: GCGGACGGCTCATTATAACA Probe: VIC-TCATTGAATGAATTGGCCATTT′-NFQ	95 °C: 2 min; 50 cycles: 95 °C: 15 s 58 °C: 30 s 72 °C: 30 s	172 bp	[19]
Enterocytozoonbieneusi	ITS	nPCR	EBITS3: GGTCATAGGGATGAAGAG EBITS4: TTCGAGTTCTTTCGCGCTC	95 °C 5 min; 35 Cycles: 94 °C: 40 s 53 °C: 45 s 72 °C: 45 s 72 °C: 4 min	435 bp	[23,24]
			EBITS1: GCTCTGAATATCTATGGCT EBITS2.4: ATCGCCGACGGATCCAAGTG	95 °C 5 min; 30 Cycles: 94 °C: 35 s 55 °C: 40 s 72 °C: 40 s 72 °C: 5 min	390 bp	

3. Results

3.1. Light Microscopy

The stool and environmental samples were initially examined using light microscopy. A total of 12% (6/50) of the human samples were positive for *Cryptosporidium* spp., 16% (8/50) for *Blastocystis* sp., and 10% (5/50) for *G. duodenalis* (Table 2, Figure 3). Moreover, 26% (13/50) of the animal samples were positive for *Cryptosporidium* spp., 78% (39/50) for *Blastocystis* sp. [4% (8/50) of the *Blastocystis* sp. were from goats and 70% (35/50) were from sheep] and 8% (4/50) for *G. duodenalis* from sheep. Regarding the soil samples, 5% (1/20) tested positive for *Cryptosporidium* spp., and 45% (9/20) for *Blastocystis* sp. Regarding the water samples, 15% (3/20) were positive for *Cryptosporidium* spp. and 5% (1/20) for *Blastocystis* sp.

Table 2. Number and percentage of positive samples in the microscopic examination for all sources and parasites used in the study.

Type of Source	Humans		Animals		Soil		Water		Total	
Name of Parasites	**Number of Positive**	**%**	**Number of Positive**	**%**	**Number of Positive**	**%**	**Number of Positive**	**%**	**Number of Positive**	**%**
Cryptosporidium spp.	6	12%	13	26%	1	5%	3	15%	23	19.16%
Blastocystis sp.	8	16%	39 Goat: 8	78% 4%	9	45%	1	5%	57	47.5%
Giardia duodenalis	5	10%	4	8%	-	-	-	-	9	7.5%
Total	19		56		10		4		89	63.57%

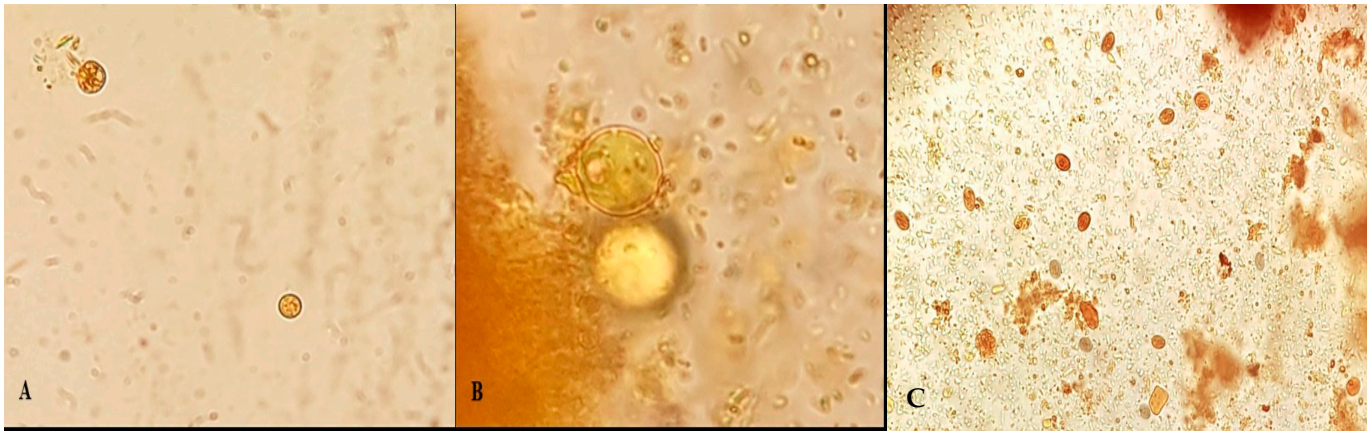

Figure 3. *Cryptosporidium* (**A**), *Blastocystis* (**B**), and *Giardia duodenalis* (**C**) from faecal samples.

Giardia duodenalis, *Enterocytozoon* spp., and *Entamoeba histolytica* were not detected by microscopy in any of the water or soil samples.

3.2. Molecular Detection

Based on qPCR analysis targeting a fragment of the SSU rRNA gene of *G. duodenalis*, 30% (15/50) of human samples were positive, 14% (7/50) of animals (all positives were from sheep), and 5% (1/20) of soil samples. Nested PCR of longer *bg* and *tpi* gene fragments amplified one of the qPCR positive sheep samples, which belonged to assemblage A.

