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Genotyping of *Cryptosporidium parvum* in Immunocompromised Patients Using GP60 Analysis in Wasit, Iraq

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Abstract

The protozoan *Cryptosporidium parvum* causes cryptosporidiosis, an opportunistic parasite infection that can cause severe, sometimes fatal diarrhea in immunocompromised people. It is essential to comprehend the genetic diversity of *C. parvum* circulating in these high-risk communities in order to guide focused prevention and control initiatives. The genetic diversity of *C. parvum* isolates infecting immunocompromised individuals in Wasit governorate was examined in this cross-sectional investigation . 50 samples collected from Al-Karama hospital and health centers, 20 (40.0%) of the fifty suspected cases of cryptosporidiosis had *Cryptosporidium* Oocysts in their stool samples, which were examined under a microscope using modified Ziehl-Neelsen staining. Following molecular detection, which yielded 35 (70.0%) results, 10 samples were used for nested PCR that targeted the *C. parvum* glycoprotein 60 (gp60) gene. Sequence analysis and phylogenetic reconstruction using the Neighbor-Joining method revealed the presence of multiple *C. parvum* genotypes .The *Cryptosporidium parvum* gp60-IId.allele (KX397563.1) was linked to the human isolates IQH.1, IQH.3, IQH.4, IQH.5, and IQH.8. *Cryptosporidium parvum* gp60-IIc.allele (FJ839876.1) was linked to the human isolates IQH.2, IQH.6, IQH.9, and IQH.10. The *Cryptosporidium parvum* gp60-IIa allele (DQ192508.1) was linked to the *Cryptosporidium parvum* human isolate IQH.7. Sequence similarity between the detected genotypes and reference isolates in the GenBank database ranged from 98.65% to 100%.

Key Words: *C. parvum,*Sequence analysis , glycoprotein 60 (gp60) gene , immunocompromised

1.Introduction

Among the several species identified in the genus *Cryptosporidium*, The most significant apicomplexan parasite is *Cryptosporidium parvum*. in terms of zoonotic potential by far, *Cryptosporidium spp.* is widespread throughout the world, including in underdeveloped

countries, rural areas of developed countries or urban centers [1]. It is a worldwide cause of diarrheal illness in juvenile ruminants and humans[2]. Due to the fact that there are both direct (from animal to person and from individual to individual) and Indirectly (by consuming tainted food or drink) modes of transmission, the epidemiology of the parasite is complicated [3]. *Cryptosporidium* is found all over the world and can infect a wide variety of animal species. One major public health problem is The spread of diseases by zoonotic means from animals to humans [1].One of the main reasons for diarrhea in both immunocompromised and non-immunocompromised people is the *cryptosporidium species* [4]. In more than 40 countries worldwide, this prevalence has been found at rates ranging from 0.7 to 20%. It has been noted that this prevalence is greater in undeveloped or emerging countries, where social and personal cleaning practices are not fully implemented and sanitation measures are not take [5]. Since the oocyst, the infectious stage of *Cryptosporidium* becomes contagious Soon after it is ejected together with the host's stool, direct transmission is preferred [1]. Oocysts, which are excreted by infected hosts and can linger in the surroundings and stay contagious for a long time are the initial cause of infection. The two methods used for *Cryptosporidium* typing are sequencing one or additional gene markers or locus fragment typing has a variable tandem repeat count (VNTR).It has recently been demonstrated that a method depend on seven VNTR loci is reliable, instructive, and cost-effective for type *C. parvum* samples [6].

2.Materials and Methods

2.1. The study's design and sample collecting

The current a cross-sectional investigation was conducted in the Iraqi province of Wasit. from 1st January to 30th April, 2025.Fifty stool samples were taken from immunocompromised patients in Al-Karama hospitals and health centers who were suspected of having cryptosporidiosis and had diarrheal symptoms.

2.2. Microscopic examination

The World Health Organization's modified Ziehl- Neelsen (ZN) Cryptosporidium oocysts were detected in specimens of stool using the staining method. In short, Stool smears underwent air drying, methanol fixing, and carbol fuchsin staining., Methylene blue was used as a counterstain after being decolored with 1% acid-alcohol.. The slides were inspected for the presence of tiny, spherical, brilliant red *Cryptosporidium* oocysts using a light microscope at 1000x magnification [7].

