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IDENTIFICATION OF *CRYPTOSPORIDIUM* SPP. INFECTIONS IN CHILDREN WITH PERSISTENT DIARRHEA BY MODIFIED ZIEHL-NEELSEN STAIN METHOD AND PCR TECHNIQUE.

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ABSTRACT

Background: Cryptosoridiosis is an intestinal infection caused by intracellular protozoan parasite *Cryptosporidium*, which may appear as a persistent diarrhea in children, that may lead to malnutrition, growth stunt and wasting. Since *Cryptosporidium* disease does not produce a specific clinical syndrome which allows to distinguish it from other intestinal infections; therefore, the final diagnosis requires a microscopic test for detection of the parasite in stool. **Methods:** Prevalence of cryptosporidiosis in children was studied in Najaf province by microscopic examination of stool samples by modiefied acid stain method in 140 children with persistent diarrhea, and the positive cases were confirmed by using SSU rRNA-based PCR-detecting technique. **Results:** The prevalence of cryptosporidiosis in children with persistent diarrhea was 12.85%, and the infection rate was higher in males (13.5%) than in females (12.1) and those with age less than 1 year (13.57%) than that of other age groups, and in children of the rural areas (14.3%) than that of the urban areas (10.5%). And the highest infection rate was recorded in May (3.57%). **Conclusion:** *Cryptosporidium* spp. is one of the most common enteric parasite that associate with persistent diarrhea in children.

KEYWORDS: Cryptosporidiosis, *Cryptosporidium*, modified acid-fast stain, SSU rRNA gene.

1.INTRODUCTION

Cryptosporidium is a protozoan belongs to the phylum Apicomplexa, and it is an obligate intracellular parasites, that own an apical compound which helps it in the penetration of host cell.[1] Many studies proved that *Cryptosporidium* is one among the four real pathogens which cause diarrhea in children with a prevalence of 8-10%.[2] and that has a great impact on mortality of children.[3] *Cryptosporidium* can cause persistent diarrhea, which leads to malnutrition, growth stunting, and wasting, and the infection called cryptosporidiosis.[4] It distributed in more than 40 countries around the world.[5] There are many methods to detect cryptosporidiosis, such as modified Ziehl–Neelsen stain (acid-fast stain) method which detects *Cryptosporidium* oocysts in stool, it is the most commonly used method for the diagnosis of cryptosporidiosis.[6]

Modern molecular techniques, the PCR, is the most sensitive technique.[7] The rRNA (SSU rRNA) gene or the *Cryptosporidium* oocyst wall protein (COWP) gene are used to determine *Cryptosporidium* species by PCR technique.[8]

Since *Cryptosporidium* disease does not produce a specific clinical syndrome which allows to distinguish it from other intestinal infections, therefore, final diagnosis requires a microscopic test for detection of the parasite in stool.[9]

The study aim is to measure the prevalence of *Cryptosporidium* infection among children with persistent diarrhea in Najaf province.

The objective of this study was achieved by the following methods

1-Detection of *Cryptosporidium* oocysts in stool samples by modified acid-fast stain method.

2-Using SSU rRNA-based PCR-detecting technique to confirm the detection of the parasite.

2.MATERIALS AND METHODS

2.1. Collection of Stool Samples

This study was carried out during the period from the beginning of January till the end of June 2016.

Stool samples were collected from 140 children attending Al-Zahra'a Teaching Hospital and suffering from persistent diarrhea, of both genders (74 males and

66 females), their ages range between (<1) year to 15 years. Each sample was placed in a screw capped container used for collection of stool samples, labeled with the number and the date of collection, and some of the samples were transported in cooling box, to the department of Microbiology at the Faculty of Medicine /University of Kufa for the purpose of the study.

2.2. Examination by modified Ziehl–Neelsen stain method

2.2.1. Dye preparation: Preparation of the stain (modified Ziehl–Neelsen) was done according to the way of (10):

***The composites:**

1- Basic carbolfucsin stain (4gm). 2- Phenol (8gm). 3- Ethanol 95% (20 ml). 4- Distilled water (100 ml).

