

Prevalence of *Cryptosporidium spp.* among Patients with Diarrhea at Wasit Province/ Iraq

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Abstract

Cryptosporidium is an important obligate enteric protozoan parasite that infects humans and wild range of animals with morbidity and mortality especially among immuno-suppressed individuals^[1] This study carried out in al-kut city /Iraq in two major hospital Al- kut hospital for gynecology ,obstetric and pediatric and al-Karamma teaching hospital which included collection of stool samples from diarrheal patients from October / 2020 – January / 2021 , (109) stool samples taken from patients with different ages to both gender examined by microscopic examination(Modified ziehl nelson) and multiplex PCR technique, the results showed the percentage of *cryptosporidium spp.* infection by using M.Z.N stain was 44(40.4%) while by using PCR technique was 38(39.6%) and 4(4.2%) for *C.parvum* and *C.hominis* respectively .

The rate of infection is significantly associated with residence when using M.Z.N stain and PCR for *C.parvum*, also significant associate among those who >5 years old. water source for drinking were significant associated factors, other factors (gender, education level) Showed no significant association with infection, the present study aimed to detection of *Cryptosporidium spp.* and possible association between the infection and some risk factors by using microscopic examination and detecting DNA of *Cryptosporidium* parasite by multiplex PCR technique.

Keywords: *Cryptosporidium*, M.Z.N stain, multiplex PCR *Cryptosporidiosis*.

Introduction

Cryptosporidium is a common parasite that infects intestinal epithelial cells in humans and a variety of animals worldwide and is an emerging infectious disease of importance to public health around the world ^[2]This disease burden varies between and within countries/regions, diarrhea is a common clinical symptom of *cryptosporidiosis* in infected hosts, varying depending on their health status, In humans, immune-competent individuals usually experience self-limiting diarrhea; however, immune-compromised individuals, particularly those with HIV infection suffer from intractable diarrhea^[3],^{4]} The *Cryptosporidium* is the second-highest priority Organisms/Biological Agents at the National Institutes

of Health (NIH), and was found this parasite is the fifth leading cause of death from diarrhea in children under the age of five years, About 84.4% of deaths due to *cryptosporidium* infection are in this age^[4, 5]. yearly 1 million deaths caused by *cryptosporidiosis* resulted in over 50,000 deaths caused by the Apicomplexan phylum that is” phenotypically and genotypically diverse” species of *Cryptosporidium* which are parasitized and located” intracellular and extracytoplasmic on the microvillus of the excretory system and epithelial respiratory tract” of humans, fish, amphibians, birds, and mammals^[6]And in the humans caused worldwide diarrhea disease that similar to cholera disease which considered by the Centers for Disease Control and Prevention (CDC)

as an emerging pathogen, this disease is mostly not noticed and asymptomatic but might be presented with mild to severe diarrhea and fever which is self-limiting and life-threatening in immunocompromised individuals like AIDS^[7, 8]. Currently, more than 30 species of the *cryptosporidium parasite* have been identified^[9]. A person can acquire cryptosporidium through several modes, such as preservation through the oral-fecal through the consumption of contaminated water and food^[10]. In addition, people can become infected through personal contact, and contaminated food, raw meat, unpasteurized milk, and contaminated juices and drinks have a role in transmitting the parasite^[11] Finally, this parasite was isolated from mucous secretions from the nose, and the patients were diagnosed with an infection, at first, it was learned that it was a respiratory disease^[12]

To determine the rate of infection in wasit province and correlation with risk factors this study was conducted.

Material and Methods

Samples collection

109 Stool diarrheal samples were collected from different ages to both gender from October 2020 to the January 2021 and they were attending to the Al-kut hospital for gynecology ,obstetric and pediatric and Al-Karama teaching hospital in Al-kut city. (Organization 1992)

Microscope examination

All stool samples were examined microscopically stained by modified Ziehl- Neelsen method^[13] The

stool sample was spread on the slide. The slides left in the open air to dry for a while 10 minutes without using a flame, the dried smear was fixed with absolute methanol for 1minutes, Carbol-Fuchsine solution was added to the slide covering the whole smear for 15 minutes, the slide was washed gently with tap water using a dropper, after this, decolorizer by acid alcohol for 30 seconds and the slide was washed off with clean water again, the methylene blue was added for 2minutes and washed again, and left to dry, the smear was examined microscopically, using the 40x and 100x (oil immersion lens) objectives and scanned thoroughly for parasite identification In this technique, the oocysts appear as pink to red, spherical to ovoid bodies on a blue or purple background^[14]

