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Research Article

Molecular detection of genetic types of *Cryptosporidium parvum* parasite causing diarrhoea in Mosul city

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Abstract
Cryptosporidium is a crucial zoonotic parasite that leads to self-limiting gastroenteritis. The primary species responsible for cryptosporidiosis are *Cryptosporidium hominis* and *C. parvum.* The objective of the study was the microscopic identification using the Modified Zeihl Neelsen and floatation using the sugar sheather solution, as well as using the molecular identification technology via the Nested Polymerase Chain Reaction (nPCR) to detect the 18SrRNA gene with DNA sequence analysis and the phylogenetic tree of some positive samples from various areas of Mosul city. Stool samples from 154 children under five with diarrhoea were collected at Ibn AlAtheer Teaching Hospital in Nineveh Governorate from July to December 2023. Samples were tested for Cryptosporidium using Modified Zeihl Neelsen stain and floatation techniques. DNA extraction and nPCR were performed for molecular analysis. The study showed that the infection percentage was 20.77% for 32 samples out of the total number of samples 154 stool samples, which were tested and microscopically identified in addition to the results of the molecular test that was conducted by using the Nested Polymerase Chain Reaction (nPCR) (32 samples were positive and 122 samples were negative) to confirm the accuracy of the microscope examination. The percentage of infection with C. parvum parasite was observed to be 18.75%, and the DNA sequencing of the infected samples was conducted. The three new isolates emerged for the first time in the Nineveh governorate and were registered at the National Center for the Biological Information (NCBI) with serial numbers (PP669700, PP669699, and PP669701).

Keywords: *Cryptosporidium parvum*, Genotypes, genetic tree, Oocyst, nPCR

INTRODUCTION

Parasitic protozoa are single-celled, eukaryotic microorganisms that have important health and economic effects. They are pathogenic to both humans and animals and are widespread globally. The severity of their impact on the host varies depending on the species, strain, and site of infection (Florin-Christensen *et al*. 2021) The Cryptosporidium belongs to Apicomplexa as it is an apex construct that assists it in penetrating the host cells (Gerace *et al*.,2019). Cryptosporidiosis, a zoonotic disease, occupies the apical surface of the intestinal epithelial cells in the lumen of the small intestine (VanDussen *et al*., 2020). For persons with good immunity capability, this parasite causes diarrhoea for a short period. In contrast, diarrhoea lasts longer for persons with poor immunity and those who suffer from immunity deficiency and infants in the developing and third world countries. Thus it becomes a danger to their life (Khudhair and Salih, 2020). Also, Cryptosporidium is responsible for severe diarrhoea, delayed growth, and poor cognitive functions in young people and individuals with poor immunity and it is also associated with colon cancer (Santin, 2020; Pinto and Vinayaks, 2021). Moreover, it is regarded as the reason behind the mortalities due to the Cryptosporidium effect for infants all over the world. Also, it is considered the main cause of diarrhoea and death for children

(Tombang *et al*., 2019).

The parasite transfers to the final host without the need for an intermediate host through two methods; either directly via direct contact with the infected individuals or animals and that is done through the transmission of the oocytes to the healthy individual (Dumaine *et al*., 2020) or indirectly, through drinking water or having a food, which is contaminated with the oocytes (Li *et al*., 2015). Furthermore, the presence of oocytes in the water is considered a great danger to general health as it results in the dissemination of epidemics on a large scale of the world and the Cryptosporidium is one of the causes of diseases that transmitted utilizing the water on a global level (Li *et al*., 2015). Also, Cryptosporidium parvum oocytes that contaminate water and food are considered highly resistant to most chemical antiseptics and the common treatment of water by chlorine. It is difficult to terminate it with these ways in addition to its tiny volume which makes it very hard to remove it through the filtering systems (Cunha *et al*., 2019; Dominguez–Uscang *et al*., 2021). More than (44) types of Cryptosporidium species and more than (120) genotypes were identified. The *Cryptosporidium parvum* and *Cryptosporidium hominis* are the most important types infecting humans (Ryan *et al*.,2021).

