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Subtypes of Cryptosporidium parvum in human in Al-Diwaniyah province, Iraq

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***Abstract*---**The current study was designed to diagnose the species of Cryptosporidium parasites in humans and to identify the subtypes of C .parvum by using the Hsp70 gene. In Al-Diwaniyah province it was conducted during the period from September 2021 to the end of February 2022. 100 stool samples were taken from individuals who differed in sex, age and place of residence. After extracting the genomic DNA of the parasite, the results of the PCR test showed that the infection rate of the parasite reached 30%. Then, N-PCR was performed on the same samples in which Cryptosporidium DNA was identified, targeting the 18SrRNA gene. Two species of Cryptosporidium identified and recorded in the NCBI-Genbank database are C.parvum:6/10 , C.hominis 4/10.While the result of Subtyping of C.parvum by using Hsp70 gene was Subtype_1 40% (2/5)and Subtype_2 60%(3/5). The identification and characterization of Cryptosporidium spp in humans was very important to avoid infection for people and healers and to implement control programs.

***Keywords*---**cryptosporidium parvum, nested-PCR, sequences, human.

Introduction

Cryptosporidiosis is a parasitic ailment caused by the *Cryptosporidium* genus, which infects fish, amphibians, reptiles, birds, and mammals (1,2). At this time, there are at least 44 species of *Cryptosporidium* and over 120 genotypes, with *C.*

hominis and *C. parvum* being the most common species infecting people (3). Cryptosporidiosis is spread mostly through the fecal-oral route, primarily through oocyte contaminated food or water, contact with sick animals, or by accident in laboratories (4,5). *Cryptosporidium* spp. can infect humans and animals who have frequent contact with workers or animals (6,7) Cryptosporidiosis causes watery diarrhea, which can be severe and last for days or weeks (8). Cryptosporidiosis is dangerous because it is resistant to numerous medications, sterilizers, and disinfectants. DNA analysis, genetic engineering, Immunofluricent Staining Technique Monoclonal Antibody, and Polymerase Chain Reaction PCR have all been used to develop parasite identification procedures (9). Genotypes can be detected and identified using PCR-based molecular diagnostic procedures. More exact definitions of host-adapted specificity, transmission mechanisms, and zoonotic potential of *Cryptosporidium* spp. can be found using molecular methods (10). Based on 18S rRNA and gp60 gene subclassification, molecular approaches including as PCR, nested PCR, and DNA sequencing were utilized to detect these parasites at the species and subtype level in this study. by employing Nested-PCR for molecular detection of *Cryptosporidium* species in cat feces and targeting 18S ribosomal RNA Following that, DNA sequencing was utilized to identify the various *Cryptosporidium* genotypes.

Materials and Methods

Parasitic samples collection

Human stool samples (n = 100) were collected at random from different health centers within Al-Diwaniyah Municipality and the Women's and Children's Hospital from September 2021 to February 2022. Stool samples were collected in clean plastic containers with a number, age, gender, area, and collection date labeled on them. The samples were subsequently transferred to the Veterinary Parasitology Laboratory in chilled bags for examination. The samples were split in half, with one kept cold at 4 °C for in vitro experiments and the other frozen at -20 °C for DNA extraction.

DNA Extraction and Molecular Analysis

- **Whole Genomic DNA Extraction**. ***Cryptosporidium* sp**.
  The Geneaid DNA Stool Kit (Geneaid, Korea) was used to extract and purify genomic DNA (gDNA) from 250 mg of each human fecal sample according to the protocol manufacturer's recommendations. The purified gDNA was then kept at 20 degrees Celsius for subsequent molecular analysis.
- **Molecular detection of *Cryprosporidium* spp.**
  The primary PCR and nested-PCR were employed to detect *Cryptosporidium* spp. DNA by targeting the srDNA (18 small-subunit rRNA gene), with two sets of outer primers (primary PCR) and inner primers (Nested-PCR), respectively, and both primer sets were modified from (11). The initial outer primers CF201 (5'-GGGTTGTATTTATTAGATAAA GAAC-3') and CR201 (5'-CTTTAAGCACTCTAATTTTCTC-3') were genus-specific, yielding 540 bp of primary PCR result for *Cryptosporidium* spp. The second inner Nested-PCR primers CPF202 (5'-GACTITTTGGTTTTGTAATTGGAATG-3') and CPR202 (5'-

