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Molecular Detection and Prevalence of *Cryptosporidium parvum*, Among Patients with Diarrhea at Al-Rifai City/Thi-Qar Province

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Abstract*:* *Cryptosporidium parvum* as genus of apicomplexan parasitic protozoa is well known as a worldwide protozoan parasite in both human and a wide range of animals and considered as one of the major causes of severe life-threatening diarrhea in immunodeficient people and self- limiting in healthy individuals. This study aimed to determine the prevalence of *C.parvum* in stool samples of diarrheic patients , this study is carried out in Thi –Qar Province / Al-Rifeadistrict in Al-Rifai general Hospital which included collection of stool samples from diarrheic patients at a period extended from October / 2017 – January / 2018 , (603) stool samples taken from patients with different ages to both sexes examined by microscopic examination and PCR technique**.** The results showed the percentage of positive samples *C. parvum* by microscopic examination was (5.1%) and negativesampleswas (94.9%), the highest infected patients found (58%) in males and lowest infected patients found (42%) in females,the highest infected patients found (64.5%) in Rural area and lowest infected patients found (35.5%) in Urbanarea, inage groupthe highest infected patients was (35.5%) inAge groupLess than (1-10years)and lowest infected patients found (9.7%) in age group (21-30) years. Results of PCR was positive sample 20 with percentage 10.4% and 172 negative samples, the percentage was (89.6%).the highest infected patients found (65%) in males and lowest infected patients found (35%) in females,the highest infected patients found (55%) in Rural area and lowest infected patients found (45%) in Urbanarea, .Age group was the highest infected patients found (45%) in age group (1-10years) and lowest infected patients found (5 %) in age group (31-40) years**.**

Keywords: *Cryptosporidium parvum* ,polymerase chain reaction (PCR) ,protozoa, Cryptosporidiosis.

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Introduction:

Diarrhea is defined as passage of watery stool for more than three times in 24 hours (1). Globally, it is estimated that there are 1.7- 4.6 billion case of diarrhea every year with 2.2 million associated deaths(2). Diarrhea can be caused by various pathogen such as bacteria, viruses and parasites(3). Protozoan parasites are important causes of diarrhea and other gastrointestinal infection in humans they include mostly *Cryptosporidium parvum*, (4). *Cryptosporidium parvum* is an gastrointestinal tract of humans it spread by fecal-oral route either direct contact with infected human, animals or taken the oocysts with contaminated food and drink(5). Cryptosporidiosis having Clinical signs include watery diarrhea, vomiting, nausea, abdominal pain and fever (6).

Cryptosporidium parvum is a genus protozoan parasites that invades the microvillus region of epithelial cells in the digestive and respiratory systems of vertebrates, it is an obligate intracellular

parasite of human and other mammals , birds , reptiles and fish .The oocysys are shed by infected hosts into environment and can survive in the adverse conditions on the environment for months until it is ingested by a new host(7). The oocysy of *C. parvum* looks spherical or ovoid and contains four naked parallel sporozoites enclosed by smooth Oocyst wall (1) the most common transmission of *C.parvum* are from animal to person, person to person and water contamination with oocyst(8). Oocyst wall consist of diverse layers; outer layer made of glycoprotein, central part consists of complex lipid material and dense inside layer composed of glycoprotein (9). The size ranges from 4 – 6 μm(10). Oocystis highly resistant to many common disinfectant that used to treat drinking water (9), it is sensitive to heat temperature of 65 ℃ inactives within 5-10 minutes,and over time of 2 hours or more,desiccation is enough to kill the Oocyst(11). Sporozoite measure is (3.5-4.2 x 0.53 - 0.60) μm , Sporozoites and Merozoites have a cylindrical ring that showed to be the site of origin for the inner membrane complex and the subpellicular microtubules, sporozoites of *C.parvum* unlike other Coccidia the sporozoites are free within the oocysts and not surrounded by sporocysts(12).

Materials and Methods:

Samples:

A total of 603 patients stool samples were collected from patients withdiarrhea who have referred to general Al- Rifae Hospital /Thi-Qar province from October / 2017 – January / 2018 . The ages were ranged from 1 months – 70 years, 320 were males and 283were females . Fecal samples were collected by using a sterile containers and then transported in to the laboratories Hospital at the laboratory the fecal samples were divided into two one portion was for the microscopic examination of parasites while the other portion of 200 mg and stored directly at -20 °C for molecular analysis conventional PCR(13).