2.3. Molecular detection and phylogenetic analysis

DNA extraction: Following the guidelines provided by the manufacturer, A commercial kit for extracting DNA (Geneaid DNA Feces Micro Kit, Taiwan) was used to obtain the stool samples' genetic DNA. Nested PCR: In accordance with[8] nested PCR was carried out to target *Cryptosporidium parvum* glycoprotein 60 (gp60) gene .The MK034696.1 *Cryptosporidium*

parvum 60 kD glycoprotein (gp60) gene, full CDs as shown in the table, was used to construct nested PCR primer for the gp60 gene in this investigation:

Primer	Sequence	Product Size
PCR- Forward Primer	CTGTATTCTCAGCCCCAGCC	879 bp
PCR- Reverse Primer	AAGCAGAGGAACCAGCATCC	
Nested Forward Primer for PCR	GGGCTCATCATCGTCATCGT	746 bp
Nested PCR- Reverse Primer	AGACACCATTGGTAGTGCCG	

Nested PCR was performed to amplify the glycoprotein 60 (gp60) gene of *Cryptosporidium parvum*. The first round of PCR utilized the PCR-Forward and PCR-Reverse primers, while the second nested PCR round employed the nested PCR-Forward and nested PCR-Reverse primers, as shown in the primer table. Each 25 µL PCR reaction contained 12.5 µL of the G2 Green Master Mix from Promega, 1 µL of each primer (10 µM), 5 µL of the extracted DNA template and nuclease-free water to reach the final volume. The cycling conditions for the first PCR round were: initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 58°C for 30 seconds and extension at 72°C for 1 minute, with a final extension at 72°C for 7 minutes. Nested PCR round used the same cycling parameters. The amplified products were visualized on a 1.5% agarose gel stained with ethidium bromide, with the expected fragment size of 746 base pairs for nested PCR.

2.4. Sequencing and phylogenetic analysis

DNA sequecing method: Positive nested PCR products were purified and subjected to sanger sequencing to obtain nucleotide sequences of *Cryptosporidium parvum* gp60 gene. Sequence assembly and editing were performed using BioEdit or similar software and the resulting sequences were compared with reference sequences in the GenBank database using the Basic Local Alignment Search Tool (BLAST) for species and genotype identification. Multiple sequence alignment was conducted using the ClustalW algorithm implemented in MEGA version 6.0 or MEGA X [9]. Phylogenetic analysis was carried out using both the Neighbor-Joining (NJ) and Unweighted Pair Group Method with Arithmetic Mean (UPGMA) methods within the same MEGA software to determine the genetic relationships among local *C. parvum* isolates and reference genotypes.

2.5. Statistical Analysis

The information was tabulated in Version 26.0 of IBM SPSS datasheet in order to carry out the statistical evaluation. To find out if there were any significant differences ,chi-square test was applied and the statistics was indicated by a likelihood value ($p \leq 0.05$) [10].

3.Result and discussion

3.1 Microscopic examination and molecular detection

According to earlier sources, *Cryptosporidium* was detected in human stool samples in the current investigation utilising clinical indicators (fever, diarrhea, and stomach pain) that differed in appearance among those affected [11]. Direct microscopic examination to identify infectious oocyst based on morphological criteria in recognising *Cryptosporidium* staining. As seen in figure (3-1), the oocyst was found to be small, round (4×6 µm) with a smooth single wall that appears brilliant red on a blue background.. The current study used Ziehl-Neelsen (ZN) light microscopy and molecular approaches to gather 50 stool samples from patients in order to investigate *Cryptosporidium parvum*. The findings demonstrated that 20 (40.0%) of the patients had positive light microscopy results, whereas 30 (60.0%) had negative results. However, the PCR findings were found in 35 out of 50 samples, or 70% of the total. On the other hand, as seen in figure (3-2) ,(3-3) and table (3-1), there were 15 negative results, or 30.0% of the total samples, and the difference was significant.