TAAATTATTAACAGAAATCCAACTACGAGC-3') were species-specific, yielding a PCR result of 165 bp for *C. parvum*.
The initial outer primers (CF201 and CR201) were used in all primary PCR reactions, which were carried out in 20 l volumes containing 10 l Hot-start Taq Master Mix (2X, Promega), 0.2 M of each primer (10X), 6 l nuclease-free water, and 30 ng/L of gDNA. An initialization cycle at 95°C for 5 minutes was followed by 35 cycles at 95°C for 35 s, 56°C for 35 s, and 72°C for 40 s, followed by a final extension at 72°C for 5 minutes.
The second cycle was carried out in the same way as the first, but with Nested-PCR primers (CPF202 and CPR202) and 5 liters of the primary PCR yield as the template for the Nested-PCR. After testing PCR results on a 1.5% agarose gel,

- **PCR for *Cryptosporidium parvum* subtypes:**
A PCR protocol was used to amplify HSP70 gene fragments from five *C.parvum* isolates from human feces, using primers complementary to the conserved nucleotide sequences in *C.parvum*. The primer was HSP70-F(AGCAATCCTCTGCCGTACAGG), HSP70-R(AAGAGCATCCTTGATCTTCT) yielding a PCR result of 587 bp for suptype C. parvum (12). PCR reaction Mix and Thermal cycling condition for *C.parvum* subtypes was master mix 10µl, Forward primers1 (10pmol) 1µl, Reverse primers1 (10pmol) 1µl, DNA template 2 µl, PCR water 6µl.

Amplicon sequencing and analysis:

10 positive PCR samples were delivered in an ice bag to Macrogen Corporation in Korea for DNA sequencing utilizing the Sanger sequencing technology and species-specific primers (CF201 and CR201). The sequences were sent to NCBI-GenBank for Genbank accession numbers after they were obtained. Then, using Molecular Evolutionary Genetic Analysis (Mega x) version 10, execute DNA sequence analysis (phylogenetic tree analysis) and multiple sequence alignment analysis using Clustal W alignment analysis.

Results

Nested-PCR optimization

We did a gradient-PCR on 8 samples of cryptosporidium spp. for N-PCR to find the best annealing temperature for 18srRNA gene , and discovered that 56 °C is the optimum annealing temperature, as shown in Fig (1).

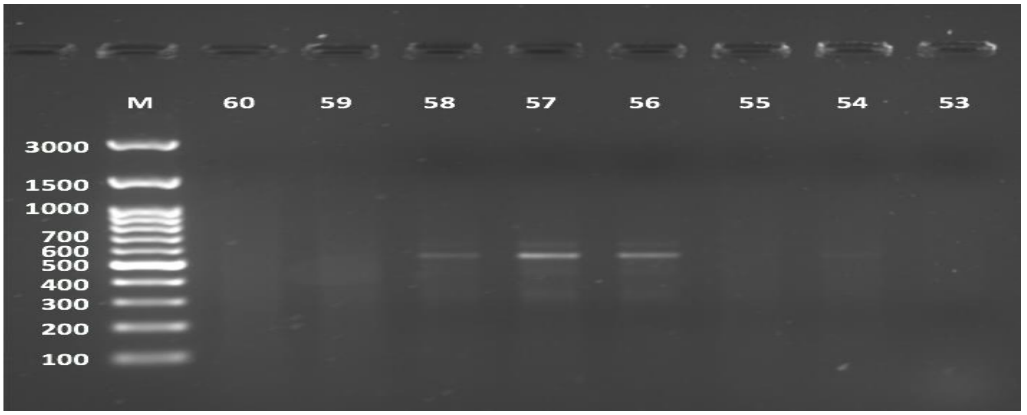

Figure 1. Agarose gel electrophoresis image (1.5 %) shows the gradient PCR protocol for *Cryptosporidium* spp. (18srRNA gene). This indicates the optimal annealing temperature which was 56 C. M is molecular marker from Genedirex

