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Original Article

Intestinal Parasitic Infections among the Pediatric Patients in a Metropolitan City of Bangladesh with Emphasis on Cryptosporidiosis

Short Title: Pediatric Intestinal Parasitic Infections.

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Original Article

Intestinal Parasitic Infections among the Pediatric Patients in a Metropolitan City of Bangladesh with Emphasis on Cryptosporidiosis.

Short Title: Pediatric Intestinal Parasitic Infections.

Abstract

Background: Gastrointestinal parasitic infections are one of the global health concerns in developing countries like Bangladesh. Among them, *Cryptosporidium* spp. plays an essential role in causing diarrhea, malnutrition, and poor cognitive function, especially in children. The study was conducted to identify the frequency of cryptosporidium cases and other parasitic agents. ***Methods***: A Cross-sectional observational study was conducted among 219 hospitalized children with diarrhea. The conventional microscopic technique was applied for parasitic detection. A particular staining procedure was performed to identify oocysts of *Cryptosporidium* spp. And PCR was conducted to determine the SSU rRNA and *gp60* gene of *Cryptosporidium.* ***Results***: Cyst of *Giardia,* ova of *Ascaris lumbricoides* (AL), *Trichuris trichiura* (TT), AL, and TT were identified in 2.3%, 1.4%, 0.5%, and 0.9% samples by wet mount preparation. The distribution of *Cryptosporidium* spp. was 1.4% and 4.1%, which was detected by the staining method and nested PCR. Factors independently associated with *Cryptosporidium* infection are unsafe water, lack of regular hand washing, and insufficiency of exclusive breastfeeding. ***Conclusions***: This is the first report to detect the frequency of Cryptosporidium and other intestinal parasites and associated factors in Chattogram city of Bangladesh.

Keywords: Intestinal Parasitic, Contagions, Diseases, Pediatric Patients, Metropolitan City of Bangladesh, Cryptosporidiosis.

Introduction

Diarrhea is defined as the passage of three or more liquid or loose stools per day or if the individual experiences more frequent passage than usual [1-3]. It is categorized clinically as i) acute watery diarrhea, which persists for several hours or days [4], ii) persistent diarrhea that lasts for fourteen days or longer, and finally [5], iii) dysentery, blood, and mucous found in diarrheal stool [6, 7]. Once diarrhea was one of the top deadly diseases [8, 9] and remains the second dominant cause of child mortality worldwide [10, 11]. Almost 1.7 billion cases of childhood diarrheal diseases are diagnosed each year globally, resulting in the annual death of approximately 525,000 children under five, which is narrated around 63% of the global

diarrhea burden [12]. Consequently, the acute diarrheal disease remains the top cause of morbidity and mortality among the pediatric population after a respiratory illness, especially in low-middle-income countries, and creates a serious public health issue [13, 14]. It is solely liable for one in eight deaths among children less than five years in Africa, Asia, and South America per annum, or a total of roughly 499,000 children every year are incriminated for diarrheal disease [13]. The far majority of which occurs in Sub-Saharan Africa, reflecting the highest child death rate in this region [14-16].

Only an episode of moderate-to-severe diarrhea has a significant repercussion on mortality and linear growth among survivors, facilitating the hazards of growth retardation, ill health, and cognitive impairment among the pediatric community [17-19]. Diarrheal diseases are a significant public health problem that affects children in developing countries where insufficient sanitation, hygiene, and portable water supply are the critical factors [13, 20-25]. In Bangladesh, one-third of the total child death burden is due to this life-threatening disease, diarrhea [26-28]. Every year, a rural child suffers from 4.6 episodes of diarrhea on average, from which about 230,000 children die [26]. Therefore, it is essential to find out the etiology with the proper diagnostic procedure, and only then can successive intervention of the disease management be possible.

Etiological Agents of Diarrhea

The rotavirus is the most prevalent among numerous viral, bacterial, and parasitic agents causing pediatric diarrhea [29, 30]. Some bacterial enteropathogens are also responsible for generating the same, such as *Shigella*, nontyphoidal *Salmonella* (NTS), Diarrhoeagenic *E. coli* (DEC), *Vibrio Cholerae*, *Campylobacter,* and *Yersinia* spp. [23, 31, 32]. Although enteric viruses and bacteria remain the predominant etiological agents [33-35], intestinal protozoal parasites are also significantly related to diarrheal disease in children, including *Cryptosporidium* spp., Giardia duodenalis *Entamoeba histolytica*, *Blastocystis hominis, and Dientamoeba fragilis* [36, 37].

Cryptosporidium as a Protozoan Diarrheal Agent

An intracellular Apicomplexan protozoan parasite belonging to the genus *Cryptosporidium* is accounted to be second only to rotavirus as the leading cause of moderate-to-severe diarrhea [38-40]. The organism attracted significance as one of the most prominent causes of diarrhea and diarrhea-causing death in young children, especially among the infant and immunodeficient individuals of developing countries worldwide [39, 41, 42]. As the annual detection rate of *Cryptosporidium*-ascribable cases is about 2.9-4.7 million in children under 2 years, the sub-Saharan African and South Asian territories face tremendous challenges in combating this infection [43].

Various species of this parasite have been identified, distinguished from each other regarding host range and public health concern [42]. Thus, *Cryptosporidium*, the ubiquitous

coccidian parasite, has over 40 established species, and of these, 20 species and subtypes account for the vast majority of the human gastrointestinal infections worldwide. Additionally, *C. homonis* and *C. parvum* are the leading culprits causing cryptosporidiosis globally [44, 45]. Transmission occurs in fecal-oral, either by zoonotic or anthroponotic transmission. *Cryptosporidium* spp. has a low infective threshold with a robust oocyst that survives adequately in moist, ambient environments and is resistant to many commonly available disinfectants such as chlorine [18, 46].

Effects of Cryptosporidiosis

Diarrhea associated with cryptosporidiosis has been linked to three fundamental mechanisms [39, 47, 48]: 1) osmotic diarrhea caused by malabsorption [49, 50]; 2) parasite-induced production of inflammatory products and host neurohumoral secretagogues [47, 48], and 3) secretory diarrhea caused by a parasite enterotoxin [51-53]. Different absorptive and secretory characteristics exist in different parts of the gastrointestinal system [54]. In immunocompetent persons, the small intestine, principally the ileum, is the primary location of Cryptosporidium infection. However, in AIDS patients, the gastrointestinal parasite distribution is more complex and extensive [55, 56].

