

Environmental and behavioral risk factors for *Cryptosporidium* infection: A molecularly confirmed study

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Abstract. *Cryptosporidium* is a leading cause of diarrhea worldwide, transmitted via contaminated water, food and contact with infected animals. The aim of the present study was to identify environmental and behavioral risk factors for *Cryptosporidium* infection. For this purpose, the present cross-sectional study was performed on 420 participants. *Cryptosporidium* oocysts were detected by modified Ziehl-Neelsen staining and confirmed by nested PCR. The study subjects were divided into the infected (296) and uninfected (124) groups. Data on residence, animal contact, drinking water source [reverse osmosis (RO) vs. non-RO] and education status were collected. Associations were assessed using the Chi-squared test and multivariable logistic regression analysis. The results revealed that the prevalence of *Cryptosporidium* infection was 70.5%. Independent factors associated with infection were animal contact [adjusted odds ratio (AOR)=2.98; 95% confidence interval (CI), 1.75-5.02; $P<0.001$] and non-RO water use (AOR=2.05; 95% CI, 1.10-3.82; $P=0.024$). An increasing age exhibited a non-significant trend (AOR=1.18 per category; 95% CI, 0.97-1.42; $P=0.097$). Residence, sex and education were not significant. The Hosmer-Lemeshow test indicated adequate model fit ($P=0.208$). On the whole, the present study demonstrates that animal contact and non-RO water use are independently associated with *Cryptosporidium* infection. Improving water quality and hygiene practices following animal contact may help reduce disease transmission.

Introduction

Cryptosporidium spp. are apicomplexan protozoan parasites that cause acute gastroenteritis and are a major cause of

childhood morbidity and mortality in low-resource settings (1). According to findings from the Global Enteric Multicenter Study (GEMS), *Cryptosporidium* is the second most common cause of moderate-to-severe diarrhea among infants and young children in Sub-Saharan Africa and South Asia, responsible for ~202,000 deaths each year (2). The pathogen is transmitted by the fecal-oral route through ingestion of contaminated water or food, or through direct contact with infected animals or humans (3). A characteristic feature of *Cryptosporidium* species is their exceptional environmental resistance due to the presence of oocysts with high resistance to chlorine disinfection. According to the World Health Organization (WHO), the log reduction value (LRV) achieved by free chlorine against *Cryptosporidium* oocysts is only 0-1, which renders conventional chlorination ineffective against this pathogen (4). Membrane filtration technologies, on the other hand, are highly effective against *Cryptosporidium* oocysts, capable of achieving LRVs of ≥ 6 (4). Chlorine resistance has been responsible for several waterborne outbreaks, notably a major one in Milwaukee (WI, USA) in 1993 involving >400,000 individuals (5).

Another critical aspect related to *Cryptosporidium* infections is the zoonotic transmission of the parasite. *Cryptosporidium parvum* is a zoonotic species commonly found in cattle. Contact with infected animals is considered a well-established risk factor for infection (5). Several studies published in recent years have demonstrated the circulation of zoonotic *Cryptosporidium* species in rural agricultural communities among both animals and humans (6). For instance, a survey conducted in Cameroon reported a high prevalence (41.5%) of *Cryptosporidium* spp. in calves, with age and water source identified as important contributing factors (7). In Iraq, particularly in Thi-Qar Province, a high prevalence of *Cryptosporidium* infection has been reported among immunocompromised populations, reaching 54.0% in patients with cancer (8). Although substantial evidence highlights the global importance of *Cryptosporidium* spp. as human pathogens, epidemiological studies have predominantly focused on pediatric populations, particularly children <5 years of age, who account for the greatest burden of cryptosporidiosis (1,2). Risk factors associated with *Cryptosporidium* infections among adolescents and adults remain relatively understudied. Therefore, the present study aimed to identify environmental and behavioral risk factors associated with *Cryptosporidium* infection in a community setting.

