

A comparative study of the effectiveness of the *N-COWP* gene in diagnosing *Cryptosporidium parvum* using PCR and nested-PCR techniques

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ABSTRACT: This study investigated the prevalence and molecular characterization of the parasite *Cryptosporidium parvum* among 100 stool samples collected from patients attending Tikrit Teaching Hospital. The study lasted from October/ 2024 to July / 2025. By using modified Ziehl-Neelsen stain, the parasite was detected in 38% of the samples when examined microscopically. The parasite was then detected using conventional PCR and nested PCR. DNA was extracted from 100 stool samples and the results showed the presence of a DNA band in 93 samples out of a total of 100. Conventional PCR diagnosis was performed using *N-COWP* gene primers on 93 samples and the results showed that the number of samples positive for the gene was 22 samples (23.7%), while the number of samples positive for the *N-COWP* gene using nested PCR on 93 samples was 7 samples (15.21%).

Keywords: *Cryptosporidium parvum*, Conventional PCR, Nested PCR, *COWP*, *N-COWP*.



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1. INTRODUCTION

Diarrhea is the second leading cause of death in children, claiming the lives of 760,000 children each year. This disease may last for several days, resulting in the body being deprived of essential water and salts required for existence.[1]. Diarrhea is increasingly common due to bacteria, viruses, or parasites, most of which are spread through contaminated water. Intestinal parasitic infections account for a significant portion of the various types of diarrhea, the most important of which is *cryptosporidiosis* [2].

Cryptosporidium is a unicellular, obligate eukaryotic protozoan intestinal parasite belonging to the phylum Apicomplexa[3, 4]. It is a pathogen that infects the gastrointestinal tract of humans and animals and is widespread worldwide. The severity of the infection varies depending on the host type, the parasite strain, and the location of infection [5]. *Cryptosporidium* oocyst wall protein (*COWP*) gene in *Cryptosporidium parvum* to a specific gene that encodes a protein found in the wall of the parasite's oocyst. This gene belongs to a larger family known as oocyst wall proteins (*COWPs*) that play a key role in maintaining the structural integrity and survival of the oocyst in harsh environments [6, 7].

2. MATERIALS AND METHODS

2.1 Samples collection

Samples were collected from patients visiting Tikrit Teaching Hospital in Salah al-Din Governorate, after obtaining approval from the hospital administration to visit the hospital and collect samples from patients. Sample collection continued from October / 2024 to April / 2025. 100 stool samples were collected from patients aged (5 months to 15 years). The stool samples were examined microscopically after staining with Ziehl-Neelsen stain to detect the presence of oocysts. Samples that were positive for microscopic examination and suspected were placed in DNA Shield, a

preservative prepared by Zymo Research, where 100-200 mg of the sample was placed in 500 microliters of preservation solution and then frozen at -20°C until the study was conducted. They were then transferred to the central laboratory at Tikrit University for the purpose of conducting experiments.

2.2 Molecular detection

Molecular studies were conducted to detect *C. parvum* from fecal samples, using a specific gene. DNA was extracted from the collected samples using the Presto Minig DNA Extraction Kit, supplied by Geneai. Purity and concentration were measured at a wavelength of 260/280 nm. The DNA was then electrophoresed on a 1% agarose gel. This step is essential to ensure that the extracted DNA is sufficiently pure and does not undergo fragmentation that would compromise its efficacy in subsequent molecular applications.

2.3 Preparation of the Primers

All primers were prepared by Macrogen and were prepared as working solutions at a final concentration of 10 pmol/μl after dissolving them in nuclease-free water for gene amplification.

2.4 The primer

Conventional PCR employed primers for the *N-COWP* gene, which was 553 bp size, forward gene F: 5'-GTAGATAATGGAAGAGAGATTGTG-3' and reverse gene R: 5'-GGACTGAAATACAGGCATTATCTTG-3' [8].