The infection ranges from asymptomatic, self-limiting diarrhea to chronic [57-59]. The disease's intensity depends on the individual host's age, nutrition, and immune status [18]. The disease disrupts the intestinal epithelium, impairs the absorptive and barrier function of the small intestine, initiates prolonged (7-14 days) and persistent (≥14 days) diarrhea [18,60]. Though cryptosporidiosis is a self-limiting disease in immunocompetent individuals, the aftermath of this illness has far-reaching threatening hazards beyond diarrheal consequences. It also interferes with nutrient absorption, resulting in chronic malnutrition, poor growth, and premature mortality, especially in developing countries [37,61]. Despite enlisting *Cryptosporidium* in its "Neglected Disease Initiative 2004" by WHO, it's one of the significant causes of diarrhea in pediatric children [62]. Several factors are related to this infection, such as host, environmental, and parasite species. Long-term contact with domestic animals, overcrowded places, poverty, poor sanitation, contaminated water sources, the immune status of the individual, and malnourishment of the children also play a dire role in this infection [40]. Recent studies highlight a reduction in overall diarrheal episodes in Bangladesh by improving water sources and sanitation behavior. Still, cryptosporidiosis and growth faltering, a recognized upshot of this infection, hasn't decreased at all [63,64].

Studies on Cryptosporidium

Epidemiologic studies have demonstrated that *Cryptosporidium* is more prevalent in developing countries than developed countries [25, 39, 41, 65]. The organism has been reported as a substantial burden causing acute diarrhea [66]. A meta-analysis study on

consequences of childhood diarrhea caused by this protozoan infection showed that in 2016, it was one of the fifth significant diarrheal etiological pathogens globally in children younger than 5 years, and acute infection attributed to more than 48000 deaths, and more than 4·2 million disability-adjusted life-years lost [18]. The significance of this intestinal parasite can easily be realized by the current Global Enteric Multicentric Study (GEMS) done on children from seven Asian and African countries where 9,439 were moderate-to-severe diarrheal cases and 13,129 control subjects, unveiling four exclusive moderate-to-severe diarrhea-causing agents namely Rotavirus, *Cryptosporidium*, Enterotoxigenic *Escherichia coli,* and *Shigella* [67].

In a longitudinal cohort study on 392 Bangladeshi slum-dwelling children (in the first two years of life) performed from 2008 to 2014, *Cryptosporidium* infection was reported to be very common (77%). The study also highlights a close correlation between poverty and stunted growth during the first two years of life [40]. A survey of over 423 fecal samples from 185 children (up to five years) in an urban slum area of Bangladesh was done, and *Cryptosporidium* oocyst was detected in 9.2% cases, where the infection was highest among the children aged less than two. Moreover, that study also observed the infection decreases with age [68]. In a prospective study, fecal samples from children under 16 years attending an outpatient clinic in Cambodia were examined for *Cryptosporidium,* where these protozoan oocysts were detected in 2.2% to 7.7% cases [60]. In Tehran, a study was done in which stool samples from below 12 years old children with diarrhea were collected, and 1.1% of cases were found positive with this infection [66]. Another Iranian study reported that *Cryptosporidium* oocysts were detected in 3.8-8% of pediatric cases and immunocompromised patients, respectively [67].

Diagnostic Modalities

The diagnostic procedure comprises the microscopic examination of the fecal sample by wet mount and staining by modified Ziehl-Neelsen (mZN) staining [68] or auramine-phenol staining technique [69]. Direct and indirect immunofluorescence assays are expensive, but oocysts are readily identified [47, 70]. An immunological method (EIA, ELISA, and ICT) provides good sensitivity over microscopy but has drawbacks like false-positive results and is unavailable in developing countries due to cost ineffectiveness [71-73]. Though commonly used and cost-effective, the microscopic procedure is labor incentive and much time-consuming, and the process exclusively relies on an individual's skill and experience [56,74]. Moreover, molecular methods are extensively used for genotyping and molecular epidemiological studies because of their higher sensitivity (detection ranges from 1-10^6 oocysts) over the traditional microscopic and immunological procedure [44,75, 76].

Various genes of *Cryptosporidium* are documented targeting its species [77], such as small subunit rRNA (SSU rRNA), *Cryptosporidium* outer wall protein (COWP), 70-kDa heat shock protein (HSP 70), thrombospondin-related adhesive protein (TRAP-C2), dihydrofolate

reductase (DHFR) and actin genes [44, 78-80]. SSU rRNA and *gp60* are the most common genetic markers for *Cryptosporidium* species identification and subtype determination, respectively [81-83]. The SSU rRNA gene is considered enormously used in genotypic differentiation between infections belonging to both humans and animals. In addition, the 60-kDa glycoprotein (gp60) gene possesses highly variable regions that permit a good deal of intraspecies sequence heterogenicity. These sites are crucial to determining many *C. parvum* and *C. homonis* subspecies [44,56]. The majority of the pathogenic strains are detected by nested assay, which is also chosen for identifying the negligible amount of oocyst (<100) in the specimen [56].

As the parasite has a long-term detrimental effect on childhood growth, nutrition, and cognitive function with no appropriate drug or vaccine strategy, study on this parasite carries much importance. To date, no such research was previously done in Chattogram city. Hence, this study was designed to detect this pathogen and other parasites as a parasitic etiological cause of diarrhea in pediatric patients in this metropolitan city.

In the current study, microscopy by wet mount preparation, concentration technique (formol-ether sedimentation technique), and staining procedure (mZN) were applied to all samples. Later on, nested PCR was performed targeting SSU rRNA and the *gp60* gene. The study would help the clinician diagnose the case properly by exploring the actual etiology of protozoa-related diarrheal illness and indirectly minimizing the empirical use of antibiotics to treat parasitic diarrhea, thus reducing the Multi-Drug Resistant problem in Bangladesh.

Objectives of the Study

i. To identify intestinal parasites microscopically by wet mount preparation. ii. To identify *Cryptosporidium* microscopically by mZN staining technique. iii. To detect the presence of *Cryptosporidium* spp. by nested Polymerase Chain reaction. iv. To compare the result of microscopy with that of PCR.

Materials and Methods

Study Design: This was a cross-sectional observational study. Place of Study: Department of Microbiology, Chittagong Medical College, Chattogram, Bangladesh and Department of Pathology and Parasitology, Chittagong Veterinary and Animal Sciences University (CVASU), Chattogram, Bangladesh. Study Period: The study was carried out from July 2019 to June 2020.

Study Population: Indoor and outdoor pediatric diarrheic patients of Chittagong Medical College Hospital and Chattogram Maa-O-Shishu Hospital Medical College, Chattogram, Bangladesh.

Eligibility Criteria

Inclusion Criteria: The study was conducted on Indoor and outdoor pediatric patients (up to 18 years) suffering from acute watery and persistent diarrhea (≥14 days) irrespective of their social status, who agreed or whose guardians had the consent to enroll their children after full explanation of the study objectives. *Exclusion Criteria*: The study precluded the pediatric patients having bloody diarrhea (with blood and mucous in it) and the adult patients. Moreover, the patients or guardians who were unwilling to sign the assent form were excluded. The macroscopic Study plan is illustrated in Figure 1.

