The incident and Molecular Identification of the Cryptosporidium Parvum in Cultivated Carp Fish (Cyprinus carpio) in Baghdad

Noor Majed Obead[1], Amer Rasool Alhaboubi[1]

[1]*Department of Parasitology, College of veterinary medicine, University of Baghdad*

KEYWORDS

Cryptosporidium, fish, Iraq, nested PCR. SSU

ABSTRACT

The current study aimed to investigate the occurrence of Cryptosporidium species by traditional and molecular methods in freshwater fish in Baghdad city. A convenient number of stomach and intestinal continent samples were collected from three types of freshwater fish from different areas of Baghdad city from November, 2023 to April, 2024. All samples were examined phenotypically using a modified Ziehl-Neelsen stain and genotypically (nested PCR technique) based on a partial sequence of 18SrRNA genes with sequencing and phylogenetic tree analysis. The overall infection rate of fish with C. parvum was 5.5% (10/180). The common carp (Cyprinus carpio) was the only fish type infected, with a rate of 16.66% (10 60). January and February had the highest infection rate, 3.33%. The molecular identification and sequencing confirmed the infections with C. parvum. Molecular speciation of the parasite confirmed microscopical primary identification results. This study suggests that fish became a new source for transmission to humans and livestock.

1. Introduction

Fish and seafood industries are developing and maintaining their productivity to meet part of the requirements for food availability, with the increase of the world's population and the constant shortage of food resources (Boyd and Davis, 2022). Aquatic life is affected by negative human activities that harm the aquatic environment (Mustafa et al., 2024). Water is one of the important media in transporting disease causes, which sometimes returns to serious epidemics (Stec et al., 2022). Infection with the protozoan parasite (Cryptosporidium) is contracted by ingestion of viable oocysts that are shed in the faeces of the host (Fayer et al., 1997), the common symptom of diarrhea mostly in children and immunocom-promised people. It infects a wide range of vertebrates and murrains including fish (Ryan et al., 2014). In fish, they have primarily confirmed the presence of Cryptosporidium nasoris in the fish intestines (Hoober et al., 1981). three species of this protozoal parasite are reported in fish: Cryptosporedum monarii (Sitjà-Bobadilla and Álvarez-Pellitero(2003), C. scopthlmi (Alvarez-Pellitero et al., 2004), and C. huwi (Ryan and Hijjawi, 2015). Infection is usually found in fresh and aquatic environments and could transmitted through fish consumption. It recently considered as a potential food fish-borne risk factor. Fish found to harbor host- zoonotic species like C.parvum, C. hominis through detecting oocysts nucleic acids of the parasite (Golomazou et al., 2021; Moratal et al., 2022). Parasite located either in the stomach or the intestine of fish and suggested to causing described as causing pathological effects in fish (Alvarez-Pellitero et al., 2004; Couso-Pérez et al., 2022) and an elevated in mortality rate, mostly in small fish (Yang et al., 2013). However, several Iraqi research on the identification and prevalence in humans and different domestic and wild hosts (Yaqoob et al., 2004; Altamimi et al., 2020), very limited studies were conducted or reported the detection of Cryptosporidium spp. in fish (Al-Taee, 2008; Ali and Al-Mahmood, 2009; Al-Sagur et al., 2015; Hussein and Mahmood, 2022). Among many protozoal and helminths screening in different host in Iraq (Abid et al., 2023; Fadel et al., 2023). No molecular identification research available on Cryptosporidium spp. in fish hosts in Iraq. The present investigation will be performed by traditional and DNA sequencing phylogenetic analysis.

2. Methodology

Ethical approval

This study was approved according to research guidelines and laboratory rules regulations of the College of Veterinary Medicine in the University of Baghdad. P.G 1296 on July 9th, 2024.

Samples collection

A content of 180 stomach and intestinal samples belonged either to wild or cultured fish in different areas of Baghdad city (Amiria, Al Radwaniyah, Al-Shawaka), during the period from November 1st 2023 to April 30th 2024. A longitudinal incision in the ventral midline extending from the head to the end of the fish, then another incision that extends from the end of the slit until the cover of the gills. The stomach and intestines were isolated and placed in a Petri dish for Sheather's sugar floating solution and modified Ziehl–Neelsen Stain with microscopic examination (X100) (Beaver and Jung ,1985). Samples collected in screw-capped stool containers with the information fish species, months, and collecting areas transported in cold bags to the department of Parasitology laboratory, COVM/, University of Baghdad

DNA Isolations

Presto™ Stool DNA Extraction Kit was used for stool DNA extraction from 40 fish samples and it was quantified by Nanodrop Spectrophotometer (Thermo, USA) with concentration ranged between 5-130 ng/µl.