DNA extraction and multiplex PCR

Genomic DNA was extracted from frozen fecal samples, Stool DNA extraction Kit from (Geneaid, Taiwan) according to the manufacturer’s instructions, all samples were treated with thermal shock for5 cycles and boiling in a water bath for each for 5 min), then incubated at 56°C for 10 min, and extend for 1 hr at 95°C. DNA was extracted and amplified by multiplex PCR targeting heat shock protein 70 (hsp70) gene using two sets of primers for detection *Cryptosporidium parvum* and *Cryptosporidium hominis* were designed in this study using NCBI-Genbank (AF221534.1 and KR296809.1) and primer 3 plus design, these primers were provided from Scientific Researcher. Co. Ltd, Iraq as the following table(1) The products amplified were by 1.5% agarose gel electrophoresis and ethidium bromide

Table (1): Primer Sequences

| Primers | Sequence 5’-3’ | | Product size |
|-----------------------|----------------|----------------------|--------------|
| C. parvum hsp70 gene | F | ATGGTGAGCAATCCTCTGCC | 618bp |
| | R | CAGTTTGGTTGTGCTCGAGC | |
| C. hominis hsp70 gene | F | TGAGGGTGAGAGAGCCATGA | 524bp |
| | R | GCATACCACCCTCAGCAGAG | |

Statistical analysis Data obtained were entered into a computer database, Statistical package for social science(SPSS)software was used for statistical analysis, data were recorded as number and percentages, Percentages were compared using the chi-squared test, $P \leq 0.05$ was considered significant^[15]

Results

Prevalence of *Cryptosporidium spp.* by microscopic examination The current study includes examination of 109 patient stool samples

with diarrhea examined by modified Ziehl–Neelsen for *Cryptosporidium spp.* by using light microscope (table 2). Showed the percentage of infected patients which were 44 (40.4%) .

Prevalence of *Cryptosporidium spp.* by molecular technique this study include examination 96 patient stool samples by using multiplex PCR showed the percentage of infected patients which were 42 (43.75%) were positive for cryptosporidiosis as (table 2)

Table (2):Prevalence of the *Cryptosporidium spp.* according to the microscopic examination and multiplex PCR results

| Methods Screening Test | Total | Results | |
|-------------------------------|-------|---------|-------|
| | | No | % |
| modified ziehl- neelsen stain | 109 | 44 | 40.4% |
| PCR technique | 96 | 42 | 43.75 |

Prevalence of the *Cryptosporidium spp.* infection according to socio -demographic characteristic

By using M.Z.N stain, the distribution of *Cryptosporidium spp.* according to gender, The highest infected patients found 19 (55.9%) in females and lowest infected patients found 32 (51.6%) in males , while by using PCR technique the distribution of *C.parvum* infection according to gender the highest percentage was found in females 14(41.2%), and lowest percentage found in males 24(38.7), and the highest rate of infection for *C. hominis* was found in males 3(4.8%) and lowest was found in females 1(2.9%)as showed in (Table 3).while according to residence by using M.Z.N stain showed in table(3) the highest infected patients found 33 (64.7%) in rural area and lowest infected patients found 18(40%) in urban area, and by using PCR technique the distribution of *C.parvum* as prevalence

of the *Cryptosporidium spp.* infection according to according to residence the highest infected patients found in rural area27(52.9%) and lowest infected patients found in urban area11(24.4%). And The distribution of *C. homini* according to residence the highest infected patients found in urban area3(6.7%) and lowest infected patients found in rural area 1(2%). As well as Prevalence of the *Cryptosporidium spp.* infection according to water source by using M.Z.N stain the highest percentage found in participant used bottled water which was 13(54%) and lowest percentage found in tap water which was 38(52.8%) While by using PCR technique the distribution of *C.parvum* according to water source the highest infected patients found in participants whose used bottle water were 14(58.3%) and lowest infected patients found in participants whose used tap water were 24(33.3%). And the distribution of *C. hominis* according to water source the highest infected patients

found in participants whose used tap water were 3 (4.2%) and lowest infected patients found in participants whose used bottle water were 1(4.2%).shown in (table 3)