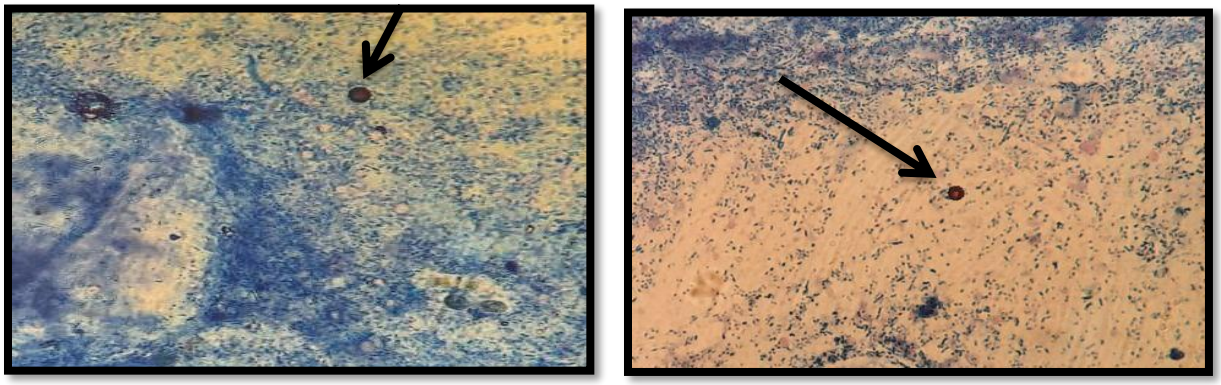


Figure (3-1): The oocyst in human fecal samples using an objective lens and Ziehl-Neelsen stain under (100x).

Table (3-1): Comparison between light microscopy and Molecular method for Diagnosis of *Cryptosporidium parvum*.

Characteristics	**light microscopy** ***n = 50***	**Molecular diagnosis** ***n = 50***	***P value***
Diagnosis of *Cryptosporidium parvum*			
Positive, *n* (%)	20 (40.0%)	35 (70.0%)	0.003 ¥ S
Negative, *n (%)*	30 (60.0%)	15 (30.0%)	

¥: Chi-square test; S: significant at P ≤ 0.05.

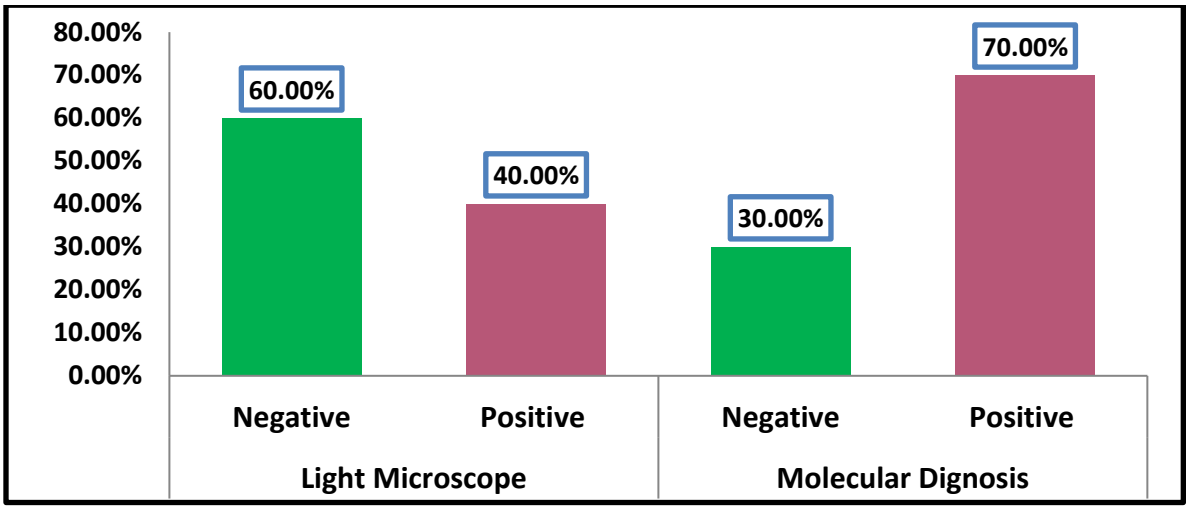


Figure (3-2): Comparison between light microscopy and Molecular method for Diagnosis of *Cryptosporidium parvum*.

