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Cryptosporidium Detection in Pediatric Diarrheal Cases: Microscopic, Immunological and Molecular Approaches in Kirkuk, Iraq

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ABSTRACT

Background: *Cryptosporidium is a protozoan parasite that causes digestive issues in children and is a major contributor to the increasing burden of waterborne parasitic infections. It is recognized as one of the primary causes of diarrheal disease among children. In low-resource settings like Iraq, poor sanitation significantly elevates the risk of outbreaks, thereby threatening child health and adding to the economic burden.* ***Purpose****: This study aimed to assess and compare the diagnostic performance of four distinct techniques: direct smear microscopy stained with modified Ziehl-Neelsen (MZN), concentration (formalin-ether sedimentation), rapid immune-chromatography, and molecular-PCR techniques for the detection of Cryptosporidium among diarrhoeic children in Kirkuk City, Iraq.* ***Methods****: A total of 150 stool samples were collected from diarrhoeic children across multiple hospitals between September 2024 and March 2025. These samples were analyzed using four diagnostic approaches.* ***Results****: Out of 150 samples, 91 (60.7%) tested positive using MZN, 96 (64%) via concentration, 103 (68.7%) through rapid immune-chromatography, and 111 (74.0%) using PCR. Compared to PCR, the sensitivity for MZN, concentration, and rapid immune-chromatography was 62.16%, 63.96%, and 74.86%, respectively; specificity was 43.59%, 35.89%, and 48.72%, respectively. Statistical analysis revealed no significant agreement for MZN and concentration (p = 0.527, 0.988), while rapid immunochromatography showed significant concordance (p = 0.007).* ***Conclusion:*** *While MZN, concentration, and rapid tests are useful, PCR remains the most sensitive and specific, offering genotyping capability, batch processing suitability, and high diagnostic accuracy, PCR is considered a valuable tool for future diagnostic and molecular epidemiological studies.*

Keywords: Cryptosporidium, Diarrhoeic Children, Rapid Immuno-Chromatography, Molecular-PCR.

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INTRODUCTION

Cryptosporidium is a widespread obligate microscopic apicomplexan parasite that infects a variety range of animals, including humans in developed and developing countries [1]It is a well-established pathogen of the gastrointestinal tract that produces cryptosporidiosis [2,3] This disease is regarded as the fifth most important food-borne parasite and the most important parasite causing watery diarrhoea in the world [4] Some epidemiological studies from Asian and African countries indicated that *Cryptosporidium* is the second most common protozoan parasite in children causing severe diarrhoea and significant morbidity [5,6] In Asia especially among low-income people, it has a high frequency of *Cryptosporidium*, particularly among young individuals [7] Today, *Cryptosporidium* is recognized as a leading cause of diarrhoea in children. Moreover, it plays a critical role in the cycle of infection, malnutrition, delayed growth, poor cognitive functions, and potentially colon cancer, depending on immune status, nutrition, and age [8–10]

Around the world, more than 44 species and 120 genotypes of *Cryptosporidium* have been identified. However, C. hominis and C. parvum have been reported to account for the vast majority of infections in humans [11,12] These two species are responsible for nearly 95% of human infections [13] *Cryptosporidium* has multiway transmission, direct faecal-oral transmission of oocysts facilitating outbreaks via person-to-person (C. hominis and C. parvum) or animal-to-person (C. parvum) contact, as well as indirect environmental transmission of oocysts through contaminated water and food [14,15] The finding of *Cryptosporidium* oocysts in stool samples is essential for diagnosing cryptosporidiosis. There are many ways for the detection of *Cryptosporidium* among infected children; earlier studies relied on modified Ziehl-Neelsen staining (modified acid-fast staining). In contrast, modern studies using immunological and molecular assays for detecting *cryptosporidium* [16] Direct smear preparation of stool or using sediment of formalin-ether technique for smear preparation and applying modified Ziehl-Neelsen staining (MZN) is an ordinary method for *Cryptosporidium* oocyst diagnosis in microscopic consideration [17,18] Although antigen detection methods using enzyme-linked immunosorbent assay (ELISA), immunofluorescence, or immune-chromatographic assays have several advantages, such as feasible sensitivity, specificity, and practicability, their high costs and short lifetime of antigen release render them transient methods for the diagnosis of acute and symptomatic cryptosporidiosis [19,20] Molecular techniques are essential to discover *Cryptosporidium* spp. because their identification cannot be made by morphological or biological characteristics alone, nor by employing adequate culture techniques. To identify isolates of *Cryptosporidium* species, only molecular methods are employed, such as DNA sequencing of PCR products [21] The targeted 18S rRNA gene is a multi-copy gene that facilitates the sensitivity of the assay and has been the target gene of a number of molecular diagnostic tools for *Cryptosporidium* spp. [22] Detection are *Cryptosporidium* spp. in clinical specimens is important, not only for identifying the cause of disease but also for epidemiological surveys, disease surveillance and food and drinking water monitoring [21]