In the nested PCR, two rounds of PCR were performed using *COWP* and *N-COWP*-specific primers, respectively. For the first step, primers targeting a *COWP* gene of 769 base pairs (forward: 5'-ACCGCTTCTCAACAACCATCTTGTCC-3'; reverse: 5' CGCACCTGTTCCCACTCAATGTAAACC-3') [9].

The second round used the *N-COWP* gene-specific primers with an expected 553 base pairs fragment length with the following forward from [8] gene 5'-GTAGATAATGGAAGAGAGATTGTG-3' and the reverse gene Cy9 5'-GGACTGAAATACAGGCATTATCTTG-3' [2]

2.5 Conventional PCR for the *NCOWP* gene

Detection of *C. parvum* at the molecular level was performed by the amplification of *N-COWP* gene of *C. parvum* using conventional PCR. The reaction mixture was prepared by adding (3 μl) template DNA, (0.75 μl) for each forward primer and reverse primer and (12.5 μl) of the prepared master mix. Finally, the reaction volume was increased to (25 μl) with (8 μl) of nuclease-free water. For amplification of *N-COWP* gene, the thermal program was: 1 cycle (95°C) for 5 min (initial denaturation). This was followed by 35 cycles of 30 seconds of denaturation at (94 °C), 30 seconds of annealing at (55 °C), and 30 seconds of elongation at (72 °C). Subsequently, reaction was completion with a final elongation at (72 °C) for 5 minutes in one cycle. Electrophoresis of samples on a 2% agarose gel was performed to confirm that the reaction worked and expected bands were generated.

2.6 Nested PCR for the *N-COWP* gene

Detection of *Cryptosporidium parvum* was done by Nested-PCR by using primers for the *COWP* and the *N-COWP* genes, in two rounds: The final reaction mixture was made up by adding (3 μL) of template DNA, 0.75 μL of each forward and reverse primers, and (12.5 μL) prepared master mix. The temperature conditions for the amplification of the *COWP* gene were set as follows: One initial denaturation at 95 °C for 5 minutes, followed by one cycle and then 30 cycles consisting of denaturation at 95 °C for 30 seconds, annealing at 58 °C for 30 seconds, and elongation at 72 °C for 30 seconds. In the final step of the reaction elongation, a single cycle was performed at 72°C for 5 minutes. In the second stage of Nested PCR the result of the first cycle was applied and the primers of the *N-COWP* gene were used. Product of the first reaction was then added (3 μL) to a new tube containing (0.75 μL) for each forward and reverse primer and (12.5 μL) of master mix, generating a reaction mixture. Finally, (8 μL) of nuclease-free water was added to increase the total reaction volume to (25 μL). The thermal settings for the thermal amplification of the *N-COWP* gene comprised an initial denaturation at 95°C for 5 minutes, followed by one cycle. This was succeeded by 30 cycles including denaturation at 94 °C for 30 seconds, annealing at 55 °C for 30 seconds, and elongation at 72 °C for 30 seconds. The reaction ended with a

solitary 5-minute elongation phase at 72°C. To confirm the reaction's success, the samples underwent electrophoresis in a 2% agarose gel, yielding data on the formation of the anticipated bands.

3. RESULTS AND DISCUSSION

Results of the study by microscopic examination of modified Ziehl-Neelsen stain, Samples give positive result 38% and 62% negative result, as shown in Table (1).

Table 1- *C. parvum* infection rates using Ziehl-Neelsen stain

Examined sample	Positive result	percentage	Negative result	Percentage
100	38	38%	62	62%

Stool samples containing *Cryptosporidium* oocysts were examined under microscope following staining, and the oocysts, which were round to oval and stained purple and red in color, were distinguished from the rest of the stool, which appeared blue. Results of the present study are consistent with a study done in Mosul, where 35% of 200 stool samples were found to be infected. [10]. Our study found higher prevalence than other study in same organ such as detection of 8.2% out of 300 stool samples collected from Samarra General Hospital [11]. Such prevalence of the parasite currently may be a threatening health hazard for people in the governorate. The parasite is likely spread through the consumption of contaminated water [12]), animal husbandry in close proximity to human habitation, use of animal faeces as fertiliser for vegetables, and direct contact with infected animals (such as sheep and cows) that shed oocysts in their stools [13]. We amplified the DNA extracted from 100 stool samples and found that extraction succeeded in 93 samples, while 7 samples showed insufficient quality DNA (Table 1). The extracted bands were electrophoresed and the results (Fig (1)) that are shown the DNA genomic DNA bands extracted from samples stool.