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Abbreviations: PCR, polymerase chain reaction; OR, odds ratio; CI, confidence interval; SSU rRNA, small subunit ribosomal RNA

Key words: *Cryptosporidium*, molecular, nested PCR, risk factors, Ziehl-Neelsen stain

Subjects and methods

Study design and population. A cross-sectional study was conducted among residents of Thi-Qar Province, Iraq. Participant enrollment, questionnaire interviews, stool sample collection, microscopic examination and molecular confirmation were conducted between January 28 and April 30, 2026 following approval by the Thi-Qar Health Directorate. Data verification and coding were performed progressively as participant recruitment and laboratory results became available. Following completion of participant enrollment and laboratory investigations on April 30, 2026, final data validation, statistical analyses, interpretation of the findings and manuscript preparation were completed during early May, 2026. A total of 420 participants presenting with gastrointestinal symptoms and attending primary healthcare centers were enrolled. Participants were classified into two groups according to the presence of *Cryptosporidium* infection in their stool samples, as determined by modified Ziehl-Neelsen staining and nested polymerase chain reaction (PCR): *Cryptosporidium*-positive cases (n=296) and *Cryptosporidium*-negative controls (n=124).

Ethics approval. The study protocol was approved by the Research Ethics Committee of Thi-Qar Health Directorate, Ministry of Health (Approval no. 2026/27, approved on January 27, 2026), following the official institutional authorization issued by the College of Science, University of Thi-Qar (Reference no. 97, dated January 20, 2026), which supported the ethical review and approval process by the Health Directorate. Written informed consent was obtained from all adult participants and from the parents or legal guardians of minors before enrollment. Participant recruitment, questionnaire interviews, and stool sample collection commenced only after all required institutional and ethical approvals had been obtained.

Data collection. Data were collected through direct face-to-face interviews using a structured questionnaire between January 28 and April 30, 2026. Questionnaire interviews and stool sample collection were performed during the same participant visit, thereby ensuring direct linkage between the demographic and epidemiological information of the participants, and their corresponding laboratory findings. Information obtained included age, sex, education level (illiterate/educated), residence (rural/urban), the presence of domestic animals (animal contact: Yes/no) and the source of drinking water. Water sources were categorized as verified reverse osmosis (RO)-treated water or non-RO water. Commercial bottled water and filtered tap water were grouped together as information regarding RO treatment was not available for all products, and their level of protection against *Cryptosporidium* oocysts could not be assumed to be equivalent to that provided by verified RO-treated water.

Stool sample collection and microscopic examination. Fresh stool samples were collected in sterile wide-mouthed containers between January 28 and April 30, 2026 during the same visit in which participants completed the study questionnaire.

Thin fecal smears were prepared on clean glass slides by mixing a small amount of stool with a few drops of water and allowed to air dry. Smears were stained using a modified Ziehl-Neelsen stain kit (REF BS0243250; Bio Research for Medical Diagnostics). Slides were covered with carbol fuchsin for 5 min with gentle heating until steam appeared, rinsed with tap water, decolorized with acid alcohol for 10-15 sec, rinsed again with water, counterstained with methylene blue for 1 min, washed with water, and air dried. Slides were examined using an light microscope (Olympus Corporation) at x40 magnification and subsequently under 100X oil immersion objective. *Cryptosporidium* oocysts appeared as bright pink to red spherical bodies (4-6 μ m) against a blue-green background (9).

Nested PCR was performed only on microscopy-positive samples to confirm the presence of *Cryptosporidium* DNA (this was a limitation to the present study, as discussed below).