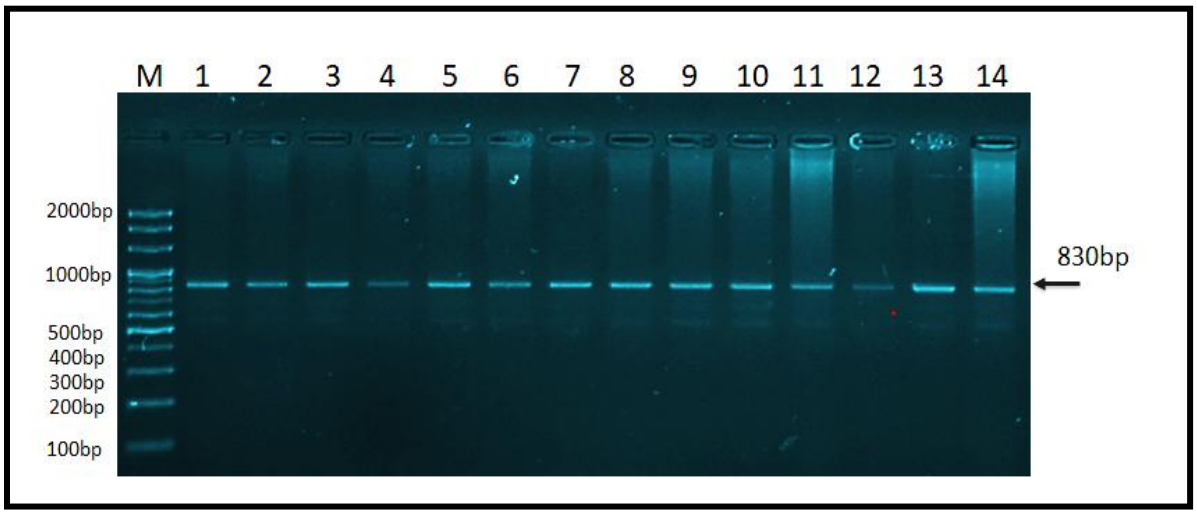


Figure (3-3): The electrophoresis of human DNA samples using nested PCR products. M: (DNA mark 2000-100 bp). There is some positive Cryptosporidium sp. at (830 bp) product size in lanes 1–14.

According to studies conducted worldwide, cryptosporidiosis is a zoonotic disease that can be found in any area with a hot climate [1]. However, rather than focusing on the prevalence of *Cryptosporidium*, More recent studies have concentrated on molecular identification , genotypic traits, methods for identifying the parasite and contrasting these techniques, separation from the natural environment and water resources, its impact on epidemics, sanitation or protective measures. Our findings were supported by a number of studies. In Wasit [12] found 32 (40%) positive cases for *Cryptosporidium* oocysts in immunocompromised patients when stool samples were examined using modified acid-fast stain. Out of 115 stool specimens from children with

diarrhea in Ramadi province, the results indicated that the percentage of infection was 39.13%[13] . The findings were comparable to those reported by [14] in Al-Qadisiyah, where 29 samples out of 100 stool specimens analysed under a microscope using the acid-fast stain (AFS) showed a 29% infection rate. In Babylon, 75 (25%) of the 300 stool samples confirmed positive for *C. parvum* [15] . Similarly, in Duhok, the results showed 16% [16] , which was less than an inconsistent with our findings. Numerous research on human feces with diarrhea were carried out in Turkey, however no parasites were found by direct microscopic inspection *Cryptosporidium spp*. were found in 2.7% (n=4) of the samples using the acid-fast staining method [17].The research population, personal cleanliness, management of sewage water, eating food that is tainted, untreated water and weather conditions were all linked to variations in *Cryptosporidium* infection rates. The season and poor economic situation are contributing factors to the current high prevalence of cryptosporidiosis [18].

