Molecular and Epidemiological Study of Cryptosporidium spp. in Mid-Euphrates Area

دراسة جزيئية و وبائية Cryptosporidium spp في منطقة الفرات الاوسط

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الخلاصة :
الهدف: تهدف الدراسة إلى الاستقصاء عن طفيلي Cryptosporidium spp. في الأطفال والتعرف على المؤثرات الوبائية المختلفة .

المنهجية: جمعت النماذج البرازية بشكل عشوائي مِنْ ٤٦٧ مِنْ الأطفال الذين راجعوا مستشفيات مِنْ أربع محافظاتٍ عراقيةٍ، صبغت المسحات البرازية بصبغة Ziehl-Neelsen المحورة، استخدمت اثنتان من تقنياتِ PCR المتقدّمة (Nested PCR and Real Time PCR) للتحري عن الانوع في كافة العينات الموجبة. تم تحليل النتائج إحصائيا بطريقة Yat's Chi-square .

النَتائج: من بين الأطفال المَفْحُوصين الـ٤٦٧،َ تبين وجود أكياس البيض لطفيلي Cryptosporidium في براز ٣٩ (٨.٣٥ %) منهم. أظهرت الاختبارات الجزيئية بأنّ الانواع Cryptosporidium parvum و Cryptosporidium hominis وَجدا في ٧٢.٩ % و٢٤.٣ % مِنْ العيناتِ الإيجابيةِ على التوالي، عينتان (٥.١ %) تُوضّحانِ عدوى مُخْتَلِطة بكلى النوعين ، بينما عيِّنة واحدة فقط (٢.٧ %) لم تظهر أيّ تضاعف مخبري للحمض النووي، الذي قَدْ يَعْني نوعَ ما آخر عدا C. parvum أو C. hominis. وقد تم تحليل النتائج إحصائيا باستخدام طريقة

الاستنتاجات: أكدتْ الدراسةُ الحاليةُ أهميةُ أنواع طفيلي Cryptosporidium spp في منطقةِ الدراسة. بالإضافة لذلك يَبْدو بأنّ النوعَ Cryptosporidium parvum المسببُ الأكثر أهمية للعدوى في المنطقةِ. طبقاً لمعرفتِنا فان هذه الدراسة هي الأولى التي سجّلَت أنواع Cryptosporidium في العراق.

التوصيات: الحاجة إلى دراسات إضافية في مناطق أخرى تشمل كل الأعمار للتعرف عل معلومات أكثر عن الوبائية الجزيئية لداء الابواغ الخبيئة في مجتمعنا، كذاك دراسته في الحيوانات لتقييم دورها في وبائيته.

Abstract:
Objectives: the aim of present study was to search for Cryptosporidium spp. in our children and tracking different epidemiological effects.
Methodology: Random fecal specimens were collected from 467 of children whom attended to hospitals from four Iraqi governorates, modified Ziehl-Neelson staining for all samples, two advanced PCR techniques (Nested PCR and Real Time PCR) were used for all positive samples to detect species. Data was analyzed by use of Yat's Chi-square test.
Results: Among the 467 examined children, Cryptosporidium Oocysts were found to be excreted in 39 (8.35%) of them. Molecular tests showed that C. parvum and C. hominis found in 72.9% and 24.3% of positive samples respectively, two samples (5.1%) explain mixed species infection, whilst one sample (2.7%) don't reveal any amplification, that may mean species other than of C. parvum or C. hominis.
Conclusions: The present study emphasized the public health importance of Cryptosporidium spp in the study area. It seems that zoonotic species (C. parvum) is the most important cause of infection in the region. According to our knowledge, this report is the first that recorded the species of Cryptosporidium in Iraq.
Recommendations: Further studies at different areas and age groups are required to investigate the molecular epidemiology of cryptosporidiosis in our community, and also in the animals to evaluate the role of zoonotic transmission in cryptosporidiosis epidemiology.
Keywords: Nested PCR, Real Time PCR, Cryptosporidium parvum, Cryptosporidium hominis.

INTRODUCTION:

Cryptosporidium spp is a parasitic protozoan that infects gastrointestinal epithelial cells of many vertebrates, including humans, globally distributed and adversely impact on human health in both developed and developing countries [1]. It causes watery diarrhea and can be fatal to immuno-compromised individuals; In children, diarrhea considered as the leading cause of death at the developing countries to those whom younger than five years of age [2,3].

