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Genotype of *Cryptosporidiumspp.* isolated from human of Al-Qadisiyah province /Iraq.

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Abstract:

The current study included examination of 100 stool sample from human was collected from AL-Qadisiyah province, from September 2018 until February 2019.the Microscopic examination result showed that oval or spherical shaped with dark pink color or red oocyst on blue ground and 22(22%) positive sample out 100 case . It was recorded that the highest rate 30% (3/10) was seen in September , but the lowest infection rate 13.3% (2/15) was seen in the month of December with no significant differences at level ($p<0.05$.) regarding to age the high rate of infection 26.31%(10/38)was found in the group less than a year , but the lowest infection rate was observed in the group(>12 years). There is no significant differences at $p<0.05$.between man and women . In this study the N- PCR in molecular examination were used ,the positive sample was13 (59.09%) out of 22 stool sample. Sequencing of a fragment of the (18s rRNA) gene (834 bp) that separated from many different area in AL-Qadisiyah government recorded (75%)9/12 samplerelated to NCBI – Blast *Cryptosporidium hominis* isolates ,(25%) 3/12 sampledisplay closed related to NCBI –Blast *Cryptosporidium parvum* .In Conclusion The current study concluded that phylogenetic tree and the homology sequences identity gives a clear differentiation of the types of *Cryptosporidium parasites* which can be isolated at high rates of human in AL- Diwaniyah province, which is likely to lead to an outbreak of Cryptosporidiosis in this province.

Keywords: *Cryptosporidium* ,human , N-PCR ,analysis , Genotype

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1. Introduction

Cryptosporidium are common gastrointestinal parasite in a broad range of hosts , such as humans being , many mammals, birds, reptiles, amphibians, and fish.The *Cryptosporidium* parasite possesses a wide genetic diversity. , Higher than 60 genotypes of *Cryptosporidium* have been identified without specific species names in addition to more than 20 known *Cryptosporidium species* are identified (Fayer,2010).In developing countries, Cryptosporidiosis is the main causes of many diarrheal diseases, nutritional deficiencies and failure in children's

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development(Ahs*et al* .,2010).In humans ,the Cryptosporidiosis are related with an immune-compromised health status such as acquired immune deficiency syndrome, leukemia, immunosuppressive therapy(Hassanien*et al* .,2012),there are many *Cryptosporidium spp*. that effect human which included *C. parvum* , *C.hominis* , *C. meleagridis* , *C.canis* , *C. felis*(Xiao, 2010).A common risk factor in the epidemiology of *Cryptosporidium parasites* in humans is direct attach with cattle(Ryan *et al* .,2014).The most important clinical signs of the infection of *Cryptosporidium parasites* is watery diarrhea, mild fever, abdominal pain and in healthy individuals. This disease is characterized by self-limiting diarrhea(El-Helalya*et al* .,2012).One of the most important ways of transmission of this protozoa is direct attach with infected animals, through person to person and by contamination of nutrients and water(Mathew *et al.*,2014).The risk of Cryptosporidiosis lies in its resistance to many drugs, sterilizers and disinfectants. The methods of parasite diagnosis have been developed using DNA analysis methods, genetic engineering , the Immunofluricent Staining Tachinque Monoclonal Antibody and Polymerase chain Reaction PCR(Lowery *et al* .,2000).

2.Material and methods

2.1 .Collection of specimens

About 100 stool sample were collected from human vary in ages and from both gender at the period extend from September 2018 to the until the end of February 2019 , Includes different areas of Qadisiyah province ., the samples are placed in sterilized containers marked with information such as name , age and sex and clinical symptom of the patient .the sample were transfer to the laboratory of the Veterinary Medicine college in University of Al -Qadissiyah for the necessary tests.

2.2 .Microscopic Examination of oocyst

Detection of *Cryptosporidium*oocysts rely on the microscopically examination of the in the stool smear and usually uses modified acid fast stain protocols , such as Zeihl-nelson stain, and the microscopic examination of the oocysts is shown as red-stained spherules. This is the best way to examine the oocysts because it is simple, fast and low cost (Silverlas*et al* .,2013).

2.3 .DNA isolation and molecular analysis

DNA was extract from 30 *Cryptosporidium* positive stool samples by used fecal DNA kit (AccuPrep® stool DNA Extraction Kit , Bioneer. Korea) , Where we followed the protocol of the manufacturer. The DNA is preserves in -20 until it is used in PCR.

