Iraqi Journal of Science, 2025, Vol. 66, No. 4, pp: 1516-1524
DOI: 10.24996/ijs.2025.66.4.11

ISSN: 0067-2904

Molecular Characterization of Human Cryptosporidium Isolates from in Kirkuk, Iraq

Inas I. Rasheed[1]*,Hussein F. Hasan[2], , Neama A. Ahmed[1]
[1]*Department of Biology, College of Science, University of Kirkuk, Iraq*
[2]*College of Pharmacy, National University for Science and Technology, Thi Qar, Iraq*

Received: 28/1/2024 Accepted: 29/5/2024 Published: 30/4/2025

Abstract

This investigation aimed to determine the prevalence of *Cryptosporidium* in children residing in Kirkuk, Iraq. DNA was extracted from 64 stool samples that had tested positive for Cryptosporidium oocysts via microscopic examination. The extracted DNA was then analyzed using 18S ribosomal RNA (rRNA) gene polymerase chain reaction (PCR) testing. Significant differences were found in the prevalence of parasite in rural community (65.6%) compared to urban community (14.17%). Whereas, non-significant differences were observed in the rate of infectivity in male (50%) as compared to (29.7%) in female. The results also revealed significant differences between infectivity rate and age groups (P=0.004). In contrast, the use of the PCR system demonstrated that this technique is a more specific and sensitive method for the detection and identification of protozoan parasites like Cryptosporidium. Overall, the results confirmed that *C.Parvum* was frequently the species demonstrated in Kirkuk.

Key words: Molecular, Cryptosporidium, Kirkuk, Iraq.

التوصيف الجزيئي لعزلة البويغة الخفية البشرية في كركوك، العراق

ايناس احسان رشيد[1]*, حسين فاضل حسن[2] , نعمة علي احمد[1]
[1]قسم علم الحياة, كلية العلوم , جامعة كركوك, العراق
[2]كلية الصيدلة, الجامعية الوطنية للعلوم والتكنولوجيا, ذي قار , العراق

الخلاصة

تم استخراج الحمض النووي من 64 عينة براز كانت نتيجة موجبة مجهريا بالاكياس البوغية للبويغة الخفية بالجين المشفر 18SrRNA بأستخدام تفاعل البوليمر المتسلسل . ثم تم تحليل الحمض النووي المستخرج باستخدام اختبار تفاعل البلمرة المتسلسل الجيني (PCR) للريبوسوم 18S (rRNA) RNA. لوحظ فروقات معنوية في نسبة انتشار الطفيلي في الريف 65.6% مقارنة بالمدينة 14.17% في حين لم يظهر فروقات معنوية في نسبة الاصابة في الذكور 50% مقارنة بالاناث 29.7%. وكشفت النتائج أيضا وجود فروق ذات دلالة إحصائية بين معدل الإصابة والفئات العمرية (P = 0.004). كما تؤكد النتائج ان البويغة الخفية في كركوك هو النوع الابواغ الخفية *C.parvum*. من ناحية أخرى، فإن استخدام تقنية تفاعل البوليمراز المتسلسل قدم دليلاً على أن هذه التقنية هي الطريقة الأكثر تحديدًا وحساسية للكشف والتعرف على الطفيليات الأولية مثل *Cryptosporidium*.

***Email:** inasihsan@uokirkuk.edu.iq

الهدف؛ يهدف هذا البحث إلى تحديد مدى انتشار طفيلي الكريبتوسبوريديوم في الأطفال المقيمين في كركوك، العراق. وأكدت النتائج أن C.Parvum هو النوع الذي ظهر في كركوك.

1. Introduction

Cryptosporidium species are intestinal protozoan apicomplexan parasites that causes gastroenteritis in a variety of vertebrate hosts worldwide [1]. *Cryptosporidium* infection is predominantly transmitted through water or food contaminated with infected oocysts, as well as through person-to-person contact [2]. *Cryptosporidium* has a complex life cycle involving multiple stages [3]. It starts with the ingestion of oocysts, the dormant form of the parasite, typically through contaminated water or food. Inside the host's intestines, the oocysts release sporozoites, which invade the intestinal cells and multiply, forming new oocysts [4,5]. These oocysts are then excreted in the host's feces, continuing the cycle by contaminating the environment [6,7,8].

