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Epidemiological and Molecular Study of the *Cryptosporidium* spp. in Some Fish Species Found in the Markets of AL-Diwaniyah Province

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Abstract

Introduction: *Cryptosporidium* spp.is an intestinal protozoan that is transmitted through water and food, and causes severe diarrhea, Cryptosporidiosis is not limited to humans, as there are many species of *Cryptosporidium* that infect a wide range of vertebrate hosts such as mammals of all species, fish, reptiles and birds.

Methods: In the current study, during the period between September 2025 and February 2026, 180 fish species were collected from the markets of Al-Diwaniyah Province, 60 fish of each species, which included both farmed fish such as *Cyprinus carpio* and non-farmed fish such as *Planiliza abu* and *Acanthopagrus latus*, with the aim of isolating the parasite *Cryptosporidium* spp., identifying it microscopically and molecularly, and determining the most widespread species through genetic sequencing and drawing its phylogenetic tree.

Results: The overall infection rate of the three fish species with the *Cryptosporidium* spp. was 14%, with the highest infection rate recorded in *Acanthopagrus latus* at 18.3%, followed by the infection rate of *Cyprinus carpio* at 11.66%, while *Planiliza abu* recorded the lowest infection rate at 10%. The highest infection rate was recorded during the months of moderate temperature, reaching 13.03% in October, and no infection was recorded during February.The results of using Nested polymerase chain reaction N-PCR technology to examine 24 fecal samples collected from the fish included in the study, which gave a positive result by direct smear method, indicated that the percentage of the SSU rRNA gene with a molecular weight of 533 bp, specific to the *Cryptosporidium* spp., reached 50%, with 12 samples.DNA sequencing technology was also used, relying on the same gene to analyze the phylogenetic tree of the local isolates. MEGA 6 software was used to determine the degree of similarity between the fish isolates and the international isolates registered with the NCBI. The results of the phylogenetic tree analysis of the local isolates revealed five species: *Cryptosporidium molnari*, *Cryptosporidium bollandi*, *Cryptosporidium xiaoi*, *Cryptosporidium parvum*, and *Cryptosporidium hominis*. The samples were registered in the international gene bank under serial numbers PZ120392, PZ120393, PZ120394, PZ120395, PZ120396, PZ120397, PZ120398, PZ120399, PZ120400, PZ120401, PZ120402, PZ120403.The registration of *Cryptosporidium molnari*, *Cryptosporidium bollandi*, and *Cryptosporidium xiaoi* marks their first recorded presence in Iraqi fish.

Conclusion: This genetic diversity in *Cryptosporidium* reflects the parasite's ability to adapt and spread in diverse aquatic environments. This serves as a vital indicator of water pollution levels and foreshadows potential health risks that may be transmitted to humans.

Keywords: Fish, *Cryptosporidium*, N-PCR, SSU rRNA gene, Iraq

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Introduction

Aquaculture is a crucial and important element in global food security in light of declining wild fish stocks due to overfishing and environmental degradation, as fish farming provides a reliable and sustainable source of protein-rich food and helps reduce pressure on natural ecosystems (1, 2). Fish of all species face many biotic and abiotic factors that undermine their immune response, jeopardizing their health and making them susceptible to various diseases (3). This leads to frequent disease outbreaks and significant economic consequences within the industry, which encompasses both farmed and wild fish populations (4). *Cryptosporidium* spp.is an intestinal protozoan that is transmitted through water and food, and causes severe diarrhea, especially in young children and people with weakened immune systems. Cryptosporidiosis is not limited to humans, as there are many species of *Cryptosporidium* that infect a wide range of vertebrate hosts such as mammals of all species, fish, reptiles and birds (5). *Cryptosporidium* has been detected in various fish species, including wild, farmed, and ornamental freshwater species. Characterization of *Cryptosporidium* species in fish relies primarily on morphological description and, most importantly, on molecular methods, which are essential for identifying species and genotypes (6).