The present study aimed to provide a comprehensive view of the evolving understanding of the taxonomy and epidemiology of the protozoan parasites *Cryptosporidium* and *Giardia* over the past 50 years. This includes developing improved detection and typing tools, the expanding range of named species, the role of zoonotic transmission, and the application of next-generation sequencing tools to better understand the genetic mechanisms for host specificity and human infectivity.

MATERIALS AND METHODS

Study area

Stool samples were collected from male and female children under five years who suffer from diarrhoea (84 males) and (70 females) who are patients of Ibn AlAtheer Teaching Hospital in the center of Nineveh Governorate for the period from the beginning of July 2023 until the end of December 2023. After obtaining the parents' consent to conduct the relevant tests of identifying Cryptosporidium, 154 samples, with a volume of 10-15 g were collected and put in sterilized plastic cups tightly sealed to keep the samples' moisture and prevent dryness. The name and sex were fixed in each case. Then, they were transported to the Laboratory of Research and Higher Studies at the College of Education for Girls and the samples were examined in terms of color, form, smell and the presence of mucus and blood. They were examined microscopically using the Modified Zeihl Neelsen stain to make sure of the presence of the parasite and also (Khudhair and Al-Niaeemi 2020) by using the floatation technique to isolate the parasite oocytes (Feire-Santos *et al*.,1999).

The solution was put in sterilized test tubes. After that, the potassium dichromate solution was added with a concentration of 2.5%, as 1 ml of the solution was added to each one of the stool samples. Then, the samples were stored at a freezing temperature of (-20) C° (khudhair & Al-Niaeemi 2020) to conduct the isolation of the DNA and the nPCR later on.

Ethical approval

According to Helsinki Declaration on Ethical Principles, the experimental research was conducted in compliance with the Health Protection guidelines of the ethical committee in the Department of Biology of Mosul University, Iraq.

Microscope test

The Modified Zeihl Neelsen (MZN) was used to diagnose the infection with Cryptosporidiosis and the stain was made as follows (khudhair and Al-Niaeemi,2020):

1-Certain quantity of stool was taken using wood sticks and it was bedded as a thin smear on the slide and the slides were left in the air to dry.

2-The smear was fixed by immersing it in ethylene with a concentration of 95% and left to dry.

3- The carbol foxin was applied to the slides for 10 minutes.

4-After that, the slides were put on hold, and left in the air to dry.

5-The slides were dyed with the blue methylene for five minutes after they were removed from the holder.

6-Slides were put on the holder once again and washed with the tap water, left to dry and examined by the microscope with a magnification power of (X40). Then, they were examined under the oil lens (X100) to search for the Cryptosporidium oocytes. After finishing the dyeing process using the MZN and identifying the oocytes, the rest of the sample was floated using the sugar sheather solution, according to (Feire-Santos *et al*.,1999).

Molecular test

First: Extraction of the DNA from *Cryptosporidium* spp. The assay procedure was conducted to extract the DNA per Favorgen Company's instruction, which was mentioned in the Kit produced in Taiwan.

Second: The preparation of agarose gel and the process of electrophoresis of the DNA:

The agarose gel was prepared with a concentration of (1.5%) by dissolving 1.5 grams of agarose in 100 ml of Tris Barate EDTA buffer (TBE) using a flask with a volume of 250 ml) with the buffer. Then, the flask was put in a microwave oven for one minute and a half until it was left to cool down. Before hardening, 3 microliters of the red gel were added and mixed well in the casting

Table 1. Primers used to identify the 18SrRNA gene of *Cryptosporidium parvum*

No.	Primer	Primer 5 sequence 5-3	Length	Product volume (bp)
1	Crypto-F1	TTCTAGAGCTAATACATGCG	20	1325
2	Crypto-R1	CCCATTTCCTTCGAAACAGGA	21	
3	Crypto-F2	GGAAGGGTTGTATTTATTAGATAAAG	26	830
4	Crypto-R2	CTCATAAGGTGCTGAAGGAGTA	22	

tray of the electrophoresis, which included a comb. The gel was left to harden for 15-20 minutes at room temperature. After that, the comb was and left with an empty and ready hole. Then, the agarose gel was placed in the relay basin and 800 ml of TBE buffer was added with a power of X1 until the agarose gel was completely immersed. After that, 7 microliters of the PCR products' results resulted from the PCR in the specified holes. As for the (100bp) ladder DNA, 5 microliters was put in the first hole in the agarose gel and a power supply (75 volts and 300 milliamperes) for 60 minutes . The gel was extracted and put in the Gel DOCEZ Documentation system by Bio-Rad in the USA, which emits ultraviolet (UV) to detect the amplification products. Then the images of the products were stored to analyze them later on.