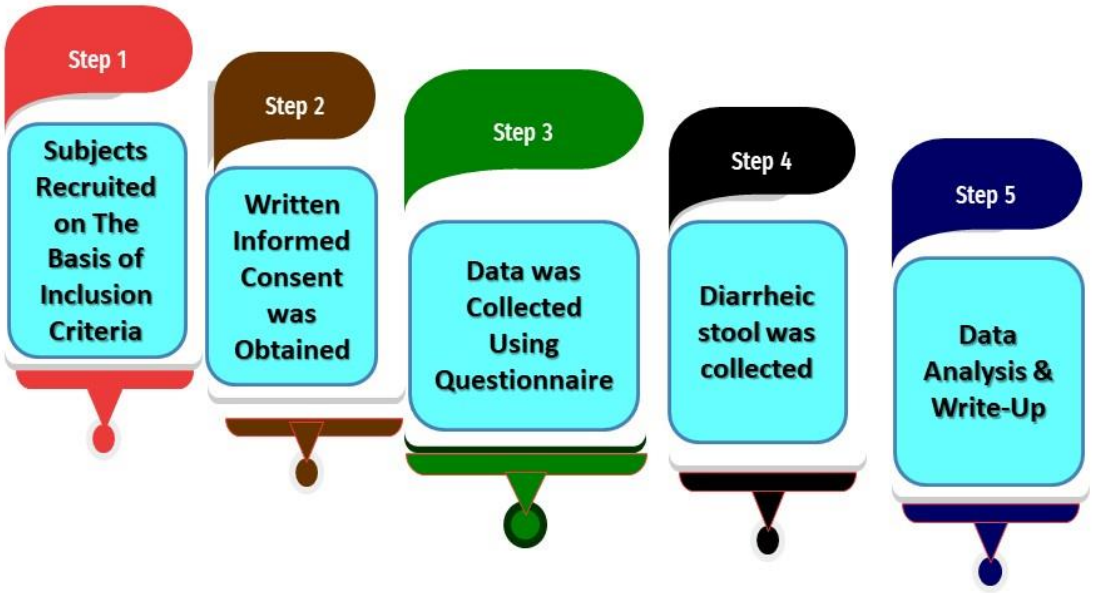

Figure 1: A Flow Chart Depicting Macroscopic Study Plan.

Ethical Approval

This study received ethical approval [Reference No.: CMC/PG/2019/592, Dated November 3, 2019] from the Institutional Review Board of Chittagong Medical College Hospital (CMCH) 57 K.B. Fazlul Kader Rd, Chattogram 4203, Bangladesh. Participation and reporting included herein were hinged on consent forms signed by patients/guardians of the pediatric diarrheic patients (children up to 18 years) before administering the questionnaire. The respondents were informed correctly using the participant's information sheet about their rights and all the relevant aspects of the study, including its aim and interview procedure. No patient was older than 18 years.

Study Sample

Diarrheal stool.

Sample Size

$n=\frac{z2pq}{d2}$

=123.2

Where n is the sample size.

z is the confidence interval (95%) z=1.96.

p is the pre-estimated prevalence of 9.2% obtained from a study performed by Ahmed in Dhaka [84], Bangladesh.

d is a marginal error (5%).

$n = (1.96)^2 x 0.092 x 0.908 / (0.05)^2$

=123.17

This means a minimum of 123 participants need to participate in this study.

Sampling Technique

Nonprobability, purposive type of sampling.

Preparation of Questionnaire

The questionnaire was prepared and accustomed from the study by Tombang, Cameroon [85]. The questionnaire was modified under the study's eligibility criteria and Bangladeshi cultural aspects. The questionnaire was comprised of a few segments. In the first segment, particulars of the patients were included; then, socio-demographic history, habitual elements, and clinical features were incorporated.

Data Collection

Indoor and outdoor diarrheic pediatric age group patients in some selected tertiary hospitals were selected. Informed written consent from patients or legal guardians was obtained after a full explanation of the outcome and purpose of the study. After taking consent, the patient's history details, including demographic information and clinical findings, were recorded in a predesigned case record form. Then stool sample was collected in a clean, leak-proof, wide-mouth container.

Method of Sample Collection

The sample was collected in a clean, leak-proof, wide-mouth container appropriately labeled with the patient's name, age, time of collection, and identification number. Then it was transported to the Department of Microbiology, and some portions of each sample were transferred to the Eppendorf tube and refrigerated at -80ºC to perform the molecular method [86]. Again, the rest of the sample was taken into two containers. One container contained a 10% formalin preserved fecal sample for direct wet mount preparation, and the other retained an unpreserved fecal sample. Afterward concentration procedure was followed with this unpreserved sample before the staining procedure.

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Laboratory Procedure

All samples were undergone to wet mount preparation by both saline and iodine preparation and staining by mZN staining Figure 2 and Figure 3. A fecal smear was made from a concentrated stool sample on a clean, grease-free glass slide. After fixation, mZN staining was done, and the slide was examined under a microscope using oil immersion. *Cryptosporidium* oocysts were identified as red, small (4 to 6 µm) sized, characteristically round or slightly ovoid, acid-fast oocyst against a blue background [87].

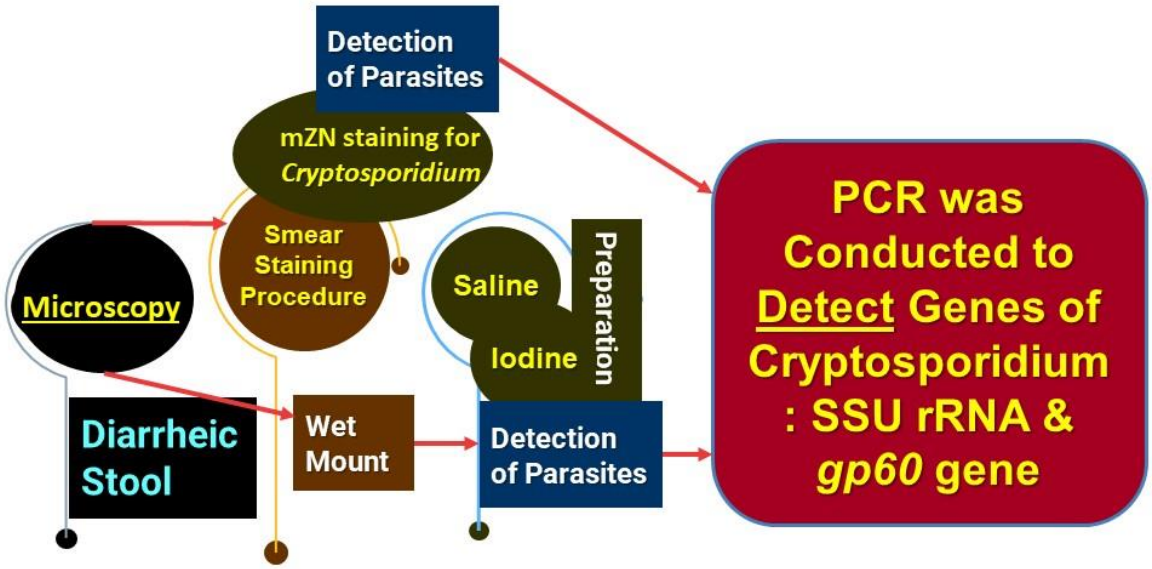

Figure 2: Illustrating the Steps of Laboratory Methods [31].