Table (3): Prevalence of the *Cryptosporidium spp.* infection according to socio -demographic characteristic

| Methods Socio-demo characteristic | Number | Microscope | | PCR technique | | | |
|---|--------|------------|------|-----------------|------|------------------|-----|
| | | | | <i>C.parvum</i> | | <i>C.hominis</i> | |
| | | + | % | + | % | + | % |
| Gender \ Male | 62 | 32 | 51.6 | 24 | 38.7 | 3 | 4.8 |
| Female | 34 | 19 | 55.9 | 14 | 41.2 | 1 | 2.9 |
| Total | 96 | 51 | 53.1 | 38 | 39.6 | 4 | 4.2 |
| P value | | 0.688 | | 0.813 | | 0.656 | |
| Residence \ Urban | 45 | 18 | 40 | 11 | 24.4 | 3 | 6.7 |
| Rural | 51 | 33 | 64.7 | 27 | 52.9 | 1 | 2.0 |
| Total | 96 | 51 | 53.1 | 38 | 39.6 | 4 | 4.2 |
| P value | | 0.015* | | 0.004* | | 0.250 | |
| Water source\ bottle | 24 | 13 | 54.2 | 14 | 58.3 | 1 | 4.2 |
| water Tap | 27 | 38 | 52.8 | 24 | 33.3 | 3 | 4.2 |
| Total | 96 | 51 | 53.1 | 38 | 39.6 | 4 | 4.2 |
| P value | | 0.906 | | 0.03* | | 1.000 | |

*Significant using Chi-squared test at 0.05 level of significance

Prevalence of the *Cryptosporidium spp.* infection according to age group

By using M.Z.N stain the highest percentage found age group (11-15) which was 15(60%) and lowest percentage found in age group (16-20) years which was 3(20%).while by using PCR technique the distribution of *C.parvum* according to age group

the highest infected patients found in age group (≤ 5 years) 19.(57.6%) and lowest infected patients found in age group >20 years 2 (13.3%) there were statistical significant between *C. parvum* and age group and the distribution of *C. hominis* according to age group the highest infected patients found in age group ≤ 5 years 3(9.1%) and lowest infected patients found in age group $20 >$ years 1 (6.7%) as shown in (table 4)

Table (4) prevalence of *cryptosporidium spp.* according to age groups.

| Methods Age | number | microscope | | PCR technique | | | |
|----------------|--------|------------|------|---------------|------|-----------|-----|
| | | | | C.parvum | | C.hominis | |
| | | + | % | + | % | + | % |
| 5≤ | 33 | 17 | 51.5 | 19 | 57.6 | 3 | 9.1 |
| 6-10 | 22 | 11 | 50 | 8 | 36.4 | 0 | 0 |
| 11-15 | 15 | 9 | 60 | 7 | 46.7 | 0 | 0 |
| 16-20 | 11 | 3 | 27.3 | 2 | 18.2 | 0 | 0 |
| 20> | 15 | 11 | 37.3 | 2 | 13.3 | 1 | 6.7 |
| Total | 96 | 51 | 53.1 | 38 | 39.6 | 4 | 4.2 |
| P value | | 0.213 | | 0.023* | | 0.364 | |

*Significant using Chi-squared test at 0.05 level of significance

Prevalence of the *Cryptosporidium spp.* infection according to educational level

By using M.Z.N stain as shown in(table 5) the highest percentage found in uneducated participant which was31(50.8%) and lowest percentage have education level (higher) which was4(80%) , while by using PCR technique the distribution of *C.parvum*

according to educational level the highest infected patients found in uneducated participant which was 25(41%) and lowest infected patients found in participants whose higher educated level 2 (40%). And the distribution of *C.hominis* according to educational level the highest infected patients found in uneducated participant which was 4(6.6%)

Table(5) prevalence of *cryptosporidium spp.* according to Educational Level

| Methods Education. L | number | microscope | | PCR technique | | | |
|-------------------------|--------|------------|------|---------------|------|-----------|-----|
| | | | | C.parvum | | C.hominis | |
| | | + | % | + | % | + | % |
| Uneducated | 61 | 31 | 50.8 | 25 | 41 | 4 | 6.6 |
| Primary | 21 | 13 | 61.9 | 7 | 33.3 | 0 | 0 |
| Secondary | 9 | 3 | 33.3 | 4 | 44.4 | 0 | 0 |
| Higher | 5 | 4 | 80 | 2 | 40 | 0 | 0 |
| Total | 96 | 51 | 53.1 | 38 | 39.6 | 4 | 4.2 |
| P value | | 0.302 | | 0.923 | | 0.495 | |

