DonnishJournals 2041-1680

Donnish Journal of Microbiology and Biotechnology Research
Vol 2(1) pp. 001-007 June, 2015.
http://www.donnishjournals.org/djmbr

Original Research Article

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

Muhammed Oda Mallah* and Nouran Raad Jomah

College of Science, Al-Muthanna University, Iraq.

Accepted 12th May, 2015.

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

**Corresponding Author: Muhammed Oda Mallah. College of Science, Al-Muthanna University, Iraq.*
Email: muhammed_mallah @yahoomail.com

children. In Iran, Stool samples collected include 104 children and adult patients with gastroenteritis. 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 60℃ 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 fitted 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.

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

Primer		Sequence
SSU rRNA	F	TCCTTGAAATGAATATTTGTGACTCG
	R	TTAATGTGGTAGTTGCGGTTGAAC
SSU rRNA Probe		VIC-TATCTCTTCGTAGCGGCGTA MGB-NFQ

Table 2: Preparation of PCR master mix

qPCR master mix	Volume
Genomic DNA	5µL
TaqMan probe (10pmol)	2
Forward primers (10pmol)	1µL
Reverse primers (10pmol)	1µL
PCR water	11 µL
Total	25µL

Table 3: Real-Time PCR Thermocycler conditions

Step	Condition	Cycle
Pre-Denaturation	95 °C 1 min	1
Denaturation	95 °C 15 sec	45
Annealing/Extension	60 °C 30 min	
Detection (Scan)		

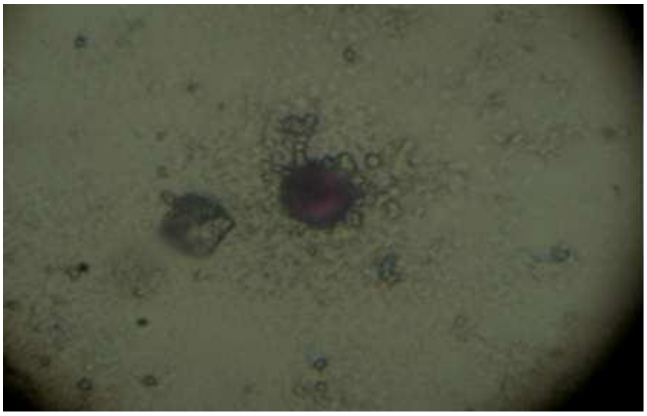

Figure (1a): Oocyst of *C.parvum* by Ziehl–Nelseen stain (100x).

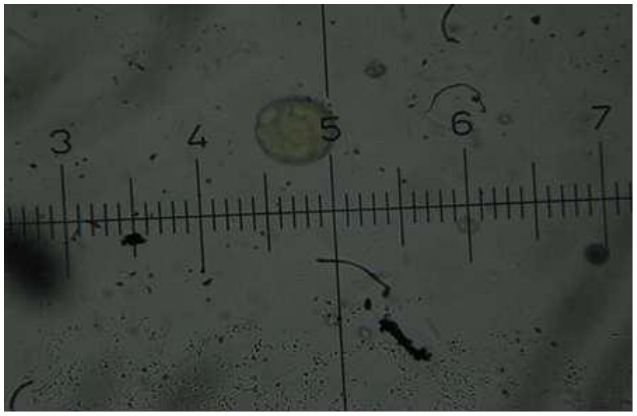

Figure (1b): Oocyst of *C.parvum* by Flotation method (100x).

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℃ freezer.

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 cleanedthe 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.

Table (4) : The relationship of age, sex and residency with infection percentage of *C.parvum* depending on Ziehl-Neelsen method.

Age (Years)	Sex				Chi-value	P-value	Geographical Location				Chi-value	P-value
	Male		Female				Rural		Urban			
	+ve	%	+ve	%			+ve	%	+ve	%		
1-3	9	42.86	5	37.80	2.28	0.05	10	47.62	4	19.05	5.16*	0.05
3-5	1	4.76	2	9.52	0.68		2	9.52	1	4.76	0.68	
5-7	0	0	1	4.76	2		1	4.76	0	0	2	
7-9	1	4.76	2	9.52	0.68		2	9.52	1	4.76	0.68	
Total	11	52.83	10	47.62			15	71.43	6	28.57		

* Significant differences at (P <0.05) .