This cycle perpetuates the spread of *Cryptosporidium*, making it a significant cause of waterborne disease. Recently molecular diagnostic tools have been used for the precise identification of Cryptosporidium species [9-12]. Fundamentally, examining genotypic variation in Cryptosporidium is important for comprehending its biology, how it spreads, and how it evolves over time. A deeper understanding in these areas can ultimately help guide approaches for preventing, containing, and managing cryptosporidiosis [13,14].

However, little attention has been paid to genotyping diversity of *Cryptosporidium* species in Iraq. Therefore, the current study aimed to characterize the prevalence and genotype variation of cryptosporidiosis in fecal samples of human in Kirkuk, Iraq.

2. Materials and Methods

2.1 Collection of samples

A total of 600 human fecal specimens in Pediatric Hospital and General Kirkuk Hospital, were collected between January 2023 and December 2023 Epidemiological data of patients concerning age, gender and place of residence were collected.

2.2 Staining of oocysts

The fecal samples were smeared on to glass slide, stained with modified Ziehl- Nielsen stain for the identification of oocysts by microscopy [15,16 ,17]. The prevalence differences in relation to epidemiological data were tested by Pearson Chi- Square and p value was demonstrated for each experiment.

2.3 Extraction of DNA

DNA was extracted using (Stool DNA extraction package Favorge, Korea) according to the manufacturer's protocol. A 20 mg frozen stool sample was incubated with 30 ml of lysis buffer (pH 8.0, 10 mM EDTA, 10 mM Tris-HCl, 1 mg/ml K-protease) at 65°C for 2 hours to solubilize it, followed by heating to 100°C for 30 minutes to denature the enzyme. Cell wreckage and proteins were removed by centrifugation (10,000 × g, 4°C, 10 min). The portion of the supernatant Contains DNA were used for PCR, and 5 µl add DNA solution to PCR mix. Assess the purity of DNA samples using optical density on a Nanodrop spectrophotometer. The purity of DNA samples was assessed by optical density using a Nanodrop spectrophotometer[18].

2.4 PCR Protocol

The PCR amplification was performed using the primers forward (GGAAATCCGTCTATCAGTGG) and reverse (CCCTCACGGTACTTGTTTGC), which were the previously reported to specifically amplify of the18SrRNA gene of *C. parvum* . The PCR reaction mixture (25µl) containing 10mM Tris-HCI, 0.2 mM of each deoxy nucleoside

triphosphate, 25 pmol of each appropriate primer, and 2.5 U of Taq DNA polymerase were used amplified using gradient Perkin- Elmer thermocycler following thermal profile which Including 32 cycles (pre-incubation at 94°C for 5 minutes, denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute, extension at 72°C for 1 minute, and further incubation at 72°C for 10 minutes). A 10 µL aliquots of the PCR amplification products were analyzed via electrophoresis on a 1% agarose gel using Tris-borate EDTA (TBE X) running buffer containing 0.045 M Tris-borate and 1 mM EDTA [19]. The PCR product bands were visualized under UV - light after staining the gel with 0.2 mg/mL ethidium bromide. This allowed separation and detection of the amplified DNA fragments by size.

3. Results and Discussion

The prevalence of infection of the *Cryptosporidium* species as demonstrated by the direct method of 600 children stool examination are shown in Table 1. Of all samples examined, 64 (10.6%) were found to be positive for cryptosporidiosis. The extraction of total DNA from all microscopically positive stool samples were used as templates PCR reaction. The results revealed that the small subunit 18SrRNA gene was amplified from 64 (79.69%.) samples and not amplified from 13 (20.31%) samples. The results of this study investigation are in agreement with results obtained in other provinces in Iraq, Babylon, Kirkuk, and neighboring countries Nigeria and Vietnam, England, Iran [20-24]. The variation in infection rates may be attributed to factors such as the characteristics of the surveyed district, the level of personal hygiene and sanitation, the number of stool samples examined, and whether the children examined were hospital patients, from families, schools, or other institutions. On the other hand, the negative PCR results in some fecal samples may be due to the presence of other pathogenic parasitic protozoa [25].

