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Original Article

Microscopic and molecular *Cryptosporidium parvum* infection in individuals with gastrointestinal symptoms in South Khorasan, Iran

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Abstract

Background: *Cryptosporidium* spp., an opportunistic protozoan affecting humans and animals, ranks as the third leading global cause of diarrheal disease. **Aims:** This study aimed to molecularly identify *C. parvum* in patients with gastrointestinal symptom in Birjand, South Khorasan province, Iran. **Methods:** *Cryptosporidium parvum* was molecularly confirmed and characterized using nested PCR and sequencing of the *18S rRNA* gene on 160 stool samples from symptomatic patients in Birjand. Phylogenetic analysis was conducted to assess genetic relationships. The resulting phylogenetic trees and haplotype networks were compared. **Results:** In this study, molecular prevalence was 8.75 (14/160). Sequencing confirmed all positives as *C. parvum*. Phylogenetic clustering revealed overlap between human isolates and animal-derived strains. **Conclusion:** Molecular epidemiology is critical for mapping *Cryptosporidium* transmission. Phylogenetic evidence supports zoonotic cycles in South Khorasan, informing targeted public health strategies.

Key words: *18S rRNA*, Birjand, *Cryptosporidium parvum*, Iran

Introduction

Cryptosporidium spp., a protozoan parasite of significant public health concern, affects humans and a wide range of animals (Checkley *et al.*, 2015). It is the causative agent of cryptosporidiosis, a zoonotic parasitic disease characterized by an oral-fecal transmission cycle (Mamedova and Karanis, 2021). This parasite poses particular risks to immunocompromised individuals and those with digestive disorders (Wang *et al.*, 2023a). *Cryptosporidium* spp. spreads easily within the environment through contaminated water, food, vegetables, and other fomites, making infection control a challenge (Taghipour *et al.*, 2019; Javanmard *et al.*, 2020; Wang *et al.*, 2023b). The life cycle of *Cryptosporidium* occurs in two stages: asexual and sexual, and takes place in a host, usually humans or animals. Infection begins after the oocysts are swallowed

and enter the body, and infection can be asymptomatic or cause a range of gastrointestinal symptoms (Walzer *et al.*, 2024). In immunocompromised individuals, cryptosporidiosis can cause chronic and severe diarrhea, which is potentially life-threatening (Ramirez *et al.*, 2004; Zilberman *et al.*, 2009; Jafari *et al.*, 2014; Mead, 2023). It can negatively affect children's health and disrupt children's cognitive development (Kotloff *et al.*, 2013). The severity of the symptoms of this disease depends on factors such as age, the state of the immune system, and type of parasite so that it can be self-limiting or dangerous for the elderly, children, and patients with immune system defects (Jafari *et al.*, 2014; Wang *et al.*, 2023b). The global prevalence of *Cryptosporidium* spp. is estimated to be 7.6% (Dong *et al.*, 2020). Totally, 90% of cases are related to the two species of *C. hominis* and *C. parvum* (Rossle and Latif, 2013). Therefore molecular techniques are essential for studying the classification

and epidemiological patterns of *Cryptosporidium* spp. (Ryan *et al.*, 2021). *Cryptosporidium* spp. signals the link between the environment, animals, and human health, and its control to prevent community spread requires preventive and management actions from a public health perspective. Therefore, the present study aimed to determine the prevalence and genetic characteristics of *C. parvum* among patients with gastrointestinal symptoms.

Materials and Methods

Ethical approval

This research study was approved with the ethics code of IR.BIRJAND.REC.1402.007 in Birjand University and in collaboration with Birjand University of Medical Sciences. Informed consent was obtained from all the study participants. Also, the patient's information was confidential and not published.

Study area and sample collection

This cross-sectional study was conducted in Birjand, the capital of South Khorasan province in eastern Iran (32.86° latitude and 59.23° east longitude) at an altitude of 1491 m above sea level (Khalili *et al.*, 2022) (Fig. 1). The city has a hot and dry climate with a population of 259,506 (Jaradat *et al.*, 2020). A total of 160 stool

samples were collected from patients with gastrointestinal symptoms (abdominal pain, constipation, epigastric pain, diarrhea) referred to hospitals and healthcare centers in Birjand. A questionnaire pertaining