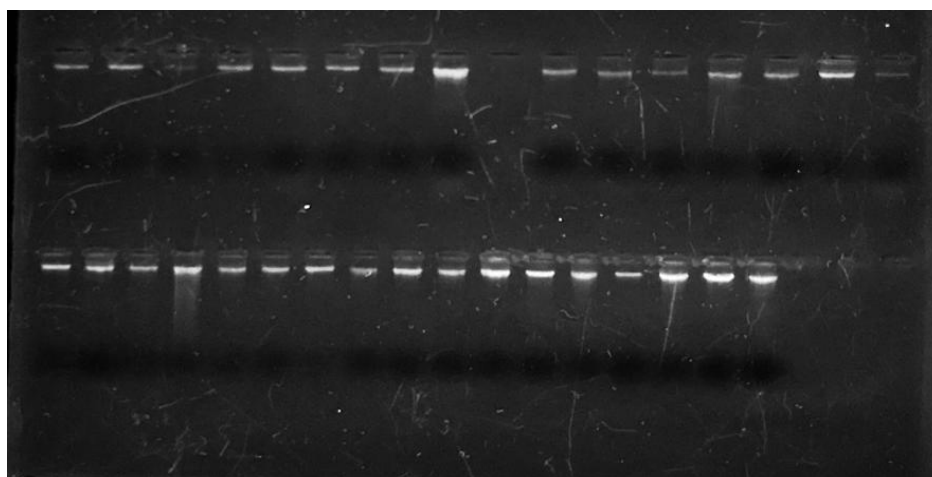


FIGURE 1. Genomic DNA packages of the samples after extraction and migration on a 1% agarose gel using the safe DNA dye Red Safe.

The results of a study conducted by [14] in Iraq to extract the parasite's DNA showed a success rate of 66.6%, which is consistent with the results of the current study. However, it is not consistent with a study conducted in Turkey in which the parasite's DNA was extracted at a rate of 4.6% [15]. The efficiency of molecular detection of *Cryptosporidium parvum* depends largely on the quality of the DNA extraction, as the presence of inhibitors in the DNA directly affects the PCR results. Conventional PCR diagnosis was performed using *N-COWP* gene primers on 93 samples and the results showed that the number of samples positive for the gene was 22 samples (23.7%) with the appearance of a band with a size of 533 base pairs compared to the standard size indicator Ladder DNA as shown in the Figure(2).

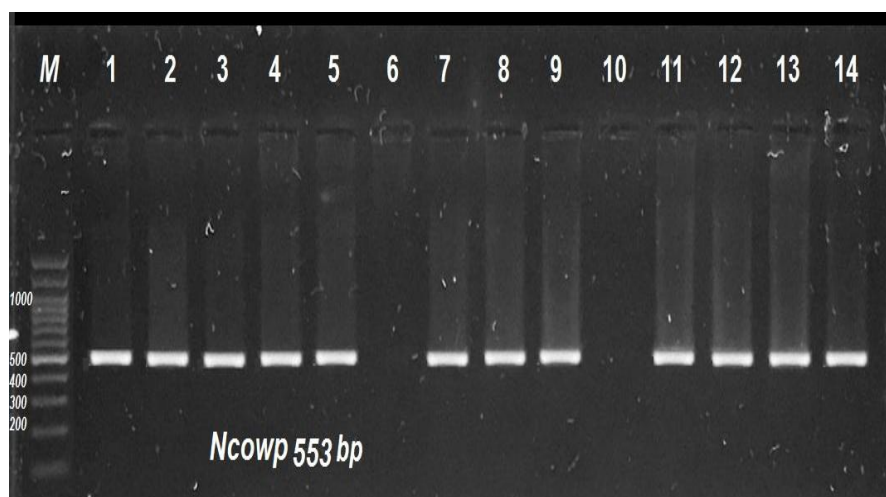


FIGURE 2.- The PCR reaction result for the *N-COWP* gene for samples (1, 2, 3, 4, 5, 7, 8, 9, 11, 12, 13, 14) represent the positive samples. The molecular size was 553 base pairs. Electrophoresis was carried out in a 2% agarose gel and the size of the standard indicator was 100 base pairs. Samples (10, 6) represent the negative samples.