Furthermore, the current study has shown an alternative application of 18SrRNA gene in the screening of cryptosporidial DNA in the stool samples of patients. The aplicon obtained by PCR resulted in amplicon of approximately size 510 bp (Figures 1,2,3,4,5,6,7). These findings are in consistent with results obtained from other studies indicating that *C. parvum* was the most prevalent species found in human [26]. According to the residency, higher rates of infectivity were encountered in rural community (65.6%) than in the urban community (14.1%) as shown in Table 2. The results revealed significant ($p= 0.460$) differences between residency and infectivity rate. From these findings it seems that the highest percentage of infectivity rate in rural area may be due to that most children came from poor families with low socioeconomic level and poor hygienic conditions. In addition, exposure to faecally transmitted organisms probably occurs continually. There are few published reports of stools survey with which to compare our findings [27,28]. Among the stool samples analyzed, 50% (32 out of 64) of those from male individuals were found to be positive for the presence of Cryptosporidium species. In contrast, the prevalence of Cryptosporidium species in stool samples from female individuals was 29.7% (19 out of 64). The comparison of these findings did not reveal a statistically significant difference between the gender of the individuals and the rate of Cryptosporidium infection. This observation aligns with the results reported in previously published studies [29, 30].

The age distribution of *Cryptosporidium* stools showed that two (3.1%) of age group 11-15, 10 (15.6%) of age group 6-10s year, 11 (17.2%) of age group under one year and 28 (13.8%) of age group 1-5 were positive. The results revealed significant differences between age groups. However, the less hygienic habits of people investigated may lead to contamination of hands, food and drinking water with cryptosporidial oocyst and to infection through eating of unwashed vegetables and fruits [31-37].

Table 1: Percentage of Infected and Non-infected Patients with *C. parvum* by using PCR

PCR positive microscopy positive	Positive		Negative		Total	
	No.	%	No.	%	No.	No.
Cryptosporidium parvum	51	79.69	13	20.313	64	100.00

Table 2: Distribution of the infected patients with *C.parvum* according to Habitation by PCR

Habitation		No. of infected with *C.Parvum*	No. of non-infected with *C.Parvum*	Total
Urban		9	3	12
		14.1%	4.7%	18.8%
Rural		42	10	52
		65.6%	15.6%	81.3%
Total		51	13	64
		79.7%	20.3%	100.0%

P value 0.460

Table 3: Distribution of with *C. parvum* according to Gender by PCR

Gender		No. of positive sample	No. of negative sample	Total
Male		32	9	41
		50.0%	14.1%	64.1%
Female		19	4	23
		29.7%	6.3%	35.9%
Total		51	13	64
		79.7%	20.3%	100.0%

P value 0.463

Table 4: Distribution of the infected patient with *C. parvum* according to age groups by PCR

Age group		No. of infected with C.parvum	No. of non-infected with C.parvum	Total
Age	<1	11	2	13
		17.2%	3.1%	20.3%
	1_5	28	2	30
		43.8%	3.1%	46.9%
	6_10	10	5	15
		15.6%	7.8%	23.4%
	11_15	2	4	6
		3.1%	6.3%	9.4%
Total		51	13	64
		79.7%	20.3%	100.0%

