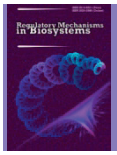

Regulatory Mechanisms in Biosystems

ISSN 2519-8521 (Print)
ISSN 2520-2588 (Online)
Regul. Mech. Biosyst.

The identification of *Cryptosporidium* species in stool samples using alternative diagnostic methods

Z. K. A. Al-Alwani*, M. M. Al Alouci*, K. A. J. Alkhazraji**

**University of Anbar, Anbar, Iraq*
*** University of Baghdad, Baghdad, Iraq*

Article info

Received 04.03.2025
Received in revised form 29.03.2025
Accepted 19.04.2025

Department of Microbiology, Faculty of Medicine, University of Anbar, Anbar, Iraq. E-mail: zainabalalwani173@uoanbar.edu.iq, mun.alouci@uoanbar.edu.iq

College of Medicine, University of Baghdad, Baghdad, Iraq. E-mail: drkhalidgit1959@gmail.com

Al-Alwani, Z. K. A., Al Alouci, M. M., & Alkhazraji, K. A. J. (2025). The identification of Cryptosporidium species in stool samples using alternative diagnostic methods. Regulatory Mechanisms in Biosystems, 16(2), e25072. doi:10.15421/0225072

Cryptosporidiosis, caused by *Cryptosporidium* species, leads to gastrointestinal issues, especially in immunocompromised individuals. Alternative diagnostic methods like PCR and ELISA offer more sensitive and accurate identification of *Cryptosporidium* in stool samples compared to traditional microscopy. We evaluated the effectiveness of alternative diagnostic methods, namely PCR, in identifying *Cryptosporidium* species in stool samples compared to traditional microscopy. This cross-sectional study was conducted from March 15, 2022 to September 15, 2023 at Ramadi Pediatric General Hospital, Ramadi General Teaching Hospital, and selected private medical laboratories. It included 320 patients (aged 18–77) with gastroenteritis symptoms. Stool samples were collected and analyzed microscopically using various staining methods, including modified Ziehl-Neelsen and fluorescence techniques. DNA extraction and nested PCR were performed to detect *Cryptosporidium* spp. Microscopic stool examination revealed a 10% infection rate with *Cryptosporidium* (32/320 samples), with higher infection rates in older adults (23.1% in 74–83 years) and retirees (17.9%). Males (10.2%) and rural residents (10.9%) had higher infection rates. Seasonal variation showed the highest rates in April (22.7%). PCR showed greater sensitivity than microscopy, detecting three additional positive samples. Diagnostic accuracy of microscopy, compared to PCR, was 90.6%, with high sensitivity (100%) and specificity (88%). Concordance between microscopy and PCR was 90.6%, with three discordant cases. Microscopy and PCR showed high concordance in diagnosing *Cryptosporidium* infections, with PCR offering higher sensitivity. The seasonal variation and higher infection rates in older adults and rural areas suggest environmental factors and compromised immunity contribute to increased susceptibility.

Keywords: *Cryptosporidium*; microscopy; PCR; gastroenteritis; prevalence; sensitivity.

Introduction

Cryptosporidium is a genus of protozoan parasites that has become an important cause of gastrointestinal illness in humans and animals. *Cryptosporidium* is an opportunistic pathogen that causes life-threatening infections in immunocompromised patients including those with HIV/AIDS, cancer patients and organ transplant recipients. In immunocompetent hosts, the clinical manifestation is usually self-limiting diarrhea, but the organism still contributes to significant morbidity and dehydration, particularly in the very young and elderly patients. *Cryptosporidium* has been identified by the World Health Organization as one of the leading causes of diarrheal disease worldwide, particularly in developing countries where sanitation and water quality are poor (Helmy & Hafez, 2022; Wang et al., 2023; Balendran et al., 2024).

