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Identification of Cryptosporidiosis in Preschool Children: Identifikasi Cryptosporidiosis pada Anak Usia Prasekolah

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Abstract

General Background: Cryptosporidiosis is a globally distributed parasitic disease that frequently causes diarrheal illness in young children and represents a persistent public health concern.**Specific Background:** Cryptosporidium parvum is one of the most significant zoonotic protozoa responsible for gastrointestinal infections, and molecular identification methods are increasingly used to clarify epidemiological patterns and genetic diversity of circulating strains. **Knowledge Gap:** Despite numerous studies on intestinal parasites, molecular data regarding local isolates and subtype distribution of C. parvum in Iraqi children remain limited. **Aims:** This study aimed to detect C. parvum in diarrheic children using nested polymerase chain reaction targeting the GP60 gene and to analyze the genetic relationships of local isolates through sequencing and phylogenetic analysis. **Results:** Among 28 fecal samples examined, 12 (42.86%) were positive for C. parvum. Sequencing analysis of positive samples revealed a high genetic similarity (approximately 99%) with global reference strains in the NCBI database, with minimal nucleotide variation. Phylogenetic analysis further classified the detected isolates into two subtype groups, IIc and IIId, with IIc representing the majority of cases. **Novelty:** This research provides the first molecular confirmation and phylogenetic characterization of these GP60 subtype groups among local Iraqi isolates deposited in the NCBI database. **Implications:** The findings contribute to the understanding of molecular epidemiology of cryptosporidiosis in Iraq and highlight the importance of expanded genotyping and surveillance studies to clarify transmission pathways and improve disease monitoring.

Keywords: Cryptosporidium Parvum, Molecular Epidemiology, Nested PCR, GP60 Gene, Pediatric Diarrhea

Key Findings Highlights

High proportion of pediatric stool samples contained detectable parasite DNA.

Genetic sequencing revealed strong similarity between local isolates and global strains.

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Two allele groups dominated the detected variants within the sampled population.

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Introduction

Vinayak et al. (2015) note that the diarrheal illnesses (particularly, the *Cryptosporidium*species, the first serious parasite organism that causes severe diarrhea) contributed to approximately 10.5% of all child deaths worldwide. Under the Eucoccidiorida Order of Apicomplexa Phylum, there is the *Cryptosporidium parvum* Family of opportunistic intracellular parasites (**Barta et al., 2012; Liu, 2017**). This parasite strikes a wide array of animals alongside people, triggering cryptosporidiosis (**Guérin and Striepen, 2020**). It ranks among the select parasites seeing rising incidence, with outbreaks increasingly routine nowadays (**Chalmers et al., 2019**).

The most common method of spreading the parasite by children below two years of age is through the consumption of infected water even though there are other methods through which the parasite is spread both directly and indirectly. *Cryptosporidium*can persist for long periods in the environment because its oocysts are protected by a thick outer shell, allowing them to withstand harsh conditions and many common disinfectants (**Robertson et al., 2020; Al-Ezzy and Kadhim, 2021**). Moreover, *Cryptosporidium* has the ability to proliferate in the small intestine microvilli, which disturbs ion homeostasis and leads to ionic loss in general (**Das et al., 2018; Mendes, 2020**). *Cryptosporidium* may also infect various sites throughout the gastrointestinal tract (**Peek et al., 2018**). Whereas, in several mild to moderate cases, cryptosporidiosis did not show any signs, severe cases may lead to vomiting, anorexia, fever, general malaise, abdominal cramping, and a great deal of watery diarrhea (**Peek et al., 2018**). The illness is common with a seroprevalence rate of 25-35% and infection rate of 1-2% across the world. The organism is capable of occurring in 1-4.5 percent of the sampled people (**Wanamaker and Grimm, 2004; Tulchinsky and Varavikova, 2014**). The organism was named blue beads due to the characteristic biopsy appearance of 1 to 5 mm-sized spherical and basophilic aggregates to appear out of the enterocytic apex in crypts or surface epithelium (**Jimenez et al., 2017; Schuetz, 2019**).

