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Microscopic and Molecular Detection of Cystoisospora spp. Among Patients with Gastro-Intestinal Disorders in Thi-Qar, Iraq

Conflict of interest: nothing to declare.
Authors' contribution: Safana Bashar Sharhan – conceptualization, data curation, investigation, methodology, project administration, resources, software, validation, writing (original draft and review & editing); Bassad Al-Aboody – conceptualization, investigation, methodology, supervision, validation, visualization, writing (original draft and review & editing).

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Abstract

Introduction. Cystoisospora spp. is gastrointestinal parasite that causes Cystoisosporiasis, one of the causes of gastro-intestinal disorders in humans. The parasite is distributed globally, and children are more at risk of infection than adults.
Purpose. This study aimed to detection the prevalence of Cystoisospora spp. in stool samples of gastro-intestinal disorders patients.
Materials and methods. 500 stool samples were collected from patients with gastro-intestinal disorders who have referred to Bint Al-Huda Hospital Teaching, Muhammad Al-Moussawi Hospital, AL-Hussein Teaching Hospital and general AL-Nasiriyah Teaching Hospital / Thi-Qar province from January / 2024 to January / 2025, with different ages for examined by microscopic examination and Nested PCR technique.
Results. The percentage of positive samples Cystoisospora spp. by microscopic examination was 3.2% and negative samples was 96.8%. The rate infected patients among females were 3.97% higher than the males were 2.02%, the highest infected patients found 3.95% in Urban area and lowest infected patients found 1.75% in Rural area. According to age group the highest infected patients was 3.94% in age group less than 1–10 years and lowest infected patients found 1.92% in age group 31–40 years and a not-infection recorded in both 51–60 and 61–70 (0.0%), while results of Nested PCR from 46 sample were positive in 15 samples with percentage 32.60% and 31 negative samples with percentage of 67.39%.
Conclusion. There is a relatively high rate of Cystoisospora spp. infection among children in Thi-Qar province. Cystoisospora spp. is one of the causes of sever diarrhea through oocyst stage.
Keywords: Cystoisospora spp., PCR, protozoa, Cystoisosporiasis, diarrheal diseases

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Микроскопическое и молекулярное выявление Cystoisospora spp. у пациентов с желудочно-кишечными заболеваниями в Ти-Каре, Ирак

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Резюме

Введение. Cystoisospora spp. – это желудочно-кишечный паразит, вызывающий цистоизоспороз, одну из причин желудочно-кишечных заболеваний у человека. Паразит распространен по всему миру. Дети подвержены большему риску заражения, чем взрослые.
Цель. Определение распространенности Cystoisospora spp. в образцах кала пациентов с желудочно-кишечными заболеваниями.
Материалы и методы. Было собрано 500 образцов кала у пациентов с желудочно-кишечными заболеваниями, обратившихся в учебную больницу Бинт Аль-Худа, больницу Мухаммада Аль-Муссави, учебную больницу Аль-Хусейна и общую учебную больницу Аль-Насирия в провинции Ти-Кар с января 2024 г. по январь 2025 г. Образцы были взяты у пациентов разного возраста для микроскопического исследования и метода вложенной ПЦР.
Результаты. Процент положительных образцов Cystoisospora spp. при микроскопическом исследовании составил 3,2%, а отрицательных – 96,8%. Частота инфицирования среди женщин была выше (3,97%), чем среди мужчин (2,02%). Наибольшее количество инфицированных пациентов (3,95%) было выявлено в городской местности, наименьшее – в сельской (1,75%). В зависимости от возрастной группы наибольшее количество инфицированных пациентов (3,94%) было выявлено в возрастной группе 1–10 лет, наименьшее – в возрастной группе 31–40 лет (1,92%). Отсутствие инфекции зарегистрировано как у лиц 51–60 лет, так и у лиц 61–70 лет (0,0%). Результаты вложенной ПЦР из 46 образцов были положительными в 15 (32,60%) случаях и отрицательными – в 31 (67,39%).
Заключение. Относительно высокий уровень инфицирования Cystoisospora spp. наблюдается среди детей в провинции Ти-Кар. Cystoisospora spp. является одной из причин тяжелой диареи на стадии ооцисты.
Ключевые слова: Cystoisospora spp., ПЦР, простейшие, цистоизоспороз, диарейные заболевания