The global infection rate range from 0.3% to 37%. Generally, both of the infection rate and clinical features are markedly increase in the children [3].

There are 20 known Cryptosporidium species. Eight fully characterized Cryptosporidium species (C. hominis, C. parvum, C. meleagridis, C. felis, C. canis, C. suis, C. muris, and C. andersoni) were detected in man [4]. Among which, only two species are of major significance to public health by causing the majority of human cases (more than 90%) both sporadic and outbreak related cases, C. hominis and C. parvum [5].

C. parvum is zoonotic and infects a wide range of animal hosts including humans, whereas C. hominis is generally restricted to humans. Therefore, the main phenotypic difference between C. hominis and C. parvum is the host range; In addition, these two Cryptosporidium species differ in geographical and temporal distribution and pathogenicity. Differential risk factors and transmission routes have also been identified [6,7].

Traditionally, identification of Cryptosporidium parasite is based on morphologic examination, mainly using modified acid-fast staining method [8]. But this approach is not reliable for delineating Cryptosporidium species because of their morphologic similarities. Alternatively, molecular tools are useful for the accurate identification of Cryptosporidium species and better understanding of population genetics of Cryptosporidium, which have important implications for studying their pathogenesis and clinical presentations [1,9].

Genetic typing of several loci, such as those encoding the 18S rRNA, actin, oocyst wall protein, and a 70- kDa heat shock protein, have been used for species identification. The small subunit ribosomal ribonucleic acid (18S rRNA)-based PCR-restriction fragment length polymorphism (PCR-RFLP) tool is preferred due to the presence of conserved regions with highly polymorphic regions in the gene, allowing the design of primerst hat can amplify most Cryptosporidium species [2,10].

Several studies agree on the higher sensitivity of PCR targeting the 18S rRNA gene, Nested PCR has been put forward as a means of improving the sensitivity of detection [11].

The real-time PCR (qPCR) method using primers derived from the CP2 gene is highly sensitive, specific, and accurate for the detection of Cryptosporidium species [12].

Molecular epidemiologic tracking species is frequently needed to assess the endemic transmission at our community level and to identify the occurrence and sources of outbreaks. In spite of their importance as they considered as an epidemiological markers, there was a marked paucity in the studies pertain Cryptosporidium species in our country.

The objectives of this study are surveillance of most important species of the parasite by use of advance molecular tests and investigation of different epidemiological impacts.

METHODOLOGY:

The study randomly included 467 of children from both sexes and different residences whom attended to hospitals from four Iraqi governorates as following: Al-Najaf governorate (AL-Zahraa Maternity and Pediatrics), Babylon governorate (Maternity and Pediatrics hospital), Al-Qadisya governorate (Maternity and Pediatrics Teaching Hospital) and Karbala governorate (Karbala Hospital for Pediatrics) the number of samples from each governorate were 132, 118, 113 and 104 respectively.

The study was performed from the beginning of August 2012 to the end of May 2013, 2 gram of fecal samples were taken from each individual, fecal smears were prepared from each sample, and stained using the modified acid-fast staining technique as in [13]. Samples that positive to Cryptosporidium were kept in 2.5% potassium dichromate solution before DNA extraction as reported by [10].

Nested PCR:

Positive fecal samples were stored at 4oC in a 2.5% aqueous potassium dichromate solution ($K_2Cr_2O_7$). Prior to purification, each sample was filtered through an 80-mesh sieve to remove large debris, and the filtrate was processed within 24 h of collection as follows: $K_2Cr_2O_7$ was removed by three cycles of precipitation with phosphate buffered saline [10].

DNA extraction and purification done by using of QIAamp DNA stool Mini Kit (QIAGEN, Hilden, Germany).

The Cryptosporidium 18S rRNA gene was amplified by a nested PCR assay. In order to highlight the presence of multiple species of Cryptosporidium in a single sample, five replicates of nested PCR were performed using different DNA volumes for the first round (10, 2, 1 and 0.5 µl), as reported elsewhere [10].

The first PCR round was performed with primer pair: forward (**5′-TTC TAG AGC TAA TAC ATG CG-3′**) and reverse (**5′-CCC ATT TCC TTC GAA ACA GGA-3′**) and the second PCR round was performed with primer pair: forward (**5′-GGA AGG GTT GTA TTT ATT AGA TAA AG-3′**) and reverse (**5′-AAG GAG TAA GGA ACA ACC TCC A-3′**).