Amplifications and primers

An amplification primer applied herein included A primarily and nested reaction PCR primers for detection Cryptosporidium spp. Small Subunit Ribosomal RNA gene. (Table .1).

Table 1: Oligonucleotides primers used for PCR reactions

Primers	Sequence 5'-3'		Product size
1st primer	SSU-F1	TTCTAGAGCTAATACATGCG	1325bp
	SSU-R1	CCCTAATCCTTCGAAACAGGA	
2nd primer	SSU-F2	GGAAGGGTTGTATTTATTAGATAAAG	815bp
	SSU-R2	AAGGAGTAAGGAACAACCTCCA	

The partial SSU rRNA gene will amplify from the genomic DNA of each sample by PCR by using two forward and reverse primers set (Xiao et al., 1999).

PCR kit components

The GoTaq® PCR Green Master Mix (Promega, USA) was applied for the primary and secondary PCR reactions. A standard volume of a total of 25µl in a PCR tube. 12.5µl of the 2X was added with 5 µl of the template. A 2µl of each primer with a concentration of 10 pmol was added, and finally, the reaction volume was with 3.5µl of Nuclease-Free water. Thermocycler conditions begin with denaturation (95°C) for 5 min, then followed by 35 cycles of denaturation (95°C) for 30sec. DNA annealing (55°C) for 30sec, extension at 72°C ,(2 min.) , and finally extension at 72°C (5min.) was followed by a hold on phase at 4°C. In the nested reaction round, 12.5µl of the 2x master mix is added to1µl of primary PCR product. Then, one µl each of 18SrRNA gene forward and reverse primers 10pmol (inner set) and 9.5µl from the water of PCR complement the reaction volume. The nested amplification protocol was used the same as above, except for the annealing temp at 57°C. All amplicons were electrophoresis through a 1.5%agarose gel alongside a 100 bp DNA marker (iNtRON, Korea), with ethidium bromide staining and ultraviolet transilluminator for visualization

DNA sequencing and phylogenetic analysis

Randomly amplicons of PCR (5 samples) were selected for sequenced at Macrogen Inc. in South Korea. The result data analysis was performed using the NCBI web. Nucleotide -BLAST® program to calculate significance and compare to the correspond sequences (Altschul et al., 1990). The constructed phylogenetic tree was carried out by using the software (MEGA 11 version) (Gharban et al., 2023).

Multiple sequence DNA fragments alignment using the ClustalW alignment tool and the genetic evolutionary distances were assessed by the UPGMA tree construct on the Max Composite Likelihood method. The tree includes all sequences with a polar clad gram using the genetic tree.

Statistical Analysis

The connection between this parasite and each of the researcher factors using the Chi-square test. Microsoft Excel 365 constructed different to be significant at levels $P \leq 0.05$ and $P \leq 0.01$ (Gharban et al., 2024).

3. Result and Discussion

Morphological examination

Morphological characteristics of *Cryptosporidium* oocysts appeared as spheroidal or ovoidal parasitic stage with a diameter ranging from 4.6-5.5 X 3.8-4.7µm, surrounded by a red circular with hallow shape and blue backdrop **(Figure 1).**