Traditional diagnostic approaches for detecting *Cryptosporidium* oocysts worldwide rely on light microscopy with Modified Ziehl-Neelsen staining, direct wet mounts, and ultrastructural techniques to visualize intracellular cysts (**Malik et al., 2013; Bones, 2017**). Nevertheless, due to different life cycle durations of *Cryptosporidium* strains and rather same appearance of this parasite, the conventional morphological and phenotypical systems cannot differentiate distinct species among humans and animals (**Wielinga et al., 2008**). Recent molecular diagnostic tools have refined *Cryptosporidium* species detection, strain subtyping, and genotyping, highlighting broad versus narrow host specificities in different strains (**Robinson and Chalmers, 2012**). Here, we deposited local positive *C. parvum* isolates into the NCBI database, classified their allelic profiles, and applied PCR-based methods to probe cryptosporidiosis at the molecular level.

Materials and Methods

Ethical approval

This study was allowed by the Scientific Committee of the College of Medicine, University of Wasit (Wasit, Iraq).

Study samples

This study enrolled 28 children with diarrhea from three government hospitals in Wasit Governorate, Iraq (Al-Kut Hospital for Gynecology, Obstetrics, and Pediatrics; Al-Karama Teaching Hospital; Al-Zahraa Teaching Hospital) between January and March 2022. Fresh stool samples were aseptically collected in disposable plastic containers from all participants, kept cool during transport, and processed for molecular analysis in the laboratory.

Molecular examination

DNA from stool samples was extracted following the manufacturer's protocol for the Stool DNA Extraction Kit (Bioneer, Korea). The concentration and purity of each of the DNA samples extracted were determined by the Nanodrop spectrophotometer. Nested PCR targeting the *GP60* gene, using two primer sets designed by **Maurya et al. (2013),** confirmed via Primer3Plus and NCBI-GenBank, and synthesized by Bioneer (Korea), was employed to detect *C. parvum*(Table 1).

Primer		Sequence (5´-3´)	Amplicon
First-step GP60 nested PCR for	F	ATAGTCTCCGCTGTATTC	480bp
Cryptosporidium parvum	R	GAGATATATCTTGGTGCG	
R		CGAACCACATTACAAATGAAG	

Table 1. **Table (1): Nested PCR primer sets targeting *C. parvum***

Nested PCR master mixes were prepared using the AccuPower® 2X PCR PreMix kit (Bioneer, Korea) in 20 µL reactions. The first round included 5 µL DNA template, 1 µL each of forward and reverse primers, and 13 µL PCR-grade water; the second round used 2.5 µL of the first-round product, 1 µL each primer, and 15.5 µL PCR-grade water. Thermal cycling (Thermocycler, Bioneer, Korea) consisted of initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation (95°C, 30 s), annealing (56°C, 30 s), and extension (72°C, 1 min), with a final extension at 72°C for 5 min. PCR products were resolved by electrophoresis on 2% agarose gels stained with ethidium bromide, visualized under UV transillumination,

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and scored as positive for a 375 bp band

Phylogenetic analysis

The PCR products that were positive were transferred to Macogen Company (Korea) to undergo DNA sequencing in the modified Sanger method. The analysis of the results was then done. Local *C. parvum* strains received designated identifiers, were submitted to NCBI GenBank (with assigned accession numbers), and underwent NCBI-BLAST comparison with reference sequences for phylogenetic tree construction.