The two PCR rounds were performed under the same conditions. Nested PCR mixtures contained 1x PCR buffer, 5 mM MgCl2, 200 lµ each deoxynucleoside triphosphate, 100 nM each primer and 1.25 U Hot Start Taq polymerase.

Cycling conditions consisting of a hot start at 94° C for 3 min followed by 35 cycles with de-naturation at 94° C for 45 seconds, annealing at 55° C for 45 seconds, and extension at 72° C for 1 minutes, and a final extension at 72° C for 7 minutes [14].

Thermocycler used was Gene Amp. PCR System 9700, Turkey.

Restriction fragment length polymorphism:

A RFLP assay was performed with two restriction enzymes, AseI and SSpI, according to the manufacturer's instructions (Ozyme, Saint Quentin en Yvelines, France).

Part of the second-round PCR products was digested with AseI and another part with SSpI, allowing the detection of different species when present. Restriction profiles were visualized by electrophoresis of digested products on a 2% agarose gel with ethidium bromide as recorded by [14]. Gel Electrophoresis device from Labnet International, USA, whilst our DNA bands were analyzed and photographed by Bio-Document Analyzer, Biometra. Turkey.

Real Time PCR:

The Real time PCR reactions were performed according to the method of [15,16]. using a Light Cycler.

DNase/RNase-free water was used in place of template DNA as a negative control. CP2 sequences of C. parvum (AY471868) and C. hominis (XM_661199) were aligned using Clone Manager Suite 7 (Sci-Ed Software, Cary, North Carolina, USA).

An aliquot (15µl) of the qPCR product was analyzed according to manufacture with specific kit (Takara Bio Inc., Shiga, Japan). We used Real Time PCR Thermocycler: MX3005p System, Agilent Technologies. Germany.

Statistical Analysis:

Data were translated into a computerized database structure. An expert statistical advice was sought for. Statistical analyses were computer assisted using SPSS version 17, variables was assessed by Yat's Chi-square test.

RESULTS:

Table (1): Percentage of Infections with Cryptosporidium spp. in the Study Area

| Governorate	NO. of Samples	Cryptosporidium Infections		C. parvum Infections		C.hominis Infections		Mixed Species Infections	
		NO.	%	NO.	%	NO.	%	NO.	%
Najaf	132	14	10.6	9	64.2	4	28.5	1	7.14
Babylon	118	7	5.9	6	85.7	1	14.2	0	_
Qadisyia	113	10*	8.85	7	70	1	10	1	10
Karbala	104	8	7.69	5	62.5	3	37.5	0	_
Total	467	39	8.35	27	72.9	9	b 24	2	5.1

* One sample belong to species other than C. parvum and C. hominis.

This table shows the infection with Cryptosporidium spp. in 39 out of 467 of the children (8.35%) by use of microscopic examination to fecal smears stained with Modified acid fast stain. All positive samples were analyzed by Nested PCR and Real Time PCR, both assays gave equal results, two samples show amplification with the primers and probes of both species, that mean mixed infection with two species. After excluding of mixed species infection (2cases), 27 out of 37 of positive samples (72.9%) were expose Cryptosporidium parvum whilst 9 (9/37) of them (24.3%) were Cryptosporidium hominis, where as one sample (2.7%) don't reveal any amplification, that may mean species other than of C. parvum or C. hominis.
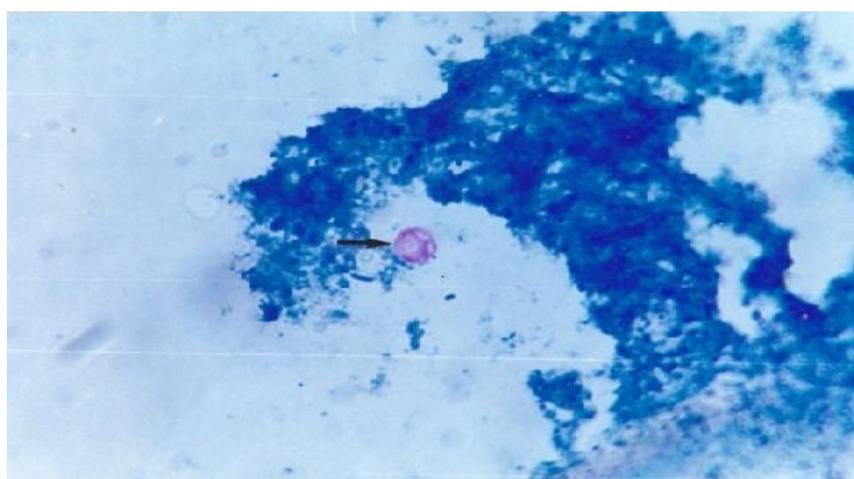

Figure (1): Oocyst of Cryptsporidium spp. in a fecal smear stained with modified Zheil-Neelsen stain.