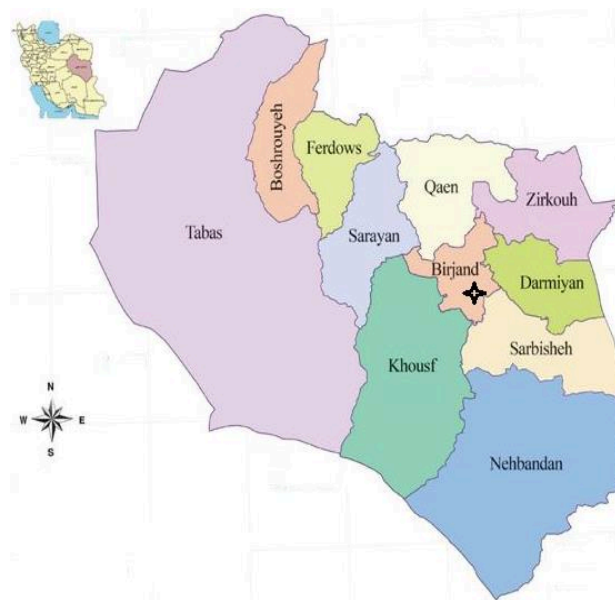


Fig. 1: Map of sampling in Birjand, South Khorasan Province, Iran

Table 1: Demographic variable characteristics of people participating in the study

Demographic variable		No. of examined participants (%)	Relative (%)	Significance level
Sex	Male	106 (66.25)	9 (8.4)	0.161
	Female	54 (33.75)	5 (9.2)	
Age	0-10	13 (8.12)	1 (7.6)	0.838
	11-20	30 (18.75)	2 (6.66)	
	21-30	31 (19.37)	6 (19.35)	
	31-40	27 (16.85)	2 (7.4)	
	41-50	36 (22.50)	1 (2.7)	
	51<	23 (14.37)	2 (8.6)	
Education	Illiterate and primitive	34 (21.25)	2 (5.8)	0.869
	High school	60 (37.50)	9 (15)	
	Academic education	66 (41.25)	3 (4.5)	
Location	City	143 (89.37)	12 (8.3)	0.639
	Village	17 (10.62)	2 (11.76)	
Job	Related ¹	6 (3.75)	2 (33.33)	0.889
	Irrelated ²	154 (96.25)	12 (7.79)	
Digestive disorder	Diarrhea	50 (31.25)	2 (4)	0.137
	Abdominal pain	103 (64.37)	11 (1.67)	
	Epigastrium	2 (1.25)	1 (50.00)	
	Constipation	5 (3.12)	0 (0)	
Animal contact	Yes	21 (13.12)	12 (57.1)	0.056
	No	139 (86.87)	2 (1.4)	
Stool consistency	Watery	7 (4.37)	1 (14.2)	0.080
	Loose	121 (75.62)	12 (9.9)	
	Soft	31 (19.37)	1 (3.2)	
	Stiff	1 (0.62)	0 (0)	

¹ Occupations at higher risk for intestinal parasitic infections include those involved in food preparation and distribution, livestock farming, and agriculture, and ² Occupations not typically associated with such infections include office workers, homemakers, self-employed individuals, and others

to examined patients (age, gender, place of residence, close contact with livestock, occupation, type of gastrointestinal disorder, faecal consistence) (Table 1). Each stool sample was aliquoted in two aliquots (10% formalin for microscopic examination, and 70% ethanol for molecular analysis) and stored at 4°C till analyses.

Microscopic examination

Smears prepared from 10% formalin-fixed stool samples (300 µL) were stained using the modified Ziehl-Neelsen (mZN) staining and of microscopically examined for *Cryptosporidium* oocysts (magnification ×1000) (Garcia, 2021).