2.4 .N-PCR .

polymerase chain reaction was used to amplify of(18s r RNA) for diagnosis of *Cryptosporidium protozoa* (Xiao *et al* .,2001)with some modifications. In the first step, partial 18S rRNA of *Cryptosporidium* was intensified in a 25 µl reaction mixture having 20 pmol of each primer (CRP-DIAG1 Forward: 5' TTC TAG AGC TAATAC ATG CG 3' and CRP-DIAG1 Reverse: 5' CAT TTC CTT CGA AAC AGG A 3'), in the first round of PCR employing the following thermal cycling protocol : first cycle of initial denaturation at 94 °C for 5 min, next by 35 cycles each of denaturation at 94°Cfor 1 min, annealing at 56°C for 1 min and extension at 72°Cfor 1 min. This was continue by last

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extension for 10 minuts b. 72°C.in the second around 1µl of the first PCR product was employ as a template and 20 pmol of primers (CRP-DIAG2 Forward: 5' GGA AGG GTT GTA TTT ATT AGA TAA AG 3' and CRP-DIAG2 Reverse: 5' AAG GAG TAA GGA ACA ACC TCC A 3') were used in 50 µl reaction mixture. The PCR reaction and cycling condition were same to the environment used for primary PCR, excepting that the temperature of annealing was at(60°C).

2.5.Sequencing

Nested polymerase chain reaction product were sent to Macrogen Co./ Korea where they were subjected to direct sequencing. *Cryptosporidium species* and subgenotype were recognized by using the BLAST search against the GenBank database.

2.6.Statistical analysis:

All statistical calculations were made using Statistical Package of Social Sciences (SPSS), version 23 (Inc., Chicago, IL, USA) computer software. Differences between different groups were analyzed using chi-square test (X2) . The level of statistical significance was set at alpha equal to 0.05 (a = 0.05). A value of P < 0.05 was considered statistically significant.(Al-Ukaelii*et al* .,1998).

3.Result.

3.1.Diagnostic Description of *Cryptosporidium spp.*

By using (MZN) stain the *Cryptosporidium spp*. oocyst were identified in human when they were examined under microscope by using oil emersion (100) lenses as in picture (1) identified as spherical -shaped or oval objects with dark pink or red color on blue space .

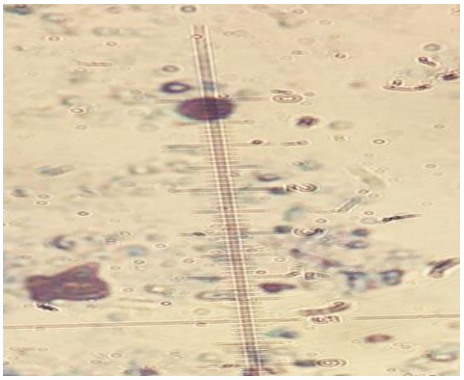

Figure (1).show *Cryptosporidium spp*. oocyst stained with(MZ N) stain(100x),

3.2.Results of microscopic examination:

In this study, 100 samples of human were examined microscopically using(MZN) stain ,where 22 (22%) samples gave positive results.

Table(1) the rate of infection of *Cryptosporidium spp*. in human

Type	Examination No.	Positive No.	Percentage %
Bovine	100	22	22

- **Infection rate of *Cryptosporidium in* human depended on the Months of study:**

In humans ,the highest infection rate 30% (3/10) was seen in September ,while the lowest 13.3% (2/15) was

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observed in December.with no significant differences at level ($p<0.05$.) , Table (2) .

Table (2) the rate of infection of *Cryptosporidium spp.* in human according to the month of study .

Month	Examination No.	Positive No.	Percentage %
September	10	3	30
October	5	1	20
November	13	3	23
December	15	2	13.3
January	20	4	20
February	28	9	28.57
Total	100	22	22
X^2	2.363(NS)		
P value	0.797		

NS: Non-significant differences at p<0.05.

- **Infection rate of *Cryptosporidium spp.* in human depended on the age:**

In the present study, we found that the highest infection rate 26.31%(10/38) was in the age group <1,while the lowest rate 8.33%(1/12)was in the age group more than 12 years.and the groups show no significant differences at level ($p<0.05$.) , Table (3)

Table(3)the rate of infection of *Cryptosporidium spp*. in human depended on human age:

Age group(years)	Examination NO.	Positive No.	Percentage %
<1	38	10	26.31
(1-5)	30	7	23.33
(6-12)	20	4	20
>12	12	1	8.33
Total	100	22	22
X^2	1.796(NS)		
P value	0.616		

NS: Non-significant differences at p<0.05.