Results

A total of 28 fecal samples were collected and a nested PCR assay was performed on them; 12 of them (42.86) were positive in general (Figure 1). Ten genomic DNAs of positive samples were phylogenetically studied using the *GP60* gene. The name of the results of the sequencing of the local isolates of *Cryptosporidium parvum* was in the following way: Local cataloged variants—such as *C. parvum* Human/IQS-1, IQS-2, IQS-4, IQS-5, and IQS strains—displayed point mutations plus aligned matches across *GP60* segments (Figure 2)

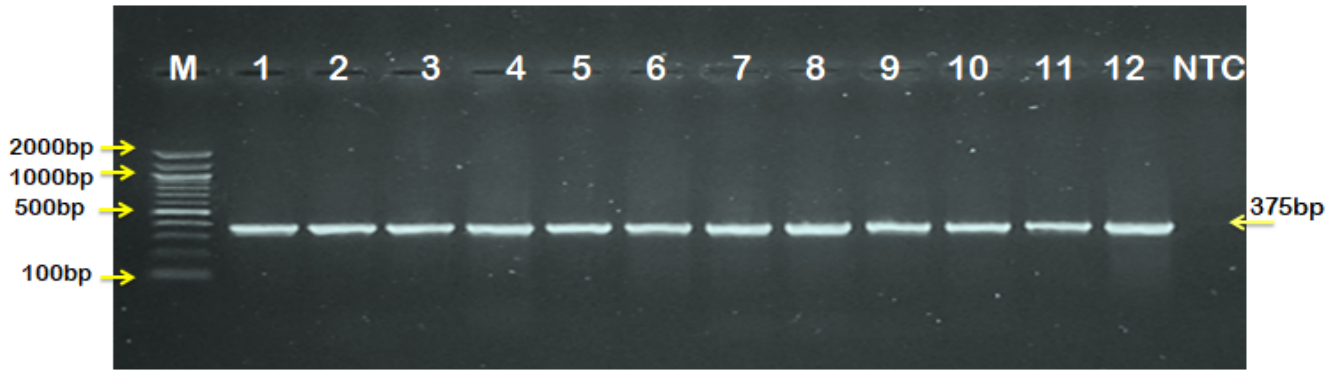

Figure 1. **Figure (1): Agarose gel electrophoresis indicates the presence of the *GP60* gene of *C. parvum* in the feces of a human being when subjected to Nested PCR analysis.**