Five species have been identified and classified as fish-specific, including *Cryptosporidium molnari*, which has

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been recorded in the gilt -headed wild bream (*Sparus aurata*) and *Cryptosporidium scophthalmi*, which was isolated from the turbot *Scophthalmus maximus* ,and *Cryptosporidium huwii* from the goby fish *Poecilia reticulate*,*Puntigrus tetrazon* ,*Paracheirodoon innesi* and *Astronotus ocellatus* , *Cryptosporidium bollandi* and *Cryptosporidium abrahamseni* Both were isolated from red-eyed tetra fish (*Moenkhausia sanctaefilomena* (7-13). Non-fish-host-specific species and genetic patterns have been identified, as the high environmental resistance of oocysts allows aquatic environments to be contaminated by oocysts originating from terrestrial hosts, typically via fecal-contaminated sewage or agricultural runoff, which then accumulate in marine organisms such as shellfish and fish (14), Zoonotic species of parasites such as *C. parvum*, and anthropogenic species such as *C. hominis*, *C. xiaoi* and *C. scrofarum* were also identified (15).

The presence of *Cryptosporium* in edible fish, however, indicates a potential risk to public health. This parasite has already been detected in fish fillets, suggesting a possible risk of cross-contamination during evisceration. Furthermore, some species of fish are consumed in certain countries without evisceration, sometimes raw or undercooked (16, 17) .Given the lack of previous studies on the *Cryptosporidium* spp. in fish found in Al-Diwaniyah Province, the current study was designed to characterize the *Cryptosporidium* using conventional and molecular methods to estimate genetic similarity within and between it and to understand its evolutionary relationship.

Material and Methods

Samples collection

During the period from September 2025 to February 2026, 180 fish species were collected from local markets in Al-Diwaniyah province, including the city center and some districts, with 60 fish of each species. These included both farmed fish, such as *Cyprinus carpio*, and wild-caught fish, which consisted of only two species *Acanthopagrus latus* and *Planiliza abu*. A separate information form was created for each fish species, recording the fish species, the date of collection, and the location of collection for each sample. The fish were dissected by making a longitudinal incision from the head to the anus. The digestive tract was separated from the rest of the body and then opened longitudinally with scissors (18). Light smears of intestinal contents were taken; a sample the size of a matchhead was placed on a glass slide, mixed with a small amount of physiological saline, spread over the entire surface of the slide, and left to air dry for 10 minutes, the prepared swabs were then fixed by immersing them in a concentrated methanol solution for 5 minutes and then left to dry at room temperature. The slide was then immersed in concentrated carbol fuchsin stain in jars and heated by placing it in an oven at 60°C for 10-15 minutes until the stain began to evaporate. The prepared smear was then washed, bleached with acidic alcohol for 30 seconds, stained with methyl blue for two minutes, washed with a water stream, and left to dry. It was then examined under a light microscope at magnifications of 10x and 40x to detect the oocysts of the *Cryptosporidium* spp.(19). At the same time, a portion of the samples from which the prepared slides appeared purple or red was stored at -20°C for molecular study.

Molecular Study

DNA was extracted according to the method described by Certad et al. [16]. The concentration and purity of the extracted DNA were then measured using a nanodrop spectrophotometer by reading the absorbance at a wavelength of 260-280 nm. The extracted DNA was then stored at 20°C in a refrigerator until N-PCR testing. The extracted DNA was amplified using primers specifically designed to amplify the 18S rRNA gene responsible for the diagnosis of the *Cryptosporidium* spp. parasite. The primers were supplied by the Korean company Bioneer, first round of PCR was performed using a pair of PCR SSUrRNA gene **F1**: 5'-ATTGGAGGGCAAGTCTGGTG-3'and **R1**: 5' TCCACCAACTAAGAACGGCC-3' then Nested-PCR were performed with a second pair of primers of SSUrRNA gene **F2**:5'- CGCGGTAATTCCAGCTCCAA-3'and **R2**: 5' TCAG CCT TG CGACCATACTC-3'. The PCR mixture was then transferred to a PCR thermocycler under thermocycler conditions. PCR result was then read by electrophoresis using a 1.5% agarose gel. The gel containing the PCR product was then examined using UV light to determine the product with a calibration ladder.