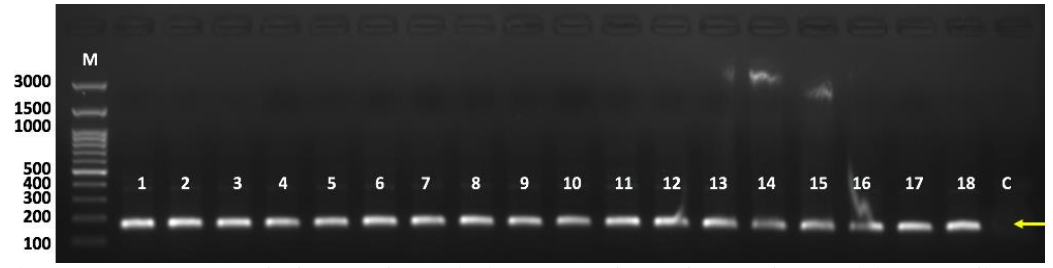

Figure 2. Agarose gel electrophoresis image (1.5 %) shows the positive amplicons of *Cryptosporidium parvum* in human. C is control negative in which similar reaction components were added except H2O was added instead of DNA. M is molecular marker from Genedirex

Cryptosporidium spp. Genomic DNA was determined in 30 out of 100 fecal samples collected from different subjects using Nested-PCR technology targeting the 18S rRNA gene. Of these, there are 10 samples of *Cryptosporidium* sp. (PCR-positive samples) were sent for Sanger DNA sequencing and thereafter, phylogenetic analysis was performed with reference strains of *C. parvum* and *C.hominis*. for the 18SrRNA gene. The sequencing results confirmed the existence of two species of *Cryptosporidium*, *C. parvum* and *C.hominis*. based on the sequencing results, *C. parvum* showed 100% similarity compared to reference *C. parvum* isolates from China, Japan and the India deposited with GenBank has (Table 1 and Figure 3). However, *C. hominis* showed lower similarity when compared to reference strains and ranged from 99.57% to 99.13%. In this study *C. parvum* was the most prevalent species in 60% (6/10), followed by *C.hominis* 40% (4/10), (Table 1 and Figure 3). They were deposited in GenBank under accession numbers (ON156750to ON156759), respectively and compared with other different world strains as shown in Table 1 and shown in Fig 3. Subtyping of five human samples positive of *C. parvum* was performed using PCR technique, followed by DNA sequencing by use of HSP70 gene. The sequencing results obtained from human samples, was Subtype_1 Where identity with global isolates from china and Sweden 99.82 and 99.45% respectively and subtype_2 with identity 100% with global isolates from UK and china .These were deposited

in the GenBank under accession numbers(ON470443 to ON470447) as in table (2)

Table 1
The NCBI-BLAST Homology Sequence identity (%) between local *Cryptosporidium* sp of human isolates and NCBI-BLAST deposited strains

Sample	Obtained Accession number	NCBI-BLAST Homology Sequence identity (%)			
		Identical to	Genbank Accession number	Country	Identity (%)
1	ON156750	*C. hominis*	JQ313988	Canada	99.57
2	ON156751	*C. hominis*	JQ313988	Canada	99.78
3	ON156752	*C. hominis*	JQ313988	Canada	99.78
4	ON156753	*C. hominis*	JQ313988	Canada	99.13
5	ON156754	*C. parvum*	MT648442	China	100
6	ON156755	*C. parvum*	MT648441	China	100
7	ON156756	*C. parvum*	MT002720	China	100
8	ON156757	*C. parvum*	MT043865	India	100
9	ON156758	*C. parvum*	MN918253	Japan	100
10	ON156759	*C. parvum*	MN918255	Japan	100

Table 2
The NCBI-BLAST Homology Sequence identity (%) between local subtypes of *Cryptosporidium parvum* of human isolates and NCBI-BLAST global deposited strains

Sequence number	Obtained accession number	Tested Host	Identical to	Subtype	Genbank Accession number	Country	Identity (%)
1	ON470443	Human	*Cryptosporidium parvum*	Subtype_2	KF577768	UK	100
2	ON470444	Human	*Cryptosporidium parvum*	Subtype_2	KC823128	China	100
3	ON470445	Human	*Cryptosporidium parvum*	Subtype_2	KC823128	China	100
4	ON470446	Human	*Cryptosporidium parvum*	Subtype_1	KC885897	China	99.82
5	ON470447	Human	*Cryptosporidium parvum*	Subtype_1	KU892574	Sweden	99.45