DNA extraction and nested PCR. Genomic DNA was extracted from 200 mg stool samples using the Presto™ Stool DNA Extraction kit (cat. no. STLD100; Geneaid Biotech Ltd.). Nested PCR targeting the SSU rRNA gene was performed using outer primers (Crypto Outer F/R, 608 bp) and inner primers (Crypto Inner F/R, 500 bp) based on the reference sequence GenBank accession number LC844811.1. Reactions were performed using GoTaq® Green Master Mix (Promega Corporation) in a T100 Thermal Cycler (Bio-Rad Laboratories, Inc.). The cycling conditions consisted of an initial denaturation 95°C/5 min; 35 cycles of 95°C/30 sec, annealing 58°C (first round) or 60°C (second round)/30 sec, extension 72°C/60 sec (first) or 45 sec (nested); final extension 72°C/5 min. PCR products were analyzed on 2% agarose gel, stained with ethidium bromide and visualized under ultraviolet illumination.

A representative subset of nested PCR-positive amplicons was submitted to Macrogen Inc. (Seoul, Korea) for bidirectional Sanger sequencing to confirm species identification. The obtained nucleotide sequences were compared with reference sequences available in the NCBI GenBank database using the BLAST program. Multiple sequence alignment was performed using ClustalW implemented in MEGA version 11.0.13, and phylogenetic associations were inferred using the UPGMA method. The representative sequences were deposited in the NCBI GenBank database under accession nos. PZ245405, PZ245406, PZ245407, PZ245408, PZ245409, PZ245410, PZ245411, PZ245412 and PZ245413.

Statistical analysis. Data were analyzed using SPSS software version 26. Categorical variables are summarized as frequencies and percentages. Associations between variables were evaluated using Pearson's Chi-squared test. Independent predictors of *Cryptosporidium* infection were identified using multivariable logistic regression analysis, including animal contact (yes/no), drinking water source (non-RO vs. RO), age category (1-5), residence (rural vs. urban), sex (female vs. male) and education level (educated vs. illiterate). Adjusted odds ratios (AORs) and their corresponding 95% confidence intervals (CIs) were calculated. Model fit was assessed using the Hosmer-Lemeshow goodness-of-fit test, with a value of $P > 0.05$ indicating adequate model fit. In addition, a P-value

Table I. Sociodemographic characteristics and potential risk factors of the study participants (n=420).

Variable	Controls (n=124) (%)	Cases (n=296) (%)	Total (n=420) (%)	P-value ^a
Age				<0.001
≤5 years	18 (14.5)	22 (7.4)	40 (9.5)	
5-14 years	12 (9.7)	40 (13.6)	52 (12.4)	
15-29 years	40 (32.3)	96 (32.4)	136 (32.4)	
30-49 years	38 (30.6)	92 (31.1)	130 (31.0)	
≥50 years	16 (12.9)	46 (15.5)	62 (14.8)	
Sex				0.281
Female	72 (58.1)	162 (54.7)	234 (55.7)	
Male	52(41.9)	134 (45.3)	186 (44.3)	
Residence				<0.001
Rural	60 (48.4)	208 (70.3)	268 (63.8)	
Urban	64(51.6)	88 (29.7)	152 (36.2)	
Animal contact				<0.001
Yes	68 (54.8)	216 (73.0)	284 (67.6)	
No	56 (45.2)	80 (27.0)	136 (32.4)	
Water source				0.007
RO	98 (79.0)	198 (66.9)	296 (70.5)	
Non-RO (bottled/filtered tap)	26 (21.0)	98 (33.1)	124 (29.5)	
Education				0.612
Educated (primary education or higher)	78 (62.9)	194 (65.5)	272 (64.8)	
Illiterate	46 (37.1)	102 (34.5)	148(35.2)	

^aData are presented as number and percentage and were analyzed using the Chi-squared test. RO, reverse osmosis.

<0.05 was considered to indicate a statistically significant difference.

Results

Characteristics of the study participants. *Cryptosporidium* infection was detected in 296 participants, whereas 124 participants tested negative, yielding an overall prevalence of 70.5%. The sociodemographic characteristics and potential risk factors of the study participants are presented in Table I. The frequency of exposure to animals and the ingestion of non-RO water was higher among the infected participants compared with the non-infected participants. Age distribution differed significantly between the two groups ($P<0.001$), indicating a significant univariate association. However, following multivariable adjustment, age was not independently associated with *Cryptosporidium* infection ($P=0.097$; Table II).