Cryptosporidium was recorded for the first time in Iraq in 1994 in diarrheic stool samples among children under five years old in Basrah Province [23] Then, between 1997 and 1998 in three hospitals in Basra, *Cryptosporidium* was isolated from stool samples of both healthy and sickle-cell anaemic patients [24] After these records, this parasite were recorded continuously in many samples in human and animal in different parts of Iraqi including Kurdistan Region by using different laboratorial techniques and some of these studies were mentioned here [25–44] The current study compares the efficacy of direct smear stained with MZN microscopy, concentration, rapid immune-chromatography and molecular-PCR detection of *Cryptosporidium*, as well as to determine the prevalence of *Cryptosporidium* in children with diarrhoea in Kirkuk City, Iraq.

METHODS

A cross-sectional study was done on stool samples, which were collected from a period between September 2024 to March 2025. Direct smear stained with MZN, concentration and rapid immune chromatography were done in the Parasitology laboratory of the hospitals where the samples were collected and the molecular study was done at the Molecular laboratory in the Medical Laboratory Department at College of Health & Medical Technology, Sulaimani. Polytechnic University, Iraq.

Sample Collection

A total of 150 stool samples were collected in a sterilised disposable 60ml plastic container from diarrheic children in three hospitals (Azadi Teaching Hospital, Al-Nasir Hospital, and Children's Hospital) in Kirkuk City. The patient's age, sex and demographic status were reported. Each sample was divided into four parts: a first small part for direct smear microscopic examination stained with MZN, the second part used for concentration (formalin-ether sedimentation) technique stained with MZN, the third part used for rapid immune-chromatography test and the final part stored at -20°C in Eppendorf tubes for molecular-PCR study [45]

Microscope Examination

For microscopic examination, direct smears were prepared after that stained with modified Ziehl- Neelsen stain and the concentration technique used formalin-ethyl acetate sedimentation method, also stained with modified Ziehl-Neelsen stain[46–48]

Immuno-chromatography

All stool samples were examined for the detection of Cryptosporidium antigen using the rapid BIOZEK Medical kit (Netherlands). This rapid immuno-chromatography tests determine the presence of Cryptosporidium qualitatively in stool samples. According to the manufacturer`s instructions briefly, 1-2mL or 1-2gm of stool in a sterile container tested within 6 hours, 2 drops (80μL) of homogenized feces were added to extraction buffer in the provided tube, then mixed vigorously for 2 minutes, then 3 full drops of extracted sample were transported to each specimen well test in the cassette and read the result with 10 minutes. (BIOZEK, Netherlands).

