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Original Research Article

Microscopic and Molecular Diagnosis of *Cryptosporidium* spp. in Human in Babylon Province, Iraq

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Abstract: ***Background*:** Considering the importance of *Cryptosporidium* spp. to public health and the country's economic situation due to the threat of an outbreak and economic losses. ***Objectives*:** this study aimed to detect *Cryptosporidium* spp. in human in Babylon province using traditional and molecular techniques. ***Methods***: this study was conducted in Babylon province from September, 2023 to March, 2024.a total of 100 human stool samples were examined microscopically for detection of *Cryptosporidium* spp. from both diarrheal and non-diarrheal patients attend hospitals and public health centers laboratories. ***Results:*** by using microscopic examination. The rate of infection with *Cryptosporidium spp.* was (25/100), 25%. According to sex, the highest infection rate in humans was in males (17/59),*28.81%* compared to females (8/41) ,19,51%. was recorded in the rate of infection between age groups, the highest rate (13/28), *46.42%* observed in < 5years of human. Rural area revealed the highest rate of infection than urban area in human (16/44),*36.63%*. In molecular diagnosis, DNA extraction was performed for 50 stool samples human 28% (14/50) C hominis 10% (5/50) C parvum 18% (9/50) by nested PCR, in order to investigate the genotypes of *Cryptosporidium spp.* from human in Babylon, five *Cryptosporidium spp.* isolates. were genetically characterized by SSU-rDNA gene sequencing. The results showed *C parvum* (1/5) *C hominis* (4/5). ***Conclusion:*** these findings suggested that infection of humans by zoonotic genotypes were the most common genotypes detected in all human samples in Babylon province, to enhance the effectiveness of the One Health approach in preventing Cryptosporidium spp. infection, it is crucial to have a deeper understanding of the environmental, epidemiological, and etiological aspects linked to *Cryptosporidium* infection. This knowledge will aid in the development of more effective risk management strategies. The future One Health plan seeks to incorporate diverse and interdisciplinary expertise.

Keywords: *Cryptosporidium* spp., human, Microscopic, molecular diagnosis, zoonotic.

INTRODUCTION

Cryptosporidiosis, one of the neglected zoonotic diseases, is a disease whose transmission depends on interactions between humans and domestic animals or surrounding wildlife reservoirs [1]. Cryptosporidium spp. infected humans and a wide range of animals, producing Cryptosporidiosis [2]. Cryptosporidiosis of mammals is a major infectious diarrheal disease affecting young livestock and humans of all age groups. Species of the apicomplexan genus Cryptosporidium *parvum*, is an important zoonotic pathogen worldwide. Parasite reproduction occurs in intestinal epithelial cells (IECs) and culminates in production of oocysts that transmit infection via the fecal-oral route. *Cryptosporidium* is a small protozoan parasite that infects the microvillous region of epithelial cells in the digestive and respiratory tract of vertebrates [3]. Oocysts, which are the infectious form of Cryptosporidium, have a high resistance to the environment, particularly Cryptosporidium parvum. This species is extensively spread and can be transmitted to people by direct contact with animals, as well as through the contamination of water and food by oocysts. Transmission of diseases from animals to humans does not occur to the same degree in both natural habitats and controlled contexts, such as intensive and confined animal farms, where animals are either more isolated from humans or have been conditioned [4]. Cryptosporidiosis is a

condition that resolves on its own, affects just the intestines, and is quite resistant to reinfection in those with a healthy immune system. Cryptosporidium spp. can cause persistent or chronic diarrhea in patients with impaired cellular immune response, such as those with AIDS, malnourishment. In these individuals, the infection can also affect the bile ducts, which can be life-threatening and hinder the absorption of antivirals, leading to treatment failure in HIV [5]. The fact that zoonotic properties of Cryptosporidium have been detected in both human with livestock because of living in farm communities, there is still a need for efforts in public health education and intervention. Health-care providers and the public need to be aware of the multiple modes of transmission of Cryptosporidium to prevent sporadic Cryptosporidium infections in asymptomatic individuals [6]. The One Health Initiative was launched in November 2009 by the One Health Commission, a national nonprofit partnership of the National Institutes of Health (NIH), Centers for Disease Control and Prevention (CDC), Food and Drug Administration (FDA), Department of Agriculture (USDA), and other global health agencies, and the Institute for Laboratory Animal Research, to identify the linkages between human, animal, and ecosystem health and to quantify the potential value of a One Health approach at the national and global levels [7]. One Health Concept (one health, one medical science, and one world) has the goal of reducing the risk of high-impact diseases in the animal-human ecosystem [8]. This multidisciplinary approach takes into account the complexity of ecosystems where humans and animals coexist. To prevent disease transmission in humans, it is necessary to control and eliminate disease in animals. This is not only to protect public health but also to protect the health and improve animal welfare, maintain food security, and reduce poverty [9].