***Preparation**

-The solution (A)

(4gm) of basic carbolfucsinin stain was dissolved in (20ml) ethanol 95%.

- The solution (B)

(8gm) of phenol was dissolved in (100ml) distilled water with constant stirring until it was completely dissolved.

The solution (B) was added to the solution (A) and the mixture was blended well by a magnetic stirrer, then it was filtered through Whatman No.1 filter paper into a dark bottle, then labeled and the date has been recorded.

2.2.2. The Procedure of modified acid-fast staining (modified Ziehl–Neelsen) method: according to (11) by (Syrbio, Switzerland) kit:

1. The smear has made from the sediment of (5gm) stool specimen that centrifuged at (1000 rpm).
2. It was left to dry.
3. It has been placed in a slide rack for fixing, then methanol was added and left for five minutes.
4. The prepared carbolfuchsin stain was applied to the slide for 20 minutes, and the slide has been heated, up to the steaming but without boiling.
5. The slide was washed with tap water.
6. Acid alcohol (HCL with 95% alcohol) has been added to the slide for decolorization.
7. The slide was washed with tap water.
8. Methylene blue (3%) was added and left for 30 seconds to one minute.
9. The slide was washed with tap water.
10. It was dried in air.
11. It was examined with standard light microscopy.
12. *Cryptosporidium* spp. oocyst can be stained red with a blue background by modified Ziehl–Neelsen stain method. *Cryptosporidium* oocyst is rounded and approximately (4-6 µm) in diameter. Potassium dichromate 2.5% was used for preservation of the positive stool specimens for *Cryptosporidium*, then it was kept at 4 ºC until using for subsequent PCR procedures.

2.3. PCR Technique for Detection of *Cryptosporidium*

2.3.1. DNA Extraction from Stool Specimens

In this part of the study, positive stool specimens for *Cryptospordium* were used, according to the modified protocol.[12] and.[13]

***Washing**

1. The tube of stool specimen with dichromate was placed on vortex.
2. (200 µl) of stool sample was transferred to an eppendrof (1.5ml) tube.
3. (800 µl) of deionized distilled water was added to the stool specimen.
4. The specimen was centrifuged with (13.000 rpm) for ten minutes.
5. The supernatant was collected and removed.
6. (1000 µl) of deionized distilled water was added, after that the tube was placed on vortex to separate the pellet.
7. The specimen was centrifuged at (14.000 rpm) for three minutes.
8. The supernatant was collected and removed.
9. The steps (6-8) were repeated for (3-5) times.

Then the specimens were transferred to a (55°C) shaker water bath, proteinase K was added to the specimens (at a final concentration of 200 µg/ml), the time was approximately (20 min), which was chilled in ice for one minute.

***Freezing and thawing**

1- The nuclei lysis solution was added to the specimens.

2- The specimens were frozen at (-170 °C) by liquid nitrogen for one

minutes (in liquid nitrogen containers) and were thawed at (98 °C) for three minutes (in a water bath).

3- Stage (2) was repeated for seven cycles.

4- The specimens were centrifuged at (13.000 rpm) for ten minutes,

and the supernatant was discarded.

5- The specimens were treated to the next steps of DNA extraction.

***DNA extraction**

A stool sample was used for the DNA extraction after cycles of freezing and thawing as mentioned above. DNA was extracted by freezing and thawing to the stool oocysts by using a DNA purification kit (Wizard Genomic DNA purification kit; Promega).

- The following steps were done to complete this adopted protocol**:**

1. (100 µl) of the protein precipitation solution was added to the specimens and placed on vortex for (20 seconds) at high speed and vigorously.
2. The specimens were put on ice for (5 minutes).
3. The specimens were centrifuged at (13.000 rpm) for (3 minutes).
4. The supernatant containing the DNA was transferred to a clean (1.5 ml.) eppendrof tube.