Molecular Study

DNA Extraction from a Stool Sample Genomic DNA was isolated from each stool sample by using the Solar Bio Stool Genomic DNA Extraction Kit (made in China). This kit is a high-performance kit designed for the efficient extraction of genomic DNA from stool samples. It provided a fast and reliable method for isolating high-quality DNA from stool, making it ideal for applications. According to the manufacturer`s instructions, Stool samples were first homogenised and subsequently processed for DNA extraction. Approximately 200 mg of each stool sample was transferred to a sterile microcentrifuge tube and thoroughly mixed with 1 mL of Reagent A, a preservation buffer. The mixture was incubated at 70°C with intermittent vertexing. Following incubation, the samples were centrifuged. Then added 800 μL of Reagent B (lysis solution) along with 2 μL of RNase to degrade RNA contaminants. The mixture was incubated at room temperature for optimal enzymatic activity. Subsequently, 20 μL of proteinase K was added. This step was followed by incubation at 65°C, then centrifugation to separate the lysed material from insoluble components. The cleared lysate was then mixed with 800 μL of Reagent C (binding buffer) and applied to a silica spin column. After a brief standing period, the column was centrifuged to allow DNA binding to the membrane, the column was washed sequentially with Rinsing Solution 1 and Rinsing Solution 2, each followed by centrifugation. A final dry spin was performed to eliminate residual ethanol and ensure complete membrane desiccation. Finally, the DNA was eluted twice using 20 μL of elution buffer per elution step, resulting in a total elution volume of 40 μL. The purified DNA was stored at −20°C until further molecular analysis.

Amplification of Extracted DNA

The polymerase chain reaction (PCR) was used to amplify a partial gene locus of 18S rRNA gene. The specific set of primers was CRU18SFc(5′GAGGTAGTGACAAGAAATAACAATACAGG-3′), CRU18SRc (5′CTGCTTTAAGCACTCTAATTTTCTCAAAG-3′) [49]

PCR was performed in a volume of 30µl (15µl of mastermix, 2µl of genomic DNA, 0.5µl of each primer and 12µl of deionized distilled water) and took place in a thermos-cycler (Biobase, China(using this cycling instructions: pre-denaturation at 94°C for 3 minutes, denaturation at 94°C for 30 seconds (35 cycles), annealing at 56°C for 30 seconds, extension at 72°C for 30 seconds and final extension at 72°C for 5 minutes, then hold at 4°C ∞. A 5µl of each PCR product was examined on 1% w/v agarose gel, stained with safety dye (Good view Solar bio, China), A 1500bp DNA ladder (Solar bio, China) was used for comparing the product and photographed by using a gel documentation system (Biobase, Bk-Ag100, China). The expected size of the PCR amplicon was ~300bp for the 18S rRNA gene.

Statical analysis

Data were entered and analysed using IBM SPSS Statistics, version 21. Descriptive statistics, including frequencies, percentages, means, and standard deviations, were used to summarise the dataset. The Pearson chi-square test was applied to assess associations between categorical variables. In cases where more than 10% of the expected cell counts were less than 5, Fisher's exact test was used as an alternative. A p-value of <0.05 was considered statistically significant. To evaluate the diagnostic accuracy of the tests in comparison with the gold standard, Receiver Operating Characteristic (ROC) curve analysis was conducted. The ROC curve plots sensitivity (true positive rate) against 1-specificity (false positive rate) across different threshold values, providing an overall measure of test performance. Furthermore, cross-tabulation was used to compare each diagnostic test with the gold standard, allowing for the calculation of sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV).

RESULTS

Demographic Study

In this study, 150 children (71 males and 79 females) participated, the ages ranging between 1 day and 14 years. The largest age group was 1 day to <1 year (n = 70), followed by 1 to <5 years (n = 48) and 5-14 years (n = 32). Gender distribution was relatively balanced across all age groups, with a slightly higher number of females overall. Tab (1).

Microscopic Examination

The direct smear using modified Ziehl-Neelsen stain (MZN) demonstrated that 91/150 (60.7%) of the samples tested positive for *Cryptosporidium* oocysts during microscopic examination. Tab (2). Compared to the PCR reference standard, microscopic direct smear stained with modified Ziehl-Neelsen (MZN) technique demonstrated sensitivity and specificity were 62.16% and 43.59% respectively (Table 3). *Cryptosporidium* oocysts appeared as pink to red round bodies against a blue background when stained with the MZN, they are about 4-6µm in diameter Fig (1).

Concentration technique

The concentration technique used formalin-ethyl acetate sedimentation method stained with modified Ziehl-Neelsen stain showed that 96/150 (64.0%) of the samples tested positive for *Cryptosporidium* oocysts during concentration technique examination. Tab (2). Compared to the PCR reference standard, concentration technique examination revealed that sensitivity and specificity were 63.96% and 35.89%, respectively. Tab)3).