The Oocyst of Cryptsporidium spp. appears red surrounded by blue background, it contain four sporozoites colored dark red.
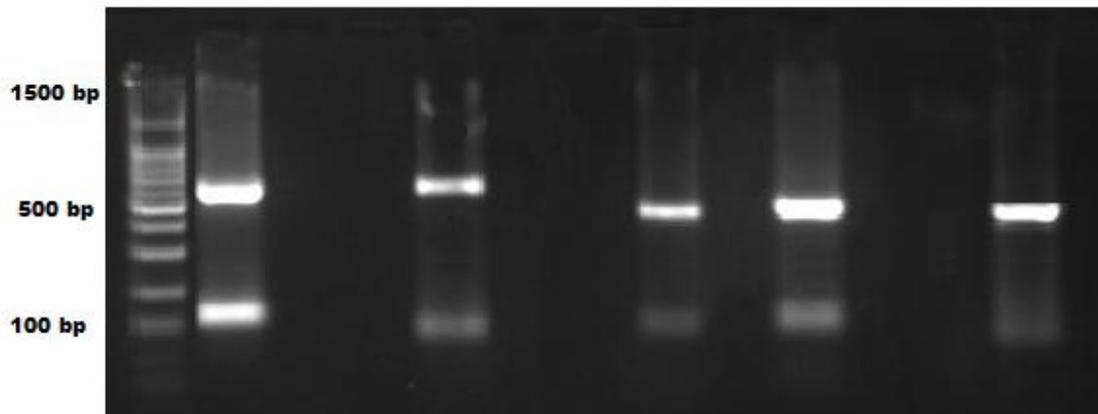
Figure (2): Gel electrophoresis after restriction with AseI and SSpI enzymes using PCR-restriction fragment length polymorphism.

The picture was photographed by Bio-Document Analyzer. Lanes 1 and 4 : C. Parvum (628 bp bands). Lanes 7,9 and 12 : C. hominis (561 bp bands).
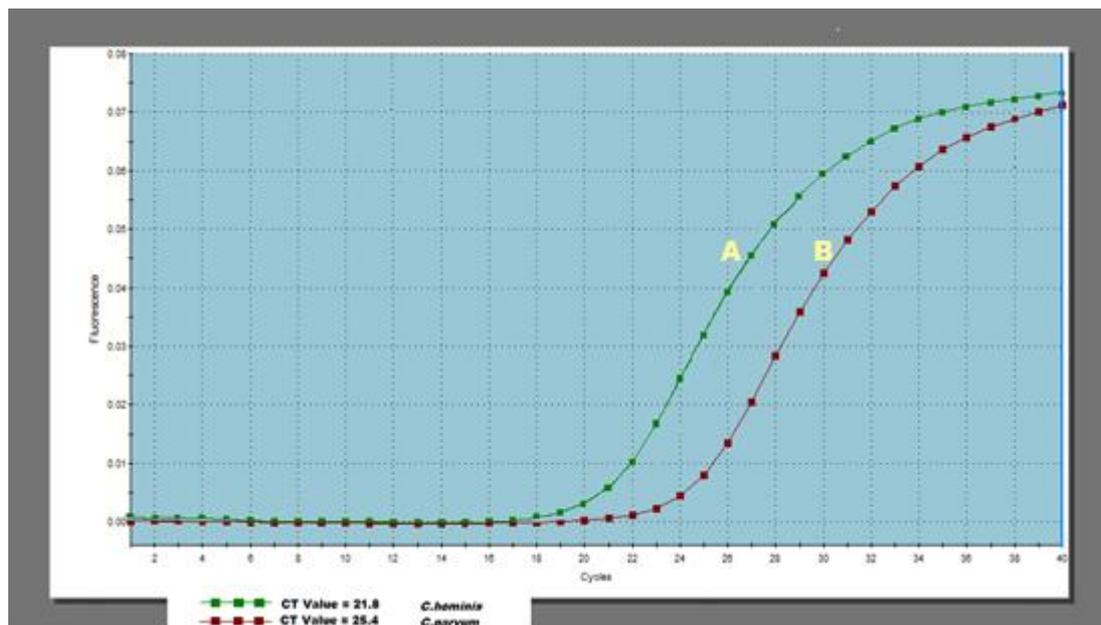
Figure (3): The Graphic Results for two Positive Cases of two species (C. parvum and C. hominis) that Obtained by Real Time PCR Thermocycler.