Direct wet:

The stool is emulsified in normal saline to allow study the parasite shape and motility(14). Stool samples observed by the preparation of direct smear methods using clean glass slides , a small drop of normal saline (0.9%) or Iodine stain put was on slide glass and mixed well with a small portion of feces using wooden stick, then was put cover slides, and examined the sample under power amplify 40X (15).

Modified Acid-Fast Staining Technique (Z-N Technique):

Staining was done according (16). Smear was made from the sediment (5gram fecal sample) of a centrifuged (1000 rpm) specimen then was placed on staining rack and flooded with carbolfuchsin 5min after hen the slide is heated to steaming with Bunsen burner, Wash the slide with tap water, declorized with 1% sulphuric acid 2 minutes, Wash the slide with tap water, flood the smear with methylene blue and stained for 1 minute, rinsed the slide with tap water, and then drained and air-dry and examined microscopically using 40x then 100x to identify the parasite.

Primer of *C. parvum*:

PCR primers were designed in this study for detection *C. parvum* based

subunit ribosomal rRNA gene by using NCBI-Genbank and primer plus design online. Then these primer was provided from Bioneer company, Korea as following (Table 1).

Table (1): Primer Sequences

Primer	Sequence (5'-3')		Product Size
18SrRNA gene *C. parvum*	F	TGGATGTCTTGGTTCTCATAACGA	504bp
	R	ACCCACTGATAGACGGATTTCC	

Genbank: *Cryptosporidium parvum* (AF015774.1).

Statistical analysis:

The statistical analysis proceeded in all groups of study, descriptive statistics analyzed by using one-way analysis of variance (ANOVA) were performed using means and standard deviations (SDs) with LSD test for continuous variables ($p \geq 0.05$) was considered to be significant, and $X^{2,}$ (P-value 0.01) was considered to be significant.

All analyses were performed with the Statistical Package for the Social Sciences SPSS for Windows (version 17.0, SPSS Inc, Chicago, III)(17).

Results:

Percentage of infectedand non-infected patients with *C.parvum* by direct smear:

The current study includes examination of 603 patient stool samples with diarrhea examined by a direct wet mount method and Ziehl–Neelsen for *C.parvum* by using light microscope (Figure 1). Showed the percentage of infected patients which were 31 positive samples with percentage (5.1%) and 572 negative samples, with the percentage was (94.9%).