Nested Polymerase Chain Reaction (nPCR)

A specific part of 18SrRNA Cryptosporidium parasite (Elsawey *et al*., 2020) by using specific primers, as shown in Table 1, was supplied by Macrogen Company.

The PCR of the type nPCR was made up of two molecular reactions. The master mix of the PCR was prepared using the master mix of the reaction kit of Addbio company, Taiwan, by calculating the volumes necessary for the reaction component of each sample, as shown in Table 2. The materials added were mixed well and distributed with a volume of (23) microliters to the PCR tubes for the PCR. After that, the DNA was extracted from the samples, with a volume of 2 microliters added to each sample tube, so the total volume in each tube became 25 microliters. Also, the PCR of the second primer was conducted following the same steps mentioned earlier and with the same conditions of the reaction except for adding the first PCR products as a template of the second PCR, as shown in Table 3.

Then, the reaction pipes were inserted into the thermocycler to perform the chain reaction using the relevant program of the reaction (Table 4). After that, the samples were loaded into the previously prepared agarose gel wells with a concentration of 1.5%, and volume index Ladder DNA (100BP) supplied by Addobio Korean company was added in the first well. Then, the samples are relayed using the Electrophoresis for 60 minutes, and the gel was photographed using the Gel Documentation system. This gel DocEZ image uses the UV to detect the amplification products.

The nitrogenous bases sequences of the samples under study were determined. The outcomes of the Second PCR for the positive samples and the primers specific to the resulting bands were sent for sequencing. The genetic sequences were read and matched with the 18SrRNA gene sequences documented in the National Center for Biotechnology Information (NCBI). and The results were analyzed using the BLAST program.

RESULTS AND DISCUSSION

The results of 154 stool samples taken from 32 children infected with *Cryptosporidium* with a percentage of 20.77% according to the microscope test conducted using the modified Zhiel Nelson stain and the floatation process, the infection percentage was(14.28%) using the Sheather's sugar solution. So, on the other hand, in the molecular test using a Nested polymerase chain reaction to obtain the molecular test results, the amplified DNA two pairs of specialized primers of the gen18SrRNA result in the primary PCR showed that there one band for three samples in the agarose gel with a molecular weight of 1325 pairs of the nitrogen bases in the DNA extracted from the Cryptosporidium oocytes, which were isolated using the floatation method with the sugar solution and this indicates that the infection with the parasite. The secondary PCR showed a band for six samples in the agarose gel with a molecular weight of 830 pairs of the nitrogen bases, indicating that the patients were infected with the parasite. The results showed that the Secondary PCR is more accurate than the primary one as 3 positive samples out of 32 samples appeared, as shown in Fig. 1, but 6 positive samples out of 32 appeared in the secondary PCR, as shown in Fig. 2.

Table 2. Components of the first PCR mix

Components	Volume (ml)
2X Master Mix	12.5
Crypt-F1 lopmol/M1	1
Crypt-R1 lopmol/M1	1
Sterilized water without enzymes (PCA grade water)	8.5
DNA extracted from the sample (DNA template)	2
Final volume	25

Table 3. Components of the second PCR mix

Components	volume (ml)
2X Master Mix	12.5
Front primer Crypt-F2 lopmol/M1	1
Reverse primer Crypt-R2 lopmol/M1	1
Sterilized water without enzymes (PCA grade water)	10
First PCR Product	0.5
Final volume	25