- **the rate of infection *Cryptosporidium spp*. in human according to sex**

In this study, which included 100 stool samples (43 males and 57 females), the infection rate was 23.25% and 21.05% males and females respectively withno significant differences at $p<0.05$ as in table (4).

Table(4)ofthe rate of infection of *Cryptosporidium spp.* in human according to sex.

Sex	Examination No.	Positive No.	Percentage %
Male	43	10	23.25
Female	57	12	21.05
Total	100	22	22
X^2	0.069(NS)		
P value	0.792		

NS: "Non-significant differences at p<0.05".

3.3 Molecular Examination results

3.3.1.Detection of *Cryptosporidium spp*. By Nested – PCR.

Nested- PCR is used for identified of *Cryptosporidium parasite* infection. the result of PCR analysis of the 18S

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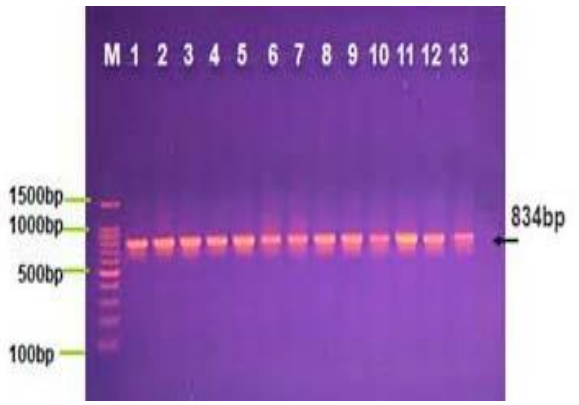
ribosomal RNA gene of *Cryptosporidium spp.* in Figure (2) **.**



(2) Agarose gel electrophoresis figure that showed the N-PCR product analysis of 18S rRNA gene in *Cryptosporidium spp.* positive samples from human . Where M: marker (1500-100bp) and lane (1-13) positive *Cryptosporidium spp*. were showed at (834bp)poly merase chain reaction product

3.4. the result of sequencing .

The nucleotides sequence result of our study proved and examined by utilizing the(NCBI – Basic Local Alignment Search Tool) (BLAST analysis) by employed nucleotide database within nucleotide query program online. Sequences verification and investigation were proved by employ references of 18s rRNA gene of *Cryptosporidium* that involved *C. parvum* , *C. hominis* , *C.bovis* , *C. andersoni*gene sequences data that reported in Gene Bank and the out groups to discovered the degrees of identity and similarity score of the 18s rRNA gene of *Cryptosporidium species* commonly that effected human and in Comparison with present study isolates strains . The results of present study local *Cryptosporidium species* (3)25% sample from human were showed closed related to NCBI – Blast *Cryptosporidium parvum* isolates , . The percentage of identity score range from (99.62- 100%) , (9)75% sample from human were display deep related to NCBI –Blast *Cryptosporidium hominis* The identity scorepercentage (99.24-100) as in figure (3). With significant difference at $p<0.05$ between this spp. as in table

(5) .Genotyping of *Cryptosporidium species* in human in Al-Qadisiyah Province.

Species	No. of isolates and %
C.hominis	**9(75)**
C.parvum	**3(25)**
Total	**12**
X^2	**6(N)**
P value	**0.014**

N : significant difference at p<0.05

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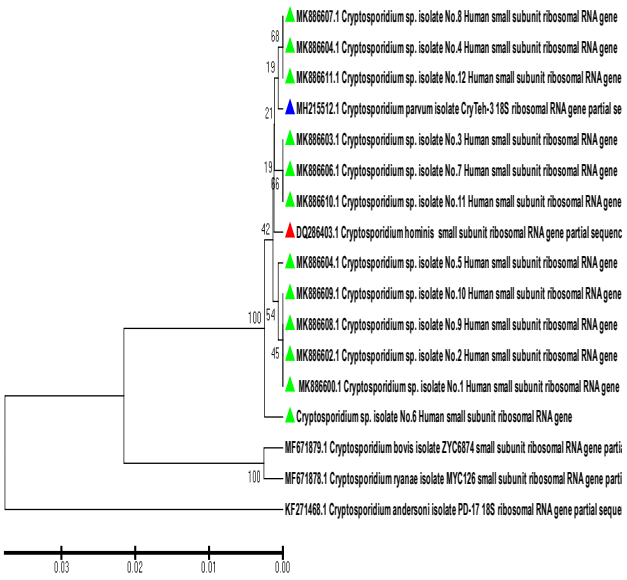