■ INTRODUCTION

Intestinal parasitic infections (IPIs) are a serious public health problem in developing countries [1]. Numerous parasites, including protozoa (e.g., Entamoeba histolytica, Giardia intestinalis, Cryptosporidium spp. and Cystoisospora belli) and helminths (e.g., Ascaris lumbricoides, Trichuris trichiura, Necator americanus, Hymenolepis nana, and Ancylostoma duodenale), can cause gastrointestinal infections [2]. The burden of IPIs is estimated approximately 3.5 billion people worldwide [3]. socioeconomic status is the key factor associated with prevalence of IPIs, with the observation that the higher prevalence of Intestinal parasitic infections is due to a low socioeconomic status, consequently leading to poor hygiene and sanitation practices [4]. Intestinal parasitic infections are closely related to poverty, unsafe drinking water, hygiene and poor sanitation, with symptoms including diarrhea, anemia, malabsorption, weight loss, dyspepsia, abdominal pain, growth retardation [5]. Cystoisospora belli is a coccidian parasite commonly associated with enteric infections in immunocompromised individuals [6]. immunocompetent individuals find it unpathological or asymptomatic, which prevents them from recognizing that they have the disease [7]. Cystoisospora spp. formerly known as Isospora spp. is an intracellular obligate protozoan parasite that primarily inhabits the intestines of its hosts, leading to significant gastrointestinal disturbances, it is known to cause acute to severe diarrhea in humans [8]. Cystoisospora spp. infection, commonly referred to as cystoisosporiasis, holds significance as a causative agent of diarrhea, particularly prevalent in tropical regions [9]. Cystoisospora belli infection occurs by ingestion of water or food contaminated with oocysts [10]. Developmental stages of Cystoisospora spp. comprise asexual multiplication, sexual reproduction, and sporogony. Definite diagnosis of cystoisosporiasis relies on identification of characteristic oocysts in stool or intestinal content. Oocysts of Cystoisospora belli can be seen by direct smear of duodenal content or the acid-fast staining procedure [11].

■ MATERIALS AND METHODS

Samples collection

A total of 500 stool samples were collected from patients with gastro-intestinal disorders who have referred to Bint Al-Huda Hospital Teaching , Muhammad Al-Moussawi Hospital, AL-Hussein Teaching Hospital and general AL-Nasiriyah Teaching Hospital / Thi-Qar province from January / 2024 to January / 2025, the ages were ranged from 13 day – 70 years, 198 were males and 302 were females. Fecal samples were collected by using a sterile containers and then transported in to the laboratory College of Science at the laboratory the fecal samples were divided into two portion the first portion was for the microscopic examination of parasites while the second portion stored directly at –20 °C for molecular analysis by Nested PCR [12].

Microscopic examination

Modified acid-fast staining technique (MZN)

The method was done according to [13] as following:

1. An appropriate amount of stool was taken to make a swab by mixing it with a few drops of water using a stick and sticking it on the slide.
2. It was immersed in carbolfushin dye for five minutes and the slide was heated by burner for a few seconds until the carbolfushin evaporates.

3. The slide was washed by drop water to remove the rest of the dye.
4. The slide was immersed in rubbing alcohol for one to three minutes.
5. The slide was washed again by drop water to remove the alcohol.
6. Finally the slide was immersed in methylene blue for one minute, and the slide is washed with water to remove the methylene blue. Airs dry the slide.
7. The slide was examined with a lens 40X and then 100X to identify Cystoisospora spp. Oocysts that appear dark pink or purple. And when using the 100X lens, special oil must be used for it.

Molecular diagnosis

The identity of Cystoisospora spp. was confirmed ,after morpholgical characterization, genetically by Nested PCR using two set of Cystoisospora spp. PCR primers were designed in this study for detection Cystoisospora spp. based on small subunit ribosomal gene (SSU rRNA) were designed in this study using NCBI-Genbank sequence and primer 3 plus design. These primers was provided from Scientific Reserch er. Co. Ltd, Iraq as following (Table1).