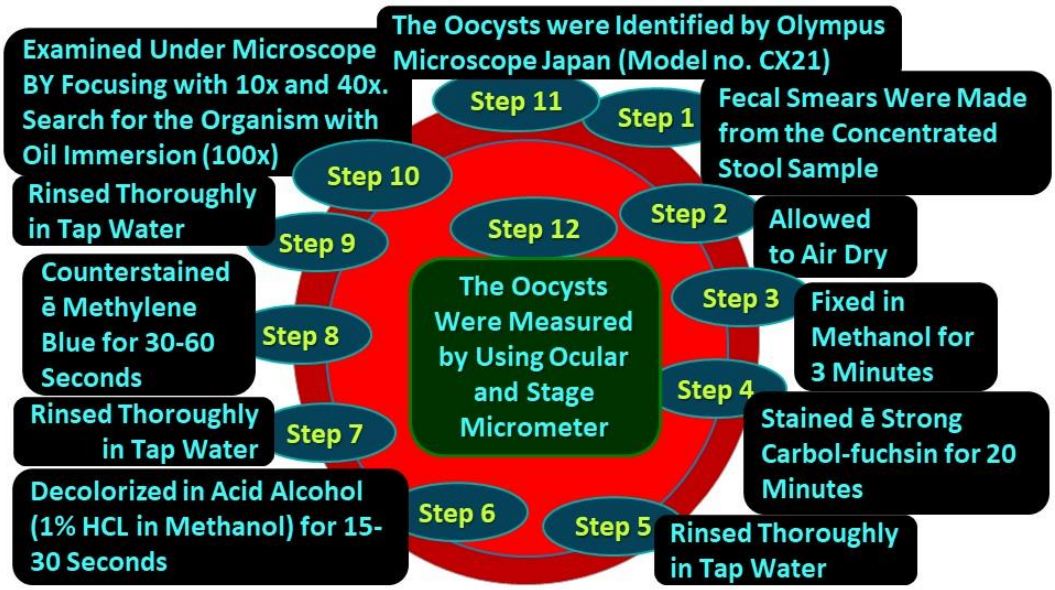

Figure 3: Illustrating the Steps of Modified Ziehl-Neelsen Stain [31].

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The Procedure of Polymerase Chain Reaction (PCR)

Process of DNA Extraction

DNA extraction was done as per the manufacturer's instruction by The Invitrogen PureLink Microbiome DNA Purification kit (Thermo Fisher Scientific), and the procedure was performed at room temperature (20–25°C). The purified DNA in the tube was preserved at -20°C for further use.

Primers used for SSU rRNA and *gp60* gene[31] are listed in Table 1.

For the SSU rRNA gene, the first pair of primers (Table 1) were used as first-round PCR to amplify the 830 bp sequence of the SSU rRNA gene, and the second pair of primers (Table 1) were used for second-round PCR to amplify a 240 bp sequence. For the *gp60* gene, first set primers (Table 1) were used as first-round PCR to amplify the 412 bp sequence, and the next pair of primers (Table 1) were used for second-round PCR to amplify the 350 bp sequence of the *gp60* gene.

Preparation of Reaction Mixture

Sterile micro-centrifuge tubes (1.5 ml) were taken and labeled with the date and identification number. Primer tubes were centrifuged for a few seconds. Then it was vortexed for 15 seconds and finally diluted with nuclease-free water to make a 1:10 dilution. For each sample, a total of 20 µl of the mixture was prepared by mixing 10 µl of master mix (mixture of dNTP, Taq Polymerase, $MgCl_2$, and PCR buffer), 1 µl forward primer, 1 µl of reverse primer, 2 µl DNA template and 6 µl of nuclease-free water.

Table 1: Primers used for SSU rRNA gene and *gp60* gene of *Cryptosporidium* species.

		Primer name	Primer sequence (5'-3')	Size
SSU rRNA gene	First Set Primer	XF2	F-GGAAGGGTTGTATTTATTAGATAAAG	830 bp
		XR2	R-AAGGAGTAAGGAACAACCTCCA	
	Second Set Primer	PSSUf	F-AAAGCTCGTAGTTGGATTTCTGTT	240 bp
		PSSUr	R-ACCTCTGACTGTTAAATACRAATGC	
gp60 gene	First Set Primer	gp15-ATG	F-ATGAGATTGTCGCCTCATTATC	412 bp
		gp15-STOP	R-TTACAACACGAATAAGGCTGC	
	Second Set Primer	gp15-15A	F-GCCGTTCCACTCAGAGGAAC	350 bp
		gp15-15E	R-CCACATTACAAATGAAGTGCCGC	

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Cyclic Condition used for Nested PCR [88] (Figure 3)