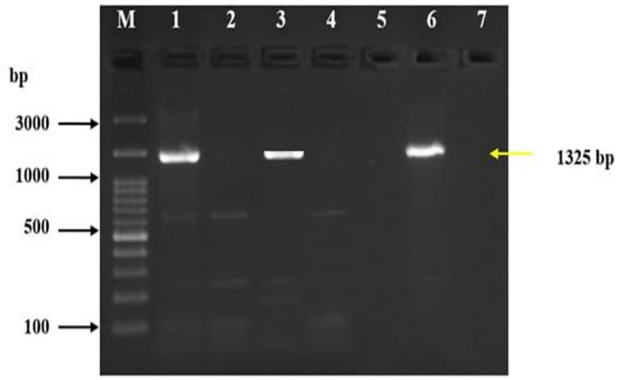

Fig. 1. *PCR for detecting 18S rRNA gene external fragments concerning detecting Cryptosporidium spp. M. represents the marker with a volume of 100. The wells 1, 3 and 6 represent positive samples with a resulting volume of 1325 bp. Well 7 stands for the negative control.*

It was also clear that the nPCR increased the opportunities for detecting small concentrations of DNA genetic substances due to the small number of oocytes in the samples. Also, the present study showed that the percentage of infection with Cryptosporidium was (18.75%) and the results of the present study were close to the study of Jomah and Malloh (2016) conducted in AlMuthanna governorate as the percentage of 18% as per the study of Gawad *et al.* (2018) in Egypt with (21.0%) and a study of Sabooni *et al*. in Qatar where the infection percentage was (17.5%) and the study of Olopade *et al*. (2023) in Nigeria that had infection percentage of (17.6%).

As for the high percentages, the study of Abbas (2023) in Iraq - Babylon governorate recorded (92%) of infection with Cryptosporidium, the study of Eraky *et al* (2014) with infection percentage of 82%, the study of Alkhanaq and Al-Hadidi (2022) in Iraq - AlKut city (39.6%) and also the study of (Elsawey *et al*.,2020), in Egypt with infection percentage of 59% and the percentage of infection with the parasite was 85.2%)in the study (Mohammad *et al*., 2021).

The reason behind the difference in the percentages of children with the parasite when using the molecular test that was noticed in the studies mentioned earlier might be due to a set of reasons, including the samples tested, type of the gene and the method used and its efficiency in terms of DNA extraction (AlObaidi, 2023), or it might be due to the type of the Kit used in extracting the DNA, the extraction method efficiency, the efficiency of the tester and the method in which the samples are stored. In additionally the main reasons were the parasite's Lower prevalence in Mosul: first of all, the shortness of the implementation period (6 months in the summer and autumn seasons), and the results were that the first two months (July-August) were the least recorded, then (Sept-Oct) were observed a increase in the infection then the highest recording observed in the research period was in (Nov - Dec) as the parasite is active when the coldness and the humidity is in the weather, by completing this research three new isolates have been recorded in Mosul city and registered in the GeneBank.

The molecular diagnosis of *C. parvum* parasite using the nPCR is one of the most important objectives of the current study as the molecular methods are characterized with simplicity and speed and through which the parasite and the source of infection can be identified as well in addition to determining the virulence factors (Trisha *et al*., 2012).

The sensitivity of the PCR might amount to the capability of identifying 20 oocytes in (1) ml of the samples (Smith *et al*., 2007), while the sensitivity of nPCR is more than (4-5) times the simple PCR method (Kato *et al*., 2003). Additionally, molecular analysis describes or identifies the genetic structure of the *C. parvum* parasite types. It was proven that the molecular methods are highly sensitive and can identify the parasite species (Xiao, 2010). Moreover, it was proven that using the gene 18SrRNA in nPCR technology gives better and more accurate results than the results of the primary polymerase chain reaction in terms of identifying the

Table 4. Steps of the program of the PCR (reaction conditions)

Step	Temperature °C	Time	No. of cycles
Primary denaturation	94	3 minutes	1
Denaturation	94	45 seconds	
Primer Annealing	55	45 seconds	35X
Primer Extension	72	1 minutes	
Final extension	72	7 minutes	1
Cooling	4	∞	-