Cryptosporidium is largely transmitted via the fecal-oral route by exposure to contaminated water or food. The parasite's unique ability to produce resistant oocysts, which can survive adverse environmental conditions such as chlorine-treated water, has led to multiple large-scale waterborne outbreaks attributed to *Cryptosporidium*. Due to its persistent nature and broad host range, timely and accurate diagnosis is critical for patient management and also for public health surveillance (Helmy & Hafez, 2022; Khan & Witola, 2023).

Historically the diagnosis of *Cryptosporidium* infection has been by microscopic examination of stained stool smears. Oocysts can primarily be visualized microscopically using modified Ziehl–Neelsen staining and auramine phenol fluorescent staining. Although straightforward and inexpensive, they are limited in sensitivity and specificity, particularly when oocyst concentrations are low or when the observer does not have enough experience. In addition, these methods cannot discriminate between species or genotypes, which is an increasingly important requirement in epidemiological studies and outbreak investigations (Loiola et al., 2021; Khan et al., 2022). Molecular diagnostic methods have been developed in order to address these limitations. Of them, polymerase chain reaction (PCR) and nested PCR have emerged as sensitive and specific techniques for the identification and species differentiation of *Cryptosporidium* species. These types of methods target conserved regions of genes, like the 18S rRNA gene, and are useful for detecting low levels of parasitic DNA in clinical samples, which can be helpful in clinical settings and research settings alike. PCR-based methods are also far more accurate, making it possible to detect asymptomatic infections and co-infections that would be missed using traditional microscopy methods (Santana et al., 2022; Tiffarent et al., 2022).

Through a comparative analysis of the merits and demerits of the two diagnostic methods, this study seeks to assist in the formulation of enhanced diagnostic guidelines for *Cryptosporidium* infection. Its aim is also to emphasize promoting molecular methods for routine clinical laboratories, especially in cases where fast and accurate diagnostics is critical. Molecular diagnostics can be included in routine practice alongside conventional testing to develop a holistic approach able to facilitate the rapid detection and appropriate management of cryptosporidiosis, decreasing disease burden and improving patient outcomes (Johansen et al., 2021; Mahmudunnabi et al., 2024; Weldemariam et al., 2024). This study aims to compare conventional microscopic techniques versus nested PCR for the detection of *Cryptosporidium* species in stool samples, especially in patients submitted to intestinal biopsies. Microscopy is still the frontline diagnostic method in many low-resource settings, but there is a growing awareness about the demand for more advanced and reliable diagnostic methodologies that can assist in the accurate diagnosis and the designing of specific treatment strategies.

Matherials and methods

The study was officially approved by the Scientific Committee of the Ramadi Health Directorate. Each participant completed a structured informal questionnaire covering personal details, symptoms,

and water source quality, and provided written informed consent. This study was conducted between March 15, 2022, and September 15, 2023, in the laboratories of Ramadi Pediatric General Hospital, Ramadi General Teaching Hospital, and selected private medical laboratories.

A cross-sectional study was carried out on 320 patients of both genders, aged between 18 and 77 years, who presented primarily with symptoms of gastroenteritis. Two stool samples were collected from each participant in airtight containers. A small amount of 0.85% normal saline was added to the samples, mixed thoroughly, and stored in cool boxes until analysis.

Stool samples were first examined macroscopically for color, consistency, and foreign bodies. Microscopic examination included diect wet mount using 0.85% NaCl (to observe motility) and 1% Lugol's iodine (to visualize internal structures), flotation with 33% $ZnSO_4$ for negative samples, and modified Ziehl-Neelsen staining. The Ziehl-Neelsen method used carbol fuchsin with heat, followed by 5% sulfuric acid and methylene blue as counterstain, allowing *Cryptosporidium* oocysts to appear as pinkish spheres against a blue background. Additional staining methods included methylene blue with safranin, phenol-auramine fluorescent staining (examined under UV microscopy at 400^X), and acridine orange staining (excited at 470 nm, emitted at 530–650 nm) to detect yeast cells and parasites using fluorescence microscopy.