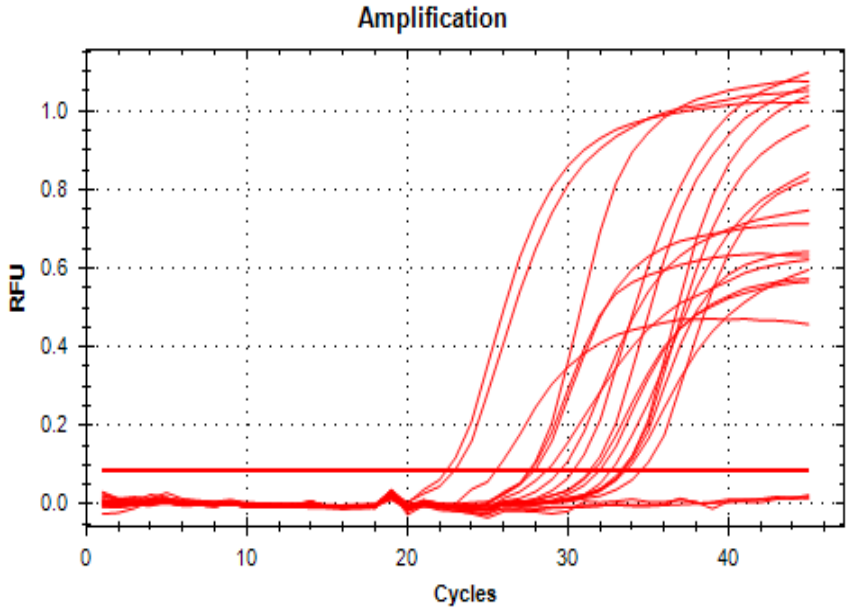

Figure (2): Real-Time PCR amplification plot of ssurRNA gene of *Cryptosporidium parvum* in positive samples.

Well	Fluor	Content	Sample	End RFU	Call
A02	FAM	Pos Ctrl	C.parvum	0.607	(+) Positive
A04	FAM	Unkn	C.parvum	0.563	(+) Positive
A05	FAM	Unkn	C.parvum	0.465	(+) Positive
B03	FAM	Unkn	C.parvum	0.629	(+) Positive
B04	FAM	Unkn	C.parvum	0.709	(+) Positive
C03	FAM	Unkn	C.parvum	1.04	(+) Positive
C04	FAM	Unkn	C.parvum	0.912	(+) Positive
D02	FAM	Unkn	C.parvum	1.06	(+) Positive
D03	FAM	Unkn	C.parvum	1.02	(+) Positive
D04	FAM	Unkn	C.parvum	0.794	(+) Positive
E02	FAM	Unkn	C.parvum	0.988	(+) Positive
E03	FAM	Unkn	C.parvum	0.554	(+) Positive
E05	FAM	Unkn	C.parvum	1.07	(+) Positive
F02	FAM	Unkn	C.parvum	1.03	(+) Positive
F03	FAM	Unkn	C.parvum	0.777	(+) Positive
G02	FAM	Unkn	C.parvum	0.732	(+) Positive
G06	FAM	Unkn	C.parvum	0.634	(+) Positive
H03	FAM	Unkn	C.parvum	0.559	(+) Positive
B01	FAM	Neg Ctrl	C.parvum	0.0117	
E01	FAM	Unkn	C.parvum	0.0168	
H02	FAM	Unkn	C.parvum	0.0141	

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

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

Age (Years)	Sex				Chi-value	P-value	Geographical Location				Chi-value	P-value
	Male		Female				Rural		Urban			
	+ve	%	+ve	%			+ve	%	+ve	%		
1-3	8	44.44	4	22.22	2.68	0.05	9	50.5	3	16.67	*6	0.05
3-5	1	5.56	3	16.67	2		4	22.22	0	0	*8	
5-7	1	5.56	1	5.56	-		2	11.11	0	0	*4	
7-9	0	0	0	0	-		0	0	0	0	-	
Total	10	55.56	8	44.44			15	83.33	3	16.67		

Real-Time PCR master mix preparation

Real-Time PCR master mix was prepared for specific primer by using **AccuPower® DualStarTM 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 Exispin centrifuge, after that transferred into MiniOpticon Real-Time PCR thermocycler.

Real-Time PCR Thermocycler conditions

Real-Time PCR thermocycler conditions was 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 (Fig. 1a,1b). The present study recorded non significant differences at level (P<0.05) in the all age groups between male and female, the highest rate of the infection in male was (42.86%) compared with female (37.80%) 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 age groups (1-3) years old compared with other age groups, the highest rate of infection in rural regions was 47.62% compared with urban (19.05%). Table (4). The results showed oocysts 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 Khalil (2000) in Mosul (20.52%), and disagreement with Mahdi *etal.,* (1996) in Bashra province-Iraq (8.6%) and with Abdulsadah *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); Abdulsadah *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); Mohammadi *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 defection was randomly (Okafor and Okunji,1994; Spausta *et al*., 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).