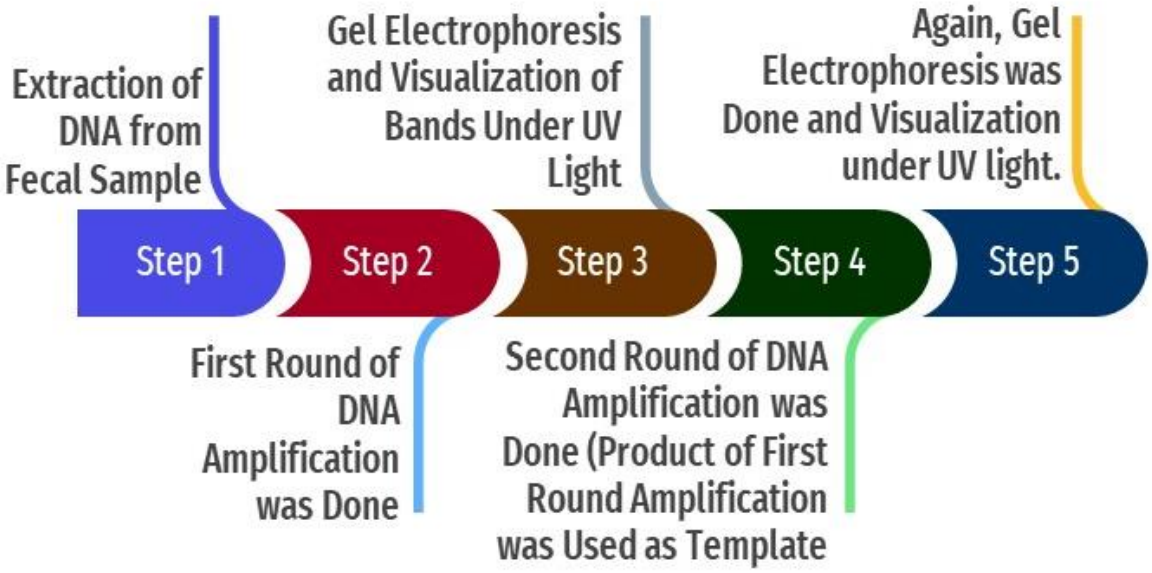

Figure 4: Flow Chart of Nested PCR.

SSU Gene of Cryptosporidium Species

1st Round PCR
Initial denaturation at 94^{0}C for 5 min followed by 30 cycles of denaturation at 94^{0}C for 45s,
primer annealing at 45^{0}C for 2 minutes,
extension at 72^{0}C for 1.5 minutes and a
final extension at 72^{0}C for 10 min.
2nd round PCR
Initial denaturation at 94^{0}C for 5 min followed by 35 cycles of denaturation at 94^{0}C for the 30s,
primer annealing at 55^{0}C for the 30s,
extension at 72^{0}C for the 30s, and a
final extension at 72^{0}C for 10 minutes.

gp60 gene

1st Round PCR
Initial denaturation at 94^{0}C for 5 min followed by 35 cycles of denaturation 94^{0}C for the 30s,
primer annealing at 55^{0}C for 45 s,
extension at 72^{0}C for 1 min and a
final extension of 72^{0}C for 10 min
2nd Round PCR
Initial denaturation 94^{0}C for 5 min followed by 30 cycles of denaturation 94^{0}C for the 30s,
primer annealing at 55^{0}C for the 30s,

extension at 72^0C for the 30s, and a
final extension at 72^0C for 10 minutes.
In the nested PCR, the amplified product of the first round of PCR is used as the template. The second set of primers was added to the reaction mixture in the second amplification (nested PCR). The PCR reactions were conducted in a thermal cycler (Applied Biosystems 2720, MA).

The amplicon size was determined by comparing the position of the amplicon concerning that of a 100 bp DNA ladder that was loaded in the adjacent well and simultaneously electrophoresis.

Interpretation

Samples were scored as PCR positive for *Cryptosporidium* spp., when PCR product of 240 bp could be detected for SSU rRNA gene. Samples were detected as PCR positive for *Cryptosporidium* spp., when 350 bp could be seen for the *gp60* gene.

Data Analysis

A questionnaire was used for each of the cases. A predesigned questionnaire systematically recorded all the relevant information (history), socio-demographic history, clinical findings, and laboratory findings of every case. The data were analyzed using the Statistical Package for Social Sciences (SPSS) version 25.0. Statistical analysis was done by standard statistical procedure; MS Excel prepared graphs and charts. The result was presented in tables and figures. A p-value <0.001 was considered significant.

Results

Distribution of The Study Population According to Their Age and Sex.

A total of 219 fecal samples were collected from pediatric diarrheic patients of two tertiary medical college hospitals. The age range of the patients was 2 months to 18 years old. At first, all samples were examined for the direct microscopic examination, then modified Ziehl-Neelsen staining, and afterward, nested PCR was done for specific target genes of Cryptosporidium. In the present study, age distribution shows that most of the study population belonged to the 1 to 5 years of age group (47%) followed by <1 year of age group (30.1%) (Table 2). Among them, 125 (57%) were male, and 94 (43%) were female. The male to female ratio was about 1.33:1.

Table 2: Distribution of the Study Population by their Age (n=219).

Age Group	Frequency	Percentage (%)
<1	66	30.1%
1-5	103	47.0%
6-10	37	16.9%
11-15	11	5.0%
15-18	02	0.9%
Total	219	100%

Microscopic Assay

a) Wet Mount Preparation: All fecal samples were first examined for direct microscopic examination by both saline and iodine preparation. Table 3 showed wet mount findings among 219 study samples where Giardia was found positive in 5 (2.3%) samples. Moreover, other parasites like helminthic eggs were found in some samples. Ova of Ascaris lumbricoides 3 (1.4%), Trichuris trichiura 1 (0.5%) and mixed infection 2 (0.9%) were detected.

Table 3: Detection of the Parasite by Wet Mount in the Study Population (n=219).

Parasites	Frequency	Percentage (%)
Giardia	05	2.3
A. lumbricoides	03	1.4
T. trichiura	01	0.5
A. lumbricoides & *T. trichiura*	02	0.9
No parasite	208	95
Total	219	100

b) Identification of Cryptosporidium by mZN Staining: The microscopic examination of the stool samples through mZN staining showed the presence of Cryptosporidium oocysts (Figure 4) in 3/219 (1.4%) samples (Table 4).

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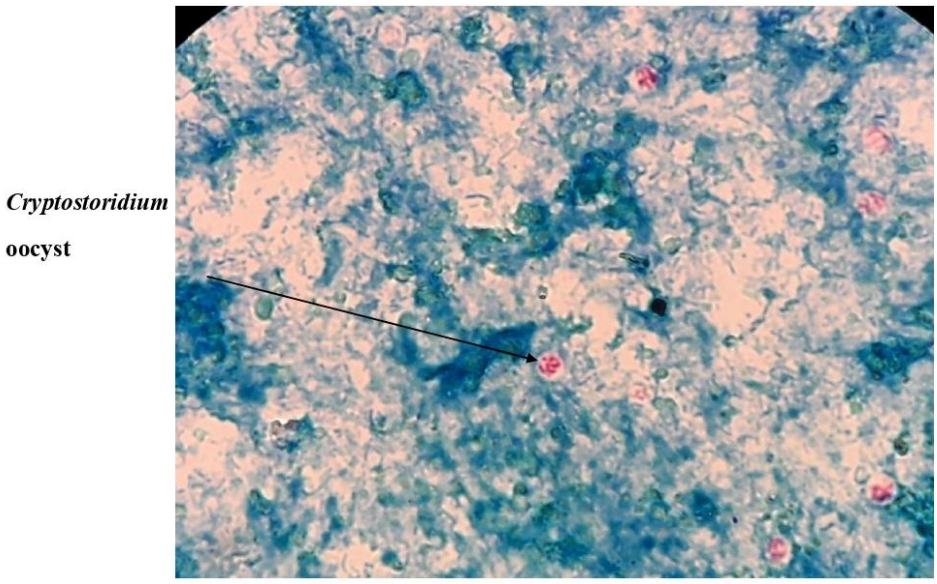

Figure 5: Cryptosporidium oocyst in mZN Stain.