DNA was extracted from 32 fecal samples using the Presto™ Stool DNA Extraction Kit (Geneaid Biotech Ltd., Taiwan). Nested PCR was then used to amplify the 18S rRNA gene of *Cryptosporidium* spp., using two sets of primers:

First round primers:

SCL1f: 5'-CTGGTTGATCCTGCCAGTAG-3'

SCL1r: 5'-TAAGGTGCTGAAGGAGTAAGG-3' (1032 bp)

Second round primers:

Sc2f: 5'-CAGTTATAGTTTACTTGATAATC-3'

Sc2r: 5'-CAATACCCTACCGTCTAAAG-3' (212 bp)

Each 50 µL PCR mixture contained: 25 µL master mix, 5 µL DNA template, 2 µL of each primer (forward and reverse), and 16 µL PCR-grade water. The thermal cycling conditions for the first round included initial denaturation at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 1 min, extension at 72 °C for 1 min, and final extension at 72 °C for 7 min. For the second round, the conditions were the same, except the annealing temperature was set at 58 °C for 45 s.

The obtained data were analyzed using the Statistical Package for the Social Sciences (SPSS) software, version 26. Descriptive statistics such as frequencies and percentages were calculated for qualitative variables, while means and standard deviations were used for quantitative data. The Chi-square test (χ^2) was employed to assess associations between categorical variables, and a p-value of less than 0.05 was considered statistically significant. Where applicable, additional statistical tests such as ANOVA were used to compare means among groups. The results are presented in tables and graphs to facilitate interpretation.

Results

Microscopic stool examination results showed a 10% infection rate with *Cryptosporidium,* with 32 out of 320 samples tested positive, while the majority (90%) tested negative. These results reflect the limited prevalence of the parasite when using traditional microscopic diagnostic methods (Table 1).

Table 1
Prevalence of *Cryptosporidium* spp. in stool samples based on microscopic diagnosis

Diagnosis result	Number of samples	Percentage
Positive	32	10.0
Negative	288	90.0
Total	320	100.0

The distribution of *Cryptosporidium* infection by age group showed that the highest infection rate was recorded in the 74–83 age group, at 23.1%, followed by the 55–73 age group, at 11.5%. The 18–36 and 37–54 age groups recorded lower infection rates, at 7.4% and 7.1%, respectively. These results reflect an increasing infection rate with age, which may indicate weakened immunity or greater risk factors in older adults (Table 2).

Table 2
Distribution of *Cryptosporidium* spp. infection by age group

Age group, years	Positive cases	Negative cases	Total samples	Infection rate, %
18–36	10	126	136	7.4
37–54	6	78	84	7.1
55–73	7	54	61	11.5
74–83	9	30	39	23.1
Total	32	288	320	10.0

The results of the prevalence of *Cryptosporidium* infection by occupation indicate that the highest infection rate was recorded among retirees at 17.9%, followed by day laborers at 11.8%. The rate was lower among employees (7.1%), students (9.6%), and housewives (8.5%). These results reflect variations in infection rates according to occupation, which may be attributed to differences in environmental exposure levels, general hygiene, and working conditions, especially among groups considered more vulnerable to sources of pollution (Table 3).

Table 3
Distribution of *Cryptosporidium* spp. infection by occupation

Occupation	Positive cases	Negative cases	Total samples	Infection rate, %
Student	5	47	52	9.6
Officer	6	78	84	7.1
Housewife	8	86	94	8.5
Retired	7	32	39	17.9
Wage Earner	6	45	51	11.8
Total	32	288	320	10.0

The distribution of *Cryptosporidium* infections by sex and residence showed that the infection rate was higher among males (10.2%) than females (9.9%). Differences were also evident between residential areas, with the infection rate being higher in rural areas (10.9%) than in urban areas (8.7%). This disparity in infection rates can be attributed to different environmental factors and health conditions between rural and urban areas, as well as the impact of socioeconomic factors on access to water and sanitation services (Table 4).