The PCR product was sent to Bioneer in South Korea for DNA sequencing using the AB DNA sequencing system. The NCBI-Genbank-Primer-Blast database software and Mega Phylogenetic tree analysis software were used to analyze the PCR sequencing results and then construct a phylogenetic tree for the species included in this study.

Statistics Analysis

The data from the current study were analyzed using the SPSS statistical package, employing the chi-square test and Fisher's exact test at $P \leq 0.05$.

Results

Microscopic examination

The results of the study, which examined 180 fecal samples taken from three different species of fish, *Cyprinus carpio*, *Planiliza abu* and *Acanthopagrus latus*, using modified Ziehl-Neelsen stain, showed that the overall infection rate with oocysts of *Cryptosporidium* ssp.(Figure 1) was 13%, with only 24 samples. The highest infection rate was recorded in *Acanthopagrus latus* at 18.3%, with 11 samples, followed by the infection rate of *Cyprinus carpio* at 11.66%, with 7 samples, while *Planiliza abu* recorded the lowest infection rate at 10%, with only 6 samples. The results of the statistical analysis indicated no significant differences between the three species of fish at the probability level $P \leq 0.05$, as shown in Table 1.

The results of the current study showed that the infection rate of fish with the *Cryptosporum* spp. was

high in October, reaching 31.03%, compared to the infection rate recorded in the rest of the year. The lowest infection rate was recorded in January, at 6.25%, No *Cryptosporidium* oocysts were observed during February .Statistical analysis showed a no significant difference in the infection rate, with a probability level of $P \leq 0.05$. (Table 2)

Molecular study

The results of using polymerase chain reaction (PCR) to examine 24 fecal samples collected from the fish included in the study, which yielded a positive result by direct smear method, indicated that the percentage of the SSU rRNA gene with a molecular weight of 533 bp, specific to the *Cryptosporidium* spp.(Figure 2), reached 50%, distributed across 12 samples: 5 samples from *Cyprinus carpio* 71% ,4 samples from *Planiliza abu* 66.6%, and 3 samples from *Acanthopagrus latus* 27% (Table 3) .The results of the statistical analysis indicated no significant differences in the percentage of SSU rRNA gene present in fish fecal samples using the direct smear method and N-PCR technique under a probability level of $P \leq 0.05$, as shown in the table.

DNA Sequence results

The DNA sequencing method was carried out to genetic species typing analysis based on small subunit ribosomal RNA gene in *Cryptosporidium* fish isolates and NCBI-Genbank related *Cryptosporidium* species isolates. The phylogenetic tree was constructed the Neighbor-Joining method in (MEGA 6.0 version). *Cryptosporidium* spp. *Acanthopagrus latus* No.1-No.3 isolate showed closed related to NCBI-BLAST *Cryptosporidium molnari* and *Cryptosporidium bollandi*. *Cryptosporidium* spp. *Planiliza abu* No.1-No.4 isolate showed closed related to NCBI-BLAST *Cryptosporidium xiaoi*, *Cryptosporidium molnari*, *Cryptosporidium hominis* and *Cryptosporidium parvum*. *Cryptosporidium* spp. *Cyprinus carpio* No.1-No.5 isolate showed closed related to NCBI-BLAST *Cryptosporidium molnari*, *Cryptosporidium bollandi*, and *Cryptosporidium parvum*. at total genetic changes (0.04-0.01%) as showed in (Figure 3). The homology sequence identity between fish *Cryptosporidium* spp. isolates and NCBI-Genbank related *Cryptosporidium* species isolate were showed genetic homology sequence identity ranged from (99.73-99.85%).

Finally, local *Cryptosporidium* fish isolates were submitted into NCBI Genbank and identified by accession numbers PZ120392, PZ120393, PZ120394, PZ120395, PZ120396, PZ120397, PZ120398, PZ120399, PZ120400, PZ120401, PZ120402, PZ120403.

Discussion

Cryptosporidium spp.is an intestinal protozoan that has received widespread attention, there are many international and local studies that have examined the oocysts of this parasite in various hosts such as mammals of all species, reptiles, birds and fish (20).