5. Then (300 µl) of isopropanol was added and mixed gently until the visible mass is thread –like strands of DNA.
6. The eppendrof tube was centrifuged at (13.000 rpm) for (2 minutes).
7. The supernatant was decanted carefully, and the eppendrof tube was drained on a clean absorbent paper.
8. Approximately (300 µl) of ethanol (70%) was added.
9. Eppendrof tube was centrifuged at (13.000 rpm) for (2 minutes), and after that, ethanol was pulled carefully.
10. Eppendrof tube was drained on a clean adsorbent paper, and the pellets were allowed in air-dry for (10-15 min.).
11. (50 µl) of DNA rehydration solution was added.
12. Rehydrated DNA was incubated at (56 °C) for one hour, the eppendrof tube solution was mixed by gently tapping.
13. The eppendrof tube with DNA was stored at (2-8 °C).

Then DNA concentration was measured by using (NanoDrop) spectrophotometer, by putting (1 µl) of each DNA sample on the lens at wave lengths of (260 nm and 280 nm), and DNA was analyzed by agarose gel electrophoresis (Maniattis et al., 1982).[14]

2.3.2. PCR Technique

The PCR technique was done according to.[15]

1. The master mixture was prepared by the kit (promega), by adding SSU-F2 primer: 5′-TTC TAG AGC TAA TAC ATG CG-3′, and SSU-R2 primer: 5′-CCC ATT TCC TTC GAA ACA GGA-3′ , for each PCR reaction, as follows:

10 x Perkin –Elmer PCR buffer ……	10 µl
dNTP (1.25) mM ……....	16µl
SSU-F2 primer (40 ng/µl) ……...	2.5 µl
SSU-R2 primer (40 ng/µl) ……...	2.5 µl
MgCl2 (25 mM) ……....6	µl
Non acylated bovine serum albumin (10 mg/mL) ……...	4 µl
Distilled Water …….…….	56.5 µl
Taq polymerase …….……	0.5 µl
Total …….……	98 µl

2. (98 µl) of Master mixture was added to each eppendrof tube.
3. Then (2 µl) of DNA suspension was added to each eppendrof tube.
4. PCR program was run on as follows:

The amplification reactions of 35 cycles were initiated by:

A- denaturation of the DNA at (94 °C) for (3 min.)
B- denaturation at (94 °C) for (45 seconds)
C- Annealing of the primer at (55 °C) for (45 seconds)
D- Extension at (72 °C) for (1 minutes)
E- An additional (7 minutes) extension at (72 °C)

5. Electrophoresis was run on (1.5%) agarose gel with (20 µl) from PCR product, using the (100-base pair (bp)) ladder as control, as follows:

Gels were worked horizontally in (0.5 X) Tris-borate buffer (TBE buffer), specimens from DNA were mixed by (1/10) volume of loading buffer and were loaded into the wells in the gel.

Agarose gel was stained with ethidium bromide (5-7 µl) for (30 – 45 minutes). TBE was added to cover the gel in Gel electrophoresis unit and was run for (2 hours) at (5 v/cm). DNA bands were visualized by UV transiluminator at a wavelength (302 nm).[14]

2.4. STATISTICAL ANALYSIS

The data were statistically processed by using SPSS version 23, by application of Chi-Square for categorical variables and descriptive statistics.

2.5.Ethical Issues

Verbal consent from all patients and surrogates of the children had been approved in addition to explanatory information about the study during interviewing and questionnaire application.

3. RESULTS

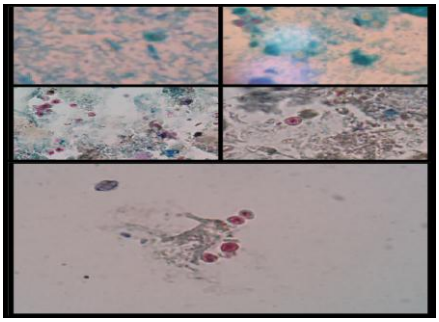
The stool samples of all the patients in this study were examined by modified Ziehl–Neelsen stain method (Figure 1-A). The total infectivity with *Cryptosporidium* spp. was (12.85%), which represents the number of positive cases (18) out of 140 patients, and the number of negative cases (non-infected) was 122 (87.15%) (Figure 1-B).

