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Molecular confirmation of *Cryptosporidium* and *Cyclospora* species in children with acute diarrhoea in Quindio region, Colombia

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Abstract

Background There are no reports with molecular confirmation of *Cryptosporidium* spp. and *Cyclospora* spp. in children consulting the emergency service due to diarrhoea in Colombia.

Methods A descriptive study was performed on 137 children who visited the Hospital San Juan de Dios Emergency Service in Armenia between April 1 and 31, 2022. Questionnaires and sampling were performed to identify parasites in the faecal samples. Fresh preparations were prepared with 1% iodine, and a modified Ziehl-Neelsen stain was used to identify pathogenic intestinal protozoa (*Cryptosporidium* spp. and *Cyclospora* spp.). PCR and sequencing of positive samples were performed to confirm infection.

Results The prevalence of *Cryptosporidium* spp. infection in children was 19,7%, and that of *Cyclospora* spp. was 10,9%. 59,2% of the children with cryptosporidiosis and 66,6% of the children with cyclosporiasis were hospitalized. PCR for *Cryptosporidium* spp. was positive in six of 28 (21%) samples and for *Cyclospora* in 11 of 15 (73%) samples. *Cyclospora* spp. SSU rRNA DNA sequences clustered 10 samples nearest to lineage A, two with lineage B, and one with lineage C.

Conclusions Cryptosporidiosis and cyclosporiasis are common in children with acute diarrhoea when consulting emergency services, and their search should be performed systematically.

Keywords *Cryptosporidium*, *Cyclospora*, Diarrhea, Prevalence, Child, Protozoa, Cryptosporidiosis, Cyclosporiasis, Polymerase chain reaction, Colombia

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Introduction

The current Colombian national guidelines for paediatric diarrhoea management, established in 2013, advise against employing coprological analysis for acute diarrhoea diagnosis [1]. This recommendation, however, stems from pre-rotavirus vaccine research and exhibited limited sensitivity, failing to identify approximately half of the causative agents [1]. A contemporary investigation into diarrheal aetiology in Colombia revealed that 57% of cases had no identifiable origin, despite employing advanced diagnostic techniques such as molecular viral detection, bacterial stool cultures, and antigenic assays for *Cryptosporidium* spp [2].

The Global Enteric Multicenter Study (GEMS) challenged the prevailing understanding of childhood diarrhoea. Specifically, this study utilized highly sensitive molecular methods and discovered that *Cryptosporidium* spp. was the second leading cause of death associated with diarrhoea, which was unexpected [3, 4]. This circumstance raises concerns regarding the underestimation of the true prevalence of this infection, as few studies have been conducted on children with diarrhoea that incorporated advanced molecular or immunological tools.

In addition to *Cryptosporidium* spp., another intestinal parasite that has become important in the public health landscape, especially in developing countries, is *Cyclospora* spp. [5, 6], which is a major cause of parasitic diarrhoea, especially in children [7, 8]. *Cyclospora* has a direct faecal-oral transmission cycle, is distributed globally, and is responsible for outbreaks of enteric diseases, mainly associated with the consumption of contaminated fresh produce [9]. Despite its importance, no studies have specifically investigated *Cyclospora* spp. as a cause of gastroenteritis in Colombian children, although an outbreak among medical students has been described in Medellín [10].

Cyclospora spp. can be identified through the examination of fresh oocysts using light microscopy, but this requires a skilled observer, as the oocysts can be confused with other parasites, such as *Blastocystis hominis*, *Endolimax nana*, and *Cryptosporidium* spp [8, 9]. To accurately identify *Cyclospora* spp. and *Cryptosporidium* spp., special stains, such as the modified Ziehl-Neelsen stain (Kinyoun method) are necessary [11–14]. These parasites have distinct differences in the size and colour of their oocysts, which aids in their differential diagnosis. *Cryptosporidium* spp. oocysts range from 3 to 5 µm, while *Cyclospora* spp. oocysts are larger, measuring 8–10 µm and have a characteristic spherical shape [13, 15, 16].

Despite the significant relevance of *Cyclospora* spp. and *Cryptosporidium* spp. for public health in Colombia, routine testing for these parasites is not commonly conducted in diagnostic laboratories [9, 16]. This is attributed to inadequate laboratory training and the limited knowledge of physicians regarding the role of these protozoa in causing diarrhoea [17]. As a result, there is a pressing need to investigate the risk factors associated with these two intestinal protozoa in children with diarrhoea. Furthermore, molecular confirmation of these infections has not been reported in Colombian children. Many species of *Cryptosporidium* have different public health implications, such as the zoonotic transmission of *C. parvum* through water, and the circulation of *C. hominis* in daycare settings [18]. It is important to determine the relative frequency of each in children with diarrhoea. Although three *Cyclospora* species have been described, their presence in Colombia remains unknown [19].

The primary objective of this study was to determine the prevalence of molecularly confirmed cyclosporiasis and cryptosporidiosis in Colombian children with acute diarrheal disease. This study was conducted at the University Hospital San Juan de Dios in Armenia and included molecular confirmation of the species of *Cryptosporidium* and *Cyclospora*. Additionally, this study aimed to identify the risk factors associated with the presence of intestinal protozoa. Owing to the scarcity of data on the molecular characterization of these diseases in children, this study sought to provide valuable insights on the topic.

Methods

Sample population and study design

The Hospital San Juan de Dios in Armenia (Quindío Department, Andean Western Region of Colombia) is a third-level departmental referral hospital that treated 1,533 children with diarrhoea symptoms in 2020. This study was a descriptive, observational, cross-sectional investigation. To assess potential sources of *Cryptosporidium* spp. and *Cyclospora* spp. infection beyond the hospital setting, information on the residential addresses of study participants was recorded. The children resided in the municipalities of Armenia, La Tebaida, Montenegro, Quimbaya, and Circasia, all within the Quindío Department. A geographical map was generated to illustrate the distribution of the study participants' residences across the region (Fig. 1).

A total of 150 stool samples were collected from paediatric patients under 15 years of age presented with acute diarrheal disease at the emergency department of the Hospital San Juan de Dios between March and May 2022. The sample size was determined based on the resources available to conduct the project. Additionally, 24 stool samples from children hospitalized for non-diarrheal conditions were analysed as controls.