Microscopic and molecular confirmation. Microscopy-positive samples subjected to nested PCR yielded the expected 500-bp amplicon. Representative microscopic and electrophoretic findings are presented in Fig. 1 (microscopy) and Fig. 2 (gel electrophoresis), respectively. Sanger sequencing of selected isolates confirmed species-level identification of *Cryptosporidium hominis* and *Cryptosporidium parvum*

among study samples. Among the sequenced isolates, 77.8% were identified as *Cryptosporidium parvum* and 22.2% as *Cryptosporidium hominis*. Representative Sanger sequencing chromatograms with sequence alignment and variant annotation are presented in Fig. 3. Sequence data were deposited in the NCBI GenBank database under accession nos. PZ245405, PZ245406, PZ245407, PZ245408, PZ245409, PZ245410, PZ245411, PZ245412 and PZ245413.

Multivariate logistic regression analysis. The results of the multivariable logistic regression analysis are presented in Table II. Following adjustment for all covariates, two variables remained significantly associated with *Cryptosporidium* infection. Participants with animal contact had significantly higher odds of acquiring the infection compared with those without animal contact (AOR=2.98; 95% CI, 1.75-5.02; $P<0.001$). Similarly, the consumption of non-RO water was associated with an ~a 2-fold increase in the odds of infection (AOR=2.05; 95% CI, 1.10-3.82; $P=0.024$). Age exhibited a positive, yet statistically non-significant association with infection (AOR=1.18 per category increase; 95% CI, 0.97-1.42; $P=0.097$). Residence, sex and educational status were not significantly associated with infection ($P>0.05$ for all variables). The Hosmer-Lemeshow goodness-of-fit test indicated that the final model demonstrated an adequate fit to the observed data ($\chi^2=10.89$, df=8; $P=0.208$).

Table II. Multivariate logistic regression analysis of factors associated with *Cryptosporidium* infection.

Variable	Adjusted OR (AOR)	95% CI	P-value
Animal contact (yes vs. no)	2.98	1.75-5.02	<0.001
Non-RO water (vs. RO)	2.05	1.10-3.82	0.024
Age (per one-category increase)	1.18	0.97-1.42	0.097
Residence (rural vs. urban)	0.69	0.41-1.18	0.175
Sex (female vs. male)	0.91	0.58-1.42	0.676
Education (primary education or higher vs. illiterate)	1.05	0.66-1.67	0.851

The model was adjusted for all variables. The Hosmer-Lemeshow test was used to determine model fit: $\chi^2=10.89$, $df=8$, $P=0.208$; Nagelkerke $R^2=0.085$. RO, reverse osmosis.

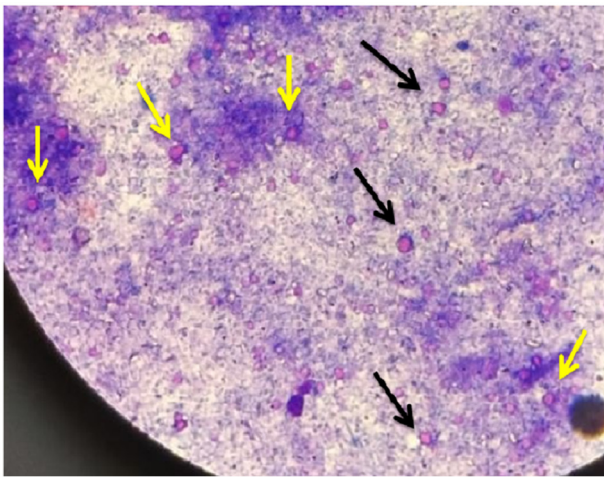


Figure 1. Microscopic appearance of *Cryptosporidium* oocysts in a human stool sample using modified Ziehl-Neelsen staining (1,000X oil immersion objective). Oocysts appear as bright pink to red spherical bodies measuring 4-6 μm against a blue-green background. Both the black and yellow arrows indicate *Cryptosporidium* oocysts; the yellow arrows were used only to improve visibility in darker areas of the image.