P value 0.004

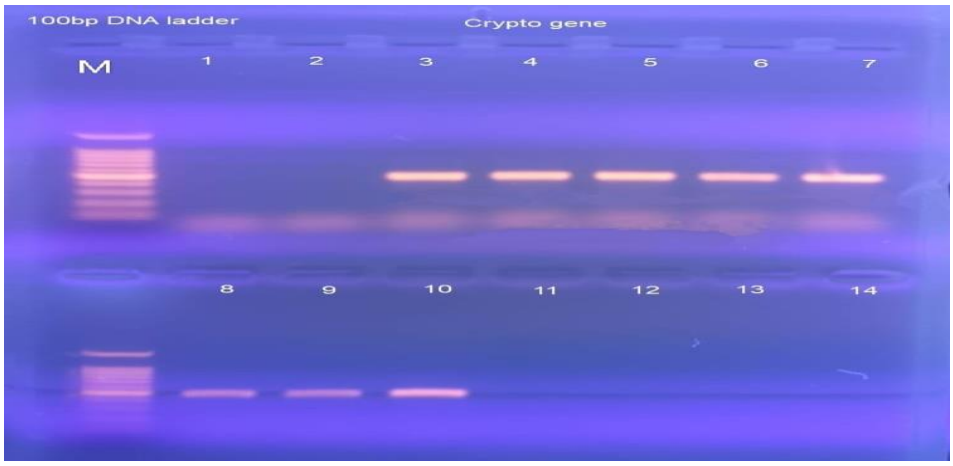

Figure 1: Agarose gel electrophoresis analysis for PCR products stained with 0.2 mg/ml of ethidium bromide for18SrRNA gene using primers species specific for *C. parvum* obtained from human stool samples. Positive samples reveal 500 bp bands. M represent (100-2000)pb DNA Lader Marker.

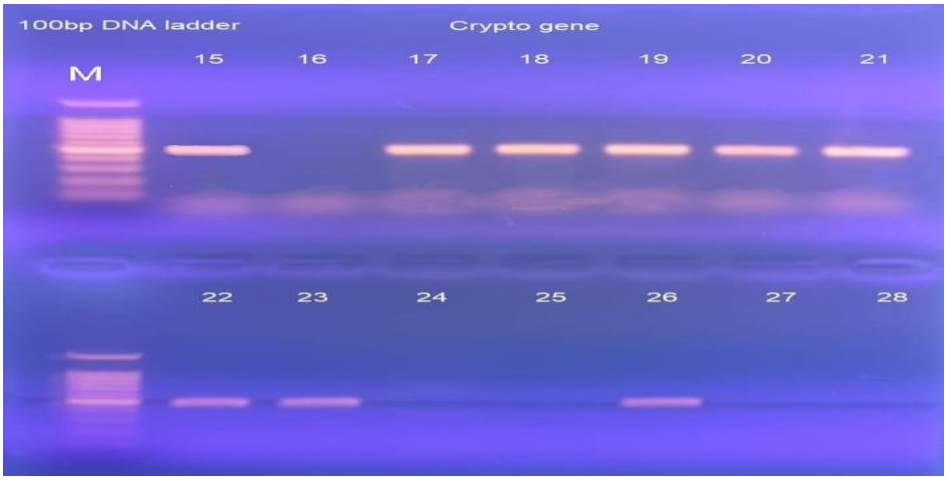

Figure 2: Agarose gel electrophoresis analysis for PCR products stained with 0.2 mg/ml of ethidium bromide for 18SrRNA gene using primers species specific for *C. parvum* obtained from human stool samples. Positive samples reveal 264 bp bands. M represent (100-2000) pb DNA Lader Marker.

Figure 3: Agarose gel electrophoresis analysis for PCR products stained with 0.2 mg/ml ethidium bromide for18SrRNA gene using primers species specific for *C.parvum* obtained from human stool samples. Positive samples reveal 500 bp bands. M represent (100-2000) pb DNA Lader Marker