Table (1) shows that the highest prevalence of *Cryptosporidium* infection appeared in the 1st age group (<1 year) at a rate of (15.38%) while the lowest infection rate appeared in the 3rd age group (11-15 years) at a rate of (5.55%).

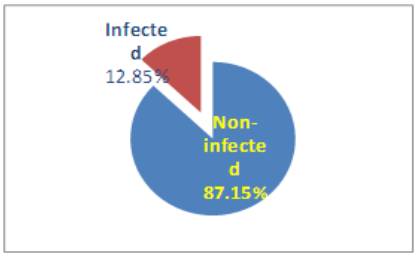
Table (2) illustrates the prevalence of *Cryptosporidium* infection in patients, according to the gender, where the infection rate was 13.5% (10/74) in males and 12.1% (8/66) in females.

Figure (2) represents the distribution of *Cryptosporidium* infections for the period from January 2016 to Jun 2016. Where the results revealed that the highest infection number was recorded in May {5 (3.57%)} while in January the lowest infection number was recorded {1 (0.71%)}.

Table (3) reveals a higher prevalence of cryptosporidiosis in rural areas (14.13%) in compare to urban areas (10.5%).



A



B

Figure (1): A-Cryptosporidium oocyst in stoolm samples stained modified Ziehl–Neelsen stain by B-Percentage of *Cryptosporidium* infected and non-infected children diagnosed by modified Ziehl–Neelsen stain method.

Table (1): Distribution of *Cryptosporidium* infections regarding to age groups.

Age Groups (year)	Number of Patients	+Ve	%	-Ve	%
(<1)	52	8	15.38	44	84.62
(1-5)	43	6	13.95	37	86.05
(6-10)	27	3	11.10	24	88.90
(11-15)	18	1	5.55	17	94.45
Total	140	18	12.85	122	87.15

$\varkappa^2 = 0.565$ P= 0.904

Table (2): Gender Distribution of *Cryptosporidium* infections.

Gender	No. of Patients	+Ve	%	-Ve	%
Male	74	10	13.50	64	86.50
Female	66	8	12.10	58	87.90
Total	140	18	12.85	122	87.15

$\varkappa^2 = 0.06$ P= 0.806

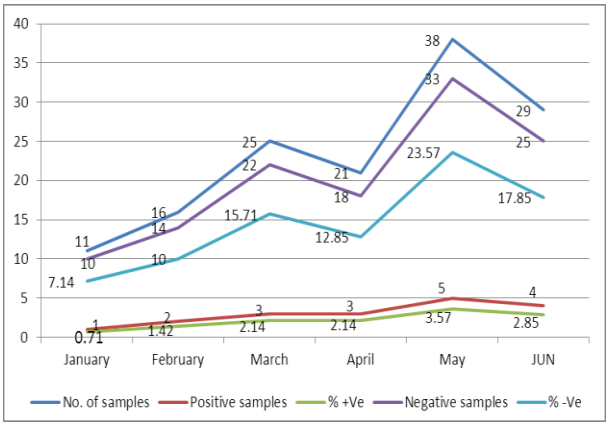


Figure (2): Monthly distribution of *Cryptosporidium* infections.

Table (3): Prevalence of *Cryptosporidium* infections in rural and urban areas.