Nevertheless, the current study's findings are different from those of the research carried out in Al Najaf AL Ashraf, Iraq by[19] who found that the overall proportion of *Cryptosporidium* infection was 58%, which was higher than our findings. Additionally, Suburban dwellers in the Duhok region of Iraq have a greater infections rate (87.6%) than urban dwellers (75%0 [20]. The sample volume, number of animals in the area, rainy seasons, the climate, sampling size are just a handful of the many factors influencing how common conflicting interests .

In reference to the molecular investigation, in the same province of our research [12] obtained identical results to ours regarding registration 48 (60%) positive by nested-PCR. Additionally, our findings closely matched those of [15] who used a PCR product evaluation of the HSP70 gene in *C. parvum* for detection and found that 50 out of 75 samples (66.66%) had the HSP70. Using PCR the greatest rate was 65% in Thi-Qar Province's Al-Rifai City [21] .The findings however were higher than those of earlier research carried out [18] in the city of AL-Diwaniyah, Iraq who reported an infection rate of 29.29% and the findings of [22] who discovered that 26% of the results were positive.

3.2. Sensitivity and specificity of the microscopically examination comparison with molecular method

PCR approach which was thought to be the gold standard method compared against the specificity and sensitivity of optical microscopy in identifying the parasite *C. parvum*. The results are shown in table (3-2). Intestinal parasite infection was diagnosed with a sensitivity of 48.6% and a specificity of 80.0%.

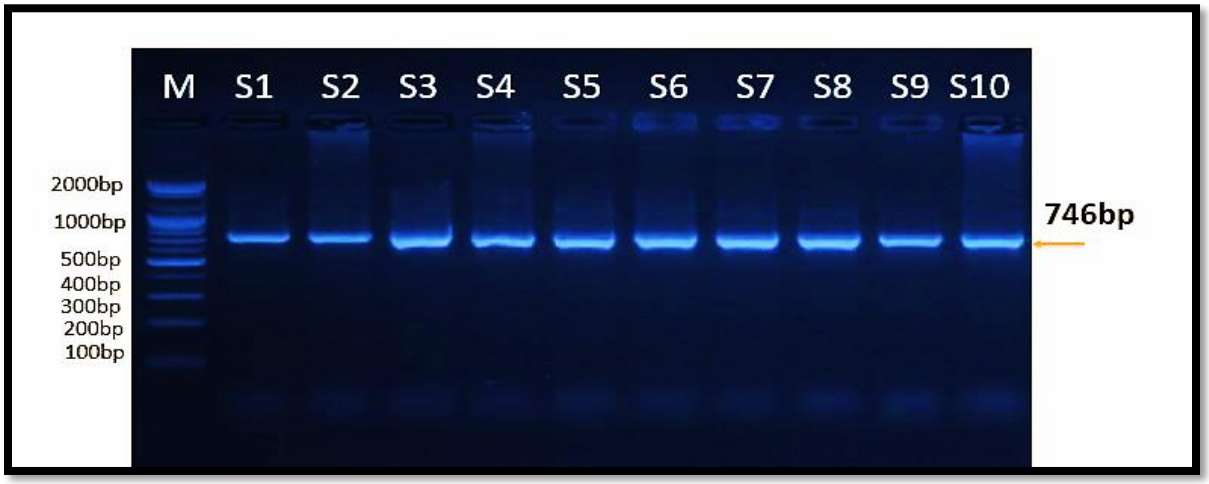
Table (3-2): Sensitivity and specificity of light microscopy in detecting *C. parvum* in comparison with PCR method

C. parvum		**Molecular method (PCR)**		**Total**
		Positive	**Negative**	
Light Microscopy	Positive	TP / 17	FP / 3	20
	Negative	FN /18	TN / 12	30
Total		**35**	**15**	**50**
Sensitivity = TP/(TP+FN) x 100 = 48.6 (48.6%) **Specificity =** TN/(TN+FP) x 100 = 80.0 (80.0%)				

In order to get around the limitations of microscopy, PCR approach has recently gained popularity in the diagnosis of cryptosporidiosis. DNA-based detection techniques have a number of benefits over microscopy include increased specificity and sensitivity , the potential for genetic typing and faster turnaround times[23] . Nested-PCR was selected as the gold standard in this investigation because to these diagnostic tendencies.