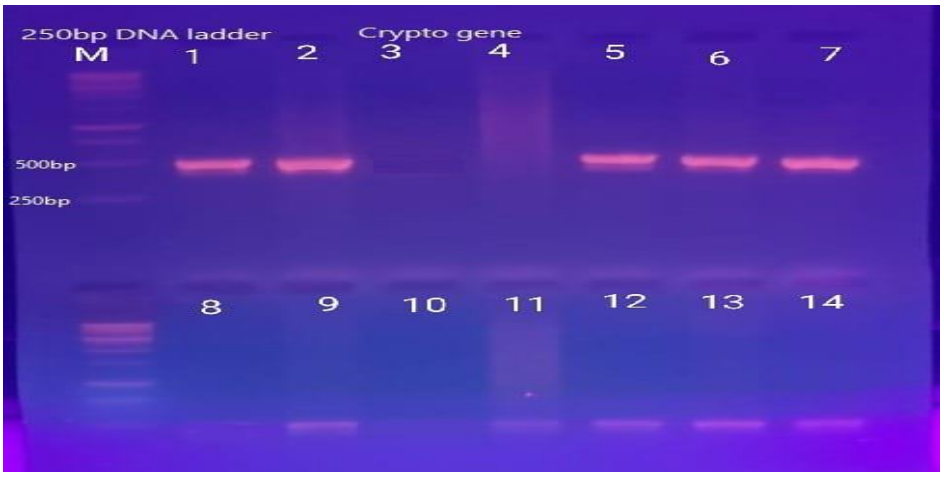

Figure 4:Agarose gel electrophoresis analysis for PCR products stained with 0.2 mg/ml of ethidium bromide for18SrRNA gene using primers species specific for *C. parvum* obtained from human stool samples. Positive samples reveal 500 bp bands. M represent (100-2000) pb DNA Lader Marker

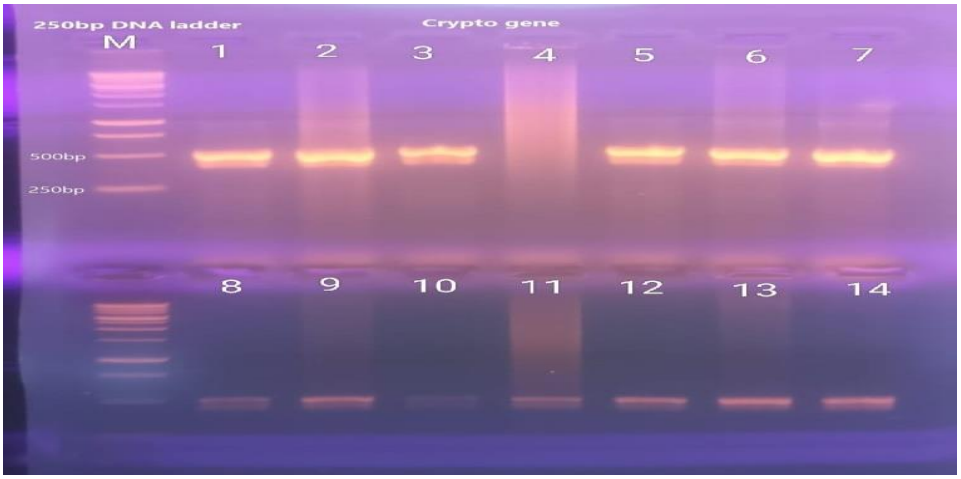

Figure 5: Agarose gel electrophoresis analysis for PCR products stained with 0.2 mg/ml of ethidium bromide for18SrRNA gene using primers species specific for *C. parvum* obtained from human stool samples. Positive samples reveal 500 bp bands. M represent (100-2000) pb DNA Lader Marker

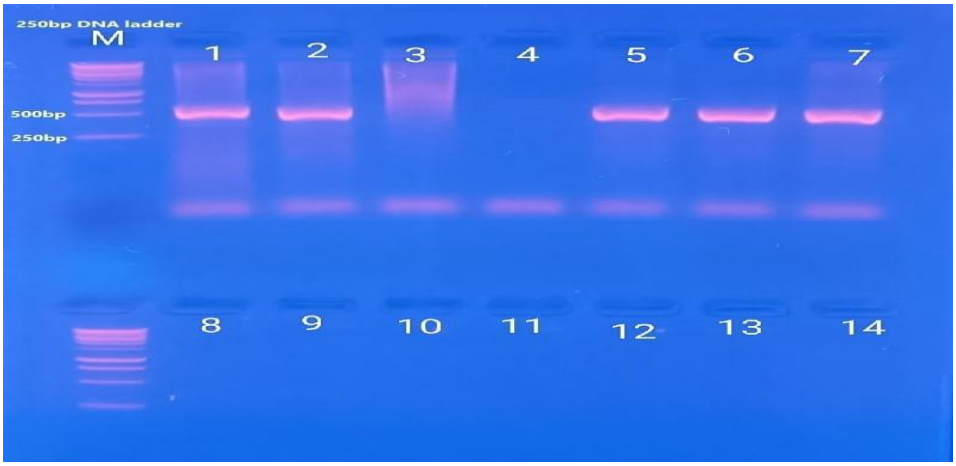

Figure 6: Agarose gel electrophoresis analysis for PCR products stained with 0.2 mg/ml of ethidium bromide for 18SrRNA gene using primers species specific for *C.parvum* obtained from human stool samples. Positive samples reveal 500 bp bands. M represent (100-2000) pb DNA Lader Marker