Rapid immune-chromatography test

The rapid immune-chromatography test indicated that 103/150 (68.7%) of the samples tested positive for *Cryptosporidium* antigen during copro-immunological examination (Table 2). Compared to the PCR reference standard, the immune-chromatography test sensitivity and specificity were 74.86% and 48.72%, respectively. Tab)3).

Molecular technique (PCR)

The molecular (PCR) approach showed that 111/150 (74.0%) of the samples tested positive for *Cryptosporidium* DNA during conventional PCR targeting the 18S rRNA gene of *Cryptosporidium* (Table 2). Molecular PCR analysis revealed that 67.6% (48/71) of the male samples and 79.7% (63/79) of the female samples tested positive for *Cryptosporidium*. However, statistical analysis indicated that the observed difference in infection rates between genders was not statistically significant (p = 0.091). Tab (4). The PCR were done by amplifying the 18S rRNA region from each stool sample. The agarose gel analysis revealed the same size for the 18S rRNA region. The amplicons were ~300bp for 18S rRNA. Fig (2) which confirmed that all the obtained DNA bands were from the same genus *Cryptosporidium.*

The comparison between direct smear microscopy and the reference standard molecular PCR revealed no statistically significant difference, with a p-value of 0.527. Similarly, the statistical analysis comparing the concentration technique to the PCR reference standard demonstrated a non-significant result (p = 0.988). In contrast, the analysis of the rapid immunochromatographic test showed a statistically significant difference when compared to PCR outcomes (p = 0.007), The corresponding Receiver Operating Characteristic (ROC) curve for the Immunological-RT test is presented in Fig (3).

According to age group, the distribution of Molecular-PCR test results demonstrated variation across the categories. Among infants aged 1 day to <1 year, 48/70 (68.6%) cases were positive and among 1 to <5 years' age group, 40/48 (83.3%) cases were positive. While in the 5-14 years' group, 23/32 (71.9%) cases were positive. The statistical analysis yielded a p-value of 0.19, indicating that the observed differences in PCR results across age groups were not statistically significant. These distributions of *Cryptosporidium* among age group were shown in Fig (4).

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Table (1): Demographic characteristics of study subjects with respect to sex and age distribution.

Age group	Gender		Total
	Male	Female	
0 - < 1years	32	38	70
1 - < 5 years	20	28	48
5 - 14 years	19	13	32
Overall	71	79	150

Table (2): Frequency and percentage of positive detection of Cryptosporidium in stool samples by sex.

Technique	Frequency	Percentage		
		Male	Female	Total
Direct smear stained with MZN	91	57.7%	63.3%	60.7%
Concentration stained with MZN	96	60.6 %	67.1%	64.0%
Rapid immune chromatography	103	44.8%	72.2%	68.7%
Molecular (PCR)	111	67.6%	79.7%	74.0%

Table (3): Sensitivity and specificity of diagnostic tests compared to PCR

Measures	Techniques		
	Microscopic	Immunological	Concentration
Sensitivity	62.16%	74.86%	63.96%
Specificity	43.59%	48.72%	35.89%
PPV*	75.82%	80.58%	73.96%
NPV**	28.81%	40.43%	25.93%

** PPV = Positive Predictive Value, *NPV= Negative Predictive Value.*

Table (4): Percentage of Cryptosporidium Positivity by Molecular PCR in Males and Females with P-Value.