PCR based techniques can detect a single cyst and also distinguish between different species and strains of parasites (Roberts and Janovy, 2000). PCR has primary been used for identification of different species and genotypes of *Cryptosporidium spp*. for taxonomical research, although there is potential for diagnostic use (Caccio *etal.,* 2005).

The present study recorded the total infection of *Cryptosporidium* by molecular assay diagnosed positive for *Cryptosporidium parvum* were genotyped on the basis of the 18S rRNA gene was 18%, this result is in agreement with Akiyoshi *etal*., (2006) and the results higher than koffie *et al*.,

(2014) in Africa (36.93) this may be due to large number of patient 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 *etal*., (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 *etal*., 2007). This study showed no significant differences between male and female ,both may have an equal chance to oocyst exposure.

Abdulsadah *etal*., (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 indiscrminate defeaction in outdoor area, or direct contact with infected animals, less health education of family (Gillespie and pearson (2001); Goh *etal*., (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 *etal*., (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 led to the contamination of drinking water sources from sewage and animal waste (Naumova *etal*., 2005; Sulaiman *etal*., 2005).

This result is in disagreement with Laupland and Church, (2005); Sallon *etal*., (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.

REFERENCES

1-Abdulsadah, A.R.; Magda, A.A. and Al-Charrakh, A.H. (2013). Prevalence of *Cryptosporidium Parvum* among Children in Iraq. *American Journal of Life Sciences*. Vol. 1, No. 6, pp: 256-260.

2-Akiyoshi, D.E.; Tumwine, J.K.; Bakeera-Kitaka, S. and Tzipori, S. (2006) . Subtype analysis of *Cryptosporidium* isolates from children in Uganda . *J. Parasitol*. 92: 1097-1100.

3-Akyon, Y.; Erguven, S.; Arikan, S.; Yurdakok, K. and Gunalp, A. (1999). *Cryptosporidium parvum* prevalence in a group of Turkish children. *Turk. J. Pediatr*. 41:189-196 .

4-AL-Braiken, F.A.; Amin, A.; Beaching, N.J.; Hommel, M. and Hart, C.A. (2003). Detection of *Cryptosporidium* among diarrhoeic and asymptomatic children in Jeddah, Saudi Arabia . *Ann. Trop. Med. Parasitol*. 97(5).

5-AL-Hindi, A.I.; Elmanama, A.A. and Elnabris, K.J. (2007). Cryptosporidiosis Among children attending Al-Nasser pediatric hospital, Gaza, Palestine. *Turk. J. Med. Sci*. 37(6): 367- 372.

6-Caccio, S.M.; Lalle, M.; Jimenez-Cardosa, E. and Pozio, E. (2005). Genotyping of *Giardia duodenalis* from humans and dogs from Mexico using a beta-giardin nested polymerase chain reaction assay. *J. Parasitol*. 91:203 -205.

7-Casimiro, A.M.; Carvalho, T.T.R. and Kanamura, H.Y. (2009). Serological evidence of *Cryptosporidium* infections in a group of pregnant women attended by the prenatal routine care at a public hospital in Sao Paulo (SP), Brazil. *Rev. Panam. Infect*. 11(2): 38-43.

8-Fayer, R.; Morgan, U. and Upton, S.J. (2000). Epidemiology of *Cryptosporidium*: transmission, detection and identification. *Int. J. Parasitol.* 30: 1305-1322.

9-Fayer, R. (2004) . *Cryptosporidium*: a water-borne zoonotic parasite. *Vet. Parasitol.* 126, 37–56.

10-Gatei, W.; Wamae, C.N.; Mbae, C.; Mulinge, E.; Waithera, T.; Gatika, S.M.; Kamwati, S.K.; Revathi, G. and Hart, C.A. (2006) . Cryptosporidiosis: prevalence, genotype analysis and symptoms associated with infections and children in Kenya. *AM. J. Trop. Med. Hyg.* 75(1) : 78-82.

11-Gillespie, S. and Pearson, R.D. editors. (2001). Principles and Practice of Clinical Parasitology. Jhon Wiley and Sons, L.t.d., New York. pp:139-164.

12-Goh, S.; Reacher, M.; Casemore, D.P. *et al.* (2004). Sporadic Cryptosporidiosis, North Cumbria, England, 1996–2000. *Emerg. Infect. Dis*. 10: 1007–1015 .