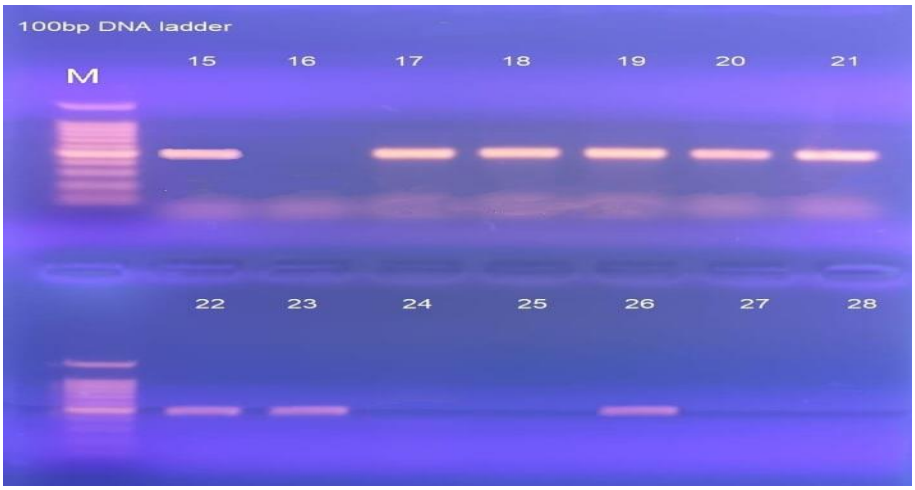

Figure 7:Agarose gel electrophoresis analysis for PCR products stained with 0.2 mg/ml of ethidium bromide for 18SrRNA gene using primers species specific for *C. parvum* obtained from human stool samples. Positive samples reveal 500 bp bands. M represent (100-2000) pb DNA Lader Marker

Conclusion

Ultimately, our findings validated that the Polymerase Chain Reaction (PCR) technique exhibits the highest sensitivity for the diagnosis of Cryptosporidium. However, alternative methods such as Ziehl-Neelsen staining and Enzyme-Linked Immunosorbent Assay (ELISA) can also be employed for the detection of this parasitic organism.

References

[1] M. Gracenea M, S. Castaño, J. Méndez, F Lucena, M. Gómez, "Faecal contamination in public pools in Barcelona province: Cryptosporidium spp. and bacterial indicators", *Journal Water Health*, vol.16, no. 5, pp.762-772.2, 2018.

[2] G.T. Keusch, C.F. Walker, J.K. Das, S. Horton, S. Kalyoussef, D. Goldman , "Giardiasis and cryptosporidiosis", *Pediatr Review,* vol.13, no. 2, pp. 837-850, 2010.

[3] V. McDonald, M.V. McCrossan, F. Petry, "Localization of parasite antigens in Cryptosporidium parvum-infected epithelial cells using monoclonal antibodies", *Cambridge Journals parasitology* , vol.110, pp. 259-268, 1995.

[4] F. Zapata, M.E. Perkins, Y.A. Riojas, T.W. Wu, S.M. Le Blancq, "The Cryptosporidium parvum ABC protein family", *Journals Molecular Biochemistry Parasitology*., vol. 120, pp. 157-161, 2002.

[5] S.P. O'Hara, J.R. Yu, J.J. Lin, "A novel Cryptosporidium parvum antigen, CP2, preferentially associates with membranous structures" , *Journal of Parasitology Research*, vol. 92, pp. 317-327, 2004.

[6] W.L. Current, N.C. Reese, "A comparison of endogenous development of three isolates of Cryptosporidium in suckling mice", *Journal of protozoology*, Vol. 33, pp. 98-108, 1986.

[7] J.M. Bjorneby, M.W. Riggs, L.E. Perryman, "Cryptosporidium parvum merozoites share neutralization-sensitive epitopes with sporozoites", *Journal of immunology*, vol. 145, pp. 298-304, 1990.