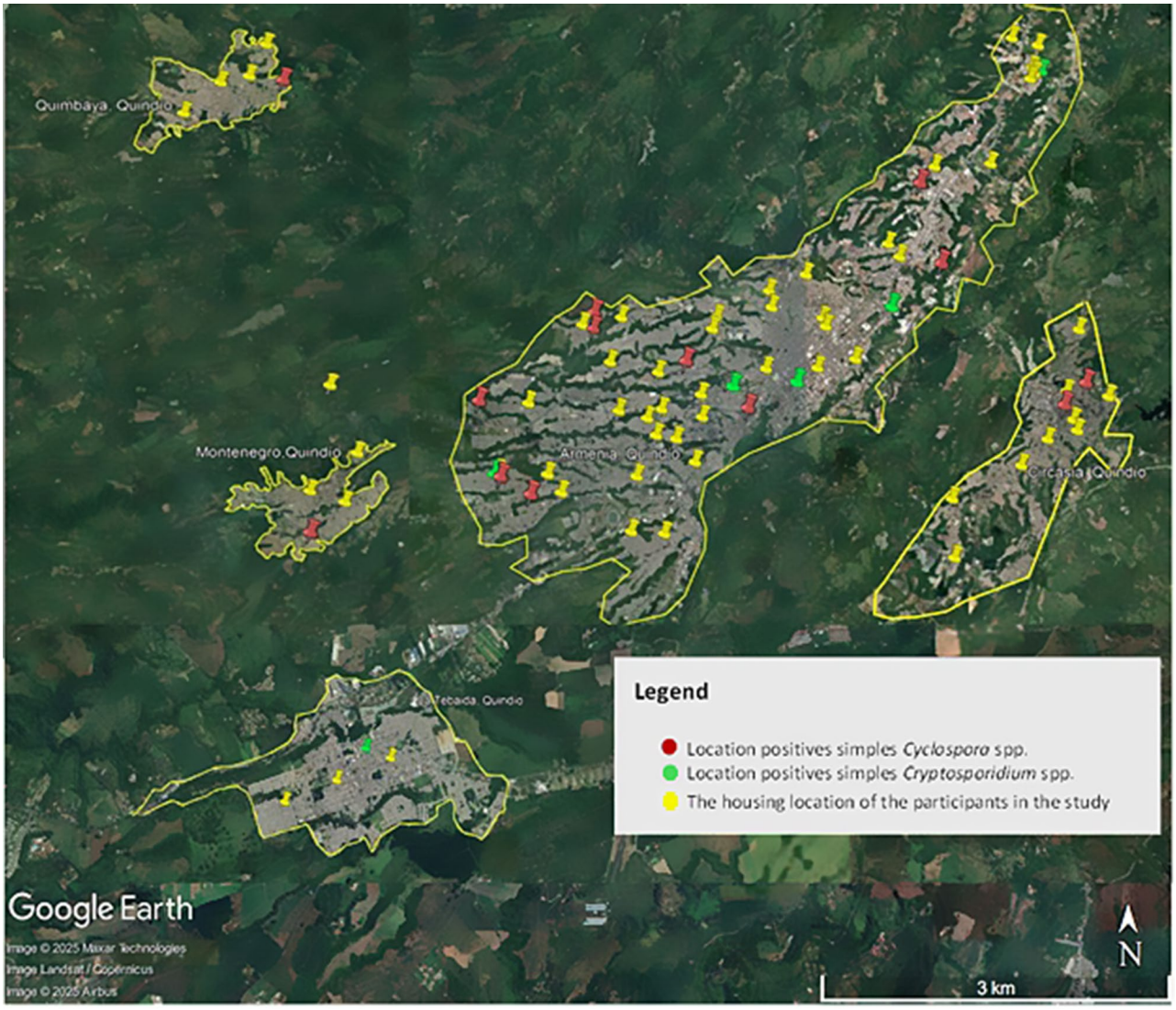

Fig. 1 Geographic distribution of study area locations in Quindío (map from Google Earth)—The map displays the municipalities of Armenia, La Tebaida, Montenegro, Quimbaya, and Circasia, where the enrolled children resided. Yellow labels indicate the housing locations of all the children included in the study. Red labels mark the residences of children who tested positive for *Cyclospora* spp., whereas green labels indicate the residences of children who tested positive for *Cryptosporidium* spp. Although all stool samples were collected at the hospital, this spatial representation provided insight into the geographic distribution of the study participants within Quindío

Ethical aspects

This study was approved by the Bioethics Committee of the Faculty of Health Sciences at the Universidad del Quindío, as documented in Act No. 17 on June 17, 2022. The study was subsequently endorsed by the Bioethics and Research Committees of the Hospital Universitario San Juan de Dios, as per their communication dated December 22, 2021.The parents or guardians of the children completed an informed consent form. All measures were respected in accordance with Ministry of Health Resolution No. 8430 of 1993. The results were provided to the parents or guardians of the children, and the indicated treatment was provided in cases of positive results.

Collection of information

The emergency department of the Hospital San Juan de Dios served as the site for collecting medical history information and stool specimens. For the purposes of this study, diarrhoea was characterized by the occurrence of three or more watery stools within a 24-hour period. The Colombian Guidelines were employed to categorize dehydration severity: Level I (mild) involved no hemodynamic alterations and approximately 3–5% body weight loss; Level II (moderate) was marked by tachycardia and roughly 6–8% body weight loss; Level III (severe) presented with hypotension, compromised perfusion, and an estimated ≥ 10% body weight loss. An axillary temperature surpassing 37.8 °C was considered indicative of fever.

Participants submitted a single stool sample on the day of hospital admission. These specimens were subsequently transported under suitable conditions to the *Centro de Investigaciones Biomédicas de la Universidad del Quindío*, where they were preserved at -20 °C for future parasitological and molecular examinations. A uniform data collection instrument was employed to document the sociodemographic and clinical details for each case, drawing upon information extracted from the hospital's medical records.

Parasitological analysis

Stool samples were collected in sterile containers with saline or phosphate-buffered saline (PBS), pH 7.4, on the day of consultation. They were transported to the laboratory in a Styrofoam cooler to maintain the appropriate conditions for subsequent parasitological and molecular analyses. Each sample was anonymized using unique codes to ensure blind evaluation by the bioanalyst, who was unaware of the patients' identities.

Diagnosis was performed by a trained professional, whose competence was externally validated by the National Institute of Health of Colombia, achieving 100% concordance in quality control tests. Stool samples were stained using the modified Ziehl-Neelsen (ZN) stain and examined under a light microscope with a 40X objective. ZN-stained plate samples of *Cryptosporidium* spp.-positive stool samples that were verified by PCR amplification of the 16s ribosomal subunit and sequenced were obtained from previous studies in our laboratory and used as positive controls in microscopy analysis. Reference stool samples containing *C. cayetanensis* were kindly donated by Dr. Ynes Ortega of the University of Georgia (USA).

Each sample was subjected to three plate reading mounts (0,83% saline, 1% Lugol, and 0,83% saline-eosin solution). Additionally, two concentration techniques were applied to enhance the detection of parasitic forms: the 0,7% formalin-ether concentration technique and the 0,8% zinc sulfate flotation technique. Morphological descriptions of the parasitic forms were conducted using fresh preparations stained with 1% parasitological Lugol solution and observed under a light microscope with a 40X objective. Positive samples for protozoa were preserved in a 10% formalin solution.

DNA extraction

A combination of chemical and mechanical lysis was used to extract DNA from stool samples. First, 300 µL of each sample was concentrated using the formalin-ether method [17, 18]. Then, 300 µL of each sample was shaken in a Mini Bead-Beater (Stratech, UK) machine for one minute and incubated on ice for another minute. This process was repeated ten times. Subsequently, 100 µl of protein precipitate was added, vortexed for 20 s, allowed to stand on ice for 5 min, and centrifuged at 13,500 rpm for 1 min. The supernatant was transferred to a clean tube and added and DNA purification using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI), the tube was allowed to dry and 80 µl of rehydrating DNA was added.