Using nested PCR, one human sample was found to be positive for C. *parvum* (*gp60* gene) and another for *Blastocystis* ST1. In animals, *Cryptosporidium* spp. was detected in 4% (2/50); one sheep sample was positive for *Cryptosporidium ubiquitum* (SSU rRNA gene) and another for *Cryptosporidium parvum* (SSU rRNA, *gp60* genes). We were not able to subtype the obtained *gp60* sequences due to their short length. A proportion of 8% (4/50) of animals were found to be positive for *Blastocystis* (SSU rRNA gene), one animal

with ST4, and three animals with ST10. Finally, 8% (4/50) of animals were positive for *E. bieneusi*, with three belonging to *E. bieneusi* genotype *BEB6* and one to genotype *COS-I*.

All samples were negative for *Entamoeba histolytica*.

Mixed infections were identified in 8% (4/50) of human samples, when combining microscopy and molecular detection methods (Figure 4). This included two samples that tested positive for *Giardia duodenalis* by molecular detection and *Blastocystis* sp. by microscopy. One sample was positive for both *Cryptosporidium* spp. and *G. duodenalis* by molecular detection, while another sample was positive for *Cryptosporidium* spp., *G. duodenalis*, and *Blastocystis* sp., all detected by molecular methods.

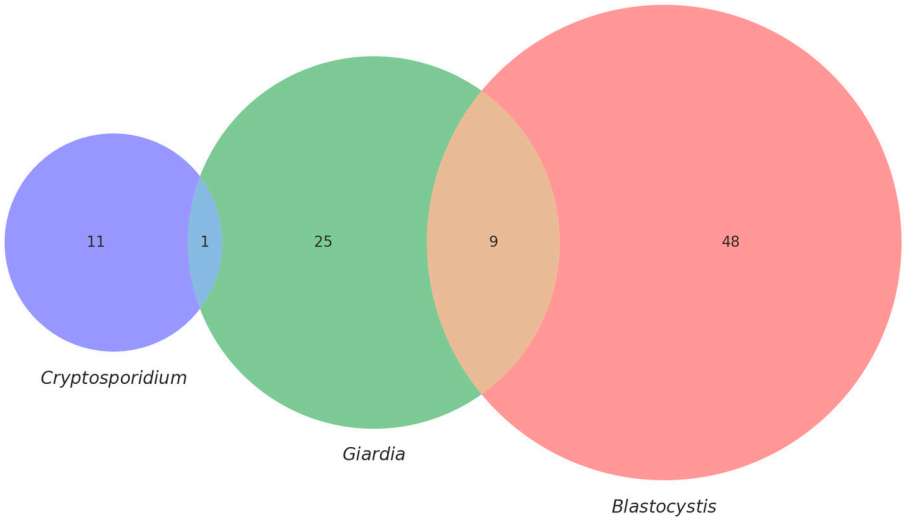

Figure 4. Venn diagram showing the overlap of intestinal protozoan infections among 50 human stool samples. *Blastocystis* was identified by microscopy, *Giardia duodenalis* by qPCR, and *Cryptosporidium* spp. by nested PCR. Only a limited number of co-infections were detected, and no triple infections were observed.

Regarding animal samples, 34% (17/50) had co-infections. Twelve animals were infected with *Cryptosporidium* spp. and *Blastocystis* spp. by microscopy-based detection, five with *G. duodenalis* and *Blastocystis* spp., this included three samples found positive for *G. duodenalis* and *Blastocystis* spp. microscopically, one sample which tested positive for both parasites molecularly and, lastly, one animal that tested positive for mixed infection, both microscopically and molecularly. Moreover, two animal samples tested positive for *Blastocystis* spp. by microscopy and *E. bieneusi* by PCR (Table 2).

4. Discussion

This study provides insights into the occurrence and genetic diversity of intestinal protozoan parasites in a rural village in Iraq. It highlights the environmental presence and hence zoonotic potential of *Cryptosporidium* spp., *Giardia duodenalis*, *Blastocystis* spp., and *Enterocytozoon bieneusi*. Small ruminants are economically important animals in Iraq and are reared primarily on small- and medium-scale herds. Despite this, reports on their intestinal organisms are relatively sparse [25,26]. Herein, the occurrence rate of *Cryptosporidium* spp. in animals is lower than in previous studies, likely due to the methodologies used or the population examined. The occurrence in sheep was much higher than in goats, matching previous findings in the country [25]. This can be attributed to the free-range nature of goats as opposed to sheep [27]. The detection of *Cryptosporidium parvum* in both humans and sheep suggests zoonotic transmission within this community. Meanwhile, detection of *Cryptosporidium ubiquitum* and *E. bieneusi* in sheep hints at livestock as potential reservoirs for environmental contamination and human infection.