X axis: cycle number, **Y axis**: Log. Fluorescence intensity. **A**: amplification curve has C.T. value = 21.4 for a sample with C. hominis. **B**: amplification curve has C.T. value = 25.4 for a sample with C. parvum.

Table (2): Number of Infected Cases with Cryptosporidium spp. at Urban and Rural Areas

SPECIES	NO.OF POSITIVE SAMPLES	NAJAF Urban	NAJAF Rural	BABYLON Urban	BABYLON Rural	QADISYIA Urban	QADISYIA Rural	KARBALA Urban	KARBALA Rural	TOTAL URBAN No.	URBAN %	RURAL NO.	RURAL %
C.parvm	27	2	7	2	4	2	5	3	2	9	33.3	18	66.6
.hominis	9	2	2	0	1	1	0	2	1	5	55.5	4	44.4
Mixed Species Infection	2	1	0	0	0	0	1	0	0	1	50	1	50
Other Species	1	0	0	0	0	0	1	0	0	0	0	1	100
Total	39	5	9	2	5	3	7	5	3	15	38.4	24	61.5*

* Significant at level of P < 0.05.

   This table reveals that 61.5% (24/39) of the infection found at rural areas which was significantly higher (at level of 0.05) than that of urban areas, 38.4% (15/39).

Table (3): The Infection Rates in Different Age Groups

Age Groups than 6 Months	No. of Tested Children	No. of Infected Children	Percentage of Infection%*
Less than 6 Months	59	13	22%*
6 Months –2 Years	92	17	18.47%*
2 – 4 Years	102	4	3.92%
4 – 6 Years	75	1	1.33%
6 – 8 Years	74	2	2.7%
8 – 11Years	65	2	3.07%
Total	467	39	8.35%

* Significant at level of P < 0.05.

This table shows that the highest infection rate was 22% (13/59) at children aged less than 6 months, followed by age group 6 months to 2 years that had slightly fewer percentages 18.47 % (17/92) whilst the lowest infection rate has recorded at the age group 4 to 6 years which was 1.33% (1/75). The statistical analysis reveal significant difference among percentages of infection in relation to ages at level of 0.05.

The current study was showed that the prevalence of infection in males was 9.2% (28/302) which is higher than that of females 6.6% (11/165), statistical analysis not found any significant difference in the prevalence of infection at level of 0.05 between two sexes.

Table (4): Positive Samples Through the Months of the Year

Months	No. of Samples	NO. of Positive Samples	Percentages of Infection %
August	40	2	5
September	46	4	8.69
October	43	3	6.97
November	46	3	6.52
December	51	3	5.88
January	45	0	0
February	52	4	7.69
March	44	7	15.9*
April	53	10	18.86*
May	47	3	6.38
Total	467	39	8.35%

* Significant at level of P < 0.05

This table reveals that the prevalence of infection through the months, the highest infection rate was noticed during April 18.86% followed by that in March which was 15.9%, and the lowest infection rates was 5% in August whilst infection not recorded at January. It was found that there were significant differences at the level of 0.05 among the infection rates throughout the months.

DISCUSSION:

Cryptosporidiosis is one of the major diarrheal diseases caused by protozoan parasites and poses a significant public health problem worldwide, particularly in children [3]. Because of their importance, we are crucially need to know about at least major species of Cryptosporidium (C.parvum and C. hominis) in Iraq. For this reason, we search the parasite in a random fecal smears of children stained with modified Zheil-Neelsen stain, all positive samples (39) were analyzed with two advance molecular tests (Nested PCR and Real Time PCR) .

Infection founded in 39 out of 467 of the children (8.35%) using the microscopic examination. Similar results were recorded by Al-Braiken et al. [17] and Shalaby et al. [18] in Saudi Arabia, they reported infection of 9% and 11% of the children respectively.