PCR detection

Detection of *Cryptosporidium* spp. DNA, nested PCR was initially used to amplify the gene encoding the membrane glycoprotein GP60 [19]. The primers used for the first amplification were Crsp601F 5´-ATAGTCTCCGC TGTATTC-3´ and Crsp601R 5´-GCCGAGGAACCAG CATC-3 × (863 bp). The following primers were used for the second PCR: Crsp602F 5´-TCCGCTGTATTCTCA GAC-3´ and Crsp602R 5´-GAGATATATCTTGGTGC GGG-3 × (443 bp). The PCR mix included GoTaq Green Master Mix (Promega, Madison, WI), primers, molecular water, BSA and DNA in a final volume of 25 µl. For the nested PCR the concentrations of the reagents were the same as for the first PCR and 1 µl of the already amplified product was added The parameters of the amplification cycles were an initial denaturation at 94°C for 5 min, followed by 35 cycles starting with denaturation at 94°C for 1 min, annealing at 46°C for 1 min, followed by an extension at 72°C for 1 min, and a final extension step for 10 min. For the second PCR, denaturation of 94°C for 10 min followed by 14 cycles with an initial denaturation at 94°C for 1 min, annealing at 54°C for 30 s, and extension at 72°C for 30 s, and an additional final extension step for 5 min at 72°C. The presence of 443 bp amplified product was visualized by 1.5% agarose gel electrophoresis, performed in a horizontal chamber with 1 × TBE running buffer at 100 V. The amplified product was visualized by 1,5% agarose gel electrophoresis. Amplification of *C. cayetanensis* was carried out by conventional PCR using conventional PCR with the 5'-GCAGTCAGAGGAGGA GGCATATATATCC-3' and 5'-ATGAGAGACCTCACA GCCAAAC-3' primers, which amplify a 116 bp fragment of the 5,8 S subunit of ribosomal RNA (SSUrRNA) [20] The PCR mix consisted of 12,5 µl of GoTaq Green Master Mix (Promega), 1.5 µl of primers (10 µM), and 3 µl of DNA in a final volume of 25 µl. The amplification cycle for the first cycle consisted of denaturation at 95 °C for 2 min, followed by 40 cycles consisting of 30 s at 95 °C, annealing at 59 °C for 30 s, and 30 s at 72 °C, followed by a final extension of 5 min at 72 °C. PCR amplifications were carried out on a Veriti thermocycler (Thermo Fisher Scientific), and the resulting products were analysed by 1.5% agarose gel electrophoresis to confirm positive samples.

Positive and negative controls for PCR

DNA was obtained from *C. parvum* oocysts purchased from the University of Arizona (https://acbs.cals.arizona.edu/crypto/purchasing). The method of obtention consisted of propagation in neonatal Holstein calves, approximately 1–2 times per month. The oocysts were purified using discontinuous sucrose and caesium chloride centrifugation gradients. The purified oocysts were stored at 4 °C in an antibiotic solution containing 0,01% Tween 20, 100U of penicillin, 100 µg of gentamicin per ml. These oocysts were used as positive amplification controls for *the Cryptosporidium* spp.

Additionally, a plasmid (pUC57) containing a 116 bp insert of the 5.8 SSU rRNA subunit of *C. cayetanensis* (GenScript HK Limited, Hong Kong) served as an additional positive control.

DNA-free samples (300 µL PBS) were used as negative controls at every stage of the process, from DNA extraction to sequence analysis, to monitor potential contamination from reagents or consumables and to detect any cross-contamination between samples during handling.

To minimize the risk of contamination, strict laboratory protocols were followed, including the use of separate pre-PCR workspaces, filter tips, and dedicated pipettes. To check for the presence of potential inhibitors, negative samples were spiked with *C. parvum* and *C. cayetanensis* DNA and reanalysed.

DNA sequencing and phylogenetic analysis

Samples that were positive after PCR amplification were sent to Psomagen Inc. software (Rockville, MD, United States) for Sanger sequencing. The nucleotide sequences obtained for the *C, parvum* gp60 marker, and *C. cayetanensis* SSUr-RNA were subjected to purification and quality control. Sequences with a QV score > 20 (values above 20 generally indicate that a sequence is reliable) were curated and aligned. In addition, nBLAST was performed for both chains (Forward and Reverse) using the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm whether the sequences obtained corresponded to the amplification target under study. Subsequently, the obtained sequences were aligned with Clustal W and trees for similarity of sequences analysis were derived in Molecular Evolutionary Genetics Analysis (MEGA) software, version 6 (available at: http://www.megasoftware.net/). All sequences were aligned with the MUSCLE algorithm and phylogeny was established using MUSCLE software in the MEGA X program to establish phylogenetic inferences (http://www.megasoftware.net/).

Immunofluorescence for *Cryptosporidium* spp

Stool samples that tested positive for *Cryptosporidium* spp. using the ZN method were further analyzed using immunofluorescence. Each ZN-positive sample was homogenized for one minute, and 100 µL of the homogenate was processed for staining. Immunofluorescence staining was performed with monoclonal antibodies specific to *C. parvum* using the Easy Stain kit (Biopoint Advancing Microbiology).

Statistical analysis

The data were processed using EpiInfo version 7.2.5.0 (Centers for Disease Control and Prevention, Atlanta, USA), available at https://www.cdc.gov/epiinfo/. A descriptive analysis of the total sample was performed, followed by bivariate analysis considering as dependent variable the presence of the parasite in the faeces of the children included in the sample. Point prevalence of parasitism and odds ratios (ORs) were calculated for each analysed factor. Chi-square tests were performed to assess statistical significance, and Fisher's test was applied when required. For quantitative variables, normality was evaluated using the Kolmogorov-Smirnov test. Depending on the results, parametric tests (Student's *t*-test) or nonparametric tests (Mann-Whitney *U* test) were used to compare the means. Statistical significance was defined as an alpha value of ≤ 0.05.

Results

Presence of *Cryptosporidium* spp. and *Cyclospora* spp. in fecal samples

Of the 150 faecal samples from children who consulted the emergency department of the San Juan de Dios Tertiary Hospital in the city of Armenia within a period of three months (March to May of 2022), 31 showed the presence of *Cryptosporidium* spp. oocysts (examples of forms observed in stools are shown in Fig. 2); therefore, the prevalence of *Cryptosporidium* spp. infection was 19,7% (95% CI: 13,4–26,0%). Similarly, 15 samples were positive for *Cyclospora* spp. oocysts (examples of forms observed in stools are shown in Fig. 3) during the same period, with a prevalence of 10,9% (95% CI: 5,7–16,1%). In 24 stool samples from children hospitalized for causes other than diarrhoea, ZN staining was negative in all cases.

Concerning the clinical characteristics of children with intestinal infections caused by *Cryptosporidium* spp. and *Cyclospora* spp., 64,5% of children with cryptosporidiosis and 46,6% of children with cyclosporiasis were hospitalized (Tables 1 and 2**).** There was a statistically significant correlation between a result be informed as presence of yeast on stool with Lugol solution and positive results for *Cryptosporidium* spp. in ZN ($p = 0{,}045$). Children infected with *Cyclospora spp.* had a significantly higher prevalence of fever than uninfected children (OR 9,9; 95%CI = 1,2–77,6; $p = 0{,}0096$). Fever was present in 93,3% of the infected children compared to 58,5% in the negative group. Yeast identification did not show statistical

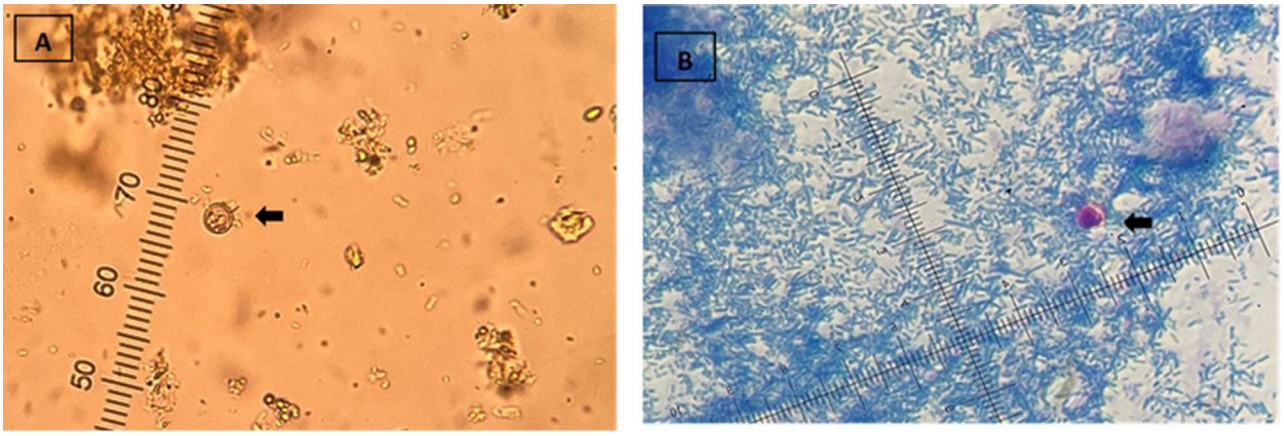

Fig. 2 Demonstrative images of children with diarrhoea who consulted the Hospital San Juan de Dios de Armenia, showing the presence of *Cryptosporidium spp.* oocysts (arrows) in stool samples. **A**. Direct visualization at a 40X objective, with a micrometer rule included for scale. **B**. Ziehl-Neelsen (ZN) staining at 100X objective, highlighting *Cryptosporidium spp.* oocysts