Table 4
Gender and residency distribution of *Cryptosporidium* spp. positive cases

Variable	Category	Positive cases	Negative cases	Total samples	Infection rate, %
Gender	male	13	115	128	10.2
	female	19	173	192	9.9
Residency	rural	21	172	193	10.9
	urban	11	116	127	8.7

The distribution of *Cryptosporidium* cases by month shows that the highest incidence rate was in April (22.7%), followed by May (12.5%) and June (12.5%), while the lowest incidence rates were in March (3.3%) and December (3.4%). These results suggest a seasonal effect on the prevalence of the parasite, as cases may be influenced by environmental factors such as temperature changes and exposure to pollutants in the summer, compared to cooler months when cases decrease (Table 5).

The results of detection of *Cryptosporidium* in biopsy wash samples using microscopy and PCR showed that 7 samples were positive by both methods (microscopy and PCR), while 15 samples were negative by both methods. Of the samples that tested negative by microscopy, only 3 were positive by PCR. These results suggest that PCR may be more sensitive in detecting *Cryptosporidium* infection than conventional microscopy (Table 6).

The microscope's diagnostic performance compared to PCR as a reference standard showed that the microscope detected 7 true positi-

ves, 3 false positives, 22 true negatives, and no false negatives. These results indicate that the microscope has a high accuracy in identifying negative cases and produces accurate positive results in some cases, but PCR remains the most accurate method for detecting *Cryptosporidium* spp. (Table 7).

Table 5
Monthly distribution of *Cryptosporidium* spp. positive cases according to seasonality

Month	Positive cases	Negative cases	Total samples	Infection rate, %
February	1	14	15	6.7
March	1	29	30	3.3
April	5	17	22	22.7
May	3	21	24	12.5
June	4	28	32	12.5
July	5	42	47	10.6
August	4	30	34	11.8
September	2	19	21	9.5
October	3	20	23	13.0
November	2	18	20	10.0
December	1	28	29	3.4
January	1	22	23	4.3
Total	32	288	320	10.0

Table 6
Detection of *Cryptosporidium* spp.
in biopsy wash samples using microscopy and nested PCR

Sample ID	Microscopy result	Nested PCR result
1	Yes	Yes
2	Yes	Yes
3	Yes	Yes
4	Yes	Yes
5	Yes	Yes
6	Yes	Yes
7	Yes	Yes
8	No	No
9	No	No
10	No	No
11	No	No
12	No	No
13	No	No
14	No	No
15	No	Yes
16	No	No
17	No	No
18	No	No
19	No	Yes
20	No	No
21	No	No
22	No	Yes
23	No	No
24	No	No
25	No	No
26	No	No
27	No	No
28	No	No
29	No	No
C1	No	No
C2	No	No
C3	No	No

Table 7
Diagnostic performance of microscopy
against nested PCR as reference standard

Diagnostic category	Count
True Positive (TP)	7
False Positive (FP)	3
True Negative (TN)	22
False Negative (FN)	0
Total	32

The diagnostic accuracy results of the microscope compared to serial PCR showed that the sensitivity was 100% (with a 95% confidence interval (CI) of 59.0% to 100.0%), indicating the microscope's high ability to identify positive cases. The specificity was 88.0% (with a 95% CI of 68.8% to 97.5%), reflecting the microscope's ability to accurately distinguish negative cases. The positive probability index (LR) was 8.3% (with a 95% CI of 2.9% to 24.1%), indicating the microscope's ability to reliably produce positive results. The positive predictive value (PV) was 70.0% (with a 95% CI of 44.7% to 87.1%), while the negative predictive value (NPV) was 100% (with a 95% CI of 84.6% to 100.0%). The overall diagnostic accuracy was 90.6% (with a 95% confidence interval ranging from 75.0% to 98.0%). These results demonstrate that the microscope is an accurate and reliable diagnostic tool, particularly in identifying negative cases with high accuracy in predicting negative outcomes (Table 8).