While there are other methods available to detect Cryptosporidium oocysts in feces, such as microscopic staining and surface antigen detection, these techniques are not enough to identify the species and genotype due to the identical appearance and antigens of the oocysts [10, 11]. Molecular methods like conventional PCR, Nested-PCR, and sequencing identify and characterize the parasite at species and subspecies levels using gp60 genes [12, 13]. The current study utilized the Nested-PCR technique to molecularly detect *Cryptosporidium* spp. in human stool by targeting the gp60 genes. Later on, the sequencing method was employed to analyze and describe the different species of *Cryptosporidium. Cryptosporidium* species are important on public health and has economic implications The country due to the risk of the outbreak of the disease and its associated conditions Economic losses. However, there are no previous epidemiological or molecular data regarding *Cryptosporidium* spp. Infection in humans in Babil Governorate.Therefore, this study aimed to detect and identify *Cryptosporidium* spp. Genotypes in humans in this region using Microscopic and molecular techniques.

MATERIALS AND METHODS

The current study utilized the Nested-PCR technique to molecularly detect Cryptosporidium spp. in human feces by targeting the gp60 genes. Later on, the sequencing method was employed to analyze and describe the different species of Cryptosporidium.

Ethical Approval

The researched was conducted in accordance with the Helsinki Declaration's ethical principles. Before taking the sample, Analytical and verbal consent from the patients was sought prior to sample collection. In order to secure this authorization, the study protocol, subject data, and consent form were assessed and authorized by the local ethics committee utilizing the document in (No.1152 in 18/7/2023).

Sample Collection

100 Stool diarrheal samples were collected from different ages to both gender from September, 2023 to March, 2024 and they were attending to the Babylon Teaching Hospital for Maternity and Children and Al Nour Children's Hospital in Babil Governorate.

Sample Procedure

Each stool sample was divided into 2 parts: 1st part has been screened using modified Ziehl–Neelsen- staining technique, and examined microscopically under 40 × ,100x magnifications for the detection of *Cryptosporidium* oocysts. The 2nd part was subjected to DNA analysis and PCR tests. were examined for the presence of Cryptosporidium oocysts using microscopy of the stool material that had been concentrated with the Sheather's sugar flotation technique Cryptosporidium-positive samples were stored in 2.5% potassium dichromate at 4°C. to used when need it.

Microscope Examination

All stool samples were examined microscopically stained by modified Ziehl- Neelsen method [14] The stool sample was spread on the slide. The slides left in the open air to dry for a while 10 minutes without using a flame, the dried smear was fixed with absolute methanol for 1minutes, Carbol-Fuchsine solution was added to the slide covering the whole smear for 15 minutes, the slide was washed gently with tap water using a dropper, after this, decolorizer by acid alcohol for 30 seconds and the slide was washed off with clean water again, the methylene blue was added for 2minutes and washed again, and left to dry, the smear was examined microscopically, using the 40x and 100x (oil immersion lens) objectives

and scanned thoroughly for parasite identification In this technique, the oocysts appear as pink to red, spherical to ovoid bodies on a blue or purple background [15].

The diameter of the parasite was measured using an ocular micrometer [16]. Oocyst measurement were measured using a Leica microscope equipped with a digital camera (ScopeImage 9.0, China). The camera software was calibrated for all microscope lenses using a 0.01 mm stage micrometer (ESM-11/Japan).

Molecular Study

DNA Extraction

Following the manufacturer's instructions, DNA was extracted directly from 50 stool samples from humans, including microscopically positive samples, for confirmation using (EasyPure®Stool Genomic DNA Kit, Transgen company, China).

Amplification of PCR

Table 1: The optimum condition of detection first reaction

No.	Phase	Tm (°C)	Time	No. of cycle
1-	Initial Denaturation	94°C	5 min	1 cycle
2-	Denaturation -2	94°C	45 Sec	35 cycle
3-	Annealing	55°C	1 min	
4-	Extension-1	72°C	1 min	
5-	Extension -2	72°C	7 min.	1 cycle

Table 2: The optimum condition of detection second reaction

No.	Phase	Tm (°C)	Time	No. of cycle
1-	Initial Denaturation	94°C	5 min	1 cycle
2-	Denaturation -2	94°C	45 Sec	35 cycle
3-	Annealing	58°C	45 Sec	
4-	Extension-1	72°C	1 min	
5-	Extension -2	72°C	7 min.	1 cycle

The Primers Preparation

The primers were lyophilized, they dissolved in the free ddH2O to give a final concentration of 100 pmol/µl as stock solution and keep a stock at -20 to prepare 10 pmol/µl concentration as work primer suspended, 10 µl of the stock solution in 90 µl of the free ddH2O water to reach a final volume 100 µl.