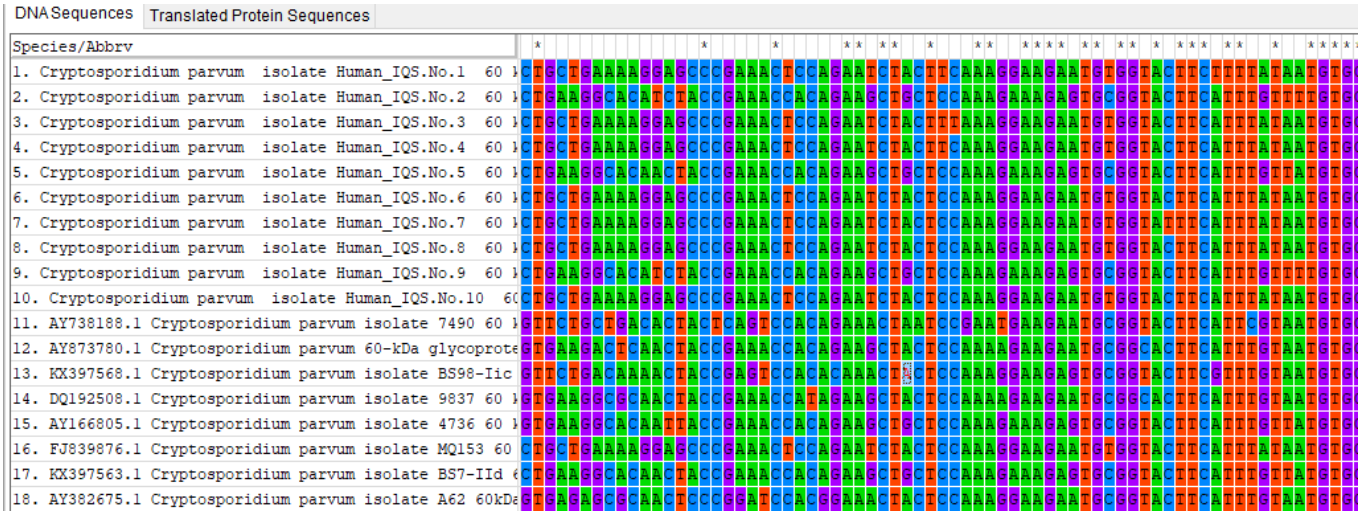

Figure 2. Lane M: DNA marker ladder (2000–100 bp); Lanes 1–12: Positive amplicons at 375 bp; NTC: No-template control

The IQS-*C. parvum*isolates 2, 5, and 9 showed significant sequence identity with the Egyptian *C. parvum*isolate (KX397563.1), as determined by NCBI-BLAST homology analysis. All local *C. parvum*subtypes subsequently clustered into the IIc (7/10 isolates) and IIId (3/10 isolates) allele groups (Figure 3).

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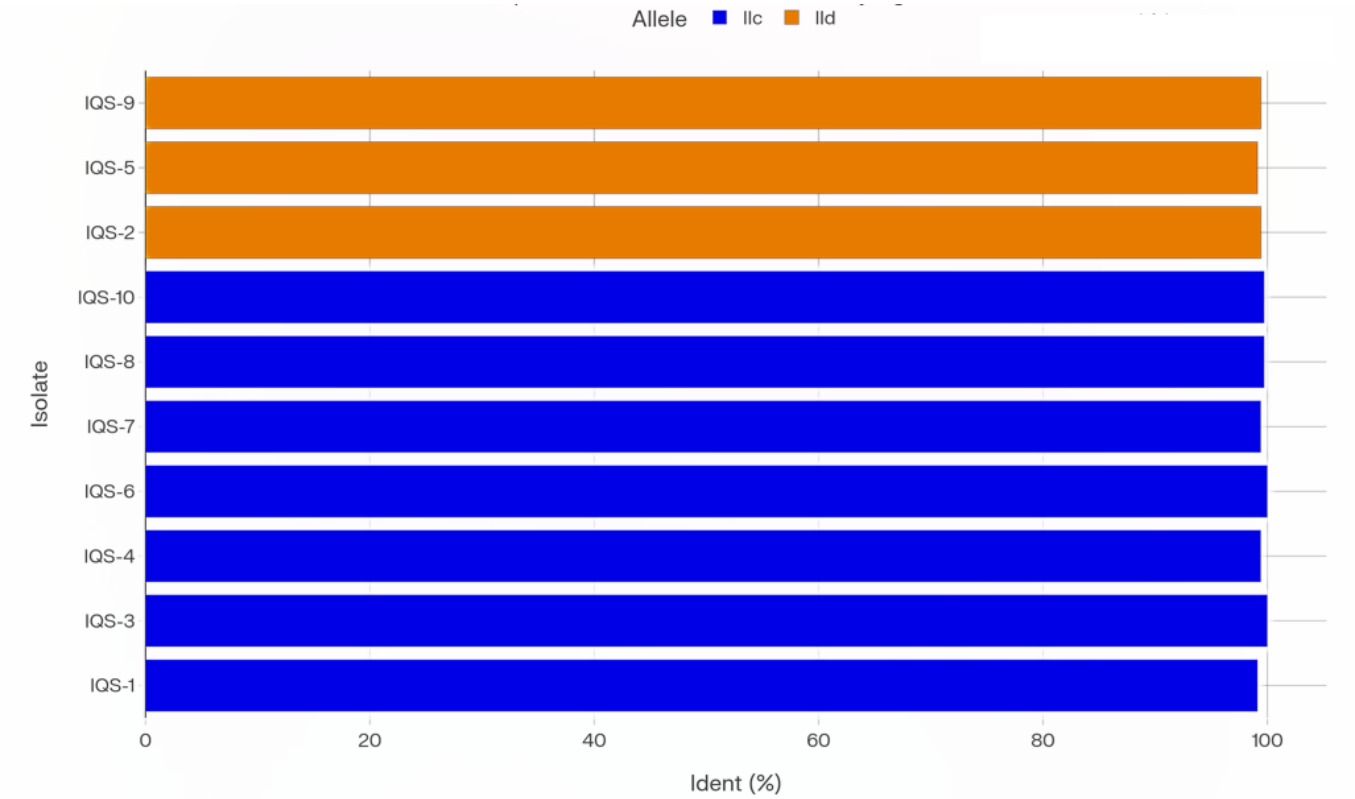