significance with a prevalence of 35,7% in the *Cyclospora* positive group and 32,3% in the negative group, with an OR of 1,1 (95% CI: 0,3–3,7; $p = 0{,}77$).

No significant differences in age were found between the children infected with *Cryptosporidium* spp. and *Cyclospora* spp. In both cases, the mean age was similar: 12 months (range: 1-168 months) for children positive for *Cryptosporidium* spp. and 12 months (range: 1-168 months) for children positive for *Cyclospora* spp.

Similarly, the duration of diarrhoea showed no significant differences between the groups infected with *Cryptosporidium* spp. or *Cyclospora* spp. (Tables 3 and 4, respectively).

Molecular confirmation and sequencing results

Unfortunately, due to lack of funding and reagents during the collection of samples, the molecular method was performed 12 months later in formalin-preserved stool samples, and it was not possible to apply this method to all positive samples. PCR for *Cryptosporidium* spp. (Fig. 4) was positive in six of 28 samples (21%), and PCR for *Cyclospora* spp. (Fig. 5) was positive in 11 of 15 samples (73%). Owing to the lack of reagents for immunofluorescence, only 10 of the 31 ZN-positive samples for *Cryptosporidium* spp. were examined using this technique. Cysts stained with a specific antibody against *C. parvum* were observed in four patients (Fig. 6).

The quality of amplification was adequate for sequencing of the amplified DNA in only one sequence of *Cryptosporidium* for species identification. This sequence clustered with that of *C. parvum* (Fig. 7).

Cyclospora sequences were aligned (Fig. 8a) with the SSU rRNA sequences of lineages A (*C. cayetanensis*), B (*C. ashfordi*), and C (*C. henanensis*). Sequence similarity analysis clustered ten samples nearest to lineage A, two with lineage B, and one with lineage C (Fig. 8b). No relationships between lineages and age of children, habit of drinking unboiled water, city, or origin were observed.

Discussion

Our study showed that at least one-third of the cases of diarrhoea in Quindío were related to two well-demonstrated intestinal protozoan pathogens, *Cryptosporidium* spp. and *Cyclospora* spp. Their clinical significance as a cause of diarrhoea in these children is supported by the results of the examination in the same period of children hospitalized for causes other than diarrhoea, where the prevalence was zero. Before beginning the present study, these two protozoan infections were unknown in our hospital. A similar situation has been described in other settings, which calls for improvement in the identification of protozoa in children with diarrhoea [21–23]. Identifying intestinal protozoan infections is relevant in terms of clinical and public health significance. Our results showed that most cases occurred in children under two years of age, suggesting that clinical manifestations occur during primary infections, which is consistent with reports from other countries [24, 25]. Timely detection is essential in children of lower age, where diarrhoea can lead to complications, such as hydro electrolytic disequilibrium, longer periods of symptomatology, and over infections [26]. *Cryptosporidium* spp. was found to be the second leading cause of death in children with diarrhoea in the largest prospective cohort study of paediatric morbidity and mortality, the Global Enteric Multicenter Study (GEMS), a case–control study in seven different African and Asian countries [5].

In Colombia, a case-control study in Bucaramanga (northwest region) found a *Cryptosporidium* spp. prevalence of 2% in children with diarrhoea; however, they did not use ZN staining, and 57% of cases remained unexplained, demonstrating the low sensitivity of the

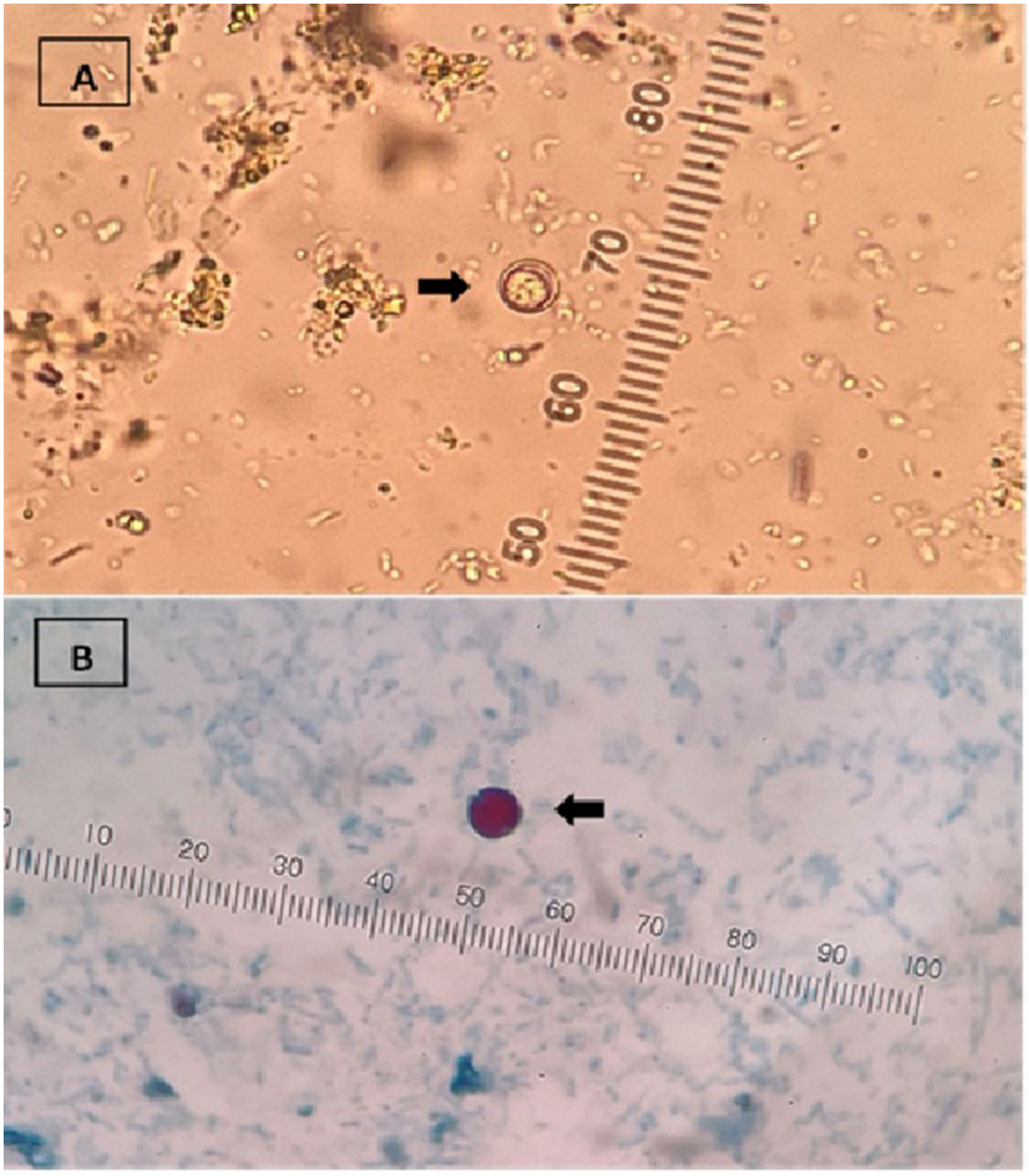

Fig. 3 Demonstrative images of children with diarrhoea who consulted the Hospital San Juan de Dios de Armenia, showing the presence of *Cyclospora spp.* oocysts (arrows) in stool samples. **A**. Direct visualization at a 40X objective, with a micrometer rule included for scale. **B**. Ziehl-Neelsen (ZN) staining at 100X objective, highlighting *Cyclospora spp.* oocysts