Residency	Patients No. (%)	+Ve	%	-Ve	%
Rural	92	13	14.13	79	85.87

Urban	48	5	10.50	43	89.50
Total	140	18	12.85	122	87.15

$\chi^2 = 0.388$ P= 0.533

The PCR technique was used to determine the presence of *Cryptosporidium* in the positive stool samples for *Cryptospordium* that diagnosed by modified Ziehl–Neelsen stain only (18 samples), by detecting specific gene locus (SSU rRNA gene) in the extracted DNA which previously extracted by modified protocol. Amplicons of ~ 550 bp were produced from these 18 samples by using the primer set, as in Figure (3) which shows an electrophoresis image with ethidium bromide-stained agarose gel to the amplification SSU rRNA gene of PCR product for *Cryptosporidium* spp. which it was extracted from the positive samples, where lane (M) is a molecular weight marker, and lanes (1,2.3,4,5,6,7,8,9,10,11,1213,14,15,16,17.18) are the positive samples at 550 pb.

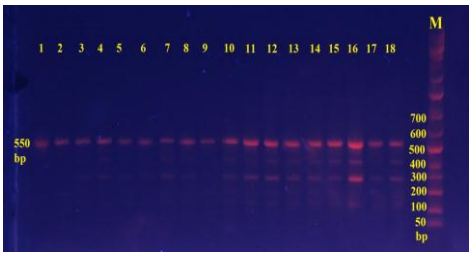


Figure (3): An ethidium bromide-stained agarose gel electrophoresis picture, showing PCR amplification products of *Cryptosporidium* SSU rRNA gene sequence (≈ 550 bp) with primers.

*Lane- M: molecular weight marker.
*Lanes: (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11,12, 13, 14, 15, 16, 17, 18) are the positive samples at 550 pb.

The PCR technique confirmed the positivity of all these positive 18 stool samples for *Cryptospordium* that diagnosed by modified Ziehl–Neelsen stain, and rvealed the 100% sensetivity of SSU rRNA-based PCR-detecting technique for detection of *cryptosporidium* in stool samples.

4. DISCUSSION

The present study is an attempt to verify the actual prevalence of *Cryptosporidium* spp. infection in Najaf province. The researchers of this study believes that the recorded number of patients with cryptosporidiosis is underestimated, and the reasons of this underestimation may be due to two main causes; first: the detection of *Cryptosporidium* oocysts in stool sample requires an expert microscopist, second: the more sensetive diagnostic procedure for detection of these oocysts such as modified Ziehl–Neelsen stain method is not used routinely for stool examination

This study showed a prevalence of (12.85%) for *Cryptosporidium* in children with persistent diarrhea diagnosed by modified Ziehl–Neelsen stain method, where this percentage agrees with the results of.[16] who reported a prevalence of (12.4%) in Baghdad. While the prevalence of (1-4%) was reported in Europe and North America, and it was (3-20%) in Australia, Asia, Africa, and Central and South America.[17] The disparity in infection prevalence of *Cryptosporidium* spp. in Iraq in comparison with other countries may be due to differences in samples size, living conditions, socioeconomic criteria, nutritional status, immune status and personal hygiene, variation in the nature of the areas, ages of the patients and type of the test which is used for the diagnosis, and the difference of temperature between one country and others and between a year to last in the same area.[16, 18]

In the present study, the oocysts stained by modified Ziehl–Neelsen stain apeared as red bodies against a dark blue background, and had a bright halo around it, these results agreed with these of.[11] and.[19] who proved that *Cryptosporidium parvum* oocysts have appeared as red spots on a blue background.

The study showed a relationship between age and infectivity prevalence; by finding a high prvalence of cryptosporidiosis among children of less than 1 year, and this may be due to a weak immiunity in this age group. This result agrees with.[20] results, in which the prevalence of *Cryptosporidium* infection was higher in children <1 year in Ramadi city. As well, it agrees with the findings of:.[21] who noticed that the infection was more prevalent in infants in Korea; and.[22] who recorded that the maximum frequency was in the age group <1 year in KwaZulu-Natal populations. Also it agrees to some extent with the results of:[23] who observed that the maximum infection rates were in age groups (1-4) and (5-8) years, and.[24] who showed that the greatest number of the reported infections in children in age group (1–9) years.