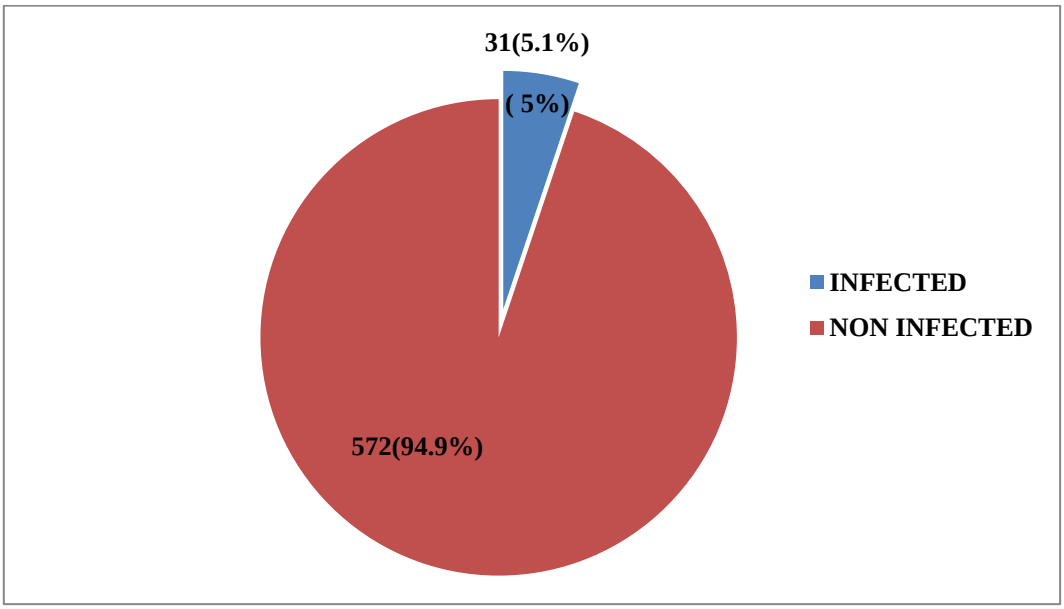


Figure (1): Showing the percentage of infected and non-infected patients with *C.parvum* by direct smear.

Distribution ofthe infected patients with *C.parvum*,according to gender by Direct smear:

(Table 2) explained distribution of *C.parvum* according to gender. The highest infected patients found (58%) in males and lowest infected patients found (42%) in females.

Table (2): Distribution of the infected patients with *C.parvum*according to gender by Direct smear.

Gender	No. of sample examanation	No.ofinfected with *C .parvum*	%
Male	320	18	58%
Female	283	13	42%
Total	603	31	100%
Cal.X2	3.115		

Tab.X2: 9.488 df: 2 P.value: 0.05

Distribution of the infected patients with *C.parvum* according to Habitation by Direct smear:

(Table 3) explained distribution of *C.parvum* according to Habitation of the highest infected patients found (64.5%) in Rural area and lowest infected patients found (35.5%) in Urbanarea.

Table (3): Percentage of distribution of the infected patients with *C.parvum*according to Habitation by Direct smear.

Habitation	No. of sample examination	No. of infected with *C .parvum*	%
Rural	305	20	**64.5%**
Urban	298	11	**35.5%**
Total	603	31	**100%**
Cal.X2	10.712		

Tab.X2: 9.488 df: 2 P.value: 0.05

Percentage of distribution of the infected patient with *C.parvum*according to Age group by direct smear:

(Table 4): Explained distribution of *C.parvum* according to age groups of the highest percentage found age group (1-10years) which was(35.5%) and lowest percentage found in age group (21-30) yearswhich was(9.7%).

Table (4): Showing the distribution of *C.parvum* according to Age group by direct smear.

Age group / year	No. of sample examanation	No.of infected with *C .parvum*	%
(1-10)	140	11	35.5%
11-20	127	5	16.1%
21-30	109	3	9.7%
31-40	102	4	12.9%
More than 40	125	8	25.8%
Total	603	31	100/%
Cal.X2		9.314	

Tab.X2: 26.296 , df:6 , P.value:0.05

Percentage of infectedand non-infected patients with *C.parvum* by PCR:

The current study included examination of 192 sample by conventional PCR, the results showed percentage of infected patients which were 20 positive samples with percentage (10.4%) and 172 negative samples, the percentage (89.6%) as shown in (Figure 2).