Infection rate in the present study was slightly lowered than that obtained by Abdul-Sada [19], he found infection rate in the children at Al-Najaf city was 13.6%, such result also was obtained by Al-Khailani [20] in Baghdad which was 14.6%. This may be due to differences in the number of children examined, type of populations studied (e.g. urban or rural), location, and year of study.

The percentage of infection at the current study was higher than that founded by Mahgoub et al. [21] in Jordan, which was 2% and Al-Delaimy et al. [22] in Malaysia, 5.2%. The variation in the prevalence may belong to number of samples, differences in hygiene measures and risk factors which may vary among countries and from one region to another.

The present study registered that 27 out of 37 of positive samples (72.9%) were expose C. parvum whilst 9 (9/37) of them (24.3%) were C. hominis.

According to our knowledge, this study was the first at least in the study areas that involved the searching of species of Cryptosporidium parasite among children and the first that recorded C. parvum or C. hominis in Iraq.

The above finding came in alignment with the majority of the global studies; the molecular epidemiological studies have shown that C.parvum is the dominant species followed by C. hominis. For instance, Al-Braiken et al. [17] in Saudi Arabia identified C. parvum and C. hominis in 37% and 42.9% of cases respectively, mixed infection with C. hominis and C. parvum in 2.9% of the infected children where as other species infection came in percentage of 2.9% (Table 1).

In Iran, Rafiei et al. [23] had registered infection rates of 68%, 25% and 6.2% from infected children with C. parvum, C. hominis and other species respectively. Similar findings were obtained in Egypt by Helmy et al.[24] they found that C. hominis predinate in humans (60·5%), followed by C. parvum (38·35%).

The domination of C. parvum may be due to lacking of the host specificity [3], that may give it a real chance to spread widely among human and animals.

The current study has showed reveals that 61.5% (24/39) of the infection found at rural areas which was significantly higher (at level of 0.05) than that of urban areas, 38.4% (15/39).). Neared finding was recorded by Sharma et al. [25] in India, they found that infection rate at rural areas (77.1%) which was significantly higher than that of urban areas (32.8%).

In China, Lu and Li [26] had similar results, they reported that infection rate in children significantly higher at rural regions (59.7%) than in urban ones (40.14%).

Children living in the 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).

The highest infection rate was 22% (13/59) at children aged less than 6 months, followed by age group 6 months to 2 years that had percentages of 18.47 % (17/92). Significant difference at level of 0.05 was founded among percentages of infection in relation to age of children.

Our results were in constant with the majority of the studies worldwide which revealed that infection rate inversely correlates with the age of children. For instance, characteristically similar finding was obtained in Iraq by Abdul-Sada [19], he found that the highest infection rate was at age group: less than 6 months (37.5%) followed by age group: 6 months to 2 years (29.1%).

In Saudi Arabia, Al-Braiken et al. [17] reported that the highest infection rate with Cryptosporidium (9%) was in children aged less than 5 years old.

In Kenya, Gatei et al. [27] The prevalence of cryptosporidiosis was highest among children aged less than one year old (5.2%) followed by among those 1-2 years of age (2%).

This may be related to bad habits of adults towards their children like putting of finger in the infant mouth and use of contaminated baby bottles and nipples which may be washed by contaminated water. Moreover, children at above age group usually checking objects by putting these objects in their mouth, especially at teething period.

The study was showed that the prevalence of infection in males was 9.2% (28/302) which is higher than that of females 6.6% (11/165), statistical analysis not found any significant difference in the prevalence of infection at level of 0.05 between two sexes.

The preponderance of the global researches in children was observed the elevation of the infection rate in male than in female. Like the study of Liu et al. [28] in China, they record infection rate 15.1% in male children which higher than that of female

children (10%) and the study of Hong et al. [29] in Mongolia, where they found infection rate 6.76% at male children which was higher than the rate 3.13% at female.

However, the studies that recorded infection rates at females higher than males were scarce; may because males more in contact with the hazards than female like external environment, playing with animals and swimming at rivers and pools.

The statistical analysis show that there were significant differences at the level of 0.05 among the infection rates throughout the months, highest infection rate occurred in April (18.86%) followed by that in March which was 15.9%.

This result was slightly neared that of Abdul-Sada [19] in Iraq, he reported that the highest infection rates was at the March followed by April. Different results were registered by Mahgoub et al. [21] in Jordan, they found that the higher infection rates were at January and February. Disagreed result was recorded by Siwila et al. [30] in Zambia where they found that the highest rates were at Jun and July. Various reasons may stand behind elevation in the prevalence of cryptosporidiosis in the current study at April and March such as increasing of the children exposure to the parasite during spring due to increasing of picnics, flies and insects spreading.