Table 1. Total numbers and infection rates of *Cryptosporidium* spp. in fishes of Al-Diwaniyah province

Species of fish	Number examined	Number infected	%
Cyprinus carpio	60	7	11.66
Planiliza abu	60	6	10
Acanthopagrus latus	60	11	18.3
Total	180	24	13

Table 2. Monthly distribution and infection rates of *Cryptosporidium* spp. in fishes

Months	Number examined	Number infected	%
Sept	27	6	22.22
Oct	29	9	31.03
Nov	29	4	13.97
Dec	31	3	9.67
Jan	32	2	6.25
Feb	32	0	0
Total	180	24	13

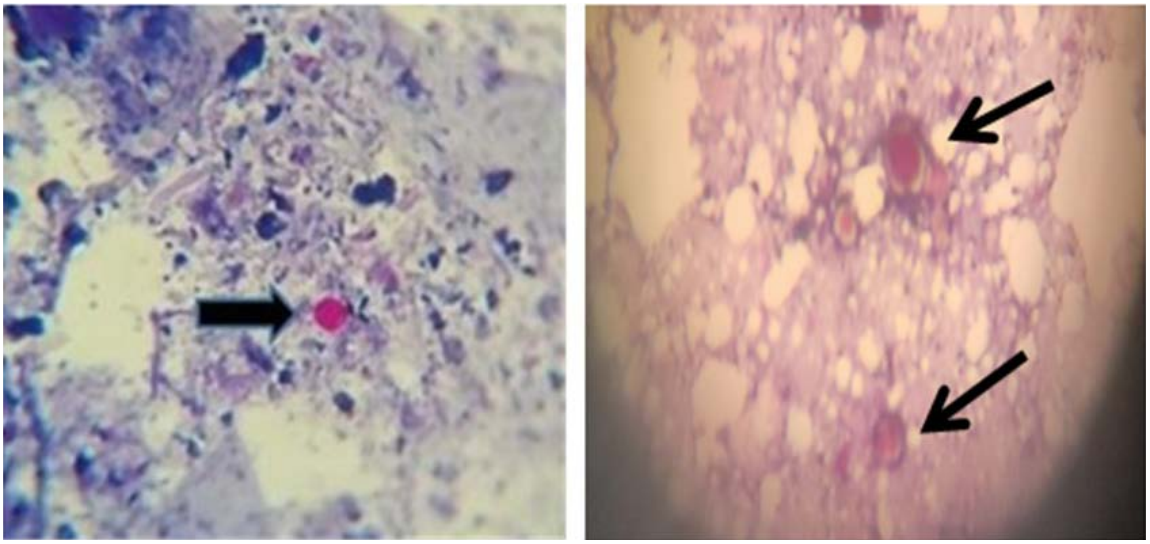

Figure 1. Oocysts of *Cryptosporidium* spp. isolated from fish (1000x)