DNA extraction and PCR procedures

Fecal samples (250 µL) were washed with phosphate-buffered saline (pH 7.2) and incubated with 300 µL of lysis buffer and 25 µL of proteinase K with Glass beads in a Bain Marie at 60°C for 30 min (vortex every 15 min). DNA extraction was performed according to the kit instruction (Favor Prep™ Stool DNA Extraction Kit, Taiwan). Nested PCR was performed to amplify the small subunit ribosomal RNA (*SSU rRNA*) gene with a fragment length of 764 bp using primer sets (18S CF2 (5'-GAC ATA TCA TTC AAG TTT CTG ACC-3') and 18S CR2 (5'-CTG AAG GAG TAA GGA ACA ACC-3'). The second PCR primer sets was 18SIF (5'-AGT GAC AAG AAA TAA CAA TAC AGG-3') and 18SIR (5'-CCT GCT TTA AGC ACT CTA ATT TTC-3') to amplify a fragment length of 323 bp (Power *et al.*, 2011). Each PCR reaction was performed in a total volume of 20 µL. The reaction mixture included 10 µL of 2X Ampliqon Master Mix (Ampliqon, Denmark), 1 µL of each primer (forward and reverse, 10 pmol/µL), 2 µL of extracted genomic DNA, and 6 µL of nuclease-free deionized water. All reactions were conducted under strict sterile conditions to ensure the accuracy and reliability of the amplification process. The first-round of conventional PCR was performed under following conditions: an initial temperature of 95°C for 5 min, followed by 35 cycles of 94°C for 40 s, 55°C for 45 s, and 72°C for 45 s, ending with a final extension at 72°C for 4 min. The second-round of PCR was set to 95°C for 5 min, followed by 30 cycles of 94°C for 35 s, 57°C for 35 s, and 72°C for 40 s, with a final extension at 72°C for 5 min. PCR products was loaded onto a 1.5% agarose gel. Positive (genomic DNA of *C. parvum* isolate with accession number OM273078 obtained from the Department of Parasitology and Mycology, Shahid Beheshti University of Medical Sciences, Tehran, Iran) and negative (nuclease-free water) controls, along with a 50 bp DNA ladder, were included. Electrophoresis was performed at 70 V for 40 min, and bands were visualized and documented using a Gel Doc system.

Sequencing and phylogenetic analyses

Following electrophoresis, specific PCR bands were excised and purified. Sanger sequencing was performed in forward directions using the inner primer (18SIF) by a

commercial service provider (Pishgam Biotech Company, Tehran, Iran) on an ABI 3730xl DNA Analyser (Applied Biosystems, USA). The resulting sequence chromatograms were then analyzed using Chromas and BioEdit software to edit and trim each sequence manually. The basic local alignment search tool (BLAST) was used to compare the modified sequences to the GenBank database (<https://blast.ncbi.nlm.nih.gov>) to identify genotypes. All sequences were deposited to GenBank with the accession number. Based on model nucleotide substitution, the Bayesian information criteria were used to draw phylogenetic tree. Consequently, the Tumora 3-Parameter option and the maximum likelihood algorithm included in the molecular evolution genetic analysis (MEGA) 11 software (<https://www.megasoftware.net/>) program were used (Tamura *et al.*, 2021). Additionally, a 1000 replication bootstrap was used to assess the statistical evidence for distance.

Statistical analysis

Data were analyzed using SPSS software, ver. 21. Descriptive statistics, including mean, standard deviation, frequency, and percentage, were used to summarize the data. To assess the association between demographic variables, including age, gender, place of residence, occupation, contact with animals, stool consistency, and type of disorder, with cryptosporidiosis infection, the Chi-square test was used to evaluate associations with infection, with 95% confidence intervals (CIs) reported.

Results

Demographic data

Of all participants (66.2% men and 33.8% women), 89.3% and 10.6% were in urban and rural areas with no contact (86.8%) with animals, respectively (Table 1).

Microscopic and molecular findings

Microscopically, 14 out of 160 stool samples (8.7%) were identified as *Cryptosporidium* (Figs. 2-3). All 14 amplified and sequenced amplicons (9 men and 5 women) were identified as *C. parvum* (accession No. PP086661-PP086674) (Table 2). The demographic variables had no significant association with prevalence of *C. parvum* infection ($P>0.05$) (Table 1). The positive cases (12/14, 85.7%) were residents of urban areas with abdominal pain (11/14, 78.5%), diarrhea (2/14, 14.2%), and epigastric pain (1/14, 7.1%) (Table 2).