Figure 3. **Figure (3): BLAST sequence identity (%) distribution across local *C. parvum* isolates compared to reference strains**

The local isolates and global isolates were compared and it was observed that the total genetic mutation of the local *C.parvum* IQS-isolates was 0-0.9% and that it had a high similarity (99% similarity) to the NCBI-BLAST *C.parvum* of the gene *GP60* (Figure 4).

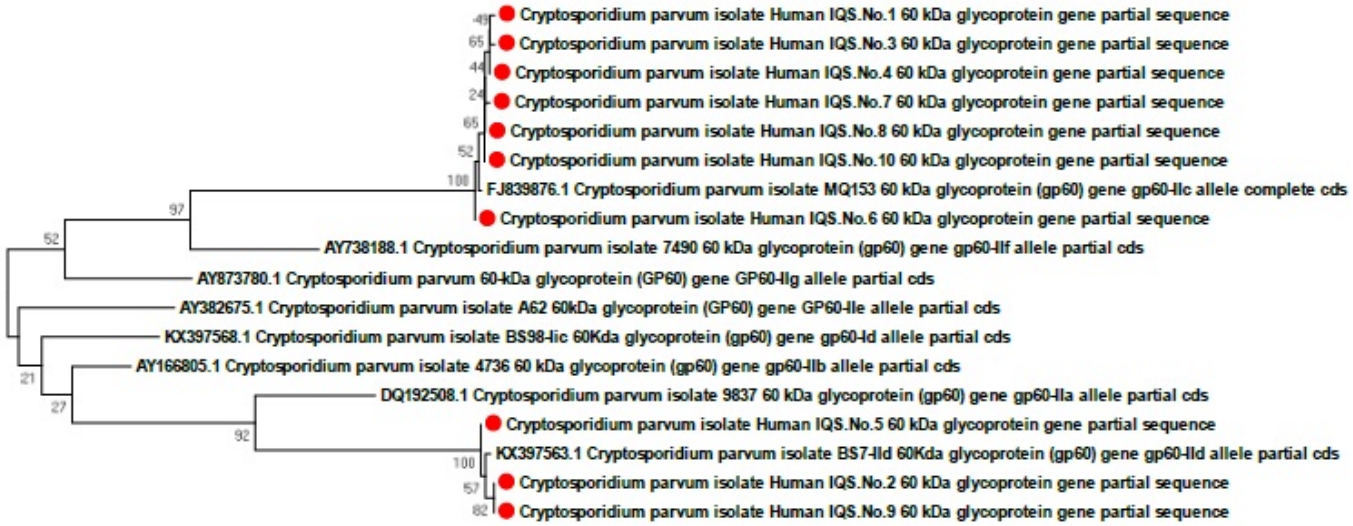

Figure 4. **Figure (4): The comparison of the NCBI-GenBank isolates using the *GP60* gene with the incomplete sequences of local *C. parvum* IQS isolates using phylogenetic tree.**

