Original Research Article

Epidemiological and Molecular Study for Distribution of Cryptosporidium parvum in Diarrheic Children in Al-Muthanna Province, Iraq

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This study was carried out in Al-Muthanna province during the period from October 2013 to May 2014 in the Educational AL-Hussein hospital in Al-Samawa, General-Al-Rumaiytha hospital, Feminine and Children's hospital in AL-Samawa and AL-Rumaiytha health centers. A total of 100 stool samples were taken from children aged 1 month to less than 10 years who were suffering from acute or persistent diarrhea examined by Ziehl-Neelsen stain and RT-PCR assay. In this study, a total of 100 fecal samples of children were examined by Ziehl-Neelsen stain, out of which 21 fecal samples had Cryptosporidium parvum with 21%. The results showed oocysts stained red bodies against a dark blue background, with a clear halo around the oocyst. Also, the present study recorded non significant differences at level (P<0.05) in all the age groups between male and female., the highest rate of the infection in male was 42.86% compared with female (23.80%) in the age group (1-3) years old. The results showed significant differences at level (P<0.05) between rural and urban regions in age groups (1-3) years old compared with other age groups, the highest rate of infection in rural regions (47.62%) compared with urban (19.05%). Also this study recorded a total of 100 fecal samples of children examined by real time PCR out of which 18 fecal samples had found Cryptosporidium parvum with 18%. The present study showed non significant differences at level (P<0.05) between male and female in all age groups, the highest rate of the infection in male was 44.44% compared with female (22.22%) in the age group (1-3) years old. Also the results showed significant differences at level (P<0.05) between rural and urban regions in all age groups compared with other age groups except (7-9) years old. The highest rate of infection in rural regions was 50.5% compared with urban (16.67%).

Keywords: C. parvum, Children, Ziehl-Neelsen, RT-PCR, Iraq.

INTRODUCTION

Cryptosporidium is considered as the major cause of diarrhea in children. Cryptosporidium is a genus of protozoan parasites that infect a wide range of vertebrates including humans and animals, and results in significant morbidity and mortality in both the developing and developed world (Fayer, 2004). Cryptosporidiosis is transmitted by faecal–oral route, or by ingestion of food and water contaminated with Cryptosporidium oocysts (Meinhardt et al., 1996). While Jelinek et al., (1997) examined 469 travelers returning to Germany with diarrhea and detected 13 (2.8%) infected with Cryptosporidium. In Mexico, Cryptosporidiosi is endemic, because of poor sanitation and crowded living conditions (Soave et al., 1989). Higher prevalence rates are to be showed more in rural regions compared to urban regions (Mak, 2004).

In Malaysia, Ahmad (1995) detected Cryptosporidium oocysts in eight out of 76 (10.5%) of Malaysian water resources which were examined. The prevalence of Cryptosporidium in children with diarrhea in the neighboring countries, such as, in Saudi Arabia, Al-Braiken et al., (2003) revealed the highest prevalence of Cryptosporidium infection among children with diarrhea who were presenting to pediatric outpatient clinics was (32%).

In Kuwait, Iqbal et al., (2001) collected 3549 stool samples, 509 children had diarrhea. Cryptosporidium oocysts were detected in 51 (10%) children with diarrhea during the period from September 1995 to August 1997. In Turkey, Akyon et al., (1999) determined Cryptosporidiosis prevalence under the age of 12 years and they detected Cryptosporidium oocysts in seven (3.5%) of the cases, this result showed that Cryptosporidium could also be a causative agent of diarrhea in

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The study revealed that Cryptosporidium infected 2.9% of the patients and other parasites observed in different percentages (Nahrevanian et al., 2007). Michel et al., (2000) recorded the infection percent as follows: (16.6%), (11.6%) and (27.9%) in Egypt for the years 1986, 1987 and 1996 respectively. While in Iraq, Abdulsadah et al., (2013) recorded that the infection percent was 33.83%. The most widely used technique for the diagnosis of Cryptosporidium is the detection of oocysts in a fecal smear (Casimiro et al., 2009).