Primers used in the study

Nested PCR

Table 3: The sequence of primer that used for detecting *cryptosporidium* spp.

Primer	Sequence	Primer sequence '3- - '5	Tm (°C)	GC%	Size of Product (bp)	Source
first reaction GP60 *Cryptosporidium* spp.						
First reaction GP60	F	ATAGTCTCCGCTGTATTC	51.7	44	bp921	
	R	GGAAGGAACGATGTATCT	53.1	44		

Table 4: The sequence of primer of nested PCR that used for detecting *C. parvum*

Primer	Sequence	Primer sequence '3- - '5	Tm (°C)	GC%	Size of Product (bp)	Source
First reaction GP60 *Cryptosporidium* spp.						
First reaction GP60	F	ATAGTCTCCGCTGTATTC	51.7	44	bp921	
	R	GGAAGGAACGATGTATCT	53.1	44		
Second reaction GP60 *Cryptosporidium parvum*						
Second reaction GP60	F	TCCGCTGTATTCTCAGCC	59.1	56	887bp	
	R	GCAGAGGAACCAGCATC	58.4	59		

DNA Sequencing

DNA sequencing was conducted to identify *Cryptosporidium* species, and five nPCR-positive local isolate products were sent to Macrogene Company in Korea to identify *Cryptosporidium* spp. A homology search was conducted using the Basic Local Alignment Search Tool available at the National Center for Biotechnology Information (NCBI, http://www.ncbi. nlm.nih.gov) and the BioEdit program, version 7.2. The results were compared with data obtained from

the GenBank and the ExPASY program (SIB Swiss Institute of Bioinformatics, Switzerland) available on the NCBI website [17].

Statistical Analysis

The data was tabulated in a datasheet of IBM SPSS version 26.0, which was utilized to do the statistical analysis. The significant differences were tested using the analysis of the person chi-square test. Statistical significance was defined as a probability value ($p \leq 0.05$) [18].

Phylogenetic Tree

To compute evolutionary distances, a phylogenetic tree was constructed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method [19, 20]. The optimal tree with the sum of branch length = 0.00795808 is shown of C. parvum. The optimal tree with the sum of branch length = 11.90711807 is shown of Cryptosporidium Canis [21, 22].

RESULTS

Microscopic Examination

The microscopy is one of the most widely used methods for the detection of Cryptosporidium oocysts in stool Specimen in human. The observations appeared that 25 out of 100 (25%) stool Specimen were found to be positive for the diarrhea causing protozoan (Cryptosporidium species). The microscopic examination results demonstrated that Cryptosporidium spp. oocyst appeared as a pink to red round bodies against blue background when stained with the modified ziehl-neelsen acid fast staining. The average size of these oocysts were (±4µm × ±5.2 µm) as shown in the Figure 1.

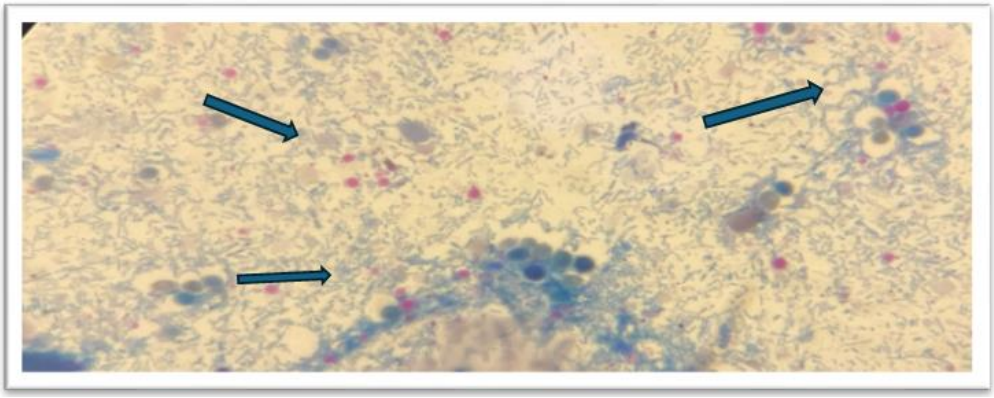

Figure-1: Morphology of Cryptosporidium spp.40x. by using Sheather's sugar solution and modified Ziehl-Neelsen stain