(3)The analysis ofPhylogenetic tree depended on small subunit ribosomal RNA gene partial sequence in local "*Cryptosporidium sp* "human isolates that used for genetic *Cryptosporidium species* identification . "The phylogenetic tree was constructed using Unweighted Pair Group method "with Arithmetic Mean (UPGMA tree) in (MEGA 6.0 version). The local *Cryptosporidium*isolate No.4, No.8 and No.12 were showed closed related to" NCBI-BLAST" *Cryptosporidium parvum* isolate (MH215512.1). The local *Cryptosporidium* isolate No.1, No.2 , No.3, No.5, No.6, No.7, No.9, No.10, and No.11 were showed deep relationship to NCBI-BLAST *Cryptosporidium hominis* (MF671879.1). "at total genetic changes (0.01-0.03%)".

5.Discussion

In this study ,100 stool samples from human vary in age age collect from different area in Al-Qadisiyah province there is 22(22%) stool samples were found positively for oocysts of *Cryptosporidium spp.*These result were close to the results of the study conducted by (Al-Alousi and Mahmood .,2012) , Which recorded an infection rate of 18.9% after the examination of 92 samples of children suffering from diarrhea between the ages of (1 - 12) years in Mosul , the result of present study higher than result recorded by(Al-Rikabi.,2012) in the province of ThiQar, were recorded a parasitic infection rate of 9% after examining 200 stool samples of children under 5 year.The high infection rate in the our study is due to several reasons, foremost among which are poor social and economic conditions, malnutrition, lack of attention to personal hygiene, deteriorating environmental conditions, ignorance and poor health culture.According to monthof study , high rate of infection was seen in September(30%) while the lowest in December(13.3%).This result agree with (Clavel*et al* .,1996) when recorded high prevalence rate of infection In the autumn. The results of the currently study identified that *Cryptosporidiosis* affects all age groups studied, with the highest incidence of parasitic infection among the age group<1year (26.31%), while the lowest percentage was among the age group (>12years)(8.33%),The results of currently study are agree with many former research (AL-Rikabi .,2012).but our result not agree with (AL-Hinidi*et al* .,2007) which indicated that the highest incidence was recorded within the age group (4-1) years. In this study, which included (100) stool samples (43 males and 57 females), the infection rate was 23.25% and 21.05% males and females severally with no significant differences at p<0.05, this agree with previous study (Lu *et al* .,2008).but our result not agree with (Adnan *et al* .,2007) which recorded a high infection rate in female (20.3%) than male (12.2%) with significant difference

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among them .Depended on Nested –PCR examination of human DNA sample,among (22) human samples positive by microscopic ,there is 13(59.09%) positive sample by N –PCR.the result of our study was close with result(11.4) detected in Tehran by (Meamar*et al* .,2007).the results of (12) DNA sequencing samples of human of the positively PCR products from different area in AL-Qadisiyah government were showed best results forward nucleotide sets as like sequencing of the 18s rRNA gene (~834). The phylogenetic tree and sequences analysis results of 18s rRNA gene of *C. parvum* showed that the,(MK886603.1 , MK886607.1, MK886611.1) and *C. parvum* formed (25)% from total infection in human stool sample , while *C. parvum* was prevalent species in 25% of stool sample from humanthis agree with(Sharma *et al* .,2013), The phylogenetic tree and sequences analysis results of 18s rRNA gene of *C. hominis* showed that the(MK886600.1 , MK886602.1 , MK886603.1 , MK886604.1 , MK886605.1, MK886606.1 , MK886608.1 MK886609.1 , MK886610.1) , Which *Cryptosporidium hominis* formed(75%) from total infection in human ,The result of present study agree with result of previous study conducted by (Sharma *et al* .,2013) ,when he recorded 75% of the isolates were *C.hominis* , also similar to the previous study in South India (Ajjampur*et al* .,2010),While the result of our study not agree with the result in Kuwait recorded by (Sulaiman*et al* .,2005)when he recorded 5% of human isolates were *Cryptosporidium hominis*.The current study concluded that phylogenetic tree and the homology sequences identity gives a clear differentiation of the types of *Cryptosporidium parasites* which can be isolated at high rates of human in AL- Diwaniyah province, which is likely to lead to an outbreak of Cryptosporidiosis in this province.

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