Genomic DNA from stool samples of patients with gastro-intestinal disorders. were extracted by using Presto™ Stool DNA Extraction Kit, Korea , and done according to company instructions.

Nested PCR thermocycler conditions by using convential PCR thermocycler system as following Initial denaturation 95 ºC for 5 min and 35 cycle of 30 sec at 95 ºC, 30 sec at 58 ºC and 2 min at 72 ºC followed by 5 min final extension at 72 ºC. PCR product was electrophoresed on 1.5% agarose gel and visualized by UV.

Sequencing and phylogenetic analysis

Multiple sequence alignment analysis of Cystoisospora spp. isolate based on small subunit ribosomal RNA gene that aligned with NCBI-Genbank submitted related Cystoisospora spp. isolates. The multiple alignment analysis was constructed using ClustalW alignment tool in (MEGA 11.0.13 version). Phylogenetic tree analysis of Cystoisospora spp. isolate based on small subunit ribosomal RNA gene that used for genetic species identification. The phylogenetic tree was constructed using Neighbor-Joining method and the evolutionary distances were computed using the Maximum Composite Likelihood method in (MEGA 11.013 version).

Statistical analysis

The data of the current study was analysed by SPSS version 26, based in using Chi-Square for independent and Odds ratio at $p<0.05$ [14].

Table 1
PCR primers were designed in this study for detection Cystoisospora spp.

Primers	Sequence 5'-3'		Product size	Genbank Reference code
Isospora spp. Inner PCR primer	F	ATCTAAGGAAGGCAGCAGGC	747bp	PP660175.1
	R	ACCCTTCCGCCAATTCCTTT		
Isospora spp. Outer Nested PCR primer	F	GTAGTTGGATTTCTGTCGGGGT	611bp	PP660175.1
	R	TGTCAATCCTTCCCATGTCTGG		

■ RESULTS

Infected and non-infected patients with Cystoisospora spp. by modified acid-fast staining technique (MZN)

Percentage of infected and non-infected patients with Cystoisospora spp. by Modified Acid-Fast staining technique (MZN). The result of examination 500 patient stool samples with gastro-intestinal disorders examined by Modified Acid-Fast staining technique using light microscope. The percentage of infected patients which were 16 positive samples with percentage 3.2% and 484 negative samples, with the percentage was 96.8%. As showed in figure 1.

Distribution of the infected patients with Cystoisospora spp., according to sex by modified acid-fast staining technique

The current reselts was recorded a significant difference at p. value <0.05, in the distributed of Cystoisospora spp. according to sex, where the infection rates are 3.97% and 2.02% among females and males respectively (Table 2).

Distribution of Cystoisospora spp. according to residency by modified acid-fast staining technique

The current results were showed a significant difference at p<0.05, in the distributed of Cystoisospora spp. according to residency, was investigated the high infected patients was in urban residence 3.95%, while in the rural residence 1.75% (Table 3).

Table 2
Distribution of the infected patients with Cystoisospora spp. according to sex by modified acid-fast staining technique

Sex	Infected patents		Total No. of examined
	No.	%	
Female	12	3.97	302
Male	4	2.02	198
Total	16	3.20	500
$TabX^2$=3.84 $CalX^2$=4.00 DF=1 p. value 0.046			

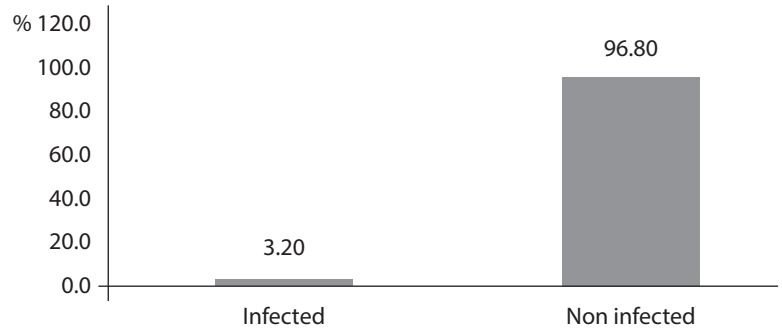

Fig. 1. The percentage of infected and non-infected patients with Cystoisospora spp.by modified acid-fast staining technique