Table 8
Diagnostic accuracy measures of microscopy
compared to nested PCR

Statistic	Value, %	95% confidence interval
Sensitivity	100.00	59.04–100.00
Specificity	88.00	68.78–97.45
Positive likelihood ratio	8.33	2.88–24.09
Negative likelihood ratio	0.00	–
Positive predictive value	70.00	44.66–87.09
Negative predictive value	100.00	84.56–100.00
Accuracy	90.62	74.98–98.02

Comparison of microscopy results with PCR-seq results showed high concordance in diagnosis, with 10 positive and 22 negative cases identified using both microscopy and PCR out of 32 samples. Thus, a total of 29 concordant cases (those with the same result obtained by both methods) were found, while there were 3 cases that were discordant between the two methods. This indicates a high concordance between the two methods for diagnosing *Cryptosporidium* spp. infections, with only a few cases showing discordant results (Table 9).

Table 9
Summary of microscopy vs. nested PCR results

Method used	Positive cases	Negative cases	Total
Microscopy	10	22	32
Nested PCR	10	22	32
Concordant (same result)	29	–	–
Discordant	3	–	–

The results showed high concordance between microscopy and PCR in diagnosing *Cryptosporidium* spp. Twenty-nine out of 32 cases were fully concordant between the two methods, representing 90.6% of the samples. In contrast, there were three discrepant cases between the two methods, representing 9.4%. These results indicate that there is a high level of concordance between the two methods in identifying parasite infection, with only a few cases showing discrepancies between them (Table 10).

Table 10
Diagnostic concordance between microscopy and nested PCR

Concordance category	Number of cases	Percentage
Fully concordant	29	90.6
Discordant	3	9.4
Total	32	100.0

Discussion

The current study sought to identify *Cryptosporidium* spp. in biopsy-derived fecal samples utilizing conventional microscopy and nested PCR. Among the 32 samples, there were 7 true positives (both techniques), 3 false positives by microscopy, and 22 true negatives but no false negatives. This has shown that nested PCR has a sensitivity of 100% and specificity of 88% with the classical assays (Hsu et al., 2021; De et al., 2022).

Our study's high sensitivity (100%) is in agreement with Leav et al's report, testifying to the sensitivity of nested PCR assays targeting the 18S rRNA gene for the detection of *Cryptosporidium* spp. in fecal specimens, particularly those with low oocyst counts. Similarly, Li et al. (2023) found that nested PCR was better than microscopy in both sensitivity and specificity. This agrees with our results that molecular detection methods show a much higher degree of sensitivity and specificity, in particular in clinical scenarios where quick and correct diagnosis is critical (Cai et al., 2021; Yue et al., 2021; Li et al., 2023).

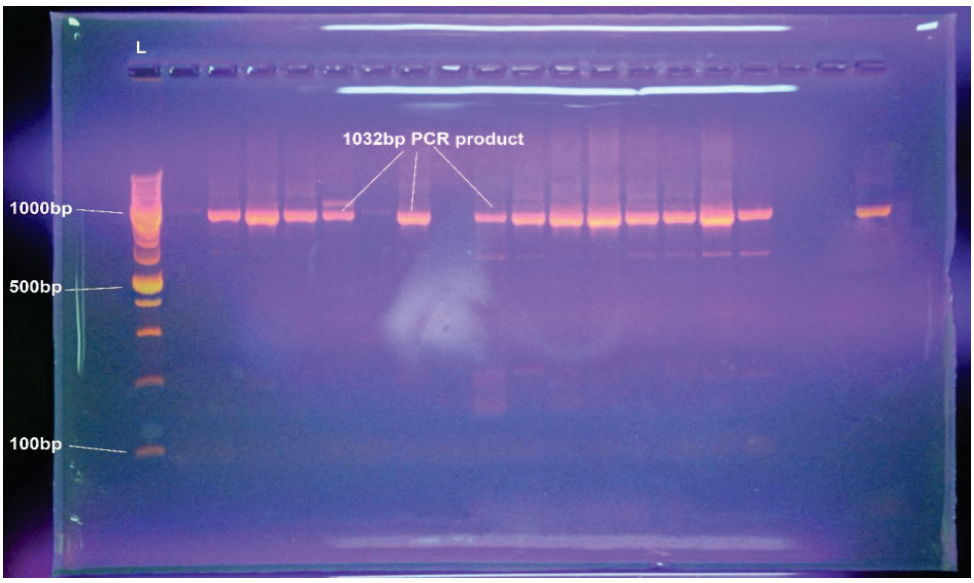

Fig. 1. Detection of *Cryptosporidium* by nested PCR, the first round produced 1032bp PCR product, which was subsequently employed for the second round as templet, the PCR product were resolved on 1.5% agarose, lane L 100bp DNA step ladder, other lanes different samples