CONCLUSIONS:

Our results provide useful information about the distribution of Cryptosporidium species, the present study demonstrated that Cryptosporidium spp. is prevalent among children at the study area. This is first report about species of the parasite in our country. Cryptosporidium parvum (zonotic species) is the most dominant species as cause of cryptosporidiosis in the region. The infection rate was markedly increase at the rural areas and inversely correlated with the age of children. The months of the years have significant effect in the prevalence.

RECOMMENDATIONS:

Future work should be focused on large scale studies to investigate the molecular epidemiology of cryptosporidiosis in our community and animals as well to assess the role of zoonotic transmission in cryptosporidiosis epidemiology.

There is an urgent need to implement an innovative and integrated control program to reduce the prevalence and intensity of these infections.

REFERENCES:

1- Snelling, W. J; Xiao, L; Ortega-Pierres, G; Lowery, C. J; Moore, J. E; Rao, J. R; Smyth, S; Millar, B. C; Rooney, P. J; Matsuda, M; Kenny, F; Xu, J. and Dooley, J. S. (2007). Review: Cryptosporidiosis in developing countries. **J. Infect. Dev.** 1(3):242-56.

2- Bouzid, M ; Hunter, P.R; Chalmers, R.M. and Tylera, K.M. (2013). Review: Cryptosporidium , Pathogenicity and Virulence. **Clin.Micro.Rev**. 26(1):115-34.

3- Ryan, U; Fayer, R. and Xiao L. (2014). Review: Cryptosporidium species in humans and animals: current understanding and research needs. **Parasitol**. 11: 101-19.

4- Xiao, L; Fayer, R; Ryan, U. and Upton, S. J. (2004). Review: Cryptosporidium taxonomy: recent advances and implications for public health. **Clin Microbiol. Rev**. 17: 72-97.

5- Caccio, S.M; Thompson, R.C; McLauchlin, J. and Smith, H.V. (2005) .Unravelling Cryptosporidium and Giardia epidemiology. **Tren. Parasitol**. 21: 430-37.

6- Caccio, S.M. and Pozio, E. (2006). Advances in the epidemiology, diagnosis and treatment of cryptosporidiosis. Exper. **Rev. Anti. Infect.** 4:429-43.

7- Xiao, L. and Feng, Y. (2008). Zoonotic cryptosporidiosis. **Immuno. Med. Microbiol**. 52:309-23.

8- Kar, S; Gawlowska, S; Daugschies, A. and Bangoura, B. (2014). Quantitative comparison of different purification and detection methods for Cryptosporidium parvum oocysts. **Vet. Parasitol**. 177: 366–70.

9- Sulaiman, I. M; Hira, P. R; Zhou, L; Al-Ali, F. M. and Al-Shelahi, F. A. (2005) Unique endemicity of cryptosporidiosis in children in Kuwait**. J. Clin. Microbiol**. 43: 2805-09.

10- Wang, R; Zhang, X; Zhu, H; Zhang, L; Feng, Y; Jian, F; et al (2011). Genetic characterizations of Cryptosporidium spp. 10 and Giardia duodenalis in humans in Henan**. Chin. Exp. Parasitol**.127: 425.

11- Jothikumar, N; Da-Silva, A.J; Moura, I; Qvarnstrom, Y. and Hill, V.R. (2008). Detection and differentiation of Cryptosporidium hominis and parvum by dual TaqMan assays**. J. Med. Microbiol**. 57:1099-105.

12- Mary, C; Chapey, E; Dutoit, E; Guyot, K. L; Hasseine, L; Jeddi, F; et al (2014). Multicentric Evaluation of a New Real- Time PCR Assay for Quantification of Cryptosporidium spp. and Identification of Cryptosporidium parvum and Cryptosporidium hominis**. J. Clinic. Microb**. 51(8):2556-63.

13- Beaver, D.C. and Jung, R.C. (1985). Animal Agents and Vectors of Human Diseases. 5th ed. Lea and Febiger.P.249.

14- Xiao, L; Morgan, U. M; Limor, J; Escalante, A; Arrowood, M; Shulaw, W; et al (1999). Genetic diversity within Cryptosporidium parvum and related Cryptosporidium species. **Appl. Envir. Microbiol**. 65: 3386-91.