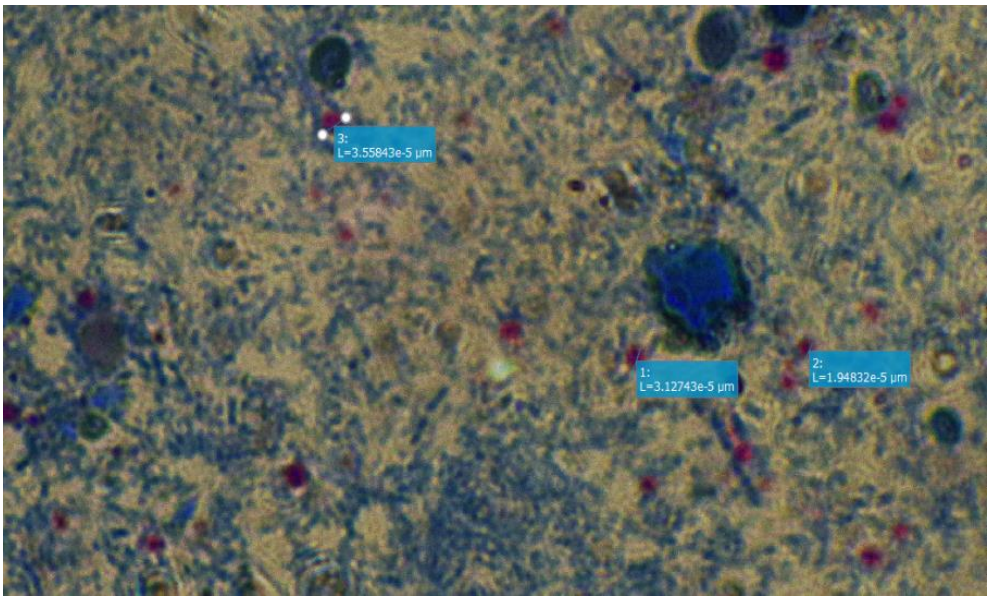

Figure-2: Morphology of Cryptosporidium spp. :100x. stain with oil immersion and using a digital camera by using Sheather's sugar solution and modified Ziehl-Neelsen

According to sex, non- Significant differences (p>0.05) was recorded in the infection rate the in humans was 17/59 (28.81%) in males while 8/41 (19.51 %) in female as shown in the figure (3), The highest infection rates were observed in humans under five years of age, with 13/28 (46.42 %) and (26-45) was 2/20 (10%) as illustrated non-Significant differences (p>0.05) was recorded in the rate of infection of in the figure (4). non- Significant differences (p>0.05) was recorded in the rate of infection of Rural areas had a higher rate of infection in human, where it was 16/44 (36.36 %) compared with urban areas 9/56 (16.07 %) Figure (5).

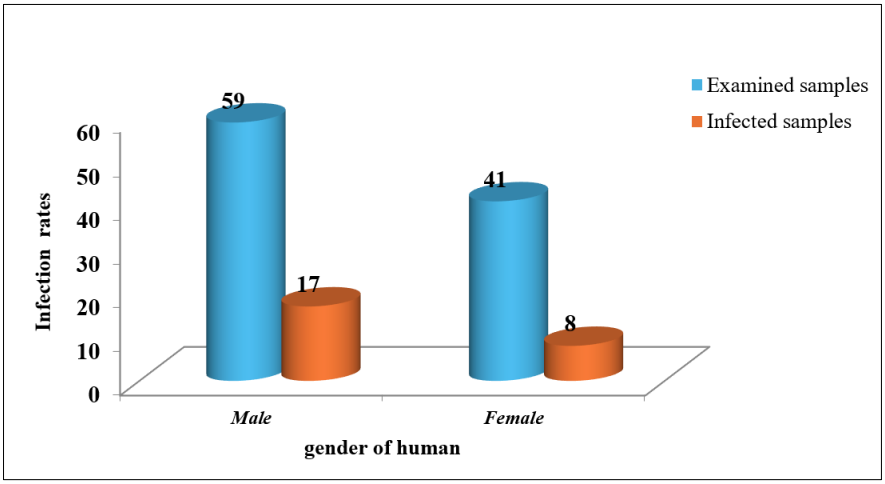

Figure-3: Infection rate of *Cryptosporidium* spp. in human according to the gender