Microscopy, on the other hand, had a lower positive predictive value (PPV = 70%) in our study, although it is cost-effective and widely used in low-resource settings. The reason for this is probably that oocysts are often misidentified, a common finding in microscopic interpretation, especially in resections that carry low parasitic loads. This result agrees with the findings of Bogan et al. (2024), which confirmed that microscopy frequently produces false-positive results that call for trained staff members for reliable assessment (Johansen et al., 2021; Robinson et al., 2023; Bogan et al., 2024).

We have confirmed that modified Ziehl–Neelsen and auramine phenol staining can also be used successfully in qualitatively visualizing *Cryptosporidium* oocysts. Despite their advantages, these methods are inferior to PCR for low parasite concentrations. The agreement between microscopy and PCR of 7 positive cases indicate the potential of microscopy as initial screening, reserving PCR for confirmation testing (Jaiswal et al., 2022; Ahmed et al., 2023; Sohrabi et al., 2023).

In contrast, Yang et al. (2022), used some different primer sets and reported a slightly reduced specificity for PCR (approximately 85%), which indicates that diagnostic performance depends largely on the design of the assay and choice of primers. This disparity illustrates the necessity of designing primers with suitable specificity and reaction conditions, as employed in the current analysis (Anantharajah et al., 2021; Yang et al., 2022; Groth-Helms et al., 2023).

Furthermore, biopsy samples rather than stool alone may have additionally increased detection sensitivity in our study. Another study by Certad et al. (2007) supported this approach, where he described that the parasitic DNA is more abundant in tissue biopsy samples and more pungent for molecular analysis (Hoffmann et al., 2021; Srirungruang et al., 2022; Rana & Pokhrel., 2023).

Overall, our results emphasize the complementary role of molecular diagnostics alongside microscopy in identifying *Cryptosporidium* spp., particularly in symptomatic patients. The use of nested PCR increases diagnostic accuracy, helps reduce misdiagnosis, and supports appropriate clinical management (Santana et al., 2022).

Conclusion

Microscopy and PCR showed strong concordance in diagnosing *Cryptosporidium* infections, with PCR providing higher sensitivity. The higher infection rates observed in older adults and rural areas, along with seasonal variation, suggest that environmental factors and compromised immunity contribute to increased susceptibility, emphasizing the need for targeted prevention measures in these vulnerable groups.

References

Ahmed, S. A. A., Quattrocchi, A., Elzagawy, S. M., Karanis, P., & Gad, S. E. M. (2023). Diagnostic performance of toluidine blue stain for direct wet mount detection of *Cryptosporidium* oocysts: Qualitative and quantitative comparison to the modified Ziehl–Neelsen stain. Diagnostics, 13(15), 2557.

Anantharajah, A., Helaers, R., Defour, J.-P., Olive, N., Kabera, F., Croonen, L., Deldime, F., Vaerman, J.-L., Barbée, C., Bodéus, M., Scohy, A., Verroken, A., Rodriguez-Villalobos, H., & Kabamba-Mukadi, B. (2021). How to choose the right real-time RT-PCR primer sets for the SARS-CoV-2 genome detection? Journal of Virological Methods, 295, 114197.

Balendran, T., Iddawela, D., & Lenadora, S. (2024). Cryptosporidiosis in a zoonotic gastrointestinal disorder perspective. Journal of Tropical Medicine, 2024(1), 6439375.