13-Gatei, W.; Wamae, C.N.; Mbae, C.; Mulinge, E.; Waithera, T.; Gatika, S.M.; Kamwati, S.K.; Revathi, G. and Hart, C.A. (2006). Cryptosporidiosis: prevalence, genotype analysis and symptoms associated with infections and children in Kenya. *AM. J. Trop. Med. Hyg.* 75(1): 78-82.

14-Gillespie, S. and Pearson, R.D. editors. (2001). Principles and Practice of Clinical Parasitology . Jhon Wiley and Sons, L.t.d., New York . pp:139-164.

15-Goh, S.; Reacher, M.; Casemore, D.P. *etal.* (2004). Sporadic Cryptosporidiosis, North Cumbria, England, 1996–2000. *Emerg. Infect. Dis*. 10: 1007–1015.

16-Iqbal, J.; Hra, P.R.; Al-Ali, F. and Philip, R. (2001). Cryptosporidiosis in Kuwaiti children: seasonality and endemicity. *Clin. Microbial. Infect*. 7: 261- 266.

17-Koffi, N'Djeti, Konan and Djè. (2014). Molecular characterization of intestinal protozoan parasites from children facing diarrheal disease and associated risk factors in Yamoussoukro*, Côte d'Ivoire*. Vol. 8(3), pp: 178-184.

18-Meinhardt, P.L.; Casemore, D.P. and Miller, K.B. (1996). Epidemiologic aspects of human Cryptosporidiosis and the role of waterborne transmission. *Epidemiol. Rev*. 18, 118–136.

19-Michel, M.Y.; Khalifa, A.M. and Ibrahim, I.R. (2000). Detection of the *Cryptosporidium parvum* antigen by Co-agglutination test and ELISA. *Eastern Mediterranean Health Journal* . 6(5): 898-907.

20-Morse, T.D.; Nichols, R.A.; Grimason, A.M.; Campbell, B.M.; Tembo, K.C. and Smith, H.V. (2007). Incidence of Cryptosporidiosis species in pediatric patients in Malawi . *Epidemiol. Infect*. In press.

21-Nahrevanian, H.; Assmar, M. and Samin, M.G.h. (2007). Cryptosporidiosis among immunocompetent patients gastroenteritis in Iran: a comparison with other enteropathogenic parasites. *J. Microbiol. Immunol. Infect*. 40:154-156.

22-Roberts, L. and Janovy, J. (2000) . Gerald D. Schmidt and Larry S. Roberts' Foundations of Parasitology, 6th ed. McGraw. Hill, Massachusetts, USA .

23-Sevinc, F.; Uslu, U. and Derinbay, O. (2005). The Prevalence of *Cryptosporidium parvum* in lambs around Konya. *Turk. J. Vet. Anim. Sci.* 29:1191-1194.

24-Sorlie, D.E. (1995). Medical biostatistics and epidemiology: Examination and Board review. First. Ed. Norwalk. Connecticut. Appleton. and Lange: 47-88.

25-Stephen, J. Hadfield ; Guy Robinson ; Kristin Elwin and Rachel, M. Chalmers. (2011). Detection and Differentiation of *Cryptosporidium spp.* in Human Clinical Samples by Use of Real-Time PCR. *J. Clin. Microbiol*.. 49. 3. 918-924 .

26-Sulaiman, I.M.; Xiao, L.; Yang, C. *et al.* (1998). Differentiating human from animal isolates of *Cryptosporidium parvum*. *Emerg. Infect. Dis*. 4: 681–685.

27- Jelinek T; Lotze M; Eichenlaub S; Löscher T. and Nothdurft HD. (1997) . Prevalence of infection with *Cryptosporidium parvum* and Cyclospora cayetanensis among international travellers. Gut. 41:801-804.

28- Soave R.; Ruiz J.; Garcia-Saucedo V.; Garrocho C. and Kean B.H. (1989). Cryptosporidiosis in a rural community in central Mexico[letter]. J. Infect. Dis. 159*:* 1160.1162.

29- Mak, J.W. (2004). Important zoonotic intestinal protozoan parasites in Asia. Trop Biomed 21: 39-50.

30- Ahmad, R.A. (1995). Pathogenic protozoa in Malaysian water resources. Sains, Malaysiana. 24: 121-127.

31- Gondim, L.F.; Gao, L. and McAllister, M.M. (2002). Improved production of *Neospora caninum* oocysts, cyclical oral transmission between dogs and cattle, and in vitro isolation from oocysts. J. Parasitol., 88: 1159-1163.