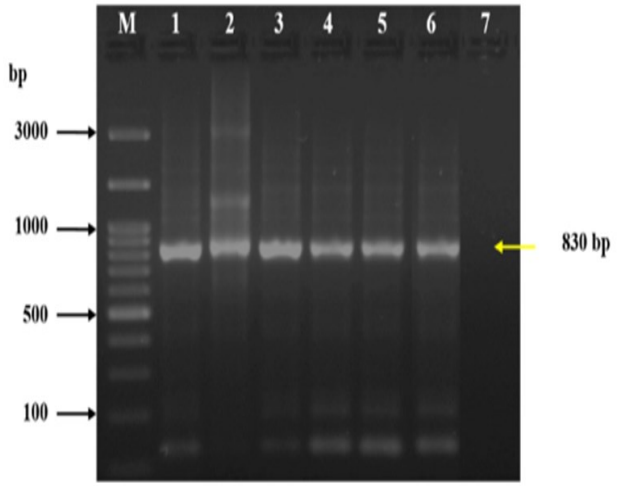

Fig. 2. *PCR to detect 18S rRNA gene internal fragments concerning detecting Cryptosporidium spp. M represents the marker with a volume of 100. Well 1 represent positive samples and with a resulting volume of 830 bp. Well 7 stands for the negative control*

species of Cryptosporidium parasite (Ruecker *et al*., 2011). The small subunit of the 18SrRNA gene detects the DNA Cryptosporidium parasite and identifies the genotypes in the human and animal samples and in the environment (Köseoğlu *et al*.,2022). The sensitivity of nPCR technology is estimated to be 100% compared to the microscope test. Also, nPCR technology makes it possible to detect the negative cases in the microscope test and the primary PCR technology (Kuzehkanan *et al*.,2011).

When the results of the molecular test with the results of the microscope test using the MZN method it is observed that the infection percentage when using nPCR is less than the infection percentage when using the microscope as the percentage when using the molecular test was 18.75%, while it was 20.77% when using the microscope and this is in conformity with the results of some studies like the study of Olopade *et al*. (2023) as the percentage of infection when using the microscopic test using MZN method was 32.1% and when using the molecular test it was 17.6% and the reason behind that might be due to the small number of oocytes in the samples or the damage of the oocytes of the parasite before extracting the DNA (Jomah and Mallah, 2016) or the reason might be the presence of some components in the sample like the iron, peleriobin, bile salts and the carbohydrates that act to attenuate the decomposition of the oocytes or inhibit the activity of the PCR if they were extracted from the targeted DNA (Oikarinen *et al*., 2009; Schrader *et al*., 2012), or the reason may be due to losing the oocytes of the parasite in the sample because of the repeated wash by the potassium dichromate that leads to negative results.

When comparing the correlation of the samples of the current study to the international isolates, as shown in Fig. 3, by finding the genetic tree, the analysis of the first and the second samples of the *C.hominis* parasite showed that there is a relationship with the Indian isolate (MW116647) with a percentage of (100%). From the other hand, results showed that there is a percentage of similarity of (99.37%) with some international samples, including the Australian isolate MG516758