This study revealed differences in prevalence of *Cryptosporidium* infection according to the gender. Difference was noted in prevalence between males (13.5%) and females (12.1%) which may indicate that males have a higher chance of infection from female. These results agree with the conclusion of.[25] who said that the higher number of cases especially in male children, may be due to a more exposure of males to the sources of contamination.

Moreover, this is in agreement with the results of (26) in Ramadi city in Iraq, which reported that males had higher infection rate (0.02 %) than females (0.019%), and this disagrees with the results of.[27] and.[28] who reported a male:female ratio of 1:2.

The seasonal distribution of *Cryptosporidium* infection was varied according to the geographic sites of the study.[29] In this study, the samples were collected during the first six months of 2016 (January, February, March, April, May and Jun). In May, the monthly distribution of *Cryptosporidium* infections was the highest (3.57%), while in January the lowest prevalence (0.71%) was recorded. The high prevalence of *Cryptosporidium* infection in May and Jun may be related to the increase of temperature (in late spring and early summer) which lead to increasing in community water consumption, for recreation and swimming during these two months, (communal bathing mainly provides optimal fecal-oral transmission), venues by younger children, low infectious dose, and high swimming densities, facilitate the transmission of infection with *Cryptosporidium*.[30]

Outbreaks of cryptosporidiosis in some countries might occur because the water supply deficiency that is common in some regions during the dry and hot season, which results in a bad hygiene or the use of alternative water sources that may harbor higher concentrations of oocysts of *Cryptosporidium*.[31] However, routine use of recreational waters by some people, including children, increases the waterborne transmission probability in summer.[32]

The results of this study showed a higher prevalence of cryptosporidiosis in rural areas (14.13%) than in urban areas (10.5%), which disagree with the results of.[33] who reported that there is no difference in prevalence of *Cryptosporidium* infections between rural and urban areas. This result in the presnt study supported by the believe of some researchers who said that the sources of infection for humans are human feces and the feces of domestic animals, mostly the cattle and sheep in particular rural areas.[34]

Differences in the prevalence of *Cryptosporidium* infection between rural and urban areas, may be due to the presence of domestic animals and directly dealing with them.[35, 36]

The present study, verified the higher yield of DNA obtained by using modified protocol and so using proteinase K for extracts D,NA from stool sample containing oocyst of *Cryptosporidium*, spp. by using PCR technique, and this agrees with.[12] [13]

The short subunit (SSU rRNA) was detected by PCR, which was carried out by using the forward primer SSU-F2 and reverse primer SSU-R2: which is specific for *Cryptosporidium* spp. to produce an amplicon of ~ 550 bp.[37]

The SSU rRNA subunit is widely used in *Cryptosporidium* detection and genotyping, because of the multi-copy nature of the subunit and presence of semi-conserved and ease the design of genus-specific primers. A review of original studies has revealed that *Cryptosporidium* spp. molecular detection and genotyping during the last three years used (SSU rRNA) as tools in 100 (86%) of 116 publications, while tools based on the *Cryptosporidium* oocyst wall protein (COWP) gene used in only 23 of 116 original publications (which is important in combined with SSU rRNA-based tools) and other genes were seldom used.[38]

Amplicons of ~ 550 bp produced from only 18 patients with postive modified acid-fast stain method by using the primer set for amplification SSU rRNA gene of PCR product for *Cryptosporidium* spp., while electrophoresis used with ethidium bromide-stained agarose gel for determining amplicons, appeared lanes positive at ~ 550 bp, this result agreed with.[38]

The 100% sensetivity of the SSU rRNA-based PCR-detecting technique for dtection of *cryptosporidium* in stool samples provide an excellent confimatory test for the results of microscopc methods as modified acid-fast stain procedures.

CONCLUSION: *Cryptosporidium* spp. is one of the most common enteric parasite that associate with persistent diarrhea in children, especially in males and those with age less than 1 year, residing the rural area, with higher rates in spring and summer.

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