15- Lee, S.U; Joung, M; Ahn, M.H; Huh, S; Song, H; Park, W.Y. and Yu, J.R. (2008). CP2 gene as a useful viability marker for Cryptosporidium parvum. **Parasitol. Res.** 102: 381-87.

16- Cheun, H; Kim, K; Yoon, S; Lee, W; Park, W; Sim, S. and Yu, J. (2013). Cryptosporidium homins Infection Diagnosed by Real-Time PCR-RFLP. **Kor. J. Parasitol**. 51(3): 353-55.

17- Al-Braiken F.A; Amin, A; Beeching, N. J; Hommel, H. and Hart, C. A. (2003). Detection of Cryptosporidium amongst diarrhoeic and Asymptomatic children in Jeddah, Saudi Arabia. **Ann . Trop. Med. Parasitol**. 97: 505-10

18- Shalaby, I; Gherbawy, Y; Jamjoom, M. and Banaja, A. (2014). Prevalence and genotyping of Cryptosporidium in stool samples collected from children in Taif City (Saudi Arabia). **Trop. Biomed**. 31(2): 215–22.

19- Abdul-Sada, K. M. (2006). Study the Epidemiology of Cryptosporidium spp in Al-Najaf city. **J.Babyl.Univ**. 13(3):583-89.

20- Al-Khailani, B. A. (1999). Study of the Epidemiology of Cryptosporidium spp in Baghdad. MS.c. Thesis. College of veterinary medicine. University of Baghdad.

21- Mahgoub, E. S; Almahbashi, A. and Abdulatif, B. (2014). Cryptosporidiosis in children in a north Jordanian paediatric hospital. **East. Mediterr. Health J**.10: 494-501.

22- Al-Delaimy, A. K; Al-Mekhlafi, H. M; Nasr N. A; Sady, H. and Atroosh, W. M. (2014). Epidemiology of Intestinal Polyparasitism among Orang Asli School Children in Malaysia. **Plo. Negl. Trop. Dis.** 8(8):3074-86.

23- Rafiei, A; Rashno, Z; Samarbafzadeh, A. and Khademvatan, S. (2014). Molecular Characterization of Cryptosporidium spp. Isolated From Immuno-compromised Patients and Children. **Jundish. J. Microbiol**. 7(4): 9183-87.

24- Helmy, Y.A; Samson, G; Noockler, K. and Zesin, K.H. (2014). Frequencies and spatial distributions of Cryptosporidium in livestock animals and children in the Ismailia province of Egypt. **Epidemiol.Infect.** 1: 1-11.

25- Sharma, P; Sharma, A; Sehgal, R; Malla, N. and Khurana, S. (2013). Genetic diversity of Cryptosporidium isolates from patients in North India. **Inter. J. Inf. Dis**. 17 (2): 601-5.

26- Lu, J. and Li. C.P. (2007). The survey of Cryptosporidium infection among young children in kindergartens in Anhui Province. **J. Inf. Dev Count**. 2(1): 331-33.

27- Gatei, W; Wamae C. N; Mbae, C; Waruru, A; Mulinge, E; Waithera, T; Gatika, S. M; Kamwati, S. K Revathi, G. and Hart,C. A. (2013). Cryptosporidiosis: prevalence, genotype analysis, and Symptom associated with infections in children in Kenya. **Am. Trop. Med. Hyg**. 75: 78-82.

28- Liu, H; Shen,Y; Yin1, J; Yu ,Z; Jiang, Y; Xu, Y; Pan, W; Hu1, Y. and Cao1, J. (2014). Prevalence and genetic characterization of Cryptosporidium. Enterocytozoon, Giardia and Cyclospora in diarrheal outpatients in china. **BMC. Inf. Dis.** 14:25- 31.

29- Hong, S; Anu, D; Jeong, Y; Abmed, D; Cho, S; Lee, W. and Lee, S. (2014). Molecular Characterization of Giardia duodenalis and Cryptosporidium parvum in Fecal Samples of Individuals in Mongolia. **Am. J. Trop. Med. Hyg**. 90(1): 43-7.

30- Siwila, J; Phiri, I. G; Enemark, H. L: Nchito, M. and Olsen, A. (2011). Seasonal Prevalence and incidence of Cryptosporidium spp. and Giardia duodenalis and associated diarrhoea in children attending pre-school in Kafue, Zambia. **Trop. Med. Hyg**. 105(2):102-8.