Table 3
Distribution of patients infected with Cystoisospora spp. according to residency Direct smear

Residency	Infected Patents		Total No. of Examined
	No.	%	
Urban	13	3.95	329
Rural	3	1.75	171
Total	16	3.20	500
TabX²=3.84 CalX²=6.25 DF=1 p. value 0.275			

Distribution of Cystoisospora spp. according to age groups by modified acid-fast staining technique

The current results were recorded a significant difference at p. value <0.05, in the distributed of Cystoisospora spp. according to age groups, was noted the highest infected patients in the age group <10 years – 3.94%, then in the age group 11–20 – 3.80%, while the lowest infection in the age group 31–40 – 1.92%, in addition, a not-infection recorded in both age groups 51–60 – 61–70 – 0.0% (Table 4).

Percentage of infected and non-infected patients with Cystoisospora spp. by nested PCR

The current study included examination of 46 sample which were 16 positive samples and 30 negative samples by Nested PCR, the results showed percentage of infected patients which were 15 positive samples with percentage 32.60% and 31 negative samples, the percentage 67.39%, as shown in figure 2 and 3.

Comparison between positive microscopic examination and nested PCR method to diagnostic Cystoisospora spp. in stool samples

The current results were recorded a significant difference at p. value <0.05, according to identification of Cystoisospora spp. by both microscopic and PCR, while, recorded a non-significant difference between two technique in diagnosis of Cystoisospora spp., as in table 5. The results showed that 16 samples of Cystoisospora spp. that were examined by microscopy were positive, as well as examination by PCR technology. The results

Table 4
Distribution of Cystoisospora spp. according to age group by modified acid-fast staining technique

Age groups	Infected patents		Total No. of examined
	No.	%	
<10 years	8	3.94	203
11–20	4	3.80	105
21–30	2	3.50	57
31–40	1	1.92	52
41–50	1	2.27	44
51–60	0	0.0	21
61–70	0	0.0	18
Total	16	3.2	500
TabX²=9.49 CalX²=10.8 DF=4 p. value 0.028			

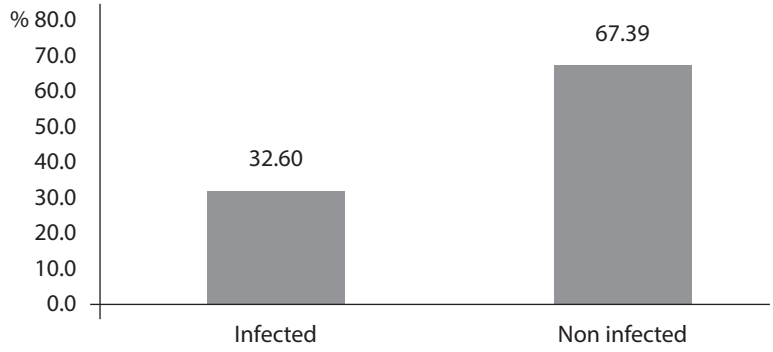

Fig. 2. The percentage of infected and non- infected patients with Cystoisospora spp. by using Nested PCR

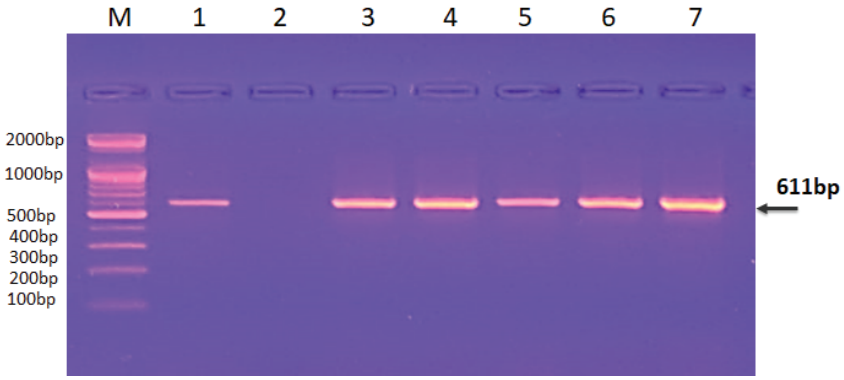

Fig. 3. Agarose gel electrophoresis image that showed the Nested PCR product analysis of small subunit ribosomal RNA gene in Cystoisospora spp. from Human stool samples. Where M: marker 2000-100bp and Lane 1–7 show some positive Cystoisospora spp. at 611bp PCR product