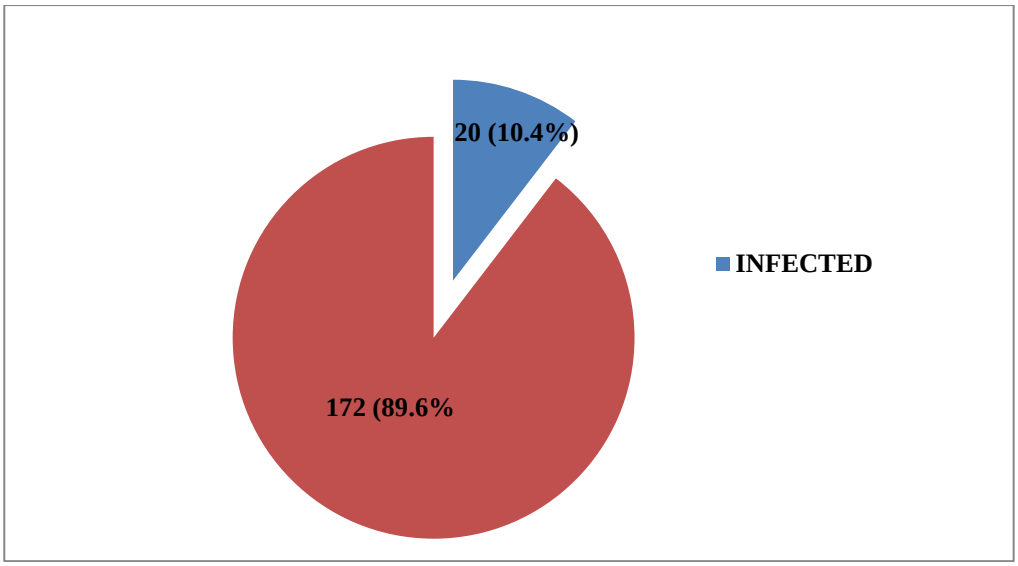


Figure (2): The percentage of infected and non- infected patients with *C . parvum*by using PCR.

Distribution of the infected patients with *C.parvum*, according to gender by PCR:

(Table 5) explained the distribution of *C.parvum* according to gender of the highest percentage was found in males (65%), and lowest percentage foundin females(35%).

Table (5): Distribution of the infected patients with *C.parvum* according to gender by using PCR .

Gender	no. of sample examination	no. of infected with *C .parvum*	%
Male	105	13	65%
Female	87	7	35%
Total	192	20	100%
Cal.X2	1.799		

Tab.X2: 9.488 df:2 P.value:0.05

Distribution of the infected patient with *C.parvum*according to Habitation by using PCR:

(Table 6). The distribution of *C.parvum* according to habitationthe highest infected patients found inRural area(55%) and lowest infected patients found in Urbanarea(45%).

Table (6): Distribution of the infected patient with *C.parvum*according to Habitation by using PCR.

Habitation	no. of sample examination	no. of infected with *C .parvum*	%
Rural	85	11	55%
Urban	107	9	45%
Total	192	20	100%
Cal.X2	2.745		

Tab.X2: 9.488 df:2 P.value:0. 05

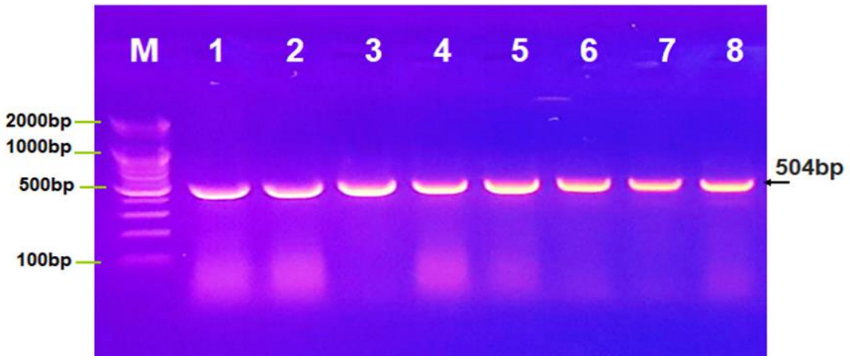
Distribution of the infected patient with *C.parvum* according to Age grope by using PCR*:*

(Table 7). The distribution of *C.parvum* according to Age group the highest infected patients found inAge group (1-10years)(45%) and lowest infected patients found in Age group (31-40) years(5%)**.**

Table (7): Distribution of the infected patient with *C.parvum*according to Age group by PCR.

Age group/ year	No. of sample examination	No.ofinfected with *C .parvum*	%
(1-10)	53	9	45%
11-20	34	3	15%
21-30	29	2	10%
31-40	37	1	5%
More than 40	39	5	25%
Total	192	20	100%
Cal.	31.665		

Tab.X2: 26.296 df:6 P.value:0.05



Figure(3): Agarose gel electrophoresis image showing the PCR product analysis of 18S ribosomal RNA gene from genomic DNA of human stool samples. Where M: Marker (2000-100bp), lane(1-8) positive samples *C. parvum* at 504bp.

Discussion:

The microscopic examination of stool samples remains the backbone of the diagnosis intestinal protozoa particularly in developing countries ,the current study included examination of 603 patient stool samples with diarrhea examined by a direct wet mount method and Ziehl–Neelsen for *C.parvum* by using light microscope.