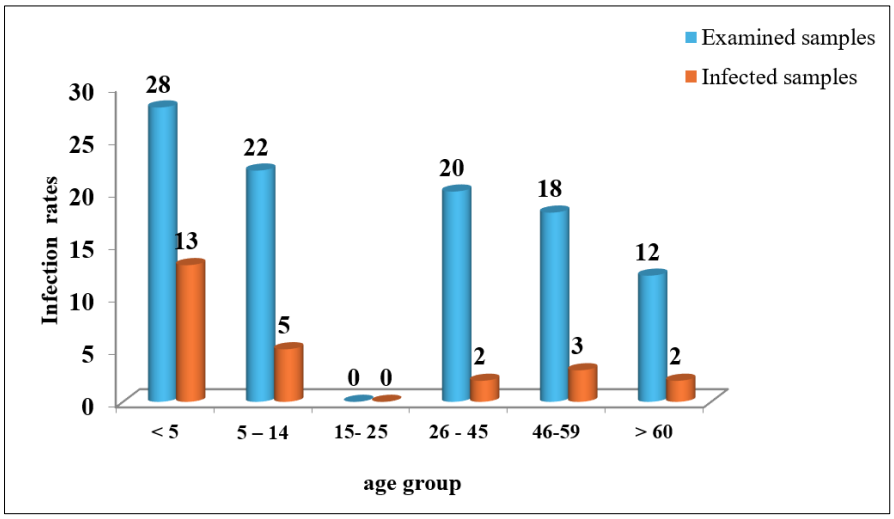

Figure-4: Infection rate of *Cryptosporidium* spp. in human according to the age group

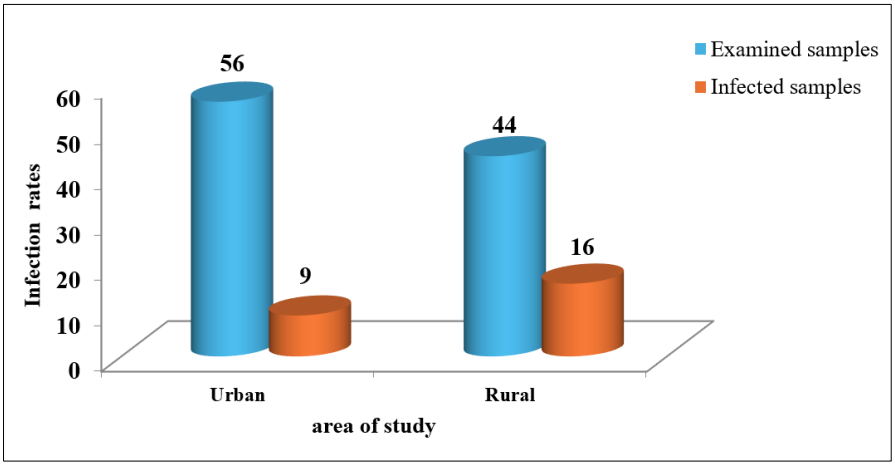

Figure-5: Infection rate of *Cryptosporidium* spp. in human according to the area of study

Molecular Diagnosis:

The molecular diagnosis results showed that the nested PCR amplification of the GP60 gene was positive for 28% (14/50) 10% (5/50) of *Cryptosporidium hominis* and 14% (9/50) of *cryptosporidium parvum* (Figures 6 to 8).