There are varieties of methods, including microscopy, immunological and molecular techniques, for the detection of Cryptosporidium oocysts. Microscopic methods include concentration techniques and staining of fecal smears. There are difficulties in distinguishing Cryptosporidium oocysts from other small particles, such as yeasts, moulds, algae, and plant debris by routine fecal examination techniques in fecal and environmental samples (Fayer et al., 2000). The most widely stain which is used have been the modified acid-fast procedures which is the gold standard for the detection of Cryptosporidium spp., It differentiates red-stained oocysts from similarly sized and shaped green-stained yeast forms (Sevinc et al., 2005).

Recently, developed PCR protocols have proven to be very specific and highly sensitive (Sulaiman et al., 1998). Also, there are no studies on this parasite in Al-Muthanna province, hence the needs for undertaking this study.

**MATERIALS AND METHODS**

**Methods**

**Patients**

This study was carried out in AL-Muthanna Province during the period from October 2013 to May 2014 in the Educational - AL-Hussein hospital in Samawa, General AL-Rumaiytha hospital, Feminine and Children's hospital public education in Samawa and Rumaiytha health centers. A total of 100 stool samples were taken from children aged 1 month to less than 10 years who were suffering from acute or persistent diarrhea and examined by Ziehl-Neelsen and RT-PCR assay.

**Fecal samples collection**

Fresh fecal samples were collected by using a disposable latex glove into sterile containers and transported into a cooled box (temperature approximately 10°C). Then, the samples were transported to the laboratory at College of Science- AL-Muthanna University, at the laboratory the fecal samples were divided into two portions, one portion was for the microscopic examination of parasites while the other portion was stored immediately at-20°C for molecular analysis (PCR).

**Concentration technique**

The present study employed just one method of concentration, Sheather's Sugar Solution to detect oocysts of parasite.

**Flotation method by Sheather's Sugar Solution**

Five grams of feces were diluted in 10ml of tap water, filtered using gauze and centrifuged at 2500 rpm for 10 min, then the supernatant was poured and the sediment was mixed in Sheather’s solution in a 15ml plastic tube, this suspension was centrifuged at 2500 rpm for 10 min, after that, the cover slip was placed touching the surface of the solution for 10-15min, then the cover slip was examined on a glass slide using microscope at 400x magnification.

Oocysts were counted, If more than 4 oocysts were observed, the same five grams sample was re centrifuged and oocysts were counted on a second and sometimes a third cover slip (Gondim, 2002). The oocysts of feces were measured with a calibrated ocular micrometer by using bright-field microscopy, the oocysts with a diameter of (4 to 6 µm) were considered to be positive for Cryptosporidium Spp. (Hammond & Long, 1973; Schares et al., 2005).

**Modified Acid-Fast Staining technique (Z-N technique) Procedure (Garcia, 1999)**

1. Sediment from concentration method was mixed, and then smear 10 µL on slide within etched circle by using transfer loop (10 µL).
2. Air-dry for at least 1 hour.
3. Fixed in Methanol for 3 to 5 minutes.
4. Stained with carbol fuchsin for 20 minutes.
5. Rinsed with tap-water for 4 minutes.
6. Decolorized with acid alcohol (HCl with 95% alcohol).
7. Rinsed with tap-water for 2 minutes.
8. Counter stain with 3% methylene blue for 30 seconds or malachite green
9. Rinsed with tap water for 2 minutes.
10. Air dry.
11. Examine by standard light microscopy.
12. By Z-N method, Cryptosporidium sp. Oocyst stain red against a blue-green background. Cryptosporidium is round and approximately (4-6) µm in diameter.

**Primers**

The Real-Time PCR primers and probe used in this study were those designed by Stephen et al., (2011) in detection of Cryptosporidium parvum and provided by (Bioneer Company, Korea) as shown in table 1.