Discussion

The present study identified animal contact and the consumption of non-RO drinking water as independent factors associated with molecularly confirmed *Cryptosporidium* infection. These findings highlight the importance of zoonotic exposure and water quality in the transmission of cryptosporidiosis within the study area. The significant association between animal contact and infection (AOR=2.98) suggests that zoonotic transmission may substantially contribute to the spread of the disease (5). The detection of both *Cryptosporidium hominis* and *Cryptosporidium parvum* suggests the coexistence of anthroponotic and zoonotic transmission pathways within the study. Furthermore, the predominance of *Cryptosporidium parvum* (77.8%) among the sequenced isolates is consistent with the observed association between animal contact and infection and suggests a possible zoonotic contribution to transmission. However, as only a subset of isolates was sequenced, this finding should be interpreted with caution. Similar findings have been reported in studies from Cameroon and

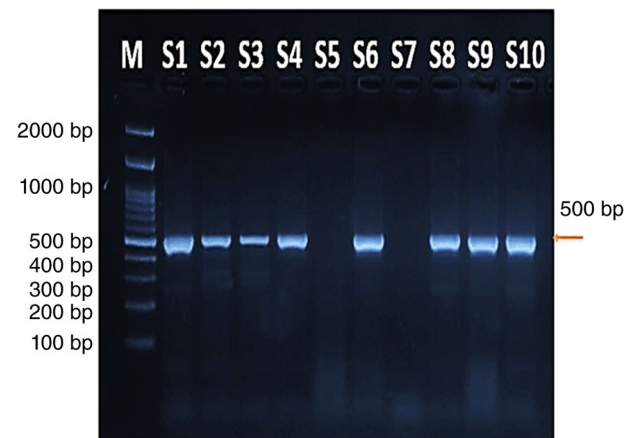


Figure 2. Agarose gel electrophoresis of nested PCR products for *Cryptosporidium* spp. detection. The lanes are as follows: Lane M, 100 bp DNA ladder; lane S1, positive control; lanes (S2-4, S6 and S8-10), representative positive patient samples (500-bp band); lanes (S5 and 7), negative control.

Romania (7,10). These observations emphasize the importance of hand hygiene following animal contact and measures that reduce environmental contamination around households. The consumption of non-RO water was also independently associated with infection (AOR=2.05). This finding supports the importance of effective water treatment in preventing cryptosporidiosis. *Cryptosporidium* oocysts are resistant to conventional chlorination, whereas reverse osmosis and other membrane filtration technologies are highly effective in their removal (4). The increased risk associated with bottled water and filtered tap water may reflect inadequate treatment, the insufficient removal of oocysts, or post-treatment contamination (3,5). Where RO systems are unavailable or unaffordable, boiling drinking water remains a practical and effective household alternative. However, the high cost of RO systems may limit their accessibility in low-income rural settings; boiling water is a practical and affordable alternative. Age exhibited a positive, yet non-significant association with infection following adjustment ($P=0.097$). Although younger children are generally considered to be at a greater risk of developing cryptosporidiosis worldwide (1,2), one possible explanation is that repeated exposure in endemic settings may