Bogan, J. E., Mason, A. K., Mishel, K., Garner, M. M., Walden, H. D. S., Childress, A., Wellehan, J. F. X., Ossiboff, R. J., & Dahlhausen, R. (2024). Comparison of sampling techniques and diagnostic tests for *Cryptosporidium serpentis* in eastern indigo snakes (*Drymarchon couperi*). American Journal of Veterinary Research, 85(10), 1–9.

Cai, S., Pataillot-Meakin, T., Shibakawa, A., Ren, R., Bevan, C. L., Ladame, S., Ivanov, A. P., & Edel, J. B. (2021). Single-molecule amplification-free multiplexed detection of circulating microRNA cancer biomarkers from serum. Nature Communications, 12(1), 3515.

De Silva, N. L., De Silva, V. N. H., Deerasinghe, A. T. H., Rathnapala, U. L., Itoh, M., Takagi, H., Weerasooriya, M. V., Kato, H., & Yahathugoda, T. C. (2022). Development of a highly sensitive nested PCR and its application for the diagnosis of cutaneous leishmaniasis in Sri Lanka. Microorganisms, 10(5), 990.

Groth-Helms, D., Rivera, Y., Martin, F. N., Arif, M., Sharma, P., & Castlebury, L. A. (2023). Terminology and guidelines for diagnostic assay development and validation: Best practices for molecular tests. PhytoFrontiers, 3(1), 23–35.

Helmy, Y. A., & Hafez, H. M. (2022). Cryptosporidiosis: From prevention to treatment, a narrative review. Microorganisms, 10(12), 2456.

Hoffmann, T., Hahn, A., Verweij, J. J., Leboulle, G., Landt, O., Strube, C., Kann, S., Dekker, D., May, J., Frickmann, H., & Loderstädt, U. (2021). Differing effects of standard and harsh nucleic acid extraction procedures on diagnostic helminth real-time PCRs applied to human stool samples. Pathogens, 10(2), 188.

Hsu, T.-K., Chen, J.-S., Tsai, H.-C., Tao, C.-W., Yang, Y.-Y., Tseng, Y.-C., Kuo, Y.-J., Ji, D.-D., Rathod, J., & Hsu, B.-M. (2021). Efficient nested-PCR-based method development for detection and genotype identification of *Acanthamoeba* from a small volume of aquatic environmental sample. Scientific Reports, 11(1), 21740.

Jaiswal, V., Brar, A. P. S., Sandhu, B. S., Singla, L. D., Narang, D., Leishangthem, G. D., & Kaur, P. (2022). Comparative evaluation of various diagnostic techniques for detection of *Cryptosporidium* infection from the faecal samples of diarrhoeic bovine calves. Iranian Journal of Veterinary Research, 23(3), 247.

Johansen, Ø. H., Abdissa, A., Zangenberg, M., Mekonnen, Z., Eshetu, B., Bjørang, O., Alemu, Y., Sharew, B., Langeland, N., Robertson, L. J., & Hanevik, K. (2021). Performance and operational feasibility of two diagnostic

tests for cryptosporidiosis in children (CRYPTO-POC): A clinical, prospective, diagnostic accuracy study. The Lancet Infectious Diseases, 21(5), 722–730.

Khan, N. U., Usman, T., Sarwar, M. S., Ali, H., Gohar, A., Asif, M., Rabbani, F., Khan, R. U., Sultana, N., Khan, N. A., Mobashar, M., Shah, A. A., & Wanapat, M. (2022). The prevalence, risk factors analysis and evaluation of two diagnostic techniques for the detection of *Cryptosporidium* infection in diarrheic sheep from Pakistan. PLoS One, 17(7), e0269859.

Khan, S. M., & Witola, W. H. (2023). Past, current, and potential treatments for cryptosporidiosis in humans and farm animals: A comprehensive review. Frontiers in Cellular and Infection Microbiology, 13, 1115522.

Li, H., Xie, Y., Chen, F., Bai, H., Xiu, L., Zhou, X., Guo, X., Hu, Q., & Yin, K. (2023). Amplification-free CRISPR/Cas detection technology: Challenges, strategies, and perspectives. Chemical Society Reviews, 52(1), 361–382.