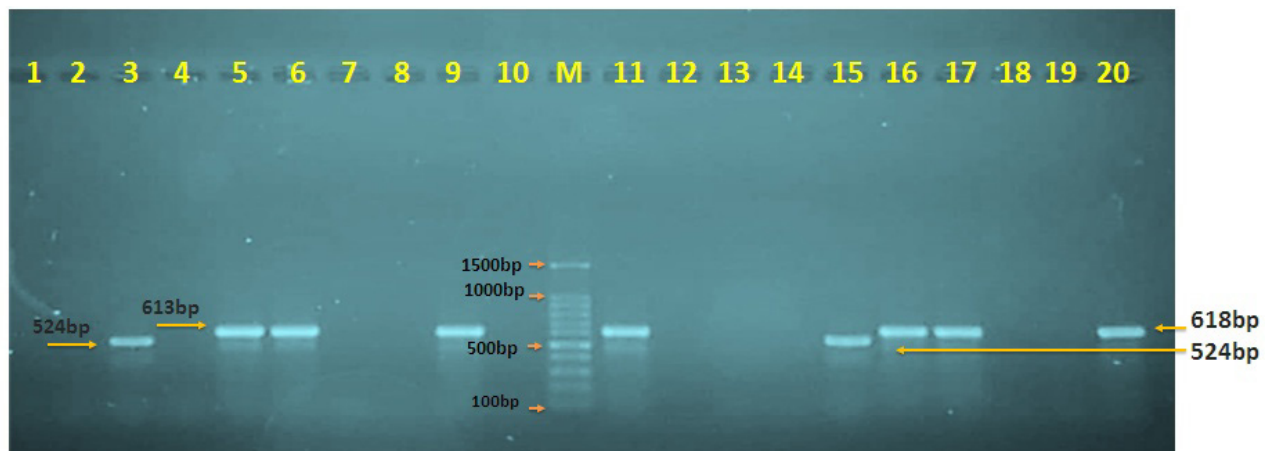


Figure (1): Agarose gel electrophoresis image that showed the Multiplex PCR product analysis of hsp70 gene in *C. parvum* and *C. hominis* from Human samples. Where, the Lane (M): DNA marker ladder (1500-100bp) and the Lane (1-20) were showed some positive hsp70 gene in *C. parvum* at 613bp and *C. hominis* at 524bp PCR product size.

Discussion

The current study included the examination of 109 diarrheal patient stool samples with examined by M. Z.N stain for *cryptosporidium* species and showed 44 (40.4%), The positive rate in this study was agreement with [8] in Al-Najaf City were recorded 29 (58%) samples positive for *cryptosporidiosis* by using M. Z.N stain in patient with diarrhea and abdominal discomfort, While our results was disagreement with [16] at Al-Dewanyia, city- Iraq that records *cryptosporidiosis* rate 4 (0.625%) in children with diarrhea by using M.Z.N stain, also [7] in Turkey which recorded 6 (3.5%) in immunocompromised patient with diarrhea using M.Z.N stain. The prevalence of *cryptosporidium spp.* infection in diarrheal patient varies substantially among countries and different geographic regions in the same country could be due to differences in climatic and sociodemographic factors and differences in the number of participants examined and type of populations studied (e.g. urban or rural), location, This may explain the

variation in positivity^[4]

The results of extracted genomic DNA from 96 stool samples were 38 (39.6%) positive for *C. parvum* agreement with [17] in Tehran which recorded 32 (1.2%) in children with diarrheal samples using nested PCR and disagreement with [18] at Al-Rifai City/Thi-Qar-Iraq that recorded 20 (10.4%) positive rate from patients with different ages to both sexes using conventional PCR, increase numbers of *C. parvum* infection 38 positive samples at al- kut city may be due to associated with contaminated drinking water supplied to these peoples with taking into account that the lowest infected dose for *C. parvum*, ranging from approximately 10 to 1,000 for healthy humans^[19] Regarding the prevalence of *Cryptosporidium hominis*, This is the first study in Al-Kut city that recorded positive cases of *C. hominis* infection in diarrheal patients were 4 (4.2%), the result was in agreement with [20] in Iran which recorded infection rate for *C. hominis* 4 (25%) in Immunocompromised Patients and Children by using PCR-RFLP assay. While,

disagreement with [21] which recorded 17 (81%) among symptomatic children in Egypt by using nested PCR. There are two important species of *Cryptosporidium* that are responsible for human infection (*C. parvum* and *C. hominis*), it is a very important issue to determine the species of this protozoan in clinical cases even though the treatment could be identical, This differences in the percentage of *C. hominis* infection may due to the variation in the sample size for each study [22] The current study showed a higher percentage of positivity among females than males although there was no statistically significant association between positivity and gender of *cryptosporidiosis* patients these results were in agreement with the findings of [23] in Jordan which record no statistically significant association between cryptosporidiosis and gender among Hemodialysis Patients in Jordan by using M.Z.N stain also [24] in Pakistan registered no significant difference between cryptosporidium spp. and gender among children by using M.Z.N stain and disagreed with the result by [25] in Nigeria which recorded statistical significant between *cryptosporidiosis* and gender by using M.Z.N stain the variation in the rate of infection between males and females may be because females are more movement and active and their contact with the external environment factors, It may lead to a lack of attention to personal hygiene and wash hands which increase the chances of being infection [26]