of 14 (87.5%) were positive and 2 samples (12.5%) were negative, and 30 samples that were examined by PCR method the results of 1 (3.33%) were positive and 29 (96.6%) were negative (Table 5).

Table 5
Comparison between positive microscopic examination and nested PCR method to diagnostic Cystoisospora spp.in stool samples

No. of sample	Microscopic examination				PCR examination			
	Positive		Negative		Positive		Negative	
	No.	%	No.	%	No.	%	No.	%
16	16	100	0	0.0	14	87.5	2	12.5
30	0	0	30	100.0	1	3.33	29	96.6
46	16	34.78	30	65.21	15	32.6	31	67.39
	p. value <0.001				p. value <0.001			
	p. value 0.369							

Phylogenetic tree analysis of the Cystoisospora spp.

The phylogenetic tree analysis of Cystoisospora spp. was investigates the genetic species typing and genetic variation between local Cystoisospora spp. five isolates and NCBI-Genbank submitted global related Cystoisospora spp. isolates, utilizing the small subunit ribosomal RNA (SSU rRNA) gene sequences. The phylogenetic tree was constructed using the Neighbor-Joining method, and evolutionary distances were calculated using the Maximum Composite Likelihood approach. Multiple sequence alignment revealed high sequence similarity in the SSU rRNA gene across the aligned Cystoisospora isolates, with only a few nucleotide substitutions observed. The phylogenetic tree analysis showed that the local Cystoisospora spp. isolates (IQN.1 to IQN.5) were closely related to the NCBI-BLAST Cystoisospora belli reference isolate, with genetic variation ranging from 0.19% to 0.39% and sequence identity of 99.61% to 99.81%. The close phylogenetic relationship between the local Cystoisospora spp. isolates and the Cystoisospora belli reference sequence suggests that the local isolates belong to the Cystoisospora belli species (Table 6 and Figures 4, 5).