3.3. Genotyping based DNA sequencing analysis

From the PCR findings 35 samples , ten samples were taken from those samples to Phylogenetic tree study using the partial sequence of the GP60 protein gene in human *Cryptosporidium parvum* isolates it is employed in genetic genotyping identification.



Figure(3-4): Nested PCR agarose gel electrophoresis gp60 protein gene at 746bp

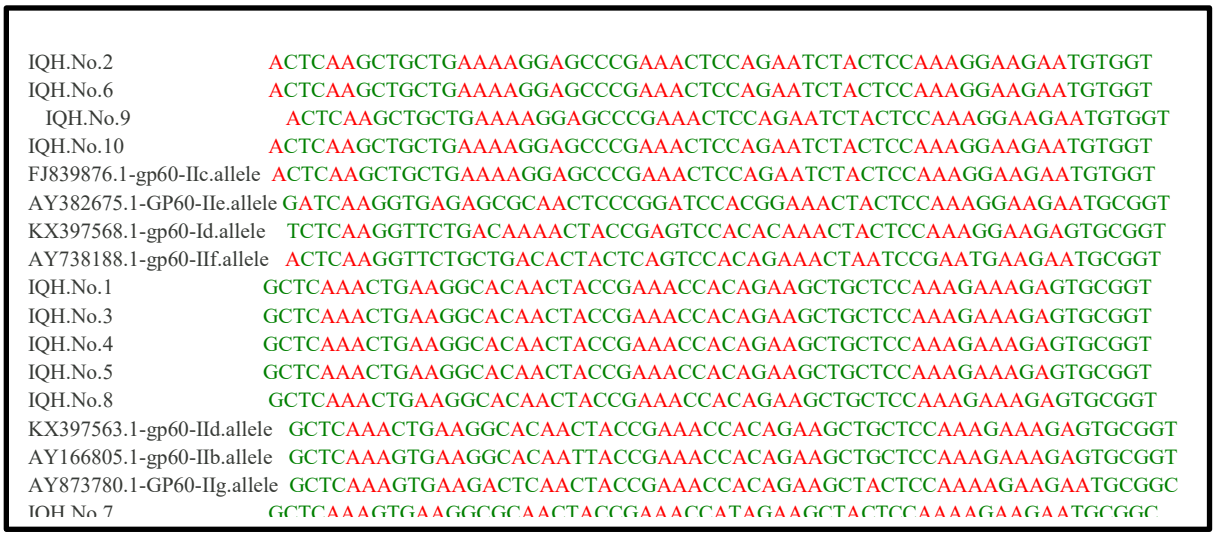


Figure (3-5): GP60 protein gene was analyzed using multiple sequence alignment in both NCBI-Genbank and local human isolates of *Cryptosporidium parvum*. The Clustal W alignment tool in MEGA 6.0 was used to create the multiple alignment analysis. The GP60 protein gene's nucleotide alignment similarity with substitution mutations is shown as (*).