**Genomic DNA Extraction**

Genomic DNA of supernatant sporulated oocyst was extracted by using AccuPrep®Genomic DNA extraction kit (Bioneer, Korea) and done according to company instruction as the following steps:

1. A 200µl of stool suspension was transferred to sterile 1.5ml microcentrifuge tube, and then added 20µl of proteinase K and mixed by vortex.
2. After that, 200µl of Binding buffer was added to each tube and mixed by vortex to achieve maximum lysis efficiency, and then all tubes were incubated at 80°C for 10 minutes.
3. A 100µl of isopropanol was added to mixture and mixed well by pipetting, and then briefly spin down to get the drops clinging under the lid. The lysate was carefully transferred into GD Binding filter column that filled in a 2 ml collection tube, and then closed the tubes and centrifuged at 8000 rpm for 1 minute.
4. Throughout lysate was discarded in disposal bottle, and then 500µl Washing buffer 1 (W1) was added to each Binding filter column, and centrifuged at 8000 rpm for 1 minute.

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Table 1: PCR primers and probe used in detection of Cryptosporidium parvum

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSU rRNA F</td>
<td>TCCTGAAATGAAATTGTGACTCG</td>
</tr>
<tr>
<td>SSU rRNA R</td>
<td>TTAATGTTGATTTGCGGTTGAAC</td>
</tr>
<tr>
<td>SSU rRNA Probe</td>
<td>VIC-TATCTCTTCGTAGCGCGTA MGB-NFG</td>
</tr>
</tbody>
</table>

Table 2: Preparation of PCR master mix

<table>
<thead>
<tr>
<th>qPCR master mix</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genomic DNA</td>
<td>5µL</td>
</tr>
<tr>
<td>TaqMan probe (10pmol)</td>
<td>2</td>
</tr>
<tr>
<td>Forward primers (10pmol)</td>
<td>1µL</td>
</tr>
<tr>
<td>Reverse primers (10pmol)</td>
<td>1µL</td>
</tr>
<tr>
<td>PCR water</td>
<td>11 µL</td>
</tr>
<tr>
<td>Total</td>
<td>25µL</td>
</tr>
</tbody>
</table>

Table 3: Real-Time PCR Thermocycler conditions

<table>
<thead>
<tr>
<th>Step</th>
<th>Condition</th>
<th>Cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-Denaturation</td>
<td>95 °C 1 min</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95 °C 15 sec</td>
<td></td>
</tr>
<tr>
<td>Annealing/Extension</td>
<td>60 °C 30 min</td>
<td>45</td>
</tr>
</tbody>
</table>

Figure (1a): Oocyst of C.parvum by Ziehl–Nelsen stain (100x).

Figure (1b): Oocyst of C.parvum by Flotation method (100x).

Genomic DNA Profile

The extracted genomic DNA was checked by using Nanodrop spectrophotometer (THERMO, USA), that checked and measured the purity of DNA through reading the absorbance at (260 /280 nm) following steps:

1. After opening up the Nanodrop software, chosen the appropriate application (Nucleic acid, DNA).
2. A dry Chem-wipe was taken and cleaned the measurement pedestals several times. Then carefully pipet 2µl of ddH2O onto the surface of the lower measurement pedestal.
3. The sampling arm was lowered and clicking OK to blank the Nanodrop, then cleaning off the pedestals.
4. After that, the pedestals are cleaned and pipet 1µl of DNA sample for measurement.

5. Throughout Washing buffer 1 was discarded in disposal bottle, and then 500µl Washing buffer 2 (W2) was added to each Binding filter column, and centrifuged at 8000 rpm for 1 minute.
6. Throughout Washing buffer 2 was discarded in disposal bottle, and then the tubes were centrifuged once more at 12000 rpm for 1 minute to completely remove ethanol.
7. After that, GD Binding filter column containing genomic DNA was transferred to sterile 1.5ml microcentrifuge tube, and then added 50µl of Elution buffer and to left stand in the tubes for 5 minutes at room temperature until the buffer is completely absorbed into the glass filter of Binding column tube.
8. Finally, all tubes were centrifuged at 8000 rpm for 1 minute to elute DNA, and storage at -20°C freezer.
Table (4): The relationship of age, sex and residency with infection percentage of *C. parvum* depending on Ziehl-Neelsen method.