diagnostic tests used [2]. Another study examined cryptosporidiosis associated with diarrhoea in Colombia, in the Amazon region of Arauca, in symptomatic children attending emergency services, using ZN modified staining, where a 46% prevalence was established [27].

In contrast with the higher frequencies seen in symptomatic children, the prevalence of asymptomatic children is lower and is detected only through molecular methods, as reported by previous studies by us and other researchers in different regions of Colombia. Among urban asymptomatic children in the same region as the present study (Quindio), the prevalence was 0% using ZN staining and 10.7% using molecular methods [28]. A similar result was obtained in the southern part of Colombia, in Popayan, using molecular methods, where a prevalence of 9,8% was identified in asymptomatic urban children aged 12–54 months [29]. In Medellin, in day care settings, the infection rate by molecular method was 2,4%

Table 1 Qualitative sociodemographic, clinical, and laboratory characteristics and analysis of their association with the presence of *Cryptosporidium* spp. detected by modified Ziehl-Neelsen staining in stool samples of children and children with negative tests who consulted the emergency department of the Hospital San Juan De Dios De Armenia (Quindio, Colombia) from April to May 2022

Characteristics	*n/N* (%) in children with positive tests versus children with negative tests for Cryptosporidium in stool	OR (IC95%)	*p*
Social security regime: Contributory vs. subsidized	16/31 (51,6%) vs. 62/119 (52,1%)	1,0 (0,4 – 2,2)	1,0
Male vs. Female Gender	15/31 (48,3%) vs. 81/119 (68%)	0,43 (0,1 – 0,9)	0,057
Urban vs. Rural	31/31 (100%) vs. 114/119 (95,8%)	Undefined	0,58
Grade II or III dehydration at the time of consultation vs. no dehydration or grade I.	14/31 (45,1%) vs. 40/119 (33,6%)	1,6 (0,7 – 3,6)	0,29
Abdominal pain	11/31 (35,4%) vs. 44/119 (36,9%)	0,9 (0,4 – 2,1)	1,0
Fever	21/31 (67,7%) vs. 72/119 (60,5%)	1,3 (0,5 – 3,1)	0,53
Emesis	13/31 (41,9%) vs. 66/119 (55,4%)	0,5 (0,2 – 1,2)	0,22
Hospitalized	20/31 (64,5%) vs. 66/119 (55,4%)	1,4 (0,6 – 3,3)	0,41
Cryptosporidium positive vs. *Cyclospora* positive	2/31 (6,4%) vs. 13/119 (10,9%)	0,5 (0,1–2,6)	0,73
Increased bacterial flora	16/30 (53,3%) vs. 61/116 (52,5%)	1,0 (0,4 – 2,3)	1,0
Yeast	**12/23 (52%) vs. 27/96 (28,1%)**	**2,7 (1–7)**	**0,045***
Mucus in stool	10/31 (32,2%) vs. 35/119 (29,4%)	1,1 (0,4 – 2,6)	0,37
Blood in stool	8/31 (25,8%) vs. 23/119 (19,3%)	1,4 (0,5 – 3,6)	0,45
Leukocytes in stool	11/30 (36,6%) vs. 56/118 (47,4%)	0,6 (0,2 – 1,4)	0,31

*Statistically significant difference ($p < 0,05$)

Table 2 Qualitative sociodemographic, clinical, and laboratory characteristics and analysis of their association with the presence of *Cyclospora* spp. detected by Ziehl-Neelsen modified staining in stool samples of children and children with negative test results who consulted the emergency department of the Hospital San Juan De Dios De Armenia (Quindio, Colombia) between April and May 2022

Features	*n/N* (%) in children with positive tests versus children with negative tests for Cyclospora spp in feces	OR (CI95%)	*p*
Social security regime: Contributory vs. subsidized	7/15 (46,6%) vs. 65/135 (48,1%)	0,9 (0,3 – 2,7)	1,0
Male vs. Female Gender	15/15 (100%) vs. 130/135 (96,3%)	Undefined	1,0
Urban vs. Rural	15/31 (48,3%) vs. 81/119 (68%)	0,43 (0,1 – 0,9)	0,057
Grade II or III dehydration at the time of consultation vs. no dehydration or grade I.	5/15 (33,3%) vs. 49/135 (36,3%)	0,8 (0,2–2,7)	1,0
Abdominal pain	6/15 (40%) vs. 49/135 (36,3%)	1,1 (0,3–3,4)	0,7
Fever	**14/15 (93,3%) vs. 79/135 (58,5%)**	**9,9 (1,2–77,6)**	**0,0096***
Emesis	5/15 (33,3%) vs. 74/135 (54,8%)	0,4 (0,1–1,2)	0,17
Hospitalized	7/15 (46,6%) vs. 79/135 (58,5%)	0,6 (0,2 – 1,8)	0,41
Increased bacterial flora	10/15 (66,6%) vs. 67/131 (51,1%)	1,9 (0,6 – 5,8)	0,28
Yeast	5/14 (35,7%) vs. 34/105 (32,3%)	1,1 (0,3–3,7)	0,77
Mucus in stool	6/15 (40%) vs. 54/133 (40,6%)	0,9 (0,3 – 2,8)	1,0
Blood in stool	11/30 (36,6%) vs. 56/118 (47,4%)	0,6 (0,2 – 1,4)	0,31
Leukocytes in stool	4/15 (26,6%) vs. 277,135 (20%)	1,4 (0,4–4,9)	0,51

*Statistically significant difference ($p < 0,05$)

and two species were identified: *C. hominis* and *C. meleagridis* [23]. The present study confirms that *C. parvum* can also cause symptomatic diarrhoea in children.

Not less important is the present description with molecular confirmation of *Cyclospora* spp. in symptomatic children with diarrhoea in Colombia. A similar frequency was reported in Nepal where 10,5% positivity was found in children with diarrhoea, whereas it was 2,2% in children without gastrointestinal symptoms [30]. We found that most *Cyclospora* small subunit ribosomal sequences in our clinical samples were nearest to lineage A (*C. cayetanensis*), two were near lineage B (*C. ashfordensis*), and one was near *C. henanensis*. To our knowledge, this is the first description of *Cyclospora* circulation with sequence similarities to these three lineages in Colombia. In the United States, the circulation of lineages A and B depends on seasonality and geographical zone [31]. Future studies should expand the analysis of the epidemiological factors associated with the circulation of these *Cyclospora* species.