Table 4: Association of microscopic findings of *Cryptosporidium* with nested PCR (n=219).

Microscopic findings	Nested PCR findings		Total	p value
	(+ve)	(-ve)		
Modified Ziehl-Neelsen staining				
Cryptosporidium (+ve)	03 (33.3%)	00 (0.0%)	03	
Cryptosporidium (-ve)	06 (66.7%)	210 (100%)	216	<0.001
Total	09	210	219	

Notes: *p* value derived from chi-square test. Figures within parentheses indicate the percentage.

Nested PCR Findings

Nested PCR detected *Cryptosporidium* oocysts in 9 (4.1%) samples (Figure 4). The bands of SSU-rRNA (240 bp) and gp60 genes (350 bp) were shown in Figures 5 and 6, respectively.

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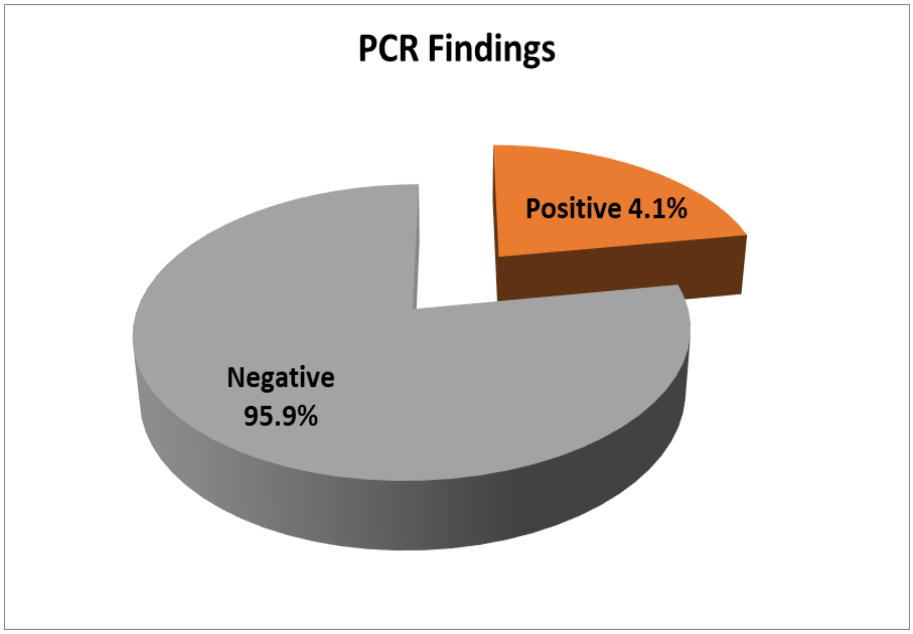

Figure 6: Distribution of *Cryptosporidium* spp. by nested PCR (n=219)

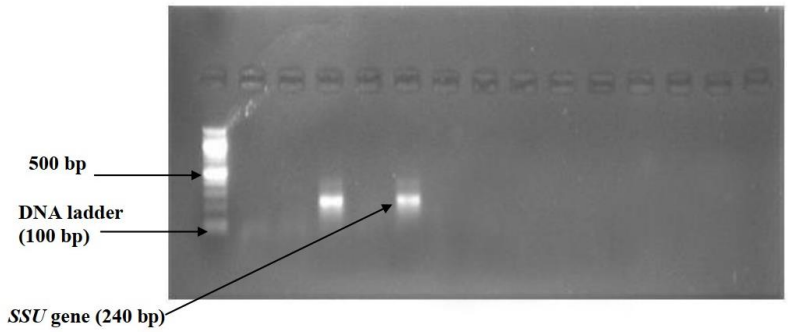

Figure 7: Bands of amplified DNA of *Cryptosporidium* spp.

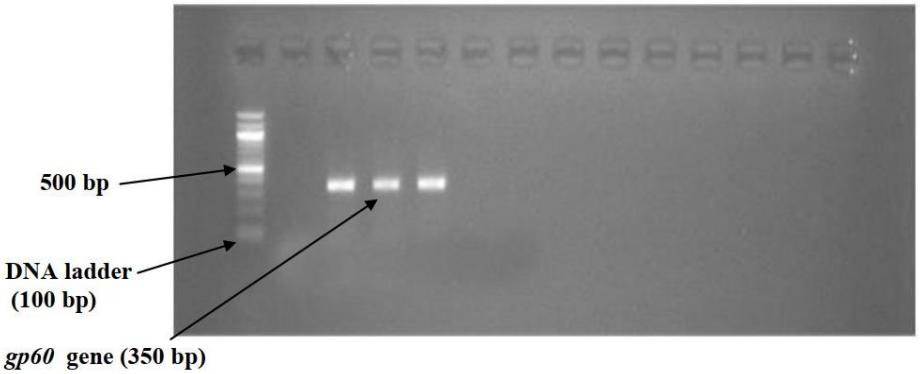

Figure 8: Bands of amplified DNA of *Cryptosporidium* spp.

Frequency of Cryptosporidium in different Age and Sex Groups.

The prevalence of *Cryptosporidium spp*. was more in the 1-5 years' age group, and male predominance was observed, but both were statistically non-significant.

Association of Microscopic Findings with that of Nested PCR.

Table 4 shows the association between microscopic and nested PCR findings of Cryptosporidium. Here Chi-square test was done, and it was found to be highly significant statistically ($p<0.001$).

Sensitivity, Specificity of *Cryptosporidium Spp. Microscopy*

The sensitivity and specificity of *Cryptosporidium Spp*. Microscopy was found to be 33.3% and 100%, respectively, taking the PCR method as the gold standard.

Associated Factors for Parasite Infestation

Most of the study population (64.8%) drink safe water, and 70.3% use regular hand washing.

Multivariate Logistic Regression to Determine the Independent Factors Associated with Cryptosporidium Infection

Table 5 summarizes the risk factors associated with cryptosporidiosis, including male gender, unsafe drinking water, irregular hand washing, rural residence, insufficiency of exclusive breastfeeding, and history of having pets. It reflected the use of unsafe drinking water (OR=0.255, P=0.043), lack of regular hand washing (OR=0.109, P= 0.001), and insufficiency of exclusive breastfeeding (OR=0.261, P=0.047) were independently associated with Cryptosporidium infection. But other factors like male gender (OR=0.938, P=0.925), rural resident (OR=0.335, P=0.097), H/O having a pet (OR=2.26, P=0.251) have no significant association with the infection.