Technique	Percentage		P-values
	Male	Female	
Molecular (PCR)	67.6%	79.7%	0.091

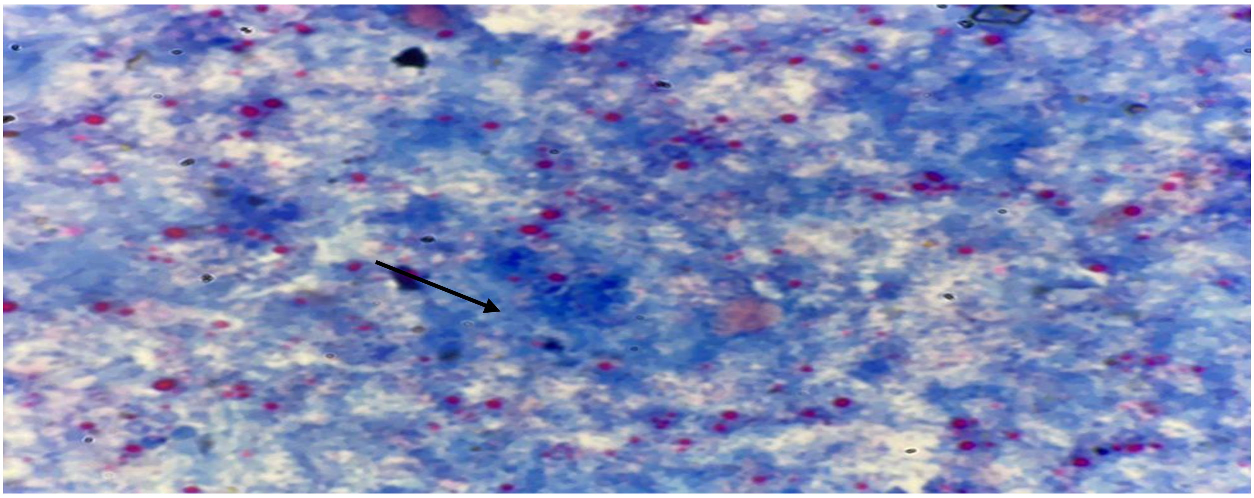

Figure (1): Photomicrograph of Cryptosporidium oocysts (arrow) in a stool sample of one-year female child, stool smear stained with MZN, 1000x

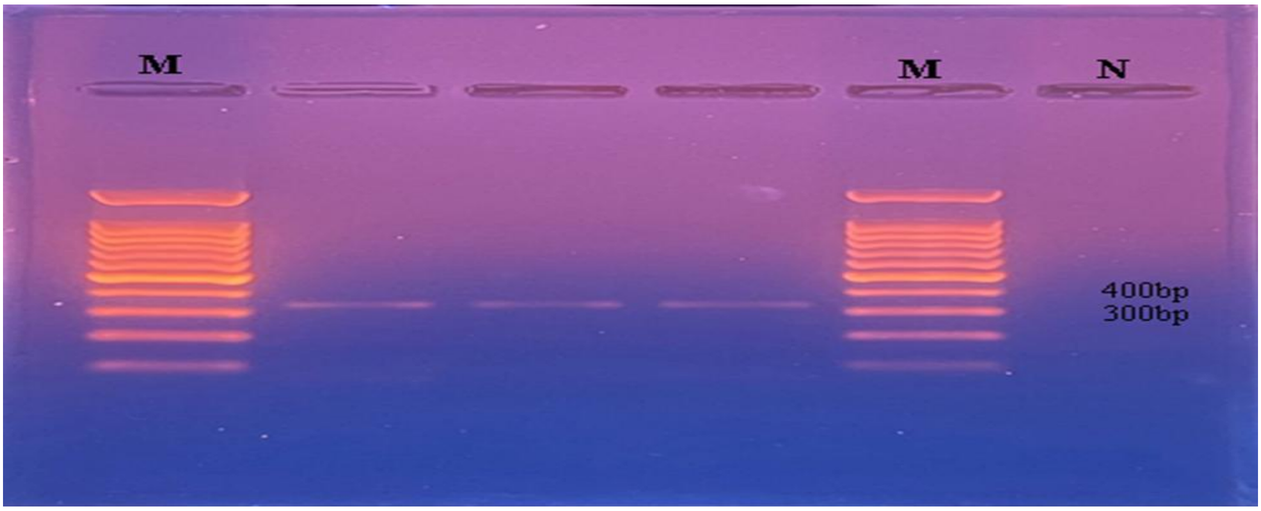

Figure (2): Agarose gel electrophoresis image showing conventional PCR product of *Cryptosporidium*, 18S rRNA gene (300bp) in human stool specimen. Bands were separated by electrophoresis on a 1% TAE agarose gel and visualized under UV light M= DNA ladder (100bp-1500bp), N= negative control.