32- Hammond, D. M. and Long, P. L. (1973). The Coccidia. 1st ed. University Park press London.

33- Schares,G.; Pantchev, N.; Barutzki, D.; Heydorn, A.O.; Bauer, C. and Conraths, F.J (2005). Oocysts of *Neospora caninum, Hammondia heydorni*, *Toxoplasma gondii* and *Hammondia hammondi* in faeces collected from dogs in Germany. Int. J Parasitol. 35: 1525–1537.

34- Garcia, L.S. (1999). Practical guide to diagnostic parasitology. ASM press, Washington DC., P: 63,102.

35- Baxby, D.; Blundell N. and Hart C. A. (1984). The development and performance of a simple, sensitive method for the detection of *Cryptosporidium* oocysts in feces. J. Hyg. (Cambridge), 92:317.323.

36- John, D.T. and Petri. (2006). Medical Parasitology 9th edition. Elsevier Inc. USA. pp:463.

37- Khalil, L. Y. (2000). Comparison of efficiency of some diagnostic tests for the disease spores hidden in lambs and kids in Nineveh province. MSC thesis, Faculty of veterinary medicine, University of Mosul, Iraq.

38- Mahdi, N.K.; Al-Sadoon, I. A. and Mohamed, A. (1996). First report of Cryptosporidiosis among Iraqi children. Eastern Mediterranean Health Journal. 2 (1),pp. 115-120.

39- Yaqoob, A.; Shubber, I. and Kawan, M. (2004). Epidemiological study of Cryptosporidiosis in Calves and Man in Baghdad . Iraqi Journal of Veterinary Medicine. 28(1), pp. 109-121.

40- Ke-Xia Wang; Chao-Pin Li ; Jian Wang and Bo-Rong Pan. (2002). Epidemiological survey of Cryptosporidiosis in Anhui Province China. World J. Gastroenterol.8 (2) : 371-374.

41- Hamedi, Y.; Safa, O. and Haidari, M. (2005). *Cryptosporidium* infection in diarrheic children in southeastern Iran. Pediatr. Infect. Dis. J.;24(1):86-8.

42- Mohammadi, B.; Falah, S.; Asgharzadeh, M. (2006). Prevalence of *Cryptosporidium* in children suffering from gastroenteritis in Ardabil hspitals. J. Ardabil Univ. Med .Sci., 6(2):176-82.

43- Okafor, J. I. and Okunji, P. O. (1994). Cryptosporidiosis in patients with diarrhoea in five hospitals in Nigeria. J. Nigeria. J. Commun. Dis. 26, No. 2, pp. 75-81.

44- Spausta, G.; Wrezkowki, A.; Ciarkowska, J.; Kniazewska, M. B.; Kalita, B.; Sikora, A.; Slimok , M.; Zmudzinska, J. and Obuchowicz A. (2000). Prevalence of *Cryptosporidium parvum* infections in children with abdominal pain". *ActaPrasitol.,* 45. 3, p: 206.

45- Ajjampur, S.S.; Gladstone, B.P.; Selvapandian, D.; Muliyil, J.P.; Ward, H and Kang, G. (2007). Molecular and spatial epidemiology of Cryptosporidiosis in children in a semiurban community in South India. J Clin Microbiol. 45: 915-920.

46- Naumova E.N.; Christodouleas J.; Hunter P.R. and Syed Q. (2005). Effect of precipitation on seasonal variability in Cryptosporidiosis recorded by the North West England surveillance system in 1990. 1999. J. Wat. Health. 3, 185-196.

47- Sulaiman I.M.; Hira P.R.; Zhou I.; Al-Ali F.M.; Shelahi F.A.; Shweiki H.M.; Iqbal J. and *et al*. (2005). Unique endemicity of Cryptosporidiosis in children in Kuwait. J. Clin. Microbiol. 43: 2805-9. *Cryptosporidium parvum*: Structural Components of the Oocyst Wall J. Robin Harris and Franz Petry The Journal of Parasitolog Vol. 85, No. 5 (Oct., 1999), pp. 839-849.

48- Sallon S.; EL- Showwa, R.; EL- Masri M.; Khalil M.; Blundell N. and Hart C.A. (1990). Cryptosporidiosis in children in Gaza. Ann. Trop. Pediatric. 11: 277.281.

49- Laupland, K.B. and Church, D.L. (2005). Population based laboratory surveillance for *Giardia* sp. and *Cryptosporidium* sp. infections in a large Canadian health region. BMC Infect. Dis. 5:72.