Table 5: Multivariate Logistic Regression to determine the independent factors associated with *Cryptosporidium* infection.

Variables	Adjusted OR	95% CI		P value
		Lower	Upper	
Male gender	0.938	0.245	3.591	0.925^{ns}
Unsafe drinking water	0.255	0.062	1.051	0.043^{s}
Irregular hand washing	0.109	0.022	0.540	0.001^{s}
Rural resident	0.335	0.087	1.290	0.097^{ns}
Insufficiency of exclusive breast feeding	0.261	0.063	1.074	0.047^{s}
H/O having pet	2.263	0.542	9.455	0.251^{ns}

CI = Confidence interval, OR = Odds ratio, s = Significant, ns = Not significant

The bar diagram shows the distribution of *Cryptosporidium* spp. by nested PCR; among 219 samples, 9 (4.1%) were *Cryptosporidium* positive.

Discussion

Intestinal parasites such as *Cryptosporidium* spp. along with other parasites are liable for diarrhea, especially among children in developing countries [89]. Though infections due to these parasites are self-limiting in immunocompetent individuals, chronicity often results in malnutrition, growth faltering, and cognitive function impairment, especially in children [90-95]. Because of these alarming effects on a child's health, it draws attention to find out the incidence of protozoan parasites responsible for childhood diarrheic disease.

In our study, 2.3% cases were found *Giardia* positive by wet mount preparation among 219 samples. The prevalence of *Giardia* infection among 0-15 years Portuguese children was 1.9% when estimated by direct microscopic examination. Nevertheless, when monoclonal ELISA techniques were utilized, the rate increased to 6.8% [96]. Furthermore, the higher rate (7.8%) of Giardia infestation was found in the younger group (0-5 years), and there was no difference observed between sexes [96]. A study among pediatric patients (<5years) in a tertiary hospital was done where *Giardia* cysts were found in 4.14% of cases [97]. In a slum area of Bangladesh, *Giardia* positive samples were found in 6.01% cases among the school-going children [98]. In Sikkim of India, a prospective study among symptomatic children (<15 years) was done where *Giardia* cysts were found in 5% of cases by wet mount [99]. About 8.2% of patients were positive for giardiasis through the direct smear method in Kashmir Valley, India [100]. Another study in Lucknow of India showed that *Giardia* was detected 15.5% by immediate wet mount preparation [101], much higher than the current study findings. This dissimilarity may be due to the large sample size (n=1680) and large age group distribution (3 to 45 years). The geographic area may also be a factor for this difference.

In wet mount film, the current study showed that ova of *Ascaris lumbricoides* 3 (1.4%), *Trichuris trichiura* 1 (0.5%), and mixed infection 2 (0.9%) were detected. A study reported 0.9% *Trichuris trichiura,* but no *Ascaris lumbricoides* were found on hospitalized pediatric diarrheic children (<5 years), according to our study [88]. Another study demonstrated 0.6% of *Trichuris trichiura* ova, but *Ascaris lumbricoides* was 9% [102]. Another study reported prevalence of *Ascaris lumbricoides* and *Trichiuris trichura* ova was 8.2% and 0.9%, respectively [103]. In our research, the relatively low number of helminths was detected because of the urban setting of the study population, who probably had better sanitation and hygiene practices. Another cause could be the ingestion of anthelminthic drugs at a regular interval.

Age and sex-specific vulnerability are essential for the prevalence of diarrheal illness, where various factors can contribute to this. These may include biochemical factors like hormones, enzymes, specific proteins, genetic or immunologic factors, food habits, culture, etc. [104].

Cryptosporidium was found positive in 1.4% of diarrheic samples by modified Z-N staining. A surveillance study on children under 12 years old in Tehran was done where the prevalence rate of the protozoa was 1.19% [66]. Another survey on pediatric children (<5 years) reported 2.3% *Cryptosporidium* oocyst [97]. A study reflected detection rate of *Cryptosporidium* spp. from the outdoor patients of ICDDR'B was 4.44% [40]. In Malaysia, the prevalence of these protozoa among diarrheic subjects (<12 years) was 4.62% by modified Ziehl-Neelsen (mZN) staining [105, 106]. In Cameroon, a hospital-based cross-sectional study among children aged <5 years reflects that the prevalence of *Cryptosporidium* infection was 13.4% in microscopy (mZN staining), having the highest detection rate at 31-60 months' group [85], which is dissimilar to this study. Several factors may play a vital role in this higher prevalence of cryptosporidiosis, such as geographic region, seasonal variation, age, personal hygiene, drinking or using untreated water, no exclusive breastfeeding, low educational status, and poor socioeconomic status of the parents.

In this study, some pitfalls are responsible for the small number of trophozoite and/or cyst and oocyst detection. Direct microscopic examination after modified Z-N staining of *Cryptosporidium* relies on morphologic recognition of small-sized oocysts that may be scanty in number. Hence, that could not be easy to find out. Again, they could be inconsistently stained, and misdiagnosis may happen. Therefore, this method is impractical to standardize as it is influenced by the microscopist's individual skills of the microscopist involved[42].

Moreover, the procedure is incapable of detecting the low parasite count. Collecting at least three stool samples on alternate days is often indicated because of intermittent shedding of protozoan cysts and oocysts. But it's difficult and inconvenient to go for three samples from the same patient. Though conventional microscopy of more than one fecal sample is still recommended to diagnose intestinal protozoa in the stool samples, its sensitivity is still low even after multiple examinations. Also, the parasite might be disguised by bile pigment and not visualized by wet mount examination [101]. But this study was done with a single fecal sample so that a low parasite concentration could be missed through microscopy. These could be the reason behind their relatively low rate of detection.

By nested PCR, *Cryptosporidium* spp. was found positive in 4.1% samples. A study was shown, where the prevalence of *Cryptosporidium* was 4.8% among children below five years of age with diarrhea [107]. A longitudinal cohort study was done on Bangladeshi slum children where the same procedure found *Cryptosporidium positive* cases in 6.3% cases [40]. A prospective study on children under 16 years showed that 7.7% of patients were detected by PCR [60]. Another study found the prevalence of this parasite is about 1.3% [108]. Improved sanitation, safe drinking water, awareness about health, and hygiene enable lower prevalence of these parasitic diseases in the community [109]. Another cause may lie in examining a single fecal specimen per individual for diagnosis, which is less sensitive than the use of multiple samples [44, 56].