Discussion

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A close relationship between *Cryptosporidium* and both acute and chronic diarrhea in children has been dem wide range of countries. In Iraq, investigations have largely depended on classic approaches like In Iraq, conventional microscopy via modified Ziehl-Neelsen (**Alali et al., 2021**) has dominated, offering scant molecular evidence. PCR-based *C. parvum* rates among diarrheal youth: Baghdad 11% (**Hussein et al., 2015**), Al-Muthanna 18% (**Mallah & Jomah, 2015**), Al-Diwaniyah 24% (**Ahmed et al., 2016**), Erbil 12% (**Azeez & Alsakee, 2017**), Al-Najaf 12.8% (**Tairsh et al., 2017**), Thi-Qar 10.42% **(Salim & Al-Aboody, 2019**). Comparable figures abroad included 10% (Netherlands), 3.77% (Ethiopia), 10.42% (Brazil), and 7.14% (Turkey) (**Wielinga et al., 2008; Adamu et al., 2010; Taghipour et al., 2011; Rolando et al., 2012; Yilmazer et al., 2017**).

Discrepancies between our findings and other local/international studies may stem from differences in targeted genes, seasonal variations in cryptosporidiosis incidence, sampling biases (sample size, selection methods, patient age), environmental parasite sources, and PCR conditions. Global reports on *Cryptosporidium* prevalence in children vary widely, with *C. hominis* predominating in South Africa (**Leav et al., 2002**), Thailand (**Tiangtip and Jongwutiwes, 2002**), Malawi (**Peng et al., 2003**), Brazil (**Bushen et al., 2007**), Kenya (**Mbae, 2008**), Peru (**Cama et al., 2008**), and South India (**Ajjampur et al., 2010**), whereas *C. parvum*was the main species in Kuwait (**Sulaiman et al., 2005**), Ethiopia (**Adamu et al., 2010**), Iran (**Taghipour et al., 2011**), and Turkey (**Yilmazer et al., 2017**).

Genotyping and subtyping efforts for *Cryptosporidium* routinely examine markers like 18S rRNA, the 70-kDa heat shock protein (hsp70), oocyst wall protein (OWP), actin, β-tubulin, TRAP, ITS1, and DHFR (**Cunha et al., 2019**). *GP60* remains a premier target for *C. parvum* subtype discrimination (**Khan et al., 2018; Yanta et al., 2021; Uran-Velasquez et al., 2022**).

Strains with identical *GP60* genotypes can vary substantially at additional genetic loci, sometimes exceeding differences seen between distinct *GP60* alleles (**Abal-Fabeiro et al., 2013**). The Ic allele, initially identified in *C. hominis*, has been found exclusively in human *C. parvum*bovine genotype isolates (**Alves et al., 2003**). The IIc subtype is enriched in short 9-serine repeats and is rare in animals, possibly due to its wide geographic distribution and partial overlap in human-animal sequence origins (**Widmer et al., 2009**).

The IId subtype is uncommon among *C. parvum*strains but linked to zoonotic cases in European countries including Italy, Hungary, Portugal, and Serbia (**Xiao and Fayer, 2008**), comprising about half of pediatric infections in Kuwait (**Sulaiman et al., 2005**). Variations in short tandem repeats and host immune responses may impose differing selective pressures on *GP60* across species, favoring short-repeat alleles in humans (**Widmer et al., 2009**).

Conclusion

In conclusion, this study revealed a notably high prevalence of *C. parvum*in Iraqi children with diarrhea and provided the first confirmation in Iraq of allelic group distributions (IIc and IIId) among local isolates. The transmission sources and pathways for *C. parvum* in Iraq have yet to be clarified. Additional studies focusing on serotyping and genotyping *Cryptosporidium* species among patients with diarrhea are crucial to address these informational voids.

Authors' Contributions

DKK gathered fecal specimens from diarrheic pediatric patients and carried out DNA isolation. BAH and GA handled the nested PCR experiments and phylogenetic evaluations. All authors contributed to genotyping the positive isolates and drafting the manuscript.

Competing Interests

There is no competing to be interested, and no funds have received to complete this work.

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