[8] N.S. Hijjawi, B.P. Meloni, M. Ng'anzo, U.M. Ryan, M.E. Olson, P.T. Cox, P.T. Monis, R.C. Thompson , "Complete development of Cryptosporidium parvum in host cell-free culture", *International Journal for Parasitology*, vol.34, pp. 769-777, 2004.

[9] B. Mahdavi Poor, J. Rashedi, M Asgharzadeh, E. Fallah, K. Hatam-Nahavandi, A. Dalimi, "Molecular Characterization of Cryptosporidium Species in Children with Diarrhea in North West of Iran", *International Journal of Molecular and Cellular Medicine*, vol. 4, no. 4, pp.235-9, 2015.

[10] Y.J. Salman, "Efficacy of some laboratory methods in detecting Giardia lamblia and Cryptosporidium parvum in stool samples", *Kirkuk Journal of Science*, vol. 9, no. 2, pp. 7-17, 2014.

[11] Y.J. Salman, and M.I. Mustafa, "Evaluation of the employment of four laboratory diagnostic methods in detecting Giardia lamblia among children in Kirkuk city", *Kirkuk Journal of Medical Sciences*, vol.1, no. 2, pp. 52-60, 2013.

[12] S.Ghaffari, and N. Kalantari, " Molecular analysis of 18S rRNA gene of Cryptosporidium parasites from patients living in Iran, Malawi, Nigeria and Vietnam", *International journal of molecular and cellular medicine*, vol.1, no. 3, pp. 153–61,2012.

[13] R. Peralta, J. Velásquez, F. Cunha, M. Pantano, F. Sodré, S. Silva, O. Astudillo, J. Peralta, S. Carnevale, "Genetic diversity of Cryptosporidium identified in clinical samples from cities in Brazil and Argentina", *Mem Inst Oswaldo Cruz*, vol,111, pp.30-6, 2016.

[14] Y. Helmy, H. Hafez, "Cryptosporidiosis: From Prevention to Treatment, a Narrative Review ", *Microorganisms journal*, vol. 12, pp, 2456, 2022.

[15] S.A. Henricksen, J.F. Pohlenz , "Staining of Cryptosporidia by a modified Ziehl- Neelsen technique ", *Acta Veterinaria Scandinavica,* vol. 22, pp. 594–6,1981.

[16] M. Bayraktar, M. Hussein, and H. Hassan, "Determination genetic variation and phylogenetic analysis in Echinococcus granulosus isolated from Iraqi sheep", *Journal of the Hellenic Veterinary Medical Society*, vol.73,no.2, 2, pp. 4245-4252,2022.

[17] L. Lazar, Sh. Al-Juboury, M. Maaroof, "Molecular detection of the ability of Biosynthesized Titanium dioxide nanoparticles to curing some genes of virulence factors of Entamoeba histolytica, " *Baghdad Science Journal*, pp. 2078- 8665, 2023.

[18] S. Hadfield, , G. Robinson, , K. Elwin, R. Chalmers, "Detection and differentiation of Cryptosporidium spp. in human clinical samples by use of real-time PCR", *Journal of Clinical Microbiology*, vol. 49, pp. 918–924,2011. DOI: 10.1128/JCM.01733-10

[19] M. Al-Shuhaib, "A minimum requirements method to isolate large quantities of highly purified DNA from one drop of poultry blood ", *Journal of Genetics*, vol. 97, pp. 87–94, 2018.

[20] A. Al-Amery, M.Al-Amery A, "Molecular diagnosis of Cryptosporidium ssp. in water buffaloes at babylon province, Iraq", *Iraqi journal of agricultural sciences*,vol. 53, no.1,pp. 147–156,2022.

[21] S. Ghaffari, N. Kalantari, "Molecular analysis of 18S rRNA gene of Cryptosporidium parasites from patients living in Iran, Malawi, Nigeria and Vietnam'', *International Journal of Molecular and Cellular Medicine*, vol. 3, pp. 153-61, 2012.