Regarding the sources of infection, this topic was previously studied by our team in Quindio and the presence

Table 3 Comparison of means between children with and without *Cryptosporidium* spp. detected by Zielh-Neelsen modified staining in stool samples of children who consulted the emergency department of the Hospital San Juan De Dios De Armenia (Quindío, Colombia) from April to May 2022

Features	Mean (range) in children with Cryptosporidium spp in stool	Mean (range) in children without Cryptosporidium sp in stool	*p*
Age (in months)	12 (1-168)	24 (1-168)	0,69
Weight (Kg)	10 (4,8–59)	12 (2,5–60)	0,48
Mother's years of schooling	12 (5–17)	11 (5–17)	0,14
Days with diarrhoea at the time of consultation	3 (1–10)	2 (1–10)	0,21

Table 4 Comparison of means between children with and without the presence of *Cyclospora* spp. detected by Zielh-Neelsen modified staining in stool samples from children who consulted the emergency department of the Hospital San Juan De Dios De Armenia (Quindio, Colombia) from April to May 2022

Features	Mean (range) in children with Cyclospora spp in stool	Mean (range) in children without Cyclospora spp in stool	*p*
Age	12 (1-168)	24 (1-168)	0,42
Weight (Kg)	10,6 (2,8–58)	12 (2,5–60)	0,36
Mother's years of schooling	12 (5–16)	11 (5–17)	0,53
Days with diarrhoea at the time of consultation	3 (1–14)	2 (1–14)	0,46

of *C. parvum* and *C. hominis* was demonstrated in water for human consumption [3] as well as in food at the school´s restaurants [29]. In the present study, the finding of *C. parvum* in one symptomatic child supports the need to study the zoonotic source (for example, cattle contamination of the river is the source of drinking water in Quindio) in future molecular epidemiological studies [32]. This can have important public health consequences because there is currently a lack of control over cattle exploitation and human settlements surrounding the border of the river, which are sources of drinking water for the city of Armenia [32]. Given that *Cryptosporidium* is resistant to chlorine treatment, additional control measures should be implemented, such as the restoration and preservation of natural barriers bordering rivers, which are sources of drinking water for cities [32]. The impact of cryptosporidiosis on Colombian infants is likely underestimated because of the lack of systematic detection of the parasite in diagnostic protocols for infectious diarrhoea [33]. Obtaining new data can be critical in the application of improved diagnostic strategies and the implementation of appropriate prevention and control practices for this parasite.

The relationship between yeast reported by Lugol staining and diagnosis of *Cryptosporidium* spp. by ZN may be explained by the size and morphology of yeasts, which can lead to confusion when samples are examined using conventional direct microscopy [34]. This leads to the recommendation of the use of ZN staining or molecular methods to correctly differentiate between these microorganisms and ensure accurate diagnosis.

The absence of DNA amplification and immunofluorescence detection in certain samples that were positive by ZN staining for *Cryptosporidium* spp. can be attributed to two factors. Firstly, a 12-month interval elapsed between sample collection and DNA extraction due to insufficient material for conducting the molecular method at the time of collection. Secondly, the immunofluorescent assay employed in this study was capable of detecting only *C. parvum*.

To improve sample integrity for molecular characterization of *Cryptosporidium* spp, a new collection method is necessary to enhance oocyst preservation and prevent DNA degradation. Recent reports indicate that storing faecal specimens containing *Cryptosporidium* spp. oocysts in 75% ethanol preserves both morphological and molecular integrity for over two years at ambient temperatures (22–38 °C), without significant structural alterations [35]. Additionally, this preservation method has been shown to facilitate successful DNA amplification using nested PCR with high sensitivity (1,25 oocysts per reaction). These findings highlight the importance of selecting appropriate preservation conditions to ensure sample viability in epidemiological and molecular investigations of *Cryptosporidium* spp. Notably, in contrast to *Crytosporidium* spp., preservation in formalin did not reduce the DNA amplification of *Cyclospora* spp.

This study underscores the significance of employing ZN staining to identify treatable pathogenic protozoa. It is imperative to consider that the administration of nitazoxanide for cryptosporidiosis treatment and trimethoprim sulfamethoxazole therapy for cyclosporiasis may yield potential benefits in ameliorating symptoms, mitigating complications in susceptible paediatric populations, and reducing the duration of hospitalization [36, 37].

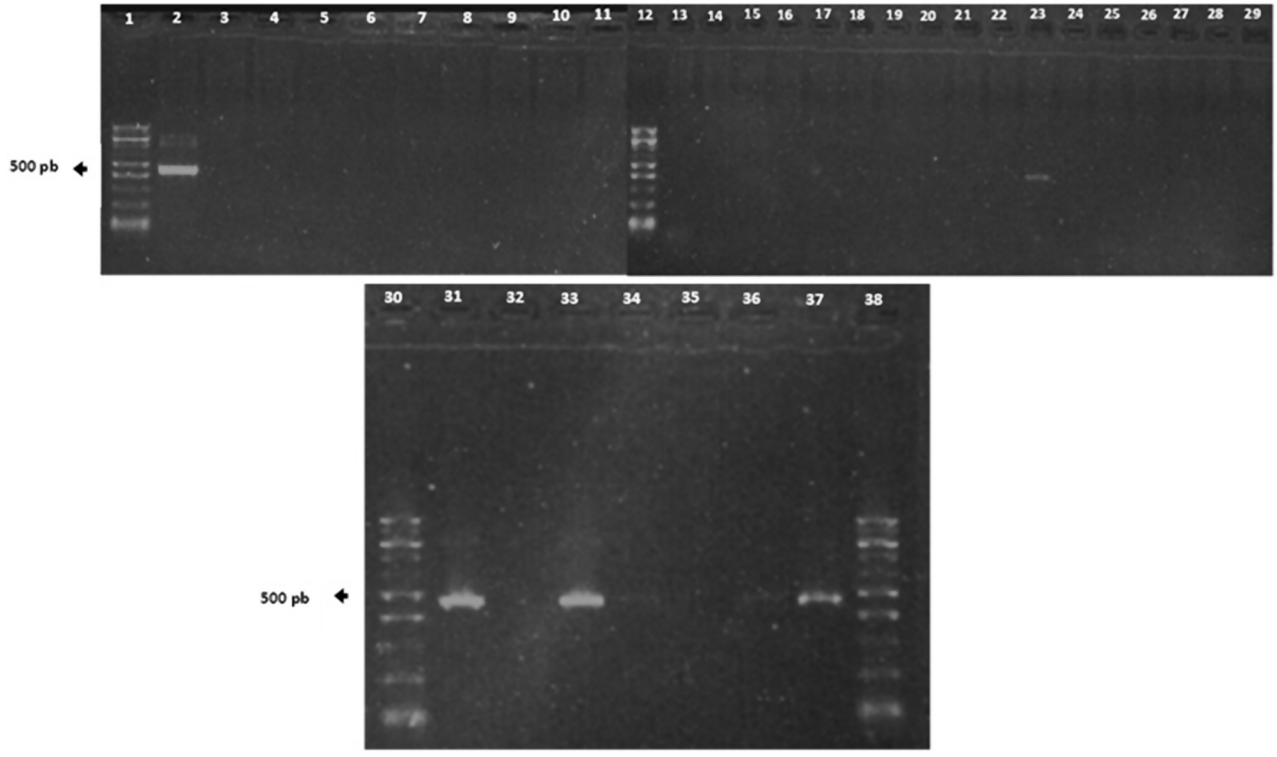

Fig. 4 Agarose gel electrophoresis of nested PCR amplification products targeting *Cryptosporidium spp.* GP160 gene. Wells: 1 molecular weight marker. 2: Positive control (reference DNA from *C. parvum*). 3: Negative control (reaction mixture without DNA). 4: Negative extraction control. 23, 31, 33, and 37: positive samples. 34 and 36: Faint bands indicate weak positive results. 5–11, 13–22 and 24–29: Negative samples