The results in microscopic examination and PCR showed the highest infection in male and lowest

infection in female, this result was agreed with study (14) in Baghdad, where They scored the highest rate of infection in males (58.5%) and lower than in females (41.5%). And agree with the study in the Al- Diwaniya province the highest rate of infection for males (6.12%), while the lowest infection for females (5.11%) but disagree with the study in Baghdad, where he scored the highest rate of infection (15.35%) in females and (12.28%) in males, and disagree in Al-Sowera which did not record any significant differences between the sexes.

The difference in the rate of infection between males and females may be due to the fact that males are the more movement and active and their contact with the external environment factors at play and of being working group in the communities, this is what makes them more relevant pathogens sick of females as males eat well and drink in public places or from street vendors, in addition to the nature of anarchism and a lack of attention to personal hygiene and wash hands which increases the chances of being infection either for Giardia and the absence of significant differences between male and females could be due to portability and having the same opportunity to infection both sexes intestinal parasites(18).

In this study found there was highest infection in rural areaswhich was (64.5%) while lowest infection urban areas was (35.5%) in Al- Rifai city in case of infection by the parasites microscopically, The result rural area more than urban area was agree with (14). Where he scored the highest rate of infection in the rural area, reaching 50.9% in Baghdad. Also agreed in Babylon province, who record the highest rate of infection in rural areas increased by 64.7% and less rate of infection in Urban areas were 35.3%, agree with(19) whom found infection in rural areas was (34.1%) while infection in Urban areas was (31.1%), agreed with (20)as the rate of infection in the rural area amounted to higher than in the cities area .and disagree with (21) how found infection in rural areas was(25.2%) infection in Urban areas was (28.3%). The reason for the high incidence of infection in rural areas due to several factors, including the absence of clean drinking water availability, and rely on river water directly as a source of water, and the absence of guidance and counseling by the authorities concerned as well as lower health and cultural level of the rural population as well as the lack of hospitals and health centers in those areas, as well as use of animal waste and human feces and sometimes as an organic fertilizer for the growth and plants and vegetables, socio- invarmental factor such as dejection level sanitation infrastructure used and water sources so that the difference in the protozoan infection in patients was insignificant with regards to the education level so, infection was less common in family with private sanitation as compared community sanitation(22).

While by using PCR technique shows the rural areas more than urban areas This result agree with what recorded by (23) in Al-Qadisyai as the rate of infection in the rural area (62.1%) higher than in the cities area (37.9%). And agree with what recorded by (24) in Al-Najaf as the rate of infection in the rural area higher than in the cities area.

The result of showed distribution of the *C.parvum* according to the ages by microscopically examination the age group (1-10years) was the highest percent (35.5%) and the lowest percent(9.7) in age group (21-30 years) ,the result agreed with (25) who found highest rate of infection in children with age group (from birth – 24 months) was (69.19%) and the difference was significant p=0.01, also, agreed with(26) who examination 115 stool samples from diarrheic children at different ages (2-6 , 6-12) years old to examine Cryptosporidiosis and she found 44.4% in children with age group (2-6) years and 27% in children with age group (6-12) years and this may be indicate the prevalence of *C. parvum* had a relationship with age and high infection rate in children can act as a great source of Cryptosporidiosis among children in Nursery and Primary schools, while in case of PCR technique found highest infection with age group (1-10 years) was (45%) and lowest infection in the age group (31-40) years was (5%) agreed(31)whom reported rate (38 %) in 1-12 years old, which may be attributed to defecation practices because these groups of children are fully independent in toilet use and are more involved in both outdoor activities and feeding (27). So this agree with (28) who was found the same results while disagree with (29) how found high infection in age groups (30-44 years), and disagree with (30) how found high infection in age groups (41-50 years), while (31) regard high infection in age groups more than (31-60), Giardiasis in this study in first age group (1-10) was 65.5%. Giardiasis occurs in all ages but is most common in early childhood, since they eat indiscriminately and have less immunity to the parasite than adults who have been exposed during their childhood(32). The results related to unsanitary practice associated with child development (e.g playing in contaminated dirt and water, sucking on dirty Finger and other objects, etc.). Their less mature immune system, especially in those < 6 years, can reduce their ability to mount strong immune defense to infectious agents(33).

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