Loiola, S. H. N., Galvão, F. L., Santos, B. M. dos, Rosa, S. L., Soares, F. A., Inácio, S. V., Suzuki, C. T. N., Sabadini, E., Bresciani, K. D. S., Falcão, A. X., & Gomes, J. F. (2021). Development of new staining procedures for diagnosing *Cryptosporidium* spp. in fecal samples by computerized image analysis. Microscopy and Microanalysis, 27(6), 1518–1528.

Mahmudunnabi, R. G., Kasetsirikul, S., Soda, N., Sallam, M., Pannu, A. S., Nguyen, N.-T., Stratton, H., & Shiddiky, M. J. A. (2024). Critical evaluation of current isolation, detection, and genotyping methods of *Cryptosporidium* species and future direction. Environmental Science: Water Research and Technology, 10(7), 1527–1551.

Rana, D. R. S. J. B., & Pokhrel, N. (2023). Effect of incorporation of bead-beating during DNA extraction for quantitative polymerase chain reaction-based detection of *Trichuris trichiura* in stool samples in community settings: A systematic review. Journal of Helminthology, 97, e15.

Robinson, G., Elwin, K., Jones, M., & Chalmers, R. M. (2023). A comparison of qPCR and microscopy for the detection and enumeration of *Cryptosporidium* oocysts from drinking water. Journal of Medical Microbiology, 72(6), 1715.

Santana, B. N., Ferrari, E. D., Nakamura, A. A., Silva, G. S. da, & Meireles, M. V. (2022). Validation of a one-tube nested real-time PCR assay for the detection of *Cryptosporidium* spp. in avian fecal samples. Revista Brasileira de Parasitologia Veterinária, 31(1), e000522.

Sohrabi, M., Nayebzadeh, H., Shokrani, H., Gohardehi, K., & Amini Farsani, Z. (2023). What is the preferred method for diagnosing cryptosporidiosis in sheep-Modified Ziehl–Neelsen or auramine O staining?. Journal of Zoonotic Diseases, 7(3), 367–370.

Srirungruang, S., Mahajindawong, B., Nimitpanya, P., Bunkasem, U., Ayuyoe, P., Nuchprayoon, S., & Sanprasert, V. (2022). Comparative study of DNA extraction methods for the PCR detection of intestinal parasites in human stool samples. Diagnostics, 12(11), 2588.

Tiffarent, R., Ekawasti, F., Nasrulloh, M. F., Imanjati, L. N., Kurniawati, D. A., Nugroho, H. A., Rizal, S., Saputra, S., & Nurcahyo, R. W. (2022). A nested-PCR assay for detection of *Cryptosporidium* spp. in cattle in Sulawesi Island, Indonesia. IOP Conference Series: Earth and Environmental Science, 1107(1), 012044.

Uppal, B., Singh, O., Chadha, S., & Jha, A. K. (2014). A comparison of nested PCR assay with conventional techniques for diagnosis of intestinal cryptosporidiosis in AIDS cases from Northern India. Journal of Parasitology Research, 2014, 706105.

Wang, X., Wang, X., & Cao, J. (2023). Environmental factors associated with *Cryptosporidium* and *Giardia*. Pathogens, 12(3), 420.

Weldemariam, D. T., Awoke, M. G., & Aklilu, M. A. (2024). Review on bovine cryptosporidiosis, its associated risk factors and diagnostics methods. SM Tropical Medicine Journal, 6(1), 8.

Yang, J., Li, D., Wang, J., Zhang, R., & Li, J. (2022). Design, optimization, and application of multiplex rRT-PCR in the detection of respiratory viruses. Critical Reviews in Clinical Laboratory Sciences, 59(8), 555–572.

Yue, H., Shu, B., Tian, T., Xiong, E., Huang, M., Zhu, D., Sun, J., Liu, Q., Wang, S., Li, Y., & Zhou, X. (2021). Droplet Cas12a assay enables DNA quantification from unamplified samples at the single-molecule level. Nano Letters, 21(11), 4643–4653.