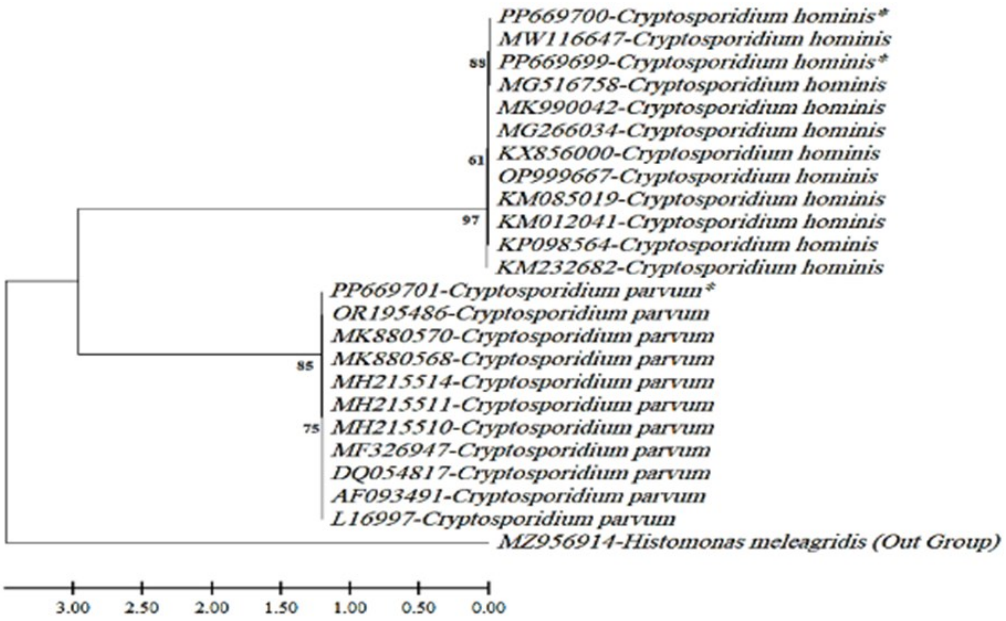

Fig. 3. *Phylogenic tree of Cryptosporidium hominis and Cryptosporidium parvum from Iraq (*). Phylogenic tree was constructed using the Maximum Likelihood method based on the Tamura-Nei model in MEGA11 software and bootstrap analysis with 1000 re-samplings. Partial DNA sequences of partial fusion 18S small subunit ribosomal RNA gene were used as input data*

and the Chinese isolates MK990042 and MG266034, respectively, and the Ethiopian sample KX856000, the sample OP999667, the isolates KM085019 and KM012041 in the United Kingdom and also the Brazilian isolate Kp098564 and the Spanish isolate KM232682.

As for the third sample of *C.Parvum*, the results of studying the genetic sequence showed that this local isolate was similar to other isolates as the results showed a relationship with a percentage of (100%) with the Iraqi isolate (OR195486). From the other hand that there is an approximation of (99.84%) with the Iraqi isolates (MK880570 and MK880568) respectively as well as the Iranian isolates (MH215514) (MH215511) and (MH215510) and the Indian isolate (MF326947) and the Korean isolate (DQ054817) and the two American isolates (AF093491)and (L16997).

The most important result of the present study is the registration of three isolates of the *C. parvum* parasite that were isolated from children's stool who are of the age less than five and for the first time in Nineveh governorate and the local isolates were registered in the name of the researcher in the International Bank of genes with the numbers that belong to (PP669701) with the serial number *C.parvum* that belong to (PP669700, PP669699) of the parasite *C.hominis* (https://www.ncbi.nlm.nih.gov/nuccore/PP669699; https://www.ncbi.nlm.nih.gov/nuccore/PP669700;https://www.ncbi.nlm.nih.gov/nuccore/PP669701)

Conclusion

In conclusion, the microscopic examination of Mosul's children stool samples revealed a higher prevalence of *Cryptosporidium* infection among children than a molecule examination. Using the nPCR technique, low concentrations of the parasite's DNA were detected, demonstrating that the secondary amplification was more accurate than the primary amplification. In addition to that, isolation and characterization of three novel strains of the pathogenic sporozoan for the first time in Nineveh Governorate, and these new isolates have been registered under the researchers' names in the numbers that belong to (PP669701) with the serial number *C.parvum*that belong to (PP669700, PP669699) of the parasite *C.hominis* in National Center for Biotechnology Information (NCBI). The isolates of the parasite *C. hominis* demonstrated a 100% similarity with the Indian isolate and a 99.84% similarity with both the Australian isolate, the two Chinese isolates, the Ethiopian isolate, the isolates from the United Kingdom, as well as the Brazilian and Spanish isolates, while The third isolate of the parasite *C. parvum* showed a 100% similarity with the Iraqi isolate and a 99.84% similarity with the two Iraqi isolates, in addition to the Iranian isolates, and the Indian, Korean, and the two American isolates.

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Conflict of interest

The authors declare that they have no conflict of interest.

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