A phylogenetic tree was constructed using MEGA11 software (<https://www.megasoftware.net/>) for all *Cryptosporidium* positive samples except one (accession No. PP086662) due to short sequence length, along with the corresponding reference strains. The phylogenetic tree placed the isolates from our study alongside animal isolates (birds and ruminants). However, the raccoon isolates do not belong to the same group as the samples

from this study, indicating a distinct evolutionary history. Phylogenetic analysis confirmed the zoonotic nature of

C. parvum and the ongoing animal-to-human transmission cycle in the studied region (Fig. 4).

Table 2: Demographic characteristics of people with cryptosporidiosis

No.	Isolation code	Sex		Age	Education	Location		Occupation	Digestive disorder	Animal contact		Faecal consistency	*Access number in gene bank
		Man	Woman			Urban	Rural			+	-		
1	BIR14	*		43	Academic education	*		Teacher	Abdominal pain		*	Soft	PP086661
2	BIR17	*		34	High school	*		Driver	Abdominal pain		*	Loosed	PP086662
3	BIR45	*		43	High school		*	Self-employment	Abdominal pain	*		Loosed	PP086663
4	BIR48	*		17	High school	*		Student	Diarrhea	*		Watery	PP086664
5	BIR58		*	21	Academic education	*		Student	Abdominal pain	*		Loosed	PP086665
6	BIR64	*		41	High school	*		Self-employment	Abdominal pain	*		Loosed	PP086666
7	BIR74		*	11	Illiterate and primitive	*		Student	Abdominal pain	*		Loosed	PP086667
8	BIR63	*		47	High school	*		Building worker	Epigastrium	*		Loosed	PP086668
9	BIR81		*	24	High school	*		Unemployed	Abdominal pain	*		Loosed	PP086669
10	BIR85	*		26	Academic education	*		Psychologist	Abdominal pain	*		Loosed	PP086670
11	BIR90	*		48	High school	*		Police officer	Abdominal pain	*		Loosed	PP086671
12	BIR101		*	14	High school	*		Student	Diarrhea	*		Loosed	PP086672
13	BIR116	*		12	Illiterate and primitive	*		Student	Abdominal pain	*		Loosed	PP086673
14	BIR160		*	30	High school		*	Housewife	Abdominal pain	*		Loosed	PP086674

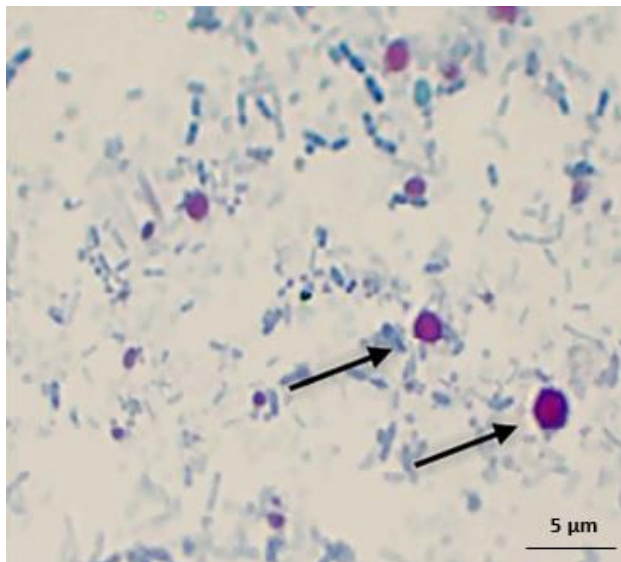


Fig. 2: *Cryptosporidium* oocysts from patients with digestive disorders referred to the hospitals and medical centers of Birjand

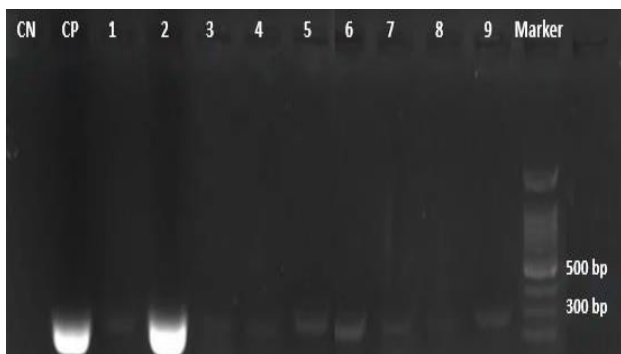


Fig. 3: PCR product electrophoresis on 1.5% agarose gel for *Cryptosporidium parvum* SSU rRNA gene. Lanes 1 to 9: PCR product obtained from SSU rRNA gene amplification, Marker 100 bp, CN: Control negative, and CP: Control positive