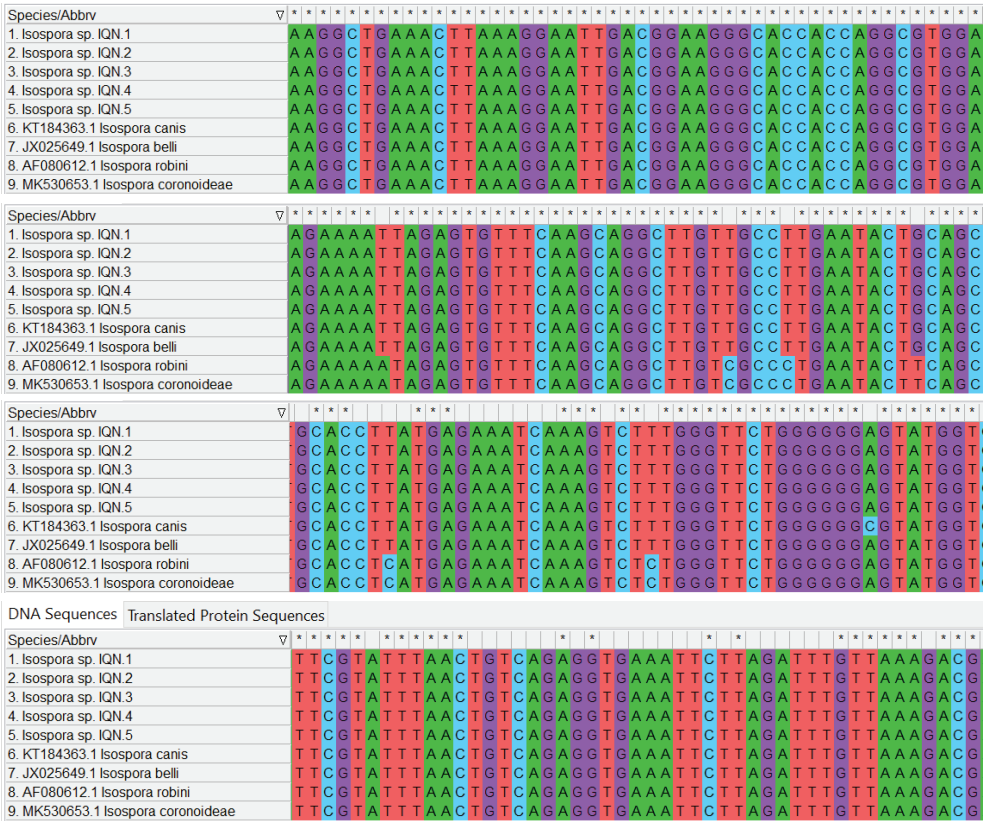

Fig. 4. Multiple sequence alignment analysis of Cystoisospora spp. isolate based on small subunit ribosomal RNA gene that aligned with NCBI-Genbank submitted related Cystoisospora spp. isolates. The multiple alignment analysis was constructed using ClustalW alignment tool in (MEGA 11.0.13 version). The analysis showed the nucleotide alignment similarity and substitution mutations in small subunit ribosomal RNA gene among all alignment sequence

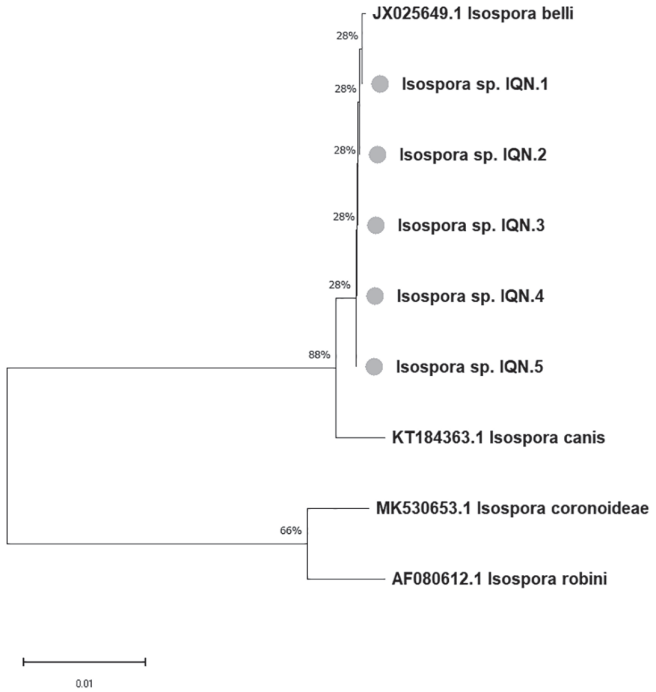

Fig. 5. Phylogenetic tree analysis of Cystoisospora spp. isolate based on small subunit ribosomal RNA gene that used for genetic species identification. The phylogenetic tree was constructed using Neighbor-Joining method and the evolutionary distances were computed using the Maximum Composite Likelihood method in (MEGA 11.013 version). The local Cystoisospora spp. IQN.1 into IQN.5 isolates were showed closed related to NCBI-BLAST Cystoisospora belli at total genetic changes (0.01%)

Table 6
NCBI-BLAST homology sequence identity between local isolates and NCBI-BLAST submitted isolates

Local isolate	Genbank Accession number	NCBI-BLAST Homology Sequence identity (%)		
		No. of Mutation	Genetic variation%	Identity (%)
Cystoisospora spp. IQN1	PQ577798.1	1	0.19%	99.81%
Cystoisospora spp. IQN2	PQ577799.1	1	0.19%	99.81%
Cystoisospora spp. IQN3	PQ577800.1	2	0.39%	99.61%
Cystoisospora spp. IQN4	PQ577801.1	1	0.19%	99.81%
Cystoisospora spp. IQN5	PQ577802.1	1	0.19%	99.81%