The *N COWP* gene is part of the *COWP* gene family, which are reliable molecular targets for parasite diagnosis due to their high specificity and consistent expression throughout the parasite's maturation stages [16]. The results of the current study are consistent with a study conducted in England, where the infection rate was 23.1% of 2,057 samples examined [9]. Nested PCR using *COWP* gene primers in the first round was performed on 93 samples. The results showed the appearance of bands measuring 769 base pairs compared to the standard size guide, ladder DNA, in 46 samples, as shown in Figure.(3)

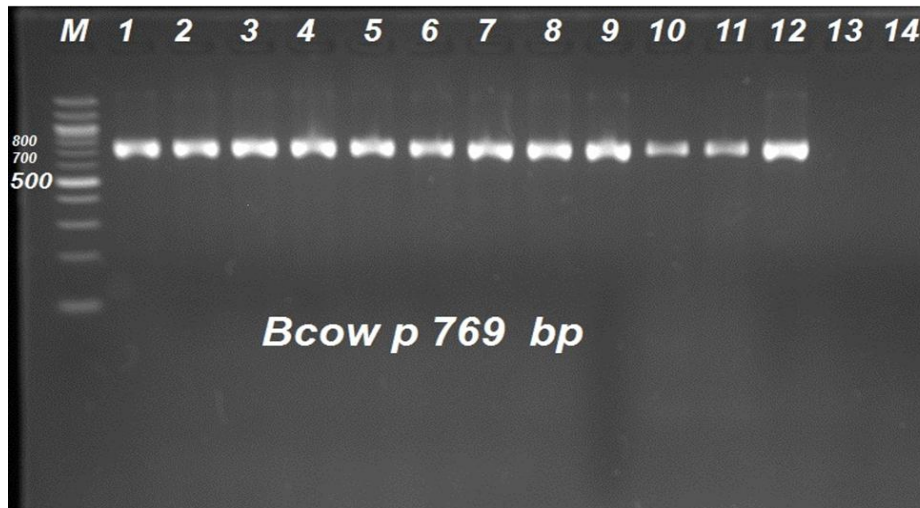


FIGURE 3.- The result of the first round of the Nested PCR reaction for the *COWP* gene for samples (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12) represent the positive samples and the size of the gene was 769 base pairs. Electrophoresis was carried out in a 2% agarose gel and the size of the standard size indicator was 100 base pairs. As for samples (14, 13), they represent the negative samples.

Nested PCR was performed using *NCOWP* gene primers in the second round on the positive samples from the first round (46 samples). The results showed the appearance of bands with a size of 553 compared to the standard size indicator DNA ladder in 7 samples.



FIGURE 4. The result of the second round of the Nested PCR reaction for the *N-COWP* gene for samples (1, 2, 10) represents the positive samples. The size of the gene was 553 base pairs. Electrophoresis was carried out in a 2% agarose gel and the size of the standard size indicator was 100 base pairs. As for samples (4, 3, 5, 6, 7, 8, 9, 11, 12, 13, 14, 15, 16, 17, 18) they represent the negative samples.

The current study is consistent with a study conducted in Karbala [17], where the incidence of *Cryptosporidium* infection using the Nested PCR technique was 5.6%.

The current study demonstrated that the use of conventional PCR was more effective in diagnosing *C. parvum* infection than nested PCR. This may be due to the compatibility of the primers used with the gene versions in local human samples, resulting in more efficient amplification, even though nested PCR is typically more sensitive in theory.

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