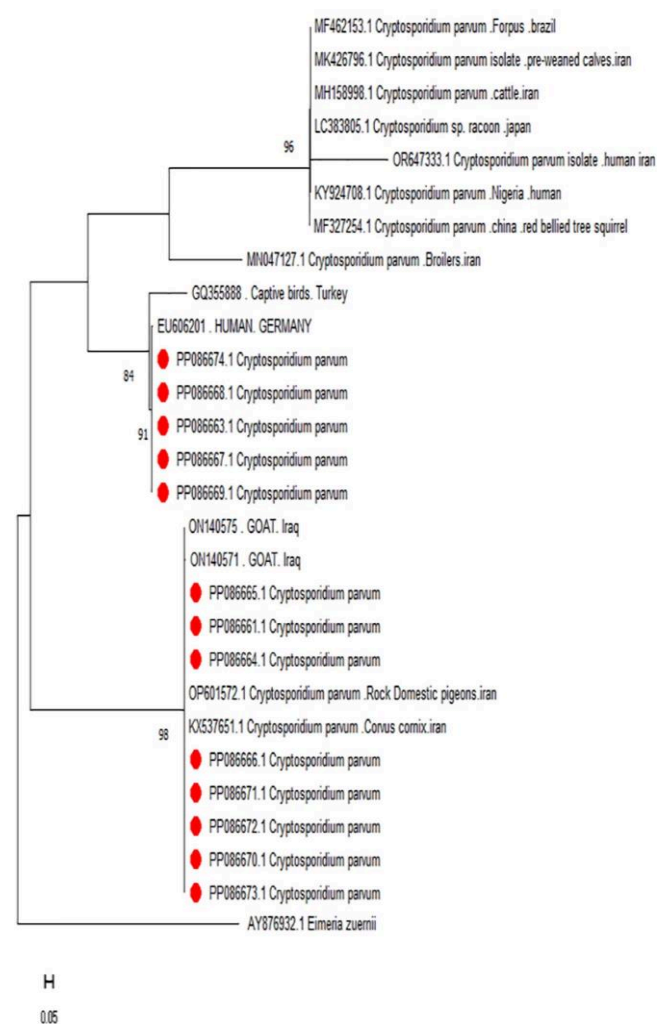


Fig. 4: The phylogenetic tree was inferred using the maximum likelihood method with the Tamura 3-parameter nucleotide substitution model in MEGA 11. Samples from this study are indicated by red circles. Bootstrap support values were calculated from 1,000 replicates to assess the statistical confidence of the inferred topology

Discussion

Identification of *Cryptosporidium* in patients with gastrointestinal disorders is important, yet prevalence rates vary across different regions and studies. Other studies indicated that *Cryptosporidium*-related diarrhea was associated with future malnutrition and higher mortality rates in low- and middle-income countries (Khalil *et al.*, 2018). In this study, the prevalence of *Cryptosporidium* infection was lower than those reported 13.49% in China (Liu *et al.*, 2014) and 13.4% in Ethiopia (Abate *et al.*, 2025). Golan Shaposhnik *et al.* (2019) and Javidmehr *et al.* (2020) reported the prevalence of *Cryptosporidium* infection in individuals with gastrointestinal disorders 3.1% and 1.6%, respectively. Factors such as use of immunosuppressive drugs, dysbiosis, dehydration, and the facilitation of *Cryptosporidium* attachment to the intestinal wall due to intestinal barrier disruption, as seen in conditions like PI and IBS, emphasizing the importance of studying cryptosporidiosis in these individuals (Shaposhnik *et al.*, 2019; Javidmehr *et al.*, 2020). These differences may be attributed to factors such as geographical and environmental variations, and differences in the population size of the study (Mirzaei, 2007; Hawash *et al.*, 2014; Liu *et al.*, 2014). Low sample size, high temperatures in the area, and people with digestive problems were likely reasons for cryptosporidiosis in this study. Researchers had thought that the oocysts of this protozoan thrived in warm, humid climates, but now they have discovered that they can survive at temperatures above 31°C and relative humidity below 63% (Wang *et al.*, 2023b).

In this study, no significant association was found between gender and the prevalence of cryptosporidiosis. However, Kargar *et al.* (2015) and Nourafab *et al.* (2022) reported a higher prevalence of *Cryptosporidium* infection among boys. Personal hygiene and health behaviors, outdoor activities, and prevalence in high-risk areas may be the reasons for this difference (Kargar *et al.*, 2015; Nourafab *et al.*, 2022).