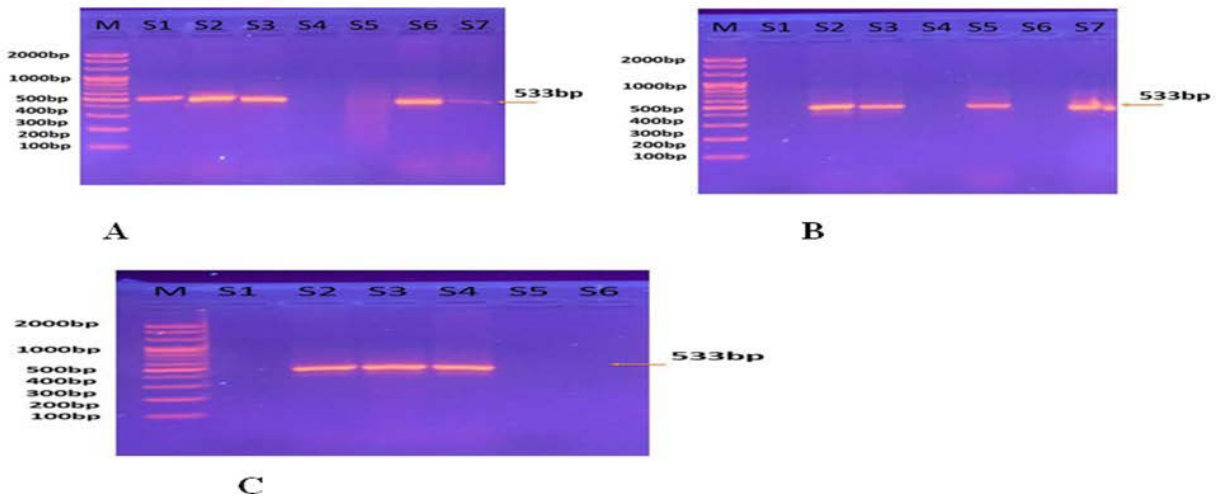

Figure 2. N-PCR amplification of the *Cryptosporidium* 18S rRNA gene (~533 bp) is demonstrated by agarose gel electrophoresis (1.5%).Lane M is a 2000 bp DNA ladder; (A) showed Positive from *Cyprinus carpio* (B) showed Positive from *Planiliza abu* (C) showed Positive *Acanthopagrus latus*

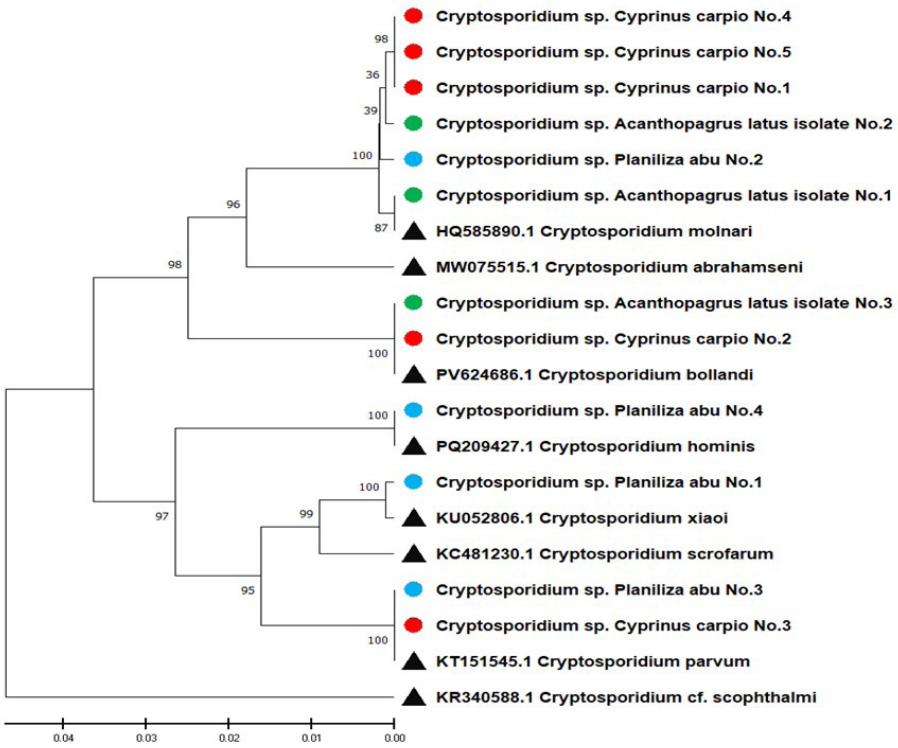

Figure 3. Phylogenetic tree analysis of *Cryptosporidium* spp

Table 3. Overall infection rate with the Cryptosporidium spp. in the studied fish, based on N-PCR

Species of fish	Positive samples using the direct swab	Positive samples N-PCR	%
Cyprinus carpio	7	5	71
Planiliza abu	6	4	66.6
Acanthopagrus latus	11	3	27
Total	24	11	50