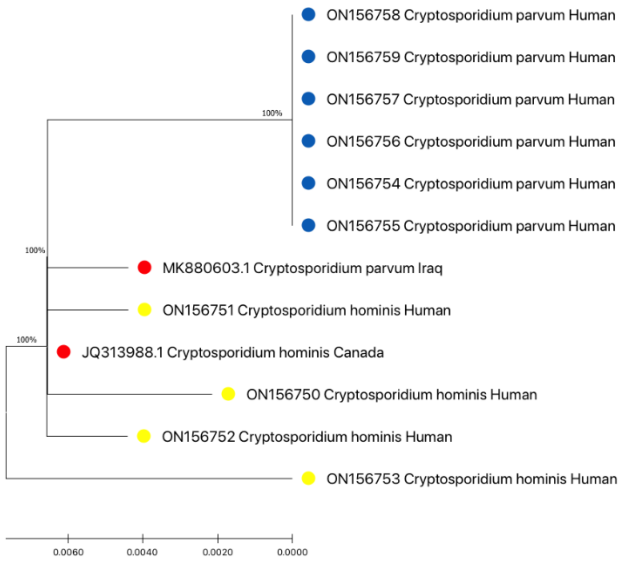

Figure 3. Phylogenetic tree analysis of the identified *Cryptosporidium* sp. targeting 18rRNA gene in human isolates. These have been deposited in the gene bank with the accession numbers followed by the identified *Cryptosporidium* spp. and the host from which were identified (Blue circles referred to *C. parvum* while yellow circles referred to *C. hominis*) while red circle referred to global isolates

Discussion

The majority of publications on cryptosporidiosis in humans and animals in Iraq were published in local journals that were not available to international scientists; however, a few findings were included in a review on cryptosporidiosis in Saudi Arabia and surrounding countries, including Iraq (13). The use of nPCR in this investigation revealed that 30% of 100 human samples tested positive for cryptosporidiosis. This research contradicts the findings of studies (14) in Egypt,(15) in Ethiopia, and(16) in Western Uganda, which found that 11.9% of Egyptian youngsters were afflicted with cryptosporidiosis. By based PCR, the prevalence of Cryptosporidium was higher in Ethiopia and Western Uganda than in this study, at 46% (86/187) and 32.4%, respectively. Other investigations, such as Study (17,18,19), reported a lower infection rate than the current study the percentages were (25%), (25%), and (23.6%), respectively. In this study, two genotypes, *C. hominis* and *C. parvum*, were detected in human stool, the incidence of *C.parvum* was 60% and *C.hominis* 40%, and the results of this study differed from the rest of the studies conducted in different regions of Iraq. Previous Iraqi studies reported a prevalence of 72.9% and 24.3% human samples in Mid-Euphrates Area(20) and(21) recorded the rate of infection 47.33% in human (46% *C.parvum* and 1.33% *C.hominis*).

While Salman found *C. parvum* in a percentage 16.28 in Kirkuk(22). Some Iranian investigations have confirmed these findings, demonstrating that *C.parvum* and *C.hominis* are the most common species in humans and that *C.parvum* is the

dominant species(23). *C.parvum* was shown to have a considerable prevalence in two investigations in Iran (24,25)and In Jordan, 50% of 44 isolates were *C.parvum* and 45% were *C.hominis*, according to a recent study (26), The preponderance of *C.parvum* in a population and the low number of *C.hominis* infections have been attributed to zoonotic transmission (27). The sequencing results for subtyping *C.parvum* obtained from human samples, was Subtype_1 Where the results showed identity with global isolates from china and Sweden 99.82% and 99.45% respectively, and subtype_2 with identity 100% with global isolates from UK and china .These were deposited in the GenBank with accession numbers from(ON470443 to ON470447) . We compared with many studies that used Hsp70 gene for the purpose of diagnosing and studying subspecies in humans and different animals. In a study conducted on the population of calves in the UK resulted On sequencing analysis, (four isolates) were identified as *C.parvum* subtype 2 which has 100% similarity to the isolate published with accession number KC823128 (28).

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Conflicts of interest

The authors declare that have been no conflicts of interest are associated with this work.

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