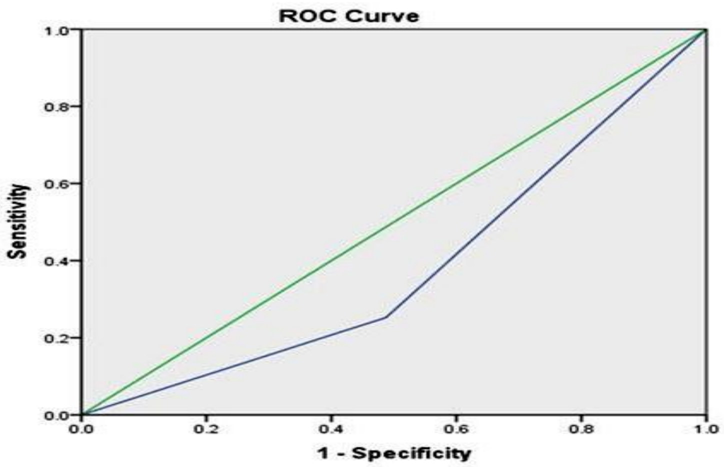

Figure (3): Roc curve for rapid immune-chromatography test virus PCR (P= 0.990). The green line represents the gold standard PCR, while the blue line indicates the rapid immunochromatographic test.

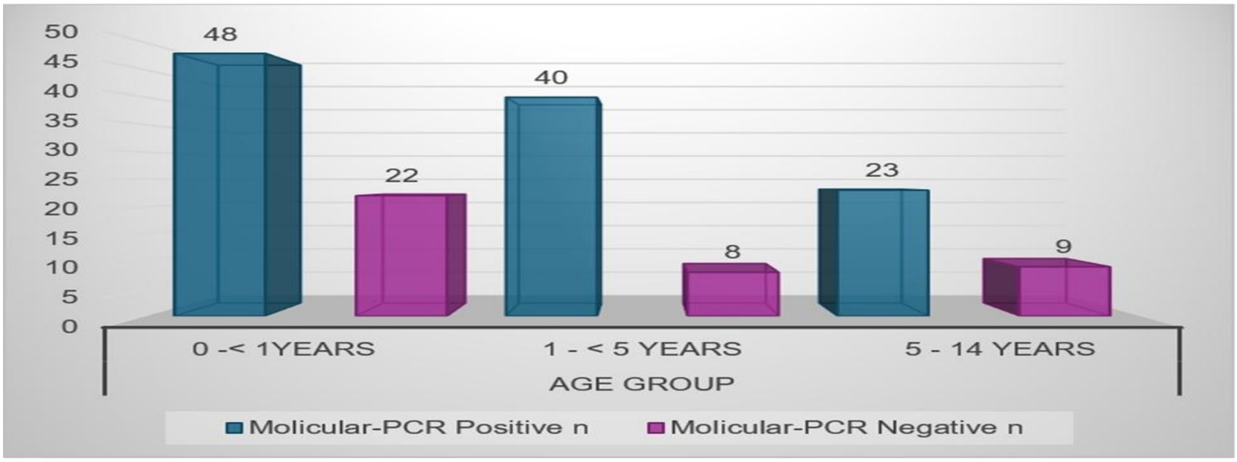

Figure (4): Distribution of *Cryptosporidium* among the age group in the present study (tested by molecular-PCR technique).

DISCUSSION

Cryptosporidium is regarded as one of the most important pathogenic parasites that affect the endothelial layer of the intestine and cause diarrheal diseases among children in poor and developing countries [50] This study compared the accuracy of four diagnostic methods: direct smear microscopy, concentration technique, both stained with MZN, rapid immune-chromatography test and molecular-PCR approach, the last one is considered the most reliable test, for detecting *Cryptosporidium* in children aged 1 day to 14 years. The results provide important information about how well each method works and highlight their strengths and limitations, especially in healthcare settings with limited resources. In this investigation, the overall rate of infection with *Cryptosporidium* among children with diarrhoea at Azadi Teaching Hospital, Al-Nasir Hospital, and Children's Hospital was 60.7% and 64.0% by examination with a microscope using direct smear and concentration technique stained with MZN. These results show a low positive rate compared with the result (74.0%) recorded by AL-Ezzy [37] in newborns to children less than 5 years old among Iraqi children living in the Baqubah City-Diyala Province. Also, lower than the result (61.68%) recorded by Askar [39] who recorded this parasite among children under 5 years old. While it shows a higher positive rate compared to the results reported by Al-Mounasi [30] in young children between 1 day - <1 year and 5 - <15 years which reached 28% and 25% respectively. Also, higher than the result (4.95%) obtained by Obayes [38] in children and adult patients in Babylon Province.