In the current study distribution of the *Cryptosporidiosis* infection according to residence by using M.Z.N stain, there was statistically significant association between positivity of *cryptosporidiosis* and residence $p=(0.015)$ this results agreement with [27] in Beni-Suef, Egypt were recorded $P\text{-value}=0.03$ in diarrheic Immunocompetent Patients and disagreement with [28] in Erbil City-Iraq were recorded no statistically significant association between positivity of *cryptosporidiosis* and residence among children from regular visitors of Raparin Hospital using M.Z.N stain. A strong significant association

was found between *C.parvum* infected and residence by using multiplex PCR $p=0.004$ the current study agreement with [29] in Mid-Euphrates Area which recorded significant association between *C.parvum* infection among children and those who living in rural area by using PCR technique. And disagreement with [30] at Wasit Province were recorded no statistical significant between *C.parvum* and residence $P\text{-value}=0.07$ among diarrhea patients by using multiplex PCR in a rural community can be subject to a higher degree of environmental exposure to potential sources of infection (e.g. contaminated water, farm animals and wildlife) and presence of grazing such as cows and sheep in rural areas compared with urban areas [31] while regarding the prevalence of *cryptosporidiosis* infection and association with water source the current study shown that the highly infection appear in participant who used bottle water in compared among who used tap water when used M.Z.N stain and there was statistically significant association recorded by using PCR technical method between *C.parvum* and water source $p=0.03$. This result disagreement with [32] in Taiz which recorded high infection occur among participant who used tap water from children of different ages by using M.Z.N stain also [23] in Turkey were recorded results that disagreement and no statistical significant between *C.parvum* and water source $p=0.33$ among Immunosuppressive and Immunocompetent Cases with Diarrhea by using PCR. These variation in results may be due to The oocysts are very resistant to chlorine, chloramines, and chlorine dioxide, which are commonly used in methods of water system disinfection, and keep vital for infection in the environment for a long time [33].

Regarding the prevalence of *cryptosporidiosis* infection and association with age groups the results in table(4) shown that the highly infection appear in age group > 5 years old and there was statistically significant association recorded by using PCR technical method between *C.parvum* and those who had been ≥ 5 years old $p=0.023$ This result disagreement with [34] in Sulimania city-Iraq which

record high infection appear in age group under 5 years old by using M.Z.N stain among children in Sulaimani Pediatric Teaching Hospital . While the result of the current study was agreement with that registered by [35]in Korea which record high infection rate occur in old age group (50–69)years old in a small rural village occupied predominantly by aged people in Hwasun-gun, Chollanam by using M.Z.N stain ,and agreement with [21]in Egypt which recorded there was statistically significant association between *C.parvum* and age group $p=0.045$ among Symptomatic Egyptian children using nested polymerase chain reaction , also disagreement with [36]in sharjah which recorded no statistically significant between *C.parvum* and age group $p=0.544$ among asymptomatic healthy expatriate workers in sharjah by using real-time PCR (qPCR). Children have more activities, inside or outside the home, and lack knowledge about personal health habits and practices, so they are more exposed to the causative agents than other age groups, the current study showed the prevalence of *cryptosporidiosis* infection and association with educational level a higher percentage of positivity among educated participant than among who have no educated although there was no statistically significant association between infection and education level this result disagreement with [25]in Nigeria which recorded statistical significant between *cryptosporidiosis* and education level $p=0.156$ among Human Immunodeficiency Virus Seropositive Patients within Kaduna- Nigeria by using M.Z.N stain and [19]in Qatar that recorded statistically significant association between educational level and parasitic infection $p= <0.001$ among immigrants in Qatar with a special focus on food handlers and housemaids by using quantitative PCR (qPCR) .The reason for the wide spread of *Cryptosporidiosis* infection among uneducated people may be regarded to fact that infected people specially children who do not have an education about the specific personal hygiene [19]

Conclusions

Our study showed that the prevalence rate of

cryptosporidium spp. was found to be higher in the rural areas and the most commonly infected in age group was >5 years old, according to the gender; the highest infection recorded in female comparison with male and there was statistically significant associated between infection and residence. This is, to the best of our knowledge, the first report from Al-Kut city to document the present *Cryptosporidium hominis* in diarrheal patients in Al-Kut city.

Conflict of Interest: This is to certify that I Dr. May Naji Al-khanaq the author of the Prevalence of *Cryptosporidium spp.* among patients with diarrhea at wasit province/ Iraq. Certify that there is no conflict of interest regarding this manuscript.(NIL)

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