<table>
<thead>
<tr>
<th>Age (Years)</th>
<th>Sex</th>
<th>Chi-value</th>
<th>P-value</th>
<th>Geographical Location</th>
<th>Chi-value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td></td>
<td></td>
<td></td>
<td>Female</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+ve</td>
<td>%</td>
<td>+ve</td>
<td>%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-3</td>
<td>9</td>
<td>42.86</td>
<td>5</td>
<td>37.80</td>
<td>2.28</td>
<td>0.05</td>
</tr>
<tr>
<td>3-5</td>
<td>1</td>
<td>4.76</td>
<td>2</td>
<td>9.52</td>
<td>0.68</td>
<td></td>
</tr>
<tr>
<td>5-7</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>4.76</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>7-9</td>
<td>1</td>
<td>4.76</td>
<td>2</td>
<td>9.52</td>
<td>0.68</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>11</td>
<td>52.83</td>
<td>10</td>
<td>47.62</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Significant differences at (P < 0.05).

Figure (2): Real-Time PCR amplification plot of ssuRNA gene of *Cryptosporidium parvum* in positive samples.

Figure (3): Real-Time PCR endpoint analysis of *Cryptosporidium parvum* in positive and negative samples.
Real-Time PCR master mix preparation

Real-Time PCR master mix was prepared for specific primer by using AccuPower® DualStar™ qPCR PreMix kit (Bioneer, Korea), and done according to company instructions as shown in table 2. These qPCR master mix reaction components mentioned above were added into TaqM TaqMan probe qPCR master mix reaction. Then these are all placed in vortex tubes for mixing the components and centrifuge for 3000rpm for 3 minutes in Exsipin centrifuge, after that transferred into MiniOpticon Real-Time PCR thermocycler.

Real-Time PCR Thermocycler conditions

Real-Time PCR thermocycler conditions were done according to primer annealing temperature and qPCR Syber green kit instructions as shown in table 3.

Real-Time PCR Data analysis

qPCR data analysis was performed by calculating the threshold cycle number (CT value) that presented the positive amplification gene in Real-time cycle number.

Statistical Analysis

The Chi-square test was used to analyse the overall prevalence data, and differences were considered significant when P< 0.05. (Sorlie, 1995).

RESULTS AND DISCUSSION

Staining Method by acid fast stain (Ziehl-Neelsen)

In the present study, a total of 100 fecal samples of children were examined by Ziehl-Neelsen, out of which 21 fecal samples had Cryptosporidium parvum with 21%. The results showed that oocysts examined by flotation method and Ziehl-Neelsen stained red bodies against a dark blue back ground, with a clear halo around the oocyst, these results are in agreement with Baxby et al., (1984); John and Petri (2006) confirmed that oocysts of Cryptosporidium parvum were visualized as red spots on blue background.

The total infection of Cryptosporidium examined by Ziehl-Neelsen was 21%, these results in agreement with Khall (2000) in Mosul (20.52%), and disagreement with Mahdi et al. (1996) in Basra province-Iraq (8.6%) and with Abdulssadah et al., (2013) in Wasit province-Iraq (33.83%) and Ahmed (2009) in Palestine (18%), this variation in infection percentage may be due to number of patients samples in screening study, differences in nature of the areas, age of patients, diagnostic method used (Yaqoob et al., 2004). Also, the results showed no significant differences between male and females, these results is agreement with Ke-XiaWang etal., (2002); Abdulssadah etal., (2013) they reported that the boys and girls had similar detectable positive rate.

The present study recorded the infection was highest in the age groups (1-3) years old, this is in agreement with Hamedi etal., (2005); Mohmammad etal.,(2006), also the infection in rural higher than urban regions due to less personal hygiene and direct contact with animals and drinking contaminated water as well as defecation was randomly (Okafor and Okunji,1994; Spausta etal., 2000).