In this study, the total rate of infection with *Cryptosporidium* was 68.7% by using a rapid immuno-chromatography test. This technique revealed a high positive rate compared with the result (38.0%) using the ELISA technique conducted by Naif [33] in children with gastroenteritis who attended the Emergency Department of Paediatrics in Al-Batool Teaching Hospital for Maternity and Paediatrics in Baqubah City. Also, the higher result (3.58%) obtained by Obayes [38] in both paediatric and adult patients from Babylon Province, the rapid immunochromatographic assay demonstrated the highest diagnostic performance among the conventional diagnostic techniques evaluated. Statistical analysis revealed no significant concordance between molecular PCR—the reference standard—and routine microscopic methods, including direct smear and concentration techniques stained with the modified Ziehl–Neelsen (MZN) stain. These findings are in agreement with those reported by Obayes [38]

In the present study, the total rate of infection with *Cryptosporidium* was 74.0% by using a molecular-PCR approach (18S rRNA). This approach revealed a low positive rate compared with the result (79.7%) reported by Rasheed [44] who used the molecular PCR technique for identification of this parasite in Paediatric Hospital and General Kirkuk Hospital from children aged between <1 to 15 years in 2003. While this technique revealed a high positive rate compared with the result (16.9%) using the molecular-PCR approach for the same gene locus recorded by Salim [31] among child patients (aged between 1-10 years) with diarrhoea at Al-Rifai City/Thi-Qar Province. Also, a higher positive rate than result 38% (28% C. homini and 10% C. parvum) found by Alasady [42] using molecular-PCR. Among the diagnostic methods evaluated, molecular PCR demonstrated the highest detection rate, reaffirming its role as the gold standard for *Cryptosporidium* detection due to its superior sensitivity and specificity.

The molecular-PCR approach not only demonstrated the highest diagnostic performance among the conventional and rapid immune-chromatography techniques but also showed statistically significant agreement with rapid immune-chromatography techniques ($p = 0.007$). This finding was further supported by ROC curve analysis (Fig. 3). This finding disagrees with the previous result recorded by Obayes [38] in which the infection rate of *Cryptosporidium* by molecular-PCR was 2.47% and showed statistical analysis no significant difference between the percentages of infection by microscopic, rapid immune-chromatography and molecular-PCR methods. These discrepancies in diagnostic sensitivity across studies may be explained by several factors, including differences in sample size, population characteristics (e.g., paediatric vs. general populations), regional epidemiological variations, parasite burden, and technical aspects related to sample handling and test implementation.

The age groups of children from 1 day to <1 year and from 1 to <5 years in the present study show the highest rate of infection 48% and 40% respectively. This result was agreed with the result obtained by Askar [39] who recorded the prevalence 61.68% of *Cryptosporidium* among children <5 years old, and agree with the result of Alasady [42] who recorded the prevalence

28%, also the result was agreed with the result recorded by Rasheed [44] 17.2% for those children <1 year and 43.8% for 1-5 years. The high rate of infection in children under five years may reflect their increased vulnerability to enteric infections due to immature immune systems, higher exposure to contaminated environments, and limited hygiene.

Although this group exhibited the highest PCR positivity rate, the association between age and *Cryptosporidium* infection was not statistically significant (p = 0.19). This finding contrasts with a study conducted in Kirkuk, Iraq, by Rasheed [44] which reported a significant association between age and infection (p = 0.004) [44]. The discrepancy may be explained by differences in sample size, population characteristics, or methodological factors, including vaiations in diagnostic approaches. Moreover, the high proportion of infants in the present sample may have affected the statistical power to detect age-related trends. These results suggest that while younger age is generally considered a risk factor for *Cryptosporidium* infection, its significance may vary depending on local epidemiological and demographic conditions.