In this work, there was higher percentage of infection in men and patients in age 21-30. Age showed no significant association with our data. These findings are consistent with those of a study conducted in Tabriz by Javidmehr *et al.* (2020) (age group of 10-19, 20-29, and over 40 years old). In contrast, Ghaedamini *et al.* (2022) found a substantial link between age groups. According to Ghaedamini *et al.* (2022) the frequency of *Cryptosporidium* infection was higher in children under 12 and 6 years-old. Playing with dirt and putting unwashed hands in the mouth were two behaviors that can put children at the highest risk of infection. Although there was no significant correlation between the present study and research by Fouladvand *et al.* (2021) showing that increasing the level of family education (especially mothers) plays a role in reducing the incidence of intestinal parasitic infections in family members (Fouladvand *et al.*, 2021). Another study showed a significant correlation between the incidence of intestinal

parasites and education level (Bahrami *et al.*, 2018). The findings of this study showed that rural people are less likely to be infected with *C. parvum* than urban residents. In a study conducted in southwestern Iran, people living in rural areas were more susceptible to intestinal parasites than their urban counterparts, which could be due to close contact with animals (Beiromvand *et al.*, 2019). The high infection rate in rural areas and on the outskirts of cities was attributed to inadequate health infrastructure (Chacín-Bonilla *et al.*, 2008; Ghenghesh *et al.*, 2012). In contrast, the elevated infection rates among urban residents linked to the anthropogenic cycle of the parasite and its reliance on a single host (Bahrami *et al.*, 2018). The difference in prevalence rates between urban and rural residents in this study and other studies can be attributed to the sample size and the number of people visiting health centers.

In our study, no significant relationship was found between the prevalence of *Cryptosporidium* infection and occupation of the participants since people who interacted with cattle had higher prevalence of *Cryptosporidium* infection. On the other hand, in earlier studies, there was a strong correlation between the prevalence of *C. parvum* infection and contact with domestic livestock (Mohammadzadeh *et al.*, 2018). Most of the participants in this study had no significant abdominal pain. In Guatemala, *C. parvum* infection was associated with symptoms such as abdominal pain and diarrhea in residents, especially children (Bentley *et al.*, 2004). In Iran, hemodialysis patients with *C. parvum* infection had also a significant association with symptoms of abdominal pain (Mohaghegh *et al.*, 2017).

The importance of molecular diagnosing *Cryptosporidium* infection can provide a more accurate understanding of the prevalence of the dominant genus and species, as well as the different routes of transmission of this protozoan, from a One Health perspective (Innes *et al.*, 2020). Both *C. hominis* and *C. parvum* infections were found in most investigations on human samples, with higher frequency of *C. parvum* (Ekici *et al.*, 2022). However, some studies suggested *C. hominis* was the dominant specie (Mohammadian *et al.*, 2019). Molecular findings confirmed *C. parvum* as the dominant species, as other studies have shown. The phylogenetic tree also indicated that *C. parvum* isolates were in the same clade as animal sequences, even though the isolates were positioned adjacent to sequences from Iran's neighboring countries (Iraq and Turkey). *C. parvum* isolates from this study were placed in a separate clade with sequences from raccoon and bovine samples, indicating evolutionary changes of *C. parvum* in these two hosts. Several studies highlighted the role of animals such as dogs, rodents, cats, sheep, cattle, and free-ranging animals in the transmission of *Cryptosporidium* infection, emphasizing its zoonotic pattern in Iran (Saki *et al.*, 2016; Dalimi and Tahvildar, 2017; Taghipour *et al.*, 2020; Taghipour *et al.*, 2021; Mirzaghavami *et al.*, 2023).

Cryptosporidium infection is prevalent in men and people with abdominal pain. There is also molecular

evidence of the possibility of a non-human transmission cycle. In addition, the sequences within a haplotype node linked to evolutionary changes related to animal isolates and some sequences occupying a distinct haplotype node underscored the significance of the One Health approach and the ongoing transmission cycle of *Cryptosporidium* infection in this area, and highlighted explicit public health implications. Future studies with larger sample sizes and using *GP60* gene may more accurately uncover the actual prevalence and essentially accurate identification and genotyping of *C. parvum* isolates.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Use of generative AI and AI-assisted technologies statement

All generated data from the present study are included in the article. No generative artificial intelligence or artificial intelligence-based technologies were used in this study.

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