The results of the current study showed that all three fish species were infected with the oocysts of the *Cryptosporidium* spp. at a total rate of 13% as determined by microscopic examination. This percentage was higher than the 5.5% recorded by Obead & Alhaboubi (21) in Baghdad for fish, including *Cyprinus Carpio, Silurus triostegus, Planiliza abu* . , infection rates may be due to factors such as fish species, age, health status, and farming system. Certad et al. (22), in a study of marine fish intended for human consumption, indicated higher infection rates than other species, larger and heavier fish were also more likely to be positive for the *Cryptosporidium* spp. due to longer exposure to contamination and the accumulation of oocysts in their intestines. A monthly variation in the infection rates *of Cryptosporidium* was also observed, with the highest infection rate occurring during the cooler months, reaching 31.03% in October, and the lowest in January, reaching 6.25 %, no infections were observed during February. This may be explained by the fact that the relatively moderate temperatures, high relative humidity, and availability of water during spring contribute to the

spread of oocysts. Furthermore, spring is a breeding season for many animals, increasing their activity and thus their exposure to sources of infection. Insects may also play a significant role in increasing infection rates, as their proliferation during spring and autumn can facilitate the mechanical transport of oocysts over greater distances, leading to a wider spread of the parasite.

The results of using N-PCR to examine 24 fecal samples collected from the fish included in the study, which gave a positive result by direct smear method, indicated that the percentage of the SSUrRNA gene with a molecular weight of 533 bp, belonging to the *Cryptosporidium* spp. parasite, reached (50%), with only 12 of the samples distributed. This percentage is higher than the percentage recorded by (21) in his study using the N-PCR method on fish, which reached 28.5%. The positive PCR result in only 12 out of 24 samples that showed positive results under microscopic examination can be attributed to either similarity to the oocysts of other parasites such as *microsporidia*. or to an error during DNA preparation and extraction.

Multiple sequence alignment analysis of the N-PCR products of the SSU rRNA gene of the *Cryptosporidium* spp. showed that all 12 samples were 99.85-99.73% identical to five *Cryptosporidium* species registered with the National Center for Biotechnology Information. *Acanthopagrus latus* isolates PZ120392 and PZ120393 showed genetic affinity with the global isolate recorded by. (23), representing *Cryptosporidium molnari* isolated from fish in Australia. Isolate PZ120394 showed genetic affinity with the Thailand isolate recorded in pearl gentian groupers by (24), representing *Cryptosporidium bolland*. Regarding *Planiliza abu* isolate that accession number PZ120395, it showed a close genetic relationship with the Chinese isolate previously recorded by (25), which represents *Cryptosporidium xiaoi* isolated from sheep, while isolate PZ120396 showed a genetic similarity with the previously recorded Australian isolate by(23), representing *Cryptosporidium molnari* . As for isolation PZ120397, it was closer to the Iraqi isolation recorded by Marhoon & Jasim(26), which isolated from birds and represents *Cryptosporidium parvum*.While isolate PZ120398 was genetically related to the Iraqi isolate recorded by AL-Dabbagh& Alhadad(27), which was isolated from humans and represents *Cryptosporidium hominis* . As for the *Cyprinus carpio* , isolates PZ120399**,** PZ120402**,** and PZ120403 showed a close relationship to the Australian isolate previously recorded by Barugahar *et al.*(23), representing *Cryptosporidium molnari,* Isolate PZ120400 showed a close relationship with the *Cryptosporidium bolland* previously recorded by Jantrakajorn *et al.* (24), However, isolate PZ120401 was closely related to the *C.parvum* (28-30).

Conclusion

Our current study reveals genetic diversity in the *Cryptosporidium*, with five different species identified, three of which (*Cryptosporidium molnari, Cryptosporidium bollandi, and Cryptosporidium xiaoi*) were first recorded in Iraq and registered in the gene bank.This genetic diversity in *Cryptosporidium* reflects the parasite's ability to adapt and spread in diverse aquatic environments. This serves as a vital indicator of water pollution levels and foreshadows potential health risks that may be transmitted to humans.

Authors' Contribution

Conceptualization: Sadiya Aziz Anah and Elham Adai Hani Hamid, Elham Adai Hani Hamid
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Supervision: Sadiya Aziz Anah and Elham Adai Hani Hamid
Validation: Elham Adai Hani Hamid and Sadiya Aziz Anah
Visualization: Elham Adai Hani Hamid and Sadiya Aziz Anah
Writing–original draft:Elham Adai Hani Hamid and Sadiya Aziz Anah
Writing–review & editing:All authors

Competing Interests

The authors declare no conflict of interest.

Ethical Approval

Not applicable.

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