Moreover, the study was performed in urban settings rather than rural or slum areas. PCR is considered a more sensitive and specific diagnostic method, but some limitations remain. There is a list of PCR inhibitors in stool samples, namely lipids, hemoglobin, bilirubin, bile salts, polysaccharides from mucous, bacteria, and food degradation products which can also affect the result of amplification. To minimize the effect of DNA inhibitors, commercial kits, including extraction columns, have been used to purify the DNA. Despite using such a kit, false-negative results may happen [110, 111].

The highest cases of *Cryptosporidium* were detected in the same age group (1 to 5 years) 7/103 (6.79%) followed by <1-year age group 2/66 (3.03%), which was not statistically significant according to p value (p=0.376). Moreover, we revealed from our study that all *Cryptosporidium* positive cases were <3 years old children. It was shown in a study that the highest prevalence of *Cryptosporidium* infection was found in 3-4 years (14.3%) followed by <1-year age group (4.5%) [107]. A study found that *Cryptosporidium* infection was predominant among children <5 years (22%) [49]. The majority of the infected cases were children <4 years of age [107]. Children at this age (1 to 5 years) develop a habit of putting unwashed hands, toys, and other objects inside their mouths.

Moreover, their compromised immunological status and poor hygiene practices lead them susceptible to intestinal infections, especially at this period [112, 113]. The infection rate decreased in higher age groups with minimum infection rate because of the improved immunological status of the individual [39, 114]. It is observed that there is a difference in detection rate with other studies. One reason could be that it was a tertiary hospital-based study rather than a rural or slum-based one. On the other side, our study's low detection rate may suggest an improved living standard of the study participants. It may be due to the improvement of the sanitation and hygiene system. Empirical use of antiparasitic drugs could be one of the reasons behind the low prevalence of intestinal parasites in our country [115, 116]. Failure to detect intestinal protozoan parasites may be due to seasonal variation and the intermittent nature of excretion of this parasite in the stool [47, 117].

In this study, male predominance was observed in the case of *Cryptosporidium Spp.* Infection was similar to Hawash et al. [118], but the opposite picture was found by another study where females [119, 120] were affected more. This discrimination is unclear because under-five children of both sexes are engaged in the same recreational activity and are likely to be exposed to the same environmental conditions but could probably be because the males constitute the majority of the study population.

Regarding this study, the association of microscopic findings with PCR for pathogens showed high statistical significance (p<0.001). The sensitivity of *Cryptosporidium* microscopy was 33.3%, and specificity was 100% in the present study. Two other studies found similar validity [121, 122].

Globally, foodborne cryptosporidiosis has been accounted for the significant reason for such infestation [123-125]. Additionally, drinking water from swimming pools, waterparks, fountains, lakes, and rivers contaminated with *Cryptosporidium* remains one of the common causes of spreading cryptosporidiosis [126-128]. This water source could be contaminated by fecal matter. In some city areas, sewage and toilet wastage may get into the surface water, which could be a potential concern for the fecal-oral transmission of this protozoan parasite. Furthermore, cryptosporidiosis has been positioned 5th among the 24 most significant foodborne parasites [129, 130]. The World Bank reported that 98% of Bangladeshi people currently have access to better-quality water. However, water quality remains poor across the country because 80% possess microbial contamination [131]. UNICEF/WHO reported that about 25% of the Bangladeshi population does not have safe drinking water resources in their homes [132]. The results of risk factor analysis support the role of having unsafe drinking water in *Cryptosporidium* infection that is statistically significant (p-value=0.043), which agrees with another study conducted in Pakistan [133]. Another critical issue is that *Cryptosporidium* oocysts are highly resistant to numerous disinfectant agents [133-135] and are killed by boiling water above 70 ^{0}C less than 1 minute [136]. In addition, multiple studies, including the WHO guidelines for drinking water quality, also suggest that those oocysts become non-viable and inactivated at 60-71.7^0 C [119, 125, 133, 138].

Another salient factor behind protozoal diarrhea is inadequate hygiene practices like handwashing [139-141]. Our study found a significant association between poor hand washing practice and cryptosporidiosis (*p* value=0.001). It may be due to improper washing of hands during the handling of infant feeding bottles. Moreover, there is a tradition of practicing force-feeding techniques with bare hands to ensure enough food intake by the children to support their growth [142, 143]. The hazard of bare hands (not properly washed) enables direct transmission of this foodborne protozoan disease [144, 145]. These malpractices welcome chances to ingest food and water contaminated with oocysts described above and are shed from infected individuals [146, 147].

Undoubtedly, breastfeeding has a widespread impact on children's health conditions, and there remains a significant correlation between breastfeeding and diarrhea [148-150]. Our study speculated that failure of exclusive breastfeeding was associated with *Cryptosporidium* infection [120, 151]. It can be explained that the mother's milk provides the bulk amount of antibodies, Immunoglobulin A (IgA), which offers passive protective immunity against various parasitic infections [120, 151, 152]. Multiple studies reported that parasitic-infested infection is almost imprisoned till 6 months of infant and when complementary foods are associated with infective diarrhea [107, 153, 154].

Though contact with animals has been reported as a risk factor for this infection [155-157] nevertheless, we observed a higher distribution of this pathogen in children without animal rearing. Our findings were similar to earlier studies [43, 158]. The finding suggests the

transmission mode could be person-to-person contact—no association of the pathogen with the rural resident, supporting some previous studies [108, 114, 159, 160].

Conclusion

Therefore, it can be concluded that the PCR method demonstrated better results than the traditional microscopic method as it yields more positive results. Using this procedure, we can adequately diagnose the parasitic causes of diarrhea among the children, bringing considerable advantages in their treatment. As a result, irrational use of antibiotics will be reduced, and the rate of antibiotic resistance will be minimized. Moreover, it can be used for research purposes to detect protozoan parasites like *Cryptosporidium*. This study gives an idea about the prevalence of protozoan causes of diarrhea in this locality or region.

Recommendation

The samples were identified positively by mZN staining for *Cryptosporidium* spp.; oocyst was successfully proved positive by molecular assay (PCR), which recommends the practice of PCR approaches and conventional microscopic procedures to overthrow the practice the diagnostic drawbacks. So, the staining method should be established in combination with wet mount preparation as routine examination for stool to detect and diagnose the parasite effectively. Moreover, molecular analysis is also recommended where facilities are available. This would help us precisely diagnose the protozoa. Further molecular analysis like DNA sequencing can be done, which will provide us with specific information about the species and genotypes, hence lending the researcher a transparent idea about the epidemiology - to identify risk factors, mode of transmission, pathogenicity, genetic diversity, etc.

Key Messages

- Parasites are one of the critical causes of pediatric diarrheic disease in our community.
- mZN staining should be introduced in routine-based detection of *Cryptosporidium* spp.
- The practice of regular hand washing and consumption of boiled water is an essential factor in minimizing parasitic infection, including cryptosporidiosis.

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