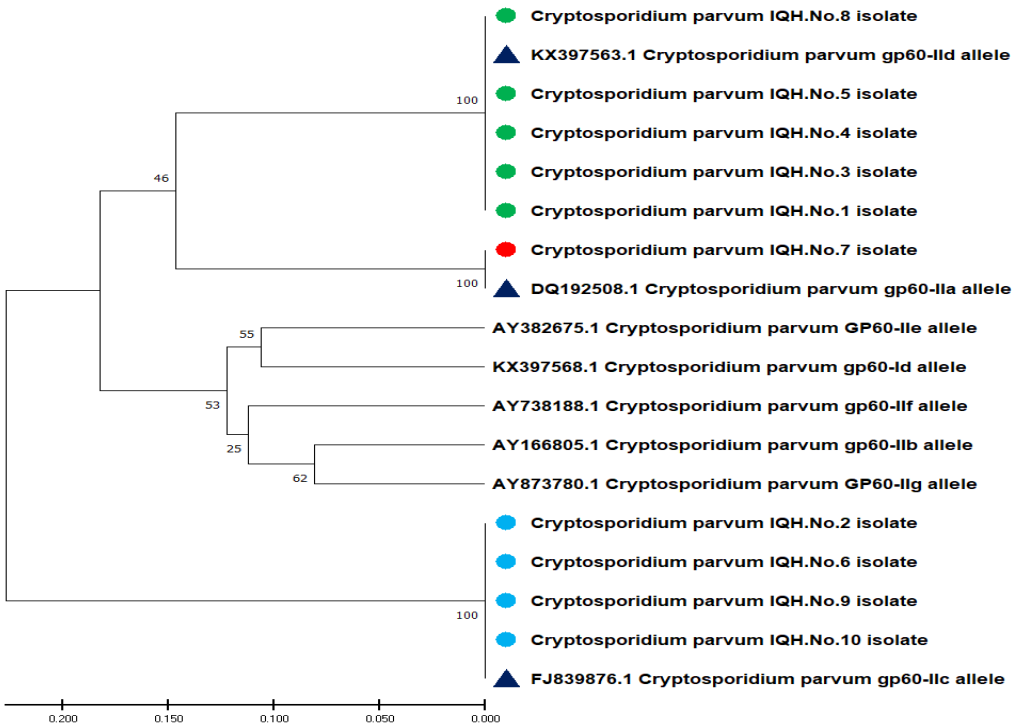


Figure (3-6): Genetic tree investigation depending on the GP60 protein gene's complete sequence in human isolates of *Cryptosporidium parvum* utilized for genetic genotyping identification. The method of the Unweighted Pair Group with Mathematical Mean (UPGMA tree) in MEGA 6.0 was used to create the phylogenetic tree at overall genomic alterations (0.200-0.050)

Table (3-3): NCBI-BLAST Homology Sequencing similarity (%) between genotypes of *Cryptosporidium parvum* submitted to NCBI-BLAST and local isolates

C. parvum isolate	**Alignment of NCBI-BLAST Homology Sequences (%)**		
	Identical genotypes	Identification number for Genbank	Personality (%)
IQH.No.1	gp60-IId.allele	KX397563.1	98.70%
IQH.No.2	gp60-IIc.allele	FJ839876.1	98.65%
IQH.No.3	gp60-IId.allele	KX397563.1	100.00%
IQH.No.4	gp60-IId.allele	KX397563.1	98.67%
IQH.No.5	gp60-IId.allele	KX397563.1	100.00%
IQH.No.6	gp60-IIc.allele	FJ839876.1	98.65%
IQH.No.7	gp60-IIa.allele	DQ192508.1	100.00%
IQH.No.8	gp60-IId.allele	KX397563.1	98.65%
IQH.No.9	gp60-IIc.allele	FJ839876.1	98.65%
IQH.No.10	gp60-IIc.allele	FJ839876.1	100.00%

The phylogenetic tree revealed that the isolates of *Cryptosporidium spp.* in this investigation were similar to sequences found in GenBank, with the Iraqi *Cryptosporidium spp.* exhibiting the highest degree of gene sequence similarity 98%–100% of strains and strains from around the world are human. The results of the phylogenetic analysis confirmed that there were only minor differences between the *Cryptosporidium spp.* Iraqi strains as well as those from other countries. The observed genetic variance could be explained by variations in the reference sequence's size and the isolates' geographic origins.

The genetic investigation included a variety of methods, including complete or partial gene sequencing using nested PCR. The investigation's findings are consistent with previous research that have demonstrated the evolutionary links and genetic diversity of *Cryptosporidium* species populations across the globe .It is thought that these isolates can spread between humans and animals through contact either direct or indirect. Our results are corroborated by earlier studies conducted in Iraq [24].

Conclusion

The study revealed the prevalence of *Cryptosporidium parvum* infection in Wasit Governorate Iraq, among immunocompromised patients. Nested PCR method was found to be the gold standard in terms of specificity. Sequence similarity between the detected genotypes and reference isolates in the GenBank database ranged from 98.65% to 100%.

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