■ DISCUSSION

Enteric pathogens are a major source of morbidity and mortality throughout the world [15]. Cystoisospora spp. is an intestinal protozoon distributed worldwide [16]. It usually causes non bloody and watery diarrhea in tropical and subtropical climates [17]. Diarrhea disease estimates 525,000 children die from diarrhea each year, which is the second greatest cause of death in children under the age of five [18]. The disease course is mild and usually transient in immunocompetent hosts. In immunocompromised individuals, the disease

can vary in severity from a chronic intermittent illness to severe life-threatening diarrheal illness [19]. Studies showed that many parasitic infections are prevalent and endemic in Iraq, however research on Cystoisospora spp. infections is limited in Iraq, with no reported data in Thi-Qar. The microscopic and molecular examination of stool samples remains the backbone of the diagnosis intestinal protozoa particularly in developing countries, the current study included examination 500 stool samples of patient with gastro-intestinal disorders examined by a Modified Acid-Fast staining technique for Cystoisospora spp. by using light microscope and Nested PCR. The microscopic examination showed that 16 (3.2%) of total samples were positive samples and 484 (96.8%) were negative samples. There are other studies that have found different rates [20] 11/354 (3.1%), [21] (9.7%). The infection with parasite may be related to worldwide distribution of this parasite comparing with other, and the transmission of these parasites occurs via fecal-oral route, either directly from person to person or indirectly by eating or drinking fecal contaminated water and food. Also this may be related to the poor living conditions and like of sanitation in studied area [22].The differences in the prevalence of parasite infection are supposed due to differences in methodology, geographical location, and type of study population, sensitivity and specificity of laboratory methods or stage of the disease [23]. The results in microscopic examination showed the highest infection in females (3.97%) and lowest males (2.02%). These results are agreed with [24] and not agreed with other study [25] in Sulaimaniyah, Iraq 156/24 (15.38) male, 156/18 (11.54) female and [26] 11 (68.8%) male, 5 (31.2%) female. Reasons for the high incidence of infection with intestinal parasites in females may be due to a combination of biological, hormonal, and socio-economic factors. The highest rate of Cystoisospora spp. infection 3.94% was found among children aged 13 day – 10 years. There are other studies that have found different rates [25] (26.92%), [27] in Egypt (2.3%), [28] (1%) and [29] in Erbil, Iraq (3.8%). Reasons for the high incidence of infection in Children may be due to their immature immunity and feeding and exploratory behaviours [30]. urban citizens reported a higher infection rate 3.95% than rural citizens 1.75%. The current results are agreed with [25] in Sulaimaniyah, Iraq 156/36 (23.08) Urban, 156/6 (3.85) Rural and [31] (137/4) Urban and (137/0) Rural. The transmission of the Cystoisospora spp. among humans is aided by environmental pollution, tainted food, tainted drinking water. The current study included examination of 46 sample by Nested PCR, the results showed percentage of infected patients which were 15 positive samples with percentage 32.60% and 31 negative samples with percentage 67.39%. There are other studies that have found different rates [32] with a range of positive (n=21) and [33] 16/354 (4.5%). That showed sensitivity PCR When compared with stool microscopy results, PCR results seemed to be more accurate, efficient and sensitive as it was able to determine positive samples that were microscopically negative, this helped to solve one important drawback of microscopy which is low sensitivity as indicated by [32]. The Comparison between positive microscopic examination and nested PCR method to diagnostic Cystoisospora spp. in stool samples the results showed that 16 samples of Cystoisospora spp. that were examined by microscopy were positive, as well as examination by PCR technology. The results of 14 (87.5%) were positive and 2 samples (12.5%) were negative The current results are agreed with [31, 33]. The reason for the higher incidence of infections in this study diagnosed by acid-fast stain compared to nested PCR may be attributed to the low and insufficient DNA concentration of Cystoisospora spp. Oocysts in the stool of positive samples which prevent DNA amplification.

■ CONCLUSION

There is a relatively high rate of Cystoisospora spp. infection among children in Thi-Qar province. Cystoisospora spp. is one of the causes of sever diarrhea through oocyst stage.

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