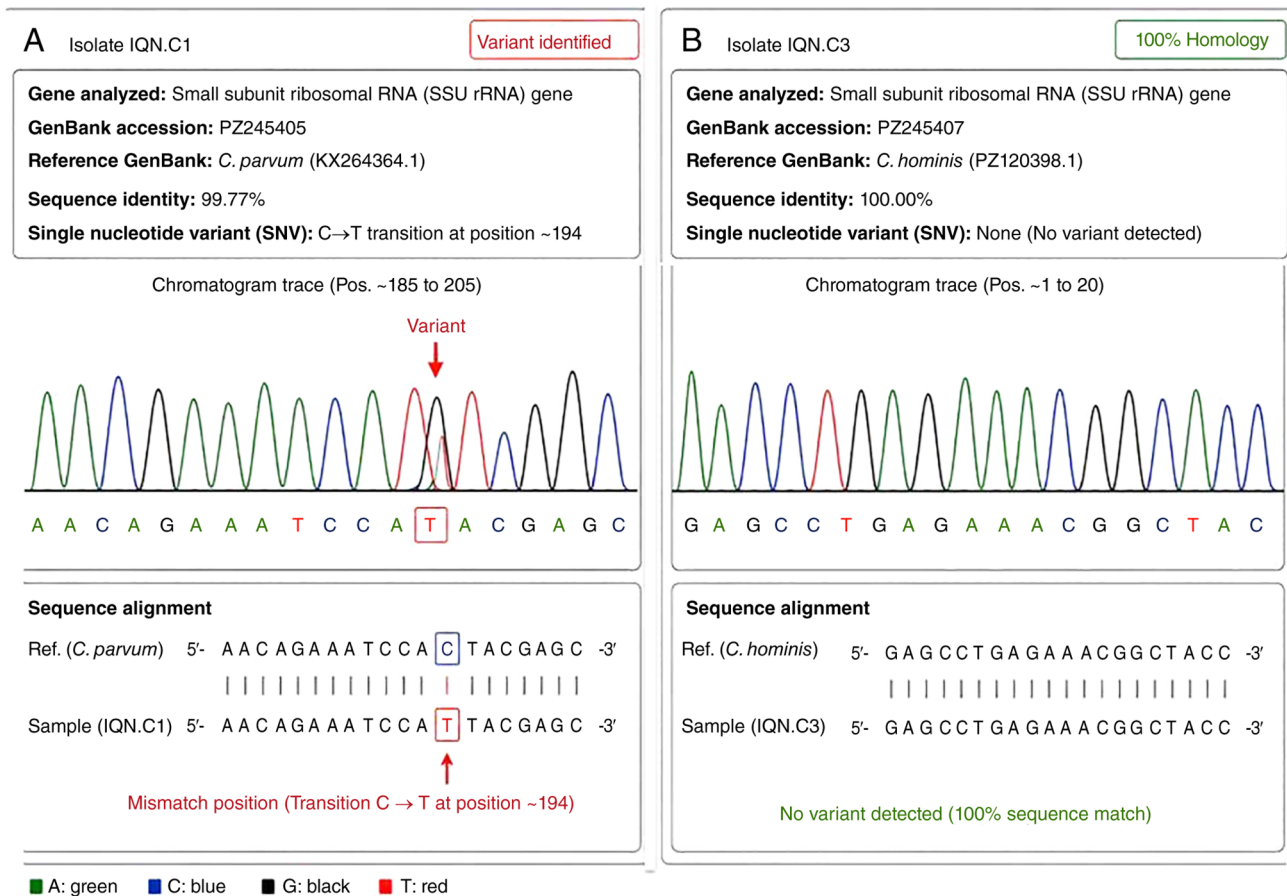


Figure 3. Representative Sanger sequencing chromatograms and sequence alignments of *Cryptosporidium* isolates. (A) Isolate IQN.C1 (GenBank Accession no. PZ245405) illustrating a single nucleotide transition (C → T) at position 194 within the non-coding SSU rRNA gene compared to the reference *C. parvum* strain (KX264364.1). The altered nucleotide peak is pointed out by a vertical arrow and boxed within the sequence text. (B) Isolate IQN.C3 (GenBank Accession no. PZ245407) demonstrating 100% sequence identity and perfect local alignment with the reference *C. hominis* strain (PZ120398.1) with no mismatches or variants. As the SSU rRNA gene is a non-protein-coding ribosomal RNA gene, cDNA coordinates, genomic coordinates, and codon numbers are not applicable.