The high prevalence of *Blastocystis* in both animals and humans align with previous reports [28,29]. There was a notable difference between the detection methods used, with microscopy-based detection identifying many more samples as positive. This is as opposed to molecular methods, with which it was not possible to amplify the corresponding gene fragment. The presence of co-infections, many of which were confirmed microscopically, could be a confounding factor here. While it is not possible to compare these findings to other studies in Iraq, previous molecular studies in neighbouring Iran showed variable *Blastocystis* occurrence rates, with one as low 5% [30,31]. As this is, to the best of our knowledge, the first molecular detection study of *Blastocystis* in the country, further studies are needed to shed light on the organism's epidemiology. This is among the first reports of ST4 in sheep in this region, offering new insights into subtype distribution in the Middle East. The detection of ST4 in sheep is of interest, as this subtype is typically linked to rodents [32,33]. A significant component of wildlife, wild rodents serve as reservoirs for numerous etiological agents, including bacteria, viruses, and microbial eukaryotes like *Blastocystis* [33,34]. It is likely that wild rodent may spread *Blastocystis* to livestock, and water sources. These results are important in terms of emphasizing the need to conduct studies on different hosts, including wildlife, to gain knowledge on interspecies transmission dynamics of ST4.

Entamoeba histolytica was not detected in any sample. Nonetheless, *Entamoeba* spp. has been detected in both microscopic and molecular-based investigations in ruminants in the country [35,36]. This discrepancy may reflect the challenging field conditions that may compromise the detection of *E. histolytica*.

Given the scarcity of molecular epidemiological studies in Iraq, direct comparisons to previous work are limited. Nonetheless, our findings are in line with some earlier reports, such as the 34% *Giardia* detection rate by microscopy in humans observed by Al-Hasnawy and Idan [37].

While *E. bieneusi* infections have been reported in birds in Iraq [38], this study contributes new evidence of its presence in livestock, expanding the known host range in the region.

Several limitations should be acknowledged. One major challenge was preserving and storing samples in a remote field setting, where access to cold-chain infrastructure is limited. Inadequate preservation may have reduced the sensitivity of both microscopy and molecular assays. This could explain certain species' absence or low detection rates in specific sample types. Additionally, although PCR-based methods were employed, only a subset of positive samples yielded high-quality sequences, limiting the depth of genetic characterization. Environmental samples, in particular, may have contained PCR inhibitors or low DNA concentrations, affecting amplification success. Our findings underline the need for standardized operating procedures for parasite sampling, preservation, and analysis in resource-limited settings. The adoption of field-friendly preservatives compatible with molecular diagnostics, along with optimized DNA extraction protocols for complex environmental matrices, would enhance data quality and comparability [39]. Another limitation of this study is its cross-sectional design, which provides only a single time point and does not capture seasonal trends or temporal changes in infection dynamics. Longitudinal studies incorporating repeated sampling from hosts and environments are needed to clarify transmission pathways, sources of reinfection, and possible seasonal patterns [40].

Despite including asymptomatic individuals and animals, future research should aim to expand the number of sampling sites, include larger sample sizes, and integrate clinical and immunological data. Such approaches would enable a more accurate assessment of the pathogenic potential and health impacts of these parasites.

Looking ahead, the high prevalence of *Blastocystis*, a common yet enigmatic member of the gut eukaryome, presents a valuable opportunity to study its interaction with the bacterial microbiota and host immune system. Future studies should consider using 16S rRNA sequencing or shotgun metagenomics to explore microbe–parasite interactions, particularly in communities with frequent co-infections [41]. These approaches could inform new strategies for diagnostics, surveillance, and intervention.

5. Conclusions

This study enriches the limited molecular data on intestinal protists (including parasites) in Iraq, offering a comprehensive view of their occurrence in humans, livestock, and the environment. The findings emphasize the importance of enhanced sampling protocols, environmental monitoring, and capacity building to improve parasite detection and control in rural, resource-limited settings.

Author Contributions: Conceptualization, A.D.T., M.M.S. and D.A.K.; methodology, Y.M.S.A.-A. and S.M.; software, Y.M.S.A.-A. and E.G.; validation, Y.M.S.A.-A., E.A.Ö., E.G. and A.D.T.; formal analysis, Y.M.S.A.-A., and E.G.; investigation, Y.M.S.A.-A., E.G. and A.D.T.; resources, A.D.T.; data curation, Y.M.S.A.-A.; writing—original draft preparation, Y.M.S.A.-A.; writing—review and editing, Y.M.S.A.-A., S.M., A.D.T., E.G., M.M.S. and D.A.K.; visualization, Y.M.S.A.-A.; supervision, A.D.T., E.G., M.M.S. and D.A.K. and E.A.Ö.; project administration, A.D.T.; funding acquisition, A.D.T. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the University of Baghdad's Ethical Committee (protocol code No. D.A. 672 and 16 March 2022." for studies involving humans. The animal study protocol was approved by the Institutional Review Board (or ethics committee) of the University of Baghdad (protocol code No. D.A. 672 and 16 March 2022) for studies involving animals.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding authors.

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Conflicts of Interest: The authors declare no conflicts of interest.

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