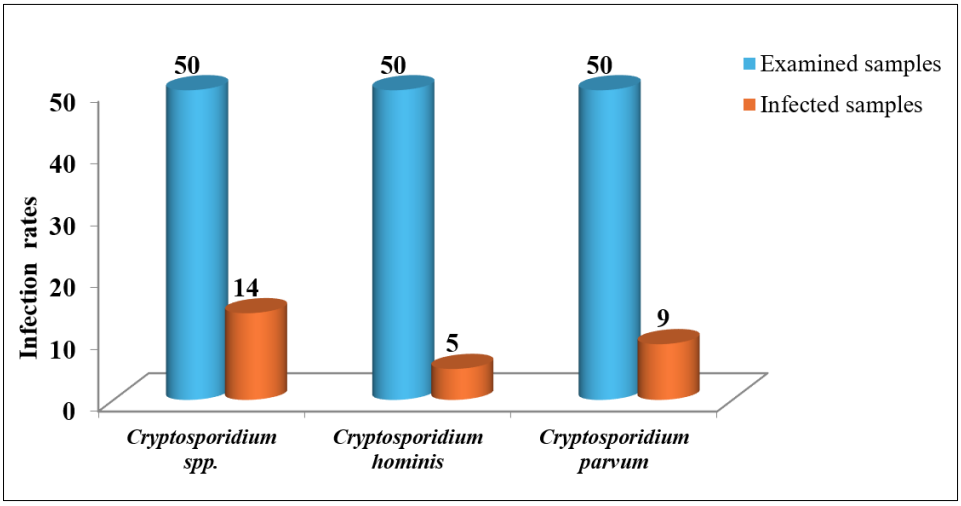

Figure 6: Infections rates of Cryptosporidium species based on the nested PCR

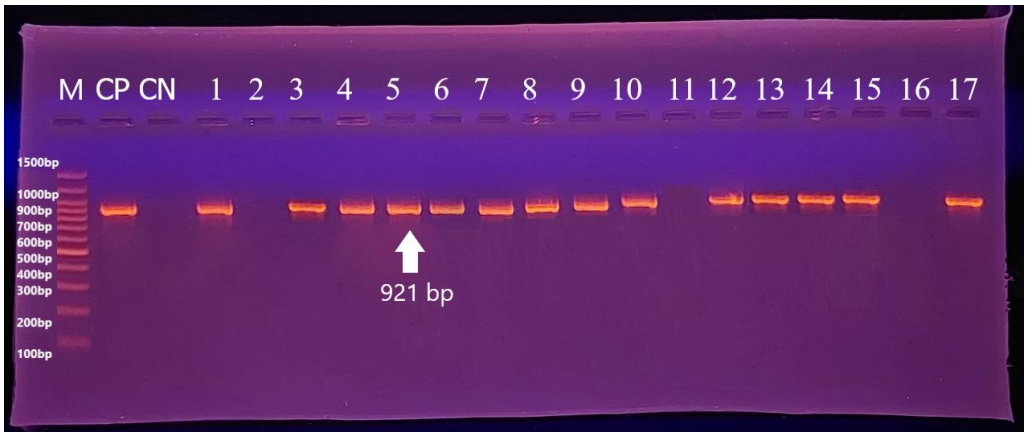

Figure 7: Agarose gel electrophoresis image shows nested PCR product for *Cryptosporidium* spp. gene GP60 921bp in human stool Specimen. Bands were fractionated by electrophoresis on a 1.5% agarose gel, and visualized under U.V. light after staining with red stain. Lane: M (M: 100bp-1500bp ladder). (M=marker) (CP= control positive) (CN= control negative)

Figure 8: Agarose gel electrophoresis image shows nested PCR product for *Cryptosporidium parvum*, GP60 gene 887bp in human stool Specimen. Bands were fractionated by electrophoresis on a 1.5% agarose gel, and visualized under U.V. light after staining with red stain. Lane: M (M: 100bp-1500bp ladder). (M=marker) (CP= control positive) (CN= control negative)

DNA Sequence Analysis of Cryptosporidium spp.

To investigate *Cryptosporidium hominis* and *Cryptosporidium parvum* in human stool Specimen in Babylon province, five isolates were genetically characterized using GP60 gene sequencing. In humans, sequencing revealed specific *C. hominis* (4/5, 80 %) and *C. parvum* (1/5, 20%), zoonotic species. *cryptosporidium* spp. isolates were submitted to the NCBI GenBank database, and GenBank accession numbers were obtained for *Cryptosporidium hominis* (4) and *Cryptosporidium parvum* (1) isolates: *C. parvum* that have been deposited in the gene bank under the accession numbers (ID: AF440621.1). and identified *C. hominis* gene (GP60) that have been deposited in the gene bank under the accession numbers (ID: MK391439.1; ID: MK391439.1; ID: MK391439.1; ID: MK391439.1) as shown in the table (6).

Table 6: DNA sequence analysis of cryptosporidium spp.

Gene: 60 kDa glycoprotein (gp60) gene							
No. of sample	**Type of substitution**	**Location**	**Nucleotide**	**Source**	**Host**	**Sequence ID with compare**	**Identities**
1	Transvertion	399	G\C	Cryptosporidium parvum	Human	ID: AF440621.1	98%
	Transvertion	400	A\C				
	Transvertion	436	A\C				
	Transition	460	G\A				
	Transition	509	T\C				
	Transition	523	A\G				
	Transition	524	A\G				
	Transition	646	A\G				
	Transition	838	C\T				
	Transvertion	842	T\A				
	Transition	846	C\T				
2	------	-----	----	Cryptosporidium hominis	Human	ID: MK391439.1	100%
3	Transition	538	A\G	Cryptosporidium hominis	Human	ID: MK391439.1	99%
4	------	-----	----	Cryptosporidium hominis	Human	ID: MK391439.1	100%
5	Transition	491	T\C	Cryptosporidium hominis	Human	ID: MK391439.1	99%
	Transvertion	496	G\T				

Phylogenetic Tree

Phylogenetic Tree Cryptosporidium Parvum

The evolutionary history was inferred using the UPGMA method [21]. The optimal tree with the sum of branch length = 0.01637916 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method [19] and are in the units of the number of base substitutions per site. The analysis involved 12 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 555 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 [20].

Phylogenetic tree Cryptosporidium

The evolutionary history was inferred using the UPGMA method [21]. The optimal tree with the sum of branch length = 0.00509168 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method [19] and are in the units of the number of base substitutions per site. The analysis involved 19 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 720 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 [20].