reduce age-related differences through the development of partial acquired immunity (6). Furthermore, Iraqi children are exposed to various microbial threats in their everyday environment (11). In the present study, residence, sex and educational status were not independently associated with infection. The loss of significance for rural residence following adjustment suggests that the higher infection rates observed in rural areas may be explained largely by differences in animal exposure and water source rather than residence itself. These findings are consistent with those of previous reports from Iraq demonstrating the presence of *Cryptosporidium* in domestic geese in Nineveh Governorate (12) and in river water and sewage in Basra Governorate (13), supporting the role of animal exposure and contaminated water in transmission.

The present study has certain limitations which should be mentioned. The cross-sectional design does not permit causal inference. Modified Ziehl-Neelsen staining is less sensitive than molecular diagnostic methods and may fail to detect low-intensity infections (14). In addition, nested PCR was performed only on microscopy-positive samples, not on all samples, potentially leading to underestimation of the true prevalence. Consequently, some associations between risk factors and infection may also have been underestimated.

Therefore, some low-burden infections may have been missed (false negatives), potentially underestimating the true prevalence of *Cryptosporidium* infection. This limitation should be considered when interpreting the findings. The relatively low Nagelkerke R^2 value (0.085) suggests that additional unmeasured factors, such as detailed hygiene practices, household crowding, or nutritional status, may contribute to infection risk. Although species identification was performed for selected isolates, subtype analysis was not conducted, limiting conclusions regarding specific transmission pathways. Finally, as the participants were symptomatic individuals attending healthcare facilities, the findings may not be generalizable to asymptomatic carriers or the wider community.

As regards public health implications, improving access to adequately treated drinking water, promoting household water treatment methods such as reverse osmosis or boiling, and encouraging proper hygiene following animal contact may be effective in reducing *Cryptosporidium* transmission. A One Health approach integrating human, animal, and environmental health sectors is likely to provide the most effective framework for prevention and control (5).

In conclusion, animal contact and consumption of non-RO drinking water were independently associated with

Cryptosporidium infection. The predominance of *C. parvum* among sequenced isolates suggests a possible zoonotic contribution to transmission; however, further molecular epidemiological studies including subtype analysis are required to confirm transmission pathways. Improving water safety and reducing exposure to animal-associated sources of infection should be central components of prevention strategies. A One Health approach may contribute to reducing the burden of cryptosporidiosis in the study area.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author. Sequence data were deposited in the NCBI GenBank database under accession nos. PZ245405, PZ245406, PZ245407, PZ245408, PZ245409, PZ245410, PZ245411, PZ245412 and PZ245413.

Authors' contributions

SRN was involved in the conceptualization of the study, and in the study methodology, investigation and formal analysis, as well as in the writing of the original draft of the manuscript and in figure preparation. BAAA supervised the study, was involved in data validation, provision of laboratory facilities, equipment, reagents and technical support, and in the writing, reviewing and editing of the manuscript. SRN and BAAA confirm the authenticity of all the raw data. Both authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study protocol was approved by the Research Ethics Committee of Thi-Qar Health Directorate, Ministry of Health (Approval no. 2026/27, approved on January 27, 2026), following the official institutional authorization issued by the College of Science, University of Thi-Qar (Reference no. 97, dated January 20, 2026), which supported the ethical review and approval process by the Health Directorate. Written informed consent was obtained from all adult participants and from the parents or legal guardians of minors prior to enrollment. Participant recruitment, questionnaire interviews and stool sample collection commenced only after all required institutional and ethical approvals had been obtained.

Patient consent for publication

Not applicable.

Competing Interests

The authors declare that they have no competing interests.

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