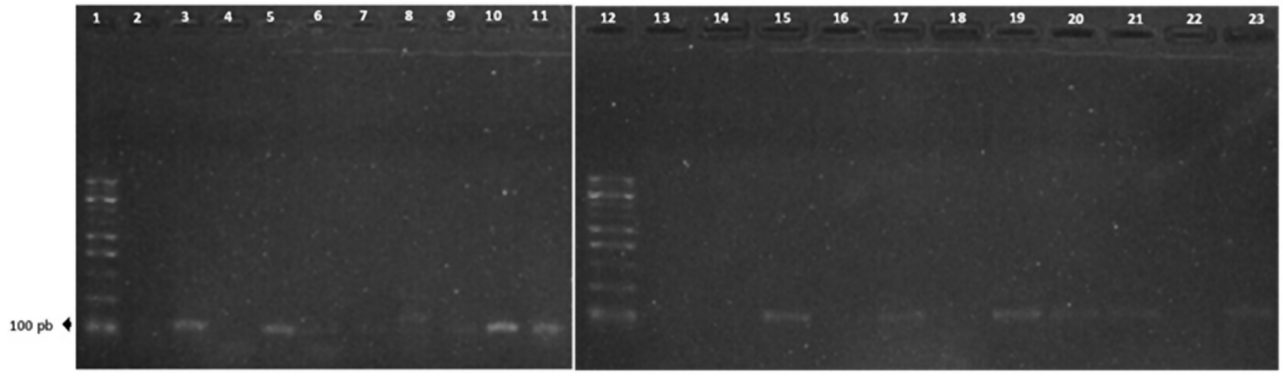

Fig. 5 PCR results for *Cyclospora* (SSU 5 S subunit). Wells 1 and 12: Molecular weight markers. 2: Negative control (mixture without DNA). 3: Positive control (plasmid DNA containing *Cyclospora* spp. sequences). 4, 13, 14, 16 and 18: negative sample. 5–11, 15, 17, 19–21 and 23: positive samples

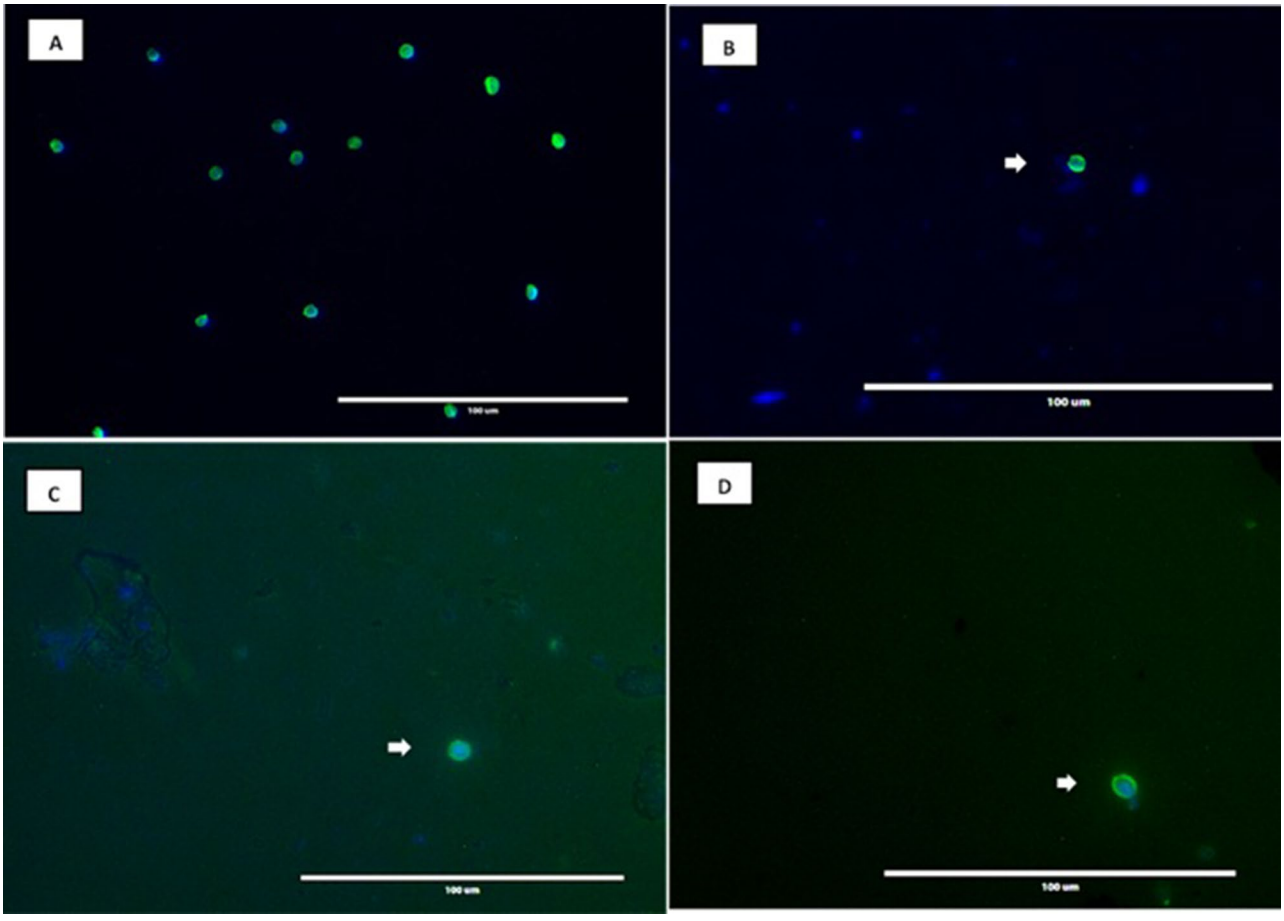

Fig. 6 Indirect immunofluorescence of *Cryptosporidium parvum* oocysts. **A**: Control oocysts of *Cryptosporidium parvum* were purchased from the University of Arizona (https://acbs.cals.arizona.edu/crypto/purchasing). The oocysts had sizes ranging from approximately 4 to 6 µm (scale bar = 100 µm). **B**, **C**, and **D**: Clinical stool samples positive for *Cryptosporidium spp*