According to the gender stratification in the present study, molecular PCR analysis revealed a higher prevalence of *Cryptosporidium* infection among females (79.7%) compared to males (67.6%); however, this difference was not statistically significant (p = 0.091). This finding is supported by the results of [39] who also observed a higher prevalence among females (65.35%) than males (58.75%) in children under five years of age in Kirkuk Province, although their results similarly lacked statistical significance. Conversely, other studies conducted in Iraq have reported differing patterns. Salim [31] found a higher infection rate in males (65%) than in females (35%) in Thi-Qar. Likewise, investigations by [35,42,44] reported higher male prevalence. Moreover, Naif [33] reported a statistically significant difference in Diyala Governorate, with a higher prevalence in males (65.79%) compared to females (34.21%). These variations in gender-specific infection rates across studies may reflect regional and demographic differences, including variations in hygiene practices, water source quality, cultural behaviours, and environmental exposures. Such discrepancies underscore the importance of further large-scale, population-based studies to clarify the role of gender in the epidemiology of *Cryptosporidium* infection in Iraq.

Routine microscopic detection of *Cryptosporidium* oocysts is the ordinary method in all parts of the world either fresh or preserved specimens can be examined using the routine stool formalin-ethyl acetate concentration and one of the modified Ziehl-Neelsen staining (modified acid-fast stains), it is one of the cheapest and fastest way for detecting *Cryptosporidium* oocysts even its sensitivity and specificity is low, so these factors that limit its routine use in low-resource settings. The routine microscopic examination many satisfactory in demonstrating the organisms in stool material, but the oocysts are directly correlated with the consistency of the stool; the oocysts are more present in diarrheic stool. Also, the detection of oocysts may be confused by some laboratory technicians. In addition, this technique cannot differentiate between different species of *Cryptosporidium* [51]

The application of immunoassays has demonstrated significant value by offering a more sensitive and reliable method for detecting *Cryptosporidium* in stool samples. These assays exhibit high specificity and sensitivity, leading to a markedly improved detection rate compared to conventional microscopic examination using direct smear staining with modified Ziehl–Neelsen (MZN). These procedures do not rely on visual identification of *Cryptosporidium* oocysts; these methods can be used for detection of the antigen in immunoassays, and this method is widely used to identify *Cryptosporidium* in water testing and outbreak situations [17,52]

The molecular-PCR approach offers alternatives for the diagnosis of *Cryptosporidium* in both clinical and environmental samples. If compared with routine microscopic examination by the conventional MZN staining method, PCR is more sensitive and easier to interpret, but it requires advanced laboratory instruments, more “hands-on” time and expertise, as well as being more expensive. Another important advantage of PCR is the ability to differentiate between different *Cryptosporidium* genotypes, which is important in outbreak situations [51]

CONCLUSION

All three procedures used in the present study (direct smear stained with MZN, concentration technique stained with MZN and rapid immune-chromatography test) are useful for the diagnosis of *Cryptosporidium* in stool samples. However, among all techniques, the molecular-PCR approach has shown the highest sensitivity toward the diagnosis of *Cryptosporidium*. Furthermore, due to its sensitivity, specificity, ability to genotype, ease of use, and adaptability to batch testing make PCR a useful tool for future diagnostic and molecular epidemiological studies.

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Ethical approval

Ethical approval was obtained from the Ethical Committee of the Medical Laboratory Department at the College of Health and Medical Technology, Sulaimani Polytechnic University, Iraq. In addition, official permissions were obtained from Azadi Teaching Hospital, Al-Nasir Hospital, and the Children's Hospital in Kirkuk City before data collection. All participants' parents were informed about the purpose and objectives of the study, and verbal informed consent was obtained from the parents before participation. Participation was entirely voluntary, and participants' parents were assured of the confidentiality and anonymity of their data. All collected information was securely stored and used exclusively for research purposes.

Statement of permission and conflict of interests

The authors declare that they have no conflict of interest.

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