Polymerase Chain Reaction (Real-Time PCR Assay)

In this study, a total of 100 fecal samples of children were examined by real time PCR out of which 18 fecal samples contained Cryptosporidium parvum with 18% Figs. (2,3). The present study showed non significant differences at level (P<0.05) between male and female in all age groups, the highest rate of the infection in male was 44.44% compared with female (22.22%) in age group(1-3) years old. Also the results showed significant differences at level (P<0.05) between rural and urban regions in all age groups compared with other age groups except (7-9) years old. The highest rate of infection in rural (50.5%) compared with urban (16.67%).

Table (5): The relationship of age, sex and geographical location with Infection percentage of C.parvum depending on Real-Time PCR method.

<table>
<thead>
<tr>
<th>Age (Years)</th>
<th>Sex</th>
<th>Chi-value</th>
<th>P-value</th>
<th>Geographical Location</th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td>Male</td>
<td></td>
<td>Female</td>
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<tr>
<td></td>
<td></td>
<td>+ve  %</td>
<td>+ve    %</td>
<td></td>
<td>+ve    %</td>
<td>+ve    %</td>
</tr>
<tr>
<td>1-3</td>
<td>8</td>
<td>44.44</td>
<td>4</td>
<td>22.22</td>
<td>9</td>
<td>50.5</td>
</tr>
<tr>
<td>3-5</td>
<td>1</td>
<td>5.56</td>
<td>3</td>
<td>16.67</td>
<td>4</td>
<td>22.22</td>
</tr>
<tr>
<td>5-7</td>
<td>1</td>
<td>5.56</td>
<td>1</td>
<td>5.56</td>
<td>2</td>
<td>11.11</td>
</tr>
<tr>
<td>7-9</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>10</td>
<td>55.56</td>
<td>8</td>
<td>44.44</td>
<td>15</td>
<td>83.33</td>
</tr>
</tbody>
</table>

Data was recorded with 100% efficiency in both rural and urban regions.

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(2014) in Africa (36.93) this may be due to large number of patients samples. Also the results recorded the highest rate of infection in the age group (1-3) years old this result is in agreement with Gatei et al., (2006); Morse et al., (2007), they attributed these results to inadequacy of sanitation or may be due to exposure to oocysts as a result of consumption of contaminated drinking water (AL-Hindi et al., 2007). This study showed no significant differences between male and female ,both may have an equal chance to oocyst exposure.

Abdulsadah et al., (2013). Infection with Cryptosporidium was the highest in rural than urban regions with significant differences (P<0.05) in all age groups, this may be due to indiscriminate defeaction in outdoor area, or direct contact with infected animals, less health education of family (Gillespie and pearson (2001); Goh et al., (2004).

**Relationship between infection percentage of C.parvum with Year months**

The present study recorded the highest rate of the infection in the November and December months while the lower rate of the infection in the January and February months in all examination methods. Table (6).

The seasonality of Cryptosporidium varied depending on the geographic locations of the studies. In the present study, the highest rates of prevalence was noticed in November and December these results are in agreement with Ajjampur et al., (2007) they recorded generally most prevalent in the rainy season because C. parvum could have also come from agricultural sources, such as that of manure application or directly from cattle, that may have been introduced in water due to rainfall and that lead to the contamination of drinking water sources from sewage and animal waste (Naumova et al., 2005; Sulaiman et al., 2005).

This result is in disagreement with Laupland and Church, (2005); Sallon et al., (1990) they reported that Cryptosporidium infections were more common in the late summer in Canada this may be due to a lot of consumption of contaminated drinking water.

**CONCLUSIONS**

The first study for diagnosis of Cryptosporidium parvum in children in Iraq by using Real Time -PCR, also recorded the highest percent of infection of Cryptosporidium parvum in male than female and in rural than urban regions. The results observed that the highest prevalence with Cryptosporidium parvum was in the age groups (1-3) years old.

**ACKNOWLEDGEMENT**

We would like to thank and appreciate the staff members of Educational AL-Hussein hospital in AL-Samawa, General- AL-Rumaiytha hospital, Feminine and Children's hospital in AL-Samawa and AL-Rumaiytha health centers for helping in completing this study.

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