[22] P.m. Bartley, J.H. Standar, F. Katzer, " Genetic characterisation of Cryptosporidium parvum in dairy cattle and calves during the early stages of a calving season", *Curr Res Parasitol Vector Borne Dis*,vol. 5, no. 1, pp. 100-160,2023.

[23] E. Brook, C. Anthony Hart, N. French, R. Christley, "Molecular epidemiology of Cryptosporidium subtypes in cattle in England", *Veterinary Journal*, vol. 179, pp. 378–382,2009.

[24] A. Hiba, S.Yahya, M. AbdulKhaliq, "Some Epidemiological Aspects of Cryptosporidium parvum Among Children Below Five Years in Kirkuk Province", *Journal of Population Therapeutics and Clinical Pharmacology*, vol. 30, no. 8. pp. 378–389,2023.

[25] H. M. Obaid, " The Efficacy of Some Detergents on Some Intestinal Parasites and Their Histopathological Effects", *Iraqi Journal of Science*, vol. 58, No.4C, pp: 2350-2363, 2017.

[26] N. Hijjawi, A. Zahedi A, M. Al-Falah, U. Ryan, "A review of the molecular epidemiology of Cryptosporidium spp. and Giardia duodenalis in the Middle East and North Africa (MENA) region", *Infection, Genetics and Evolution*, vol. 98, pp. 98:105-212,2022.

[27] M. Al-Bayati1, L. J. Jihad and Sh.A. Al-Attar, ''The effects of Gastro intestinal Parasites on haemato-biochemical parameters of sheep in Kirkuk province, Iraq", *Journal of Applied Veterinary Sciences*,Vol. 8, pp. 62-68,2023.

[28] B.A. Mohmmed, Z.K. Rasheed, Jihad L.J. and K.S. Abass, "Frequency of Giardia lamblia among Iraqi children in Kirkuk governorate" , *Systematic Reviews in Pharmac*, vol.11, no. 12, pp. 1909-1911, 2020.

[29] S. Akram, and A. Bassad, "Molecular Detection and Prevalence of, Cryptosporidium Parvum,Enta1moeba histolytica and Giardia lamblia among Patients with Diarrhea at Al- Rifea city/Thi-Qar Province", *Journal of Global Pharma Technology*, vol.12, no. 1, pp. 503-511, 2020.

[30] I. M. Al-Sagur, S. H. Mahmood, H. M. A. Al-Obaidi , " Investigation of Giardia lamblia and other parasites in tap waters potential source of transmission in some regions of Baghdad " ,*Iraqi Journal of Science*, vol 56, no.1B, pp: 337-344, 2015.

[31] AWB. Hussien, "Study of prevalence intestinal parasite in patient visit some hospital of Baghdad ", *Anbar Journal Science*, vol. 2, no. 2, pp. 1-11, 2009.

[32] S.M. Mor, and S. Tzipori, "Cryptosporidiosis in children in Sub-Saharan Africa: a lingering challenge", *Clin Infect Dis*,vol. 47, no. 7, pp. 915-21, 2008.

[33] A. Nujood, J. Mansoor J. Alkhaled, " Molecular detection of Cryptosporidium spp. in stray cats in Al-Qadisiyah governorate, Iraq", *Iraqi Journal Veterinary Science*, vol 37. no.2, pp. 369-373, 2023.

[34] J. Al-Ani. and H. Al-Warid, " Nutritional Status and Lipid Profile Among Children Infected with Giardia lamblia and Cryptosporidium", *Iraqi Journal of Science*, vol. 64, no. 6, pp. 2717–2725, Jun. 2023,

[35] S.T. Mohammed, "Role of Kefir Milk on The Pathogenesis of Entamoeba histolytica" , *Iraqi Journal of Science*, Vol. 57, No.2B, pp.1116-1124, 2016

[36] Z. Kadhim, "Some Protozoan Species Inhabiting the East Bank Sediment of River Tigris in Baghdad City" ,*Iraqi Journal of Science*, vol. 55(2B), 655-667, 2014.

[37] A. Ahmed and H. Jasim , "Frequency of intestinal parasites among school children around Al Hawija, Kirkuk city", *Biomedicine (India)This link is disabled*, vo. 43, no. 3, pp. 988–991, 2023.