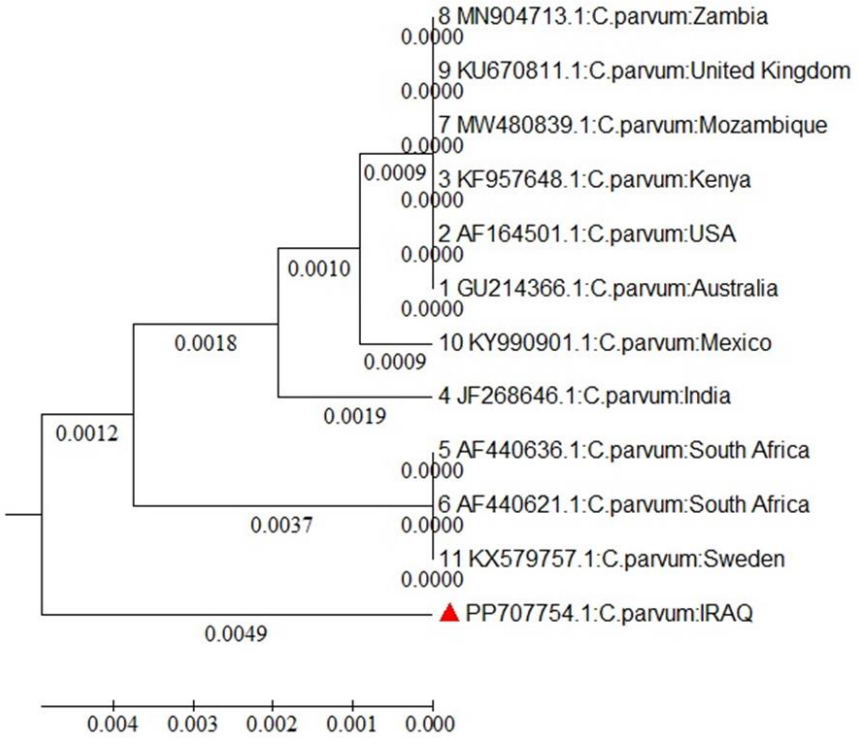

Figure 8: Phylogenetic tree analysis based on the partial sequence of gp60 gene explains identity between the local isolates of Cryptosporidium parvum in humans and NCBI-BLAST isolates

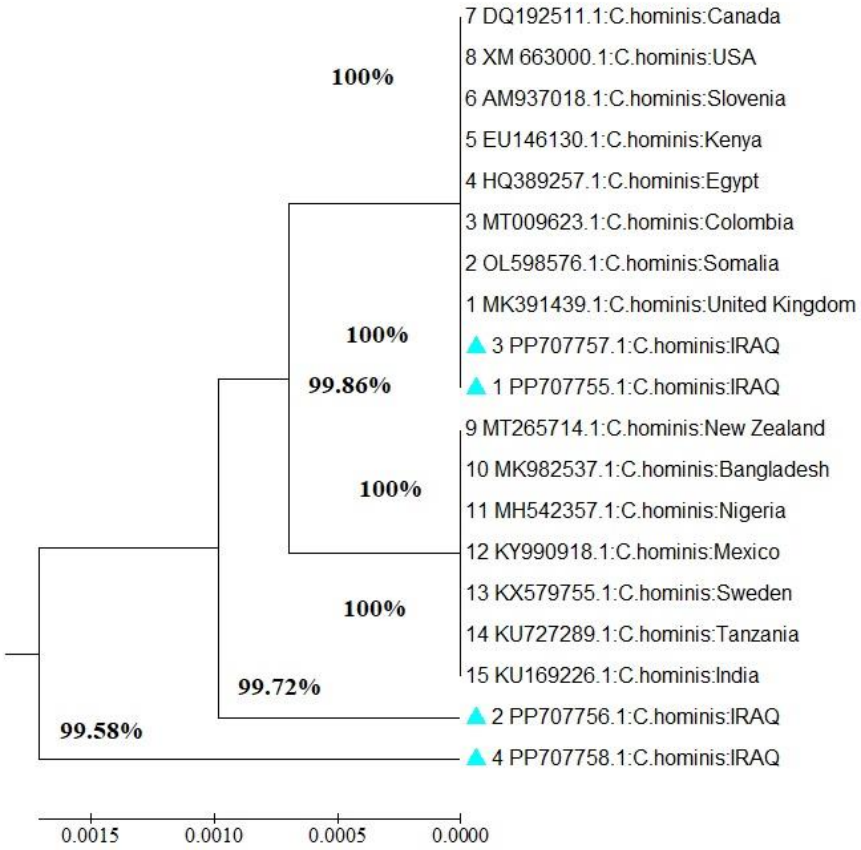

Figure 9: Phylogenetic tree analysis based on the partial sequence of gp60 gene explains identity between the local isolates of *Cryptosporidium hominis* in humans and NCBI-BLAST isolates