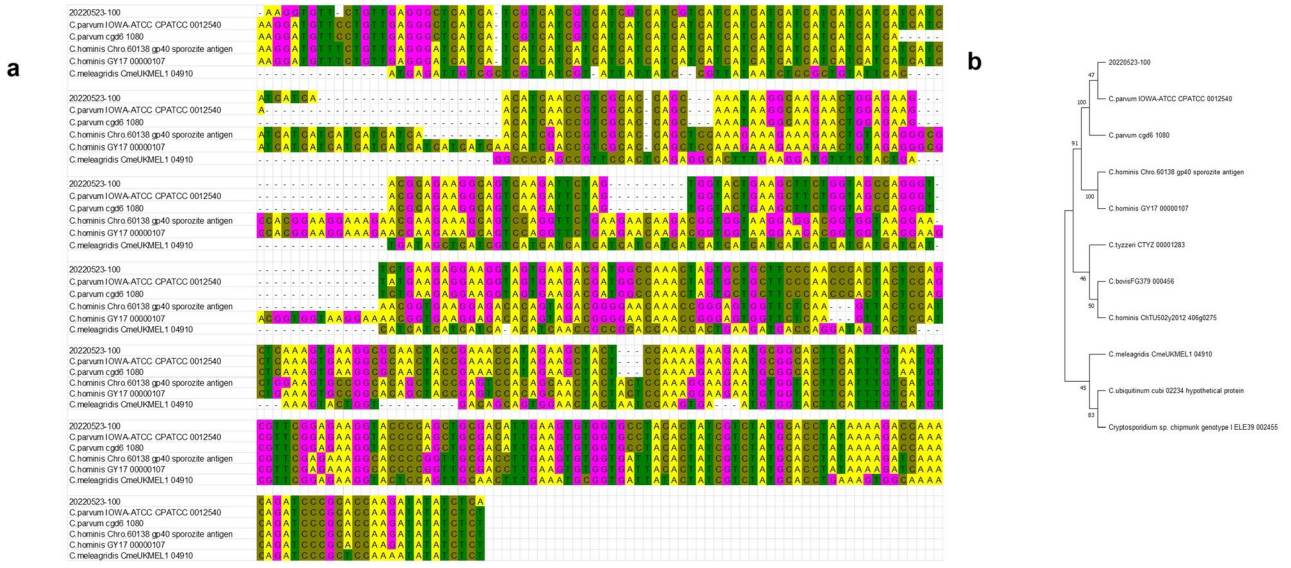

Fig. 7 **a** Alignment of one sequence *of Cryptosporidium* spp. obtained in stools from children with diarrhea in Quindio University Hospital with two reference sequences of *C. hominis*, two reference sequences of *C. parvum*, and one from *C. meleagridis*. **b** Clustering of one *Cryptosporidium* DNA sequence obtained in diarrheic clinical samples from children in Quindio Hospital and ten reference sequence of the 60 kDa glycoprotein (gp60) gene. Neighbor-Joining tree was constructed by using the Jukes-Cantor method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances are expressed in units of the number of base substitutions per site. This analysis was carried out using MEGA11 Software

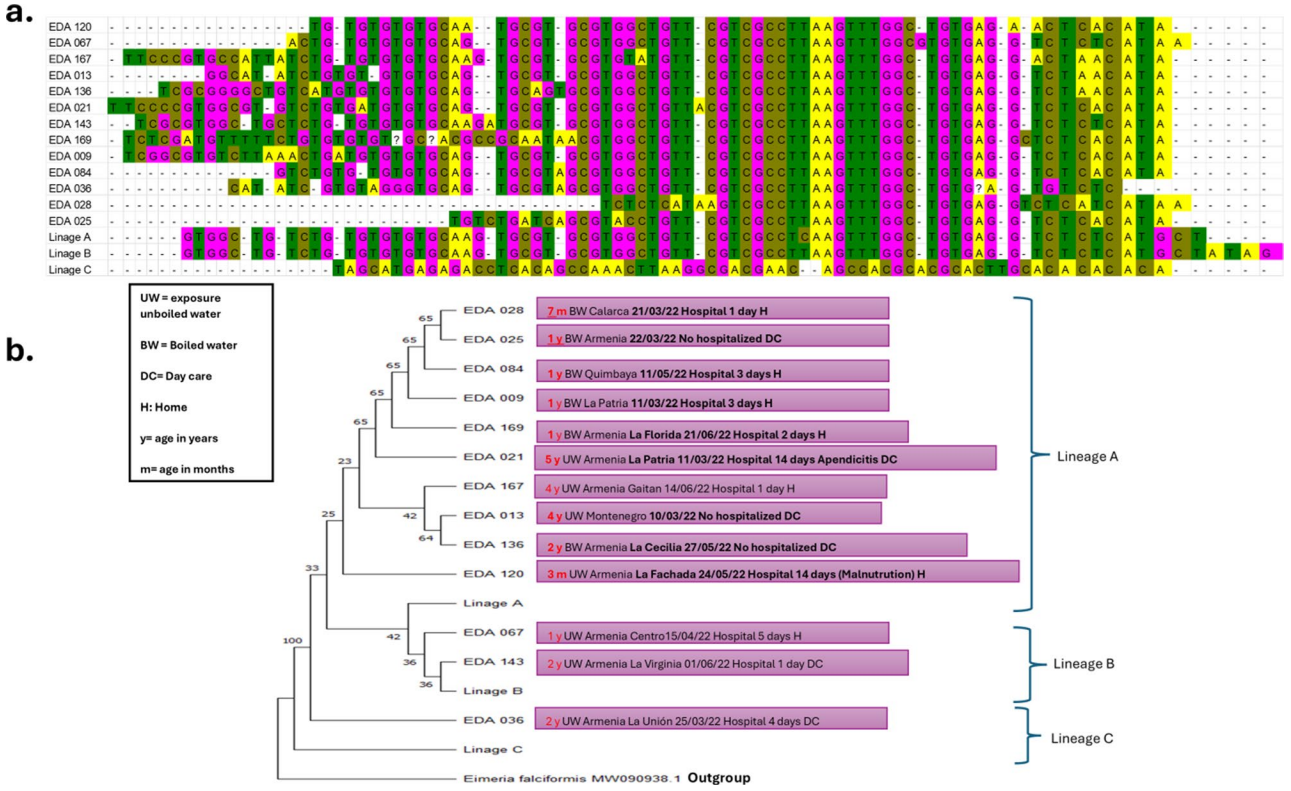

Fig. 8 **a** Alignment of 13 SSU rRNA DNA sequences of *Cyclospora* spp. obtained from stool samples from children with diarrhoea in Quindío University Hospital. **b** Clustering of 13 *Cyclospora* SSU rRNA sequences obtained from diarrheic clinical samples from children in Quindio Hospital and three SSU RNA sequences from reference *Cyclospora* lineage A (Sequence ID: RDSA01000698.1), lineage B (Sequence ID: RDRJ01001336.1), and lineage C (Sequence ID: PDMJ01000471.1). The outgroup organism was *Eimeria falciformis* (Sequence ID: MW090938.1). The UPGMA bootstrap consensus tree using the Jukes-Cantor method was inferred from 500 replicates of base substitutions per site. The analysis involved 17 nucleotide sequences. All positions with gaps and missing data were eliminated (complete deletion option). There were 30 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 Software

Conclusions

This study highlights the significant role of *Cryptosporidium* spp. and *Cyclospora* spp. as primary causes of diarrhoea in children in the Quindío region. These findings emphasize the need for systematic detection of these protozoan pathogens in diagnostic protocols, particularly for children under two years of age who are more susceptible to severe complications.

The identification of *Cyclospora* lineages and confirmation of *C. parvum* in symptomatic cases emphasize the significance of molecular methodologies in enhancing diagnostic precision and informing appropriate treatment protocols. Furthermore, the environmental and zoonotic sources of infection must be addressed through augmented public health measures, encompassing water quality surveillance and preventive strategies.

These results contribute to a better understanding of the epidemiology of these protozoan infections in Colombia and support the adoption of improved diagnostic and preventive practices to reduce their impact on vulnerable paediatric populations.

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Author contributions
JT: Formal analysis, Conceptualization, Methodology, Writing - original draft. HANC: Conceptualization, Methodology; FLS, NC: Conceptualization, Methodology, Resources, Supervision; JEGM: Conceptualization, Methodology, Formal analysis, Resources, Data Curation, Writing - review & editing, Supervision. All authors reviewed the manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
This study was approved by the Bioethics Committee of the Faculty of Health Sciences of the Universidad del Quindío in Act No. 17 of June 17, 2022.

Consent for publication
Personal data or images were not published.

Declaration of generative AI and AI-assisted technologies in the writing process
No Generative AI was employed to write this manuscript. During the preparation of this work, the authors used Paperpal to check English grammar. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

Competing interests
The authors declare no competing interests.

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