DISCUSSION

In this study, microscopic and molecular diagnosis was applied to identify *Cryptosporidium* spp.from stool Specimen of human in Babylon province. the frequency of cryptosporidiosis in humans in the present study is equal to previous studies conducted in Iraq AL- Diwaniyah city done by Al-Difaie [23] who recorded infections rate 29.29% and agreement with result of Al-yasary [24] who found that positive results was (26%) in Karbala similarly Khan [25] in Pakistan, who reported rate of 29.88%. However, this study disagree with other studies in Iraq, in which the higher rate of infection recorded by Raad, [26] in Al-Najaf AL-Ashraf and in Baghdad Province by Merdaw [27] recorded the total percentage of the positive result were 58% and 47.33%, respectively. The variation in the incidence of Cryptosporidium is related to many factors such as the differences in the population of the study, Age, sexuality, diarrheic and non-diarrheic circumstances, individual cleanliness, sewage water management, consuming contaminated foods untreated water, and metrological circumstances. The season, and poor economic status, are all factors that play a role in the current high prevalence of cryptosporidiosis [23, 25].

The result of current study agreed with another previous study in Iraq [28] in Basra in which it was found the higher infection rate 24.2% in male and 23.5% in females. The present results disagreed with [29] in Babylon in which it was found that the rate of cryptosporidiosis in males (7.55%) did not vary significantly from females(9.75%) Relationship between gender and infection with Cryptosporidium spp. was recorded by [26] in Al-Najaf AL -Ashraf where high prevalence rate in male than female was recorded (55.2%) and (44.8%) respectively. The differences in sex in our study explained possibly by that the infection was more in males than females could be due playing of male children in the gardens and farms outdoor area with soil and animals, which can increase the risk of parasite transmission and that agreed with [23, 30].

Infection rate of Cryptosporidium spp. Showed non-significant relation among age groups of patients The result of present study showed an agreement with previous studies in Iraq in Karbala done by Al-yasary [24] the maximum infection rate showed in age group 2-6 years 44%, while the minimum rate was among age group 18-25 years 12%. in Basra [28] which it was found higher infection rate in children among age group lower than one year 28.0% and the lowest infection rate was in age group among one to five years 21.6 % but in age group five to fifteen years 25.0% mentioned that in Egypt [31] referred to recording high infection rate in children lower than two years old which was 44.4% and lower prevalence of age group six to twelve years old 27% .the higher infection in children occurs due to their immune system functions which were undeveloped so intake small number of oocysts may result in cryptosporidiosis and repeated low dose infections may stimulate the immunity to Cryptosporidium which may protect children tend to have relatively more symptomatic disease than older agree with [32, 33].

Rural area revealed the highest rate of infection than urban area. agreement with studies in Australia The lowest rate of notifications was in the major cities (7.7 per 100 000 population), followed by and remote areas (17.1 per 100 000 population) [34]. High infection rates in rural areas may due to several factors including; lack of clean drinking water and dependence on river water as a direct source of water, dealing with the contaminated soils of gardens and farms with parasite cysts, breeding and contact with animals that are reservoir of the parasite, using animal waste as organic fertilizer, and the low health and cultural levels of the rural population [35].

Based on the phylogenetic tree, the Cryptosporidium spp. isolates in this study showed similarity to sequences stored in GenBank, with the highest similarity of gene sequences between the Iraqi Cryptosporidium spp. strains and strains from around the world being 99%–100% for human. The phylogenetic study results verified the presence of only slight disparities between the Iraqi strains of Cryptosporidium spp. and those originating from other countries. The genetic variance seen could be attributed to disparities in the size of the reference sequence and the geographical locations from which the isolates were obtained. Multiple techniques were employed for the genetic study, such as nPCR-based gene sequencing of either partial or entire genes. The findings of this study are consistent with previous research that has documented the presence of genetic diversity and evolutionary connections across populations of Cryptosporidium spp. across the globe. These isolates are believed to be capable of being transmitted between animals and humans, either through direct or indirect contact. The conclusions we have drawn are corroborated by prior research [24, 36, 37].

CONCLUSION

Due to the nature of human practices in the region and their close associations with animals which are regarded as a sustainable source of food, measures to control the zoonotic transmission of *Cryptosporidium* spp. is problematic. Therefore, in order to reduce the environmental contamination and to protect human and animal health in the region, improved disease prevention and control strategies in livestock need to be implemented including better hygiene and disinfection and fencing of livestock away from water sources. The success of these “One Health” initiatives requires better communication and collaboration among doctors, veterinarians, and water utilizes across.

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