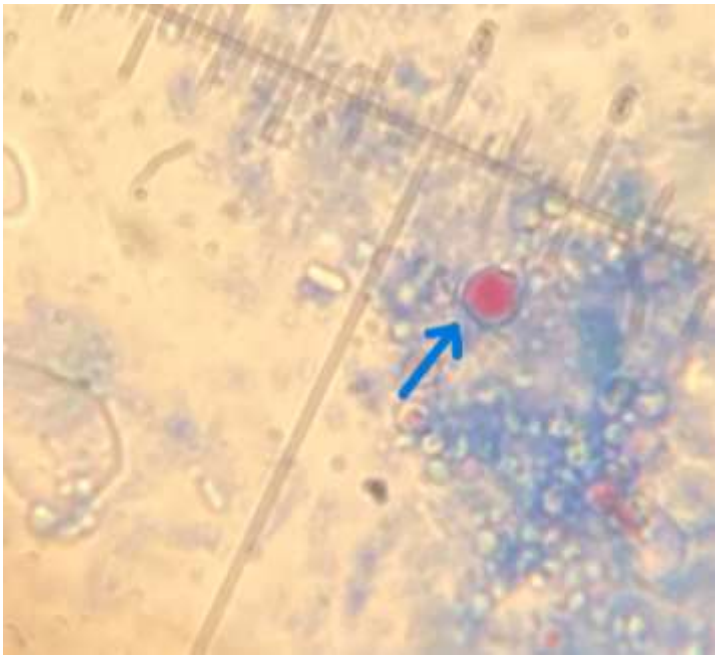

Figure 1: The fish's small intestine is thinly walled and destined by gas collection and watery fluids

Although microscopic examination of fish gastric and intestinal revealed the presence *of Cryptosporidium* oocyst. It is difficult to determine the types because they are similar in phenotypic characteristics. Therefore, Molecular analysis is critical for species differentiation. For example, *C. molnari* infects fish. 4.5 × 4.7 µm Similar in size to zoonotic species *C. parvum* that infects Cows, sheep, and goats (4.5 × 5 µm) and *C. hominis* infect humans and sheep 4.5 × 5.5 µ (Taylor *et al.,* 2016). Mehlhorn (2016) reported that all types of the *cryptosporidium* parasite ranged from 5 - 6 µm except *C. muris* larger than seven µm.

The study assessed the infection rates of *Cryptosporidium* in three fish species: *Cyprinus carpio, Silurus triostegus, and Planiliza abu*. showed in (Table 2). The fish (*Cyprinus carpio*) had the infection rate at 16.66%, while fish of the other types (*Silurus triostegus* and *Planiliza abu)* had no infections recorded. The overall infection rate across all species was 5.55%. The chi-square test for the species infection rates indicated a p-value of 0.00025, statistically significant difference in infection rates among the species.

Table 2: *Cryptosporidium* infection rate according to the fish species

Species	Total Fish	Infected Fish	Infection Rate (%)
Cyprinus carpio	60	10	16.67%

Silurus triostegus	60	0	0
Planiliza abu	60	0	0
Total	180	10	5.56
p-value			**0.000025***

*** Statistically significant (p ≤ 0.05)**

Monthly Distribution of Infected Fish with Cryptosporidium

The distribution of infections across the six months stated distinctive patterns, as shown in Table 3. The highest number of infections occurred in January and February, each with an infection rate of 10.00% (3/30). While the other months (November, December of 2023, March and April of 2024) had a lower infection rate of 3.33% (1/30). The p-value for the monthly distribution of infections is 0.18, indicating that while there were peaks in certain months, the differences in infection rates across the months were not statistically significant (p >0.05). These differences in infection rates determine the temperature influence on parasites in fish.

Table 3: Monthly distribution of infected fish with *Cryptosporidium*

Month	Total Samples	Infected Fish	Infection Rate (%)
November/2023	30	1	3.33
December/2023	30	1	3.33
January/2024	30	3	10.00
February/2024	30	3	10.00
March/2024	30	1	3.33
April/2024	30	1	3.33
Total	180	10	5.56
p-value 0.18 *			

* Statistically non-significant (p >0.05)

These values appear supportive of the premise that colder months (winter) , of January, February, are usually companion with increasing the parasite transition. Al -Jawasim and Al-AKhaled (2019) reported a 30% infection rate among humans in September, by recording a different seasonal onset in a local study. In dog's samples observed maximum infection rates in March and April, 35% (Hadi and Faraj, 2022).

Distribution of infected fish according to region

The parasite prevalence varied across the screening area. The results expose a clear prevalence inducing infection of fish in Baghdad city. The fish were sampled across three regions: Al-Amiria, Al Radwaniyah, and Al-Shawaka. The distribution of infections varied by region, with Al Radwaniyah showing the highest infection rate, followed by Al-Amiria and Al-Shawaka. (Table 4). summarizes the distribution of infected fish across the regions with infection rates expressed as percentages. Al Radwaniyah had the highest infection rate at 25.00%, while Al-Shawaka had the lowest at 10.00%. Al-Amiriya had a moderate infection rate of 15.00%. The p-value for the regional distribution of infections is 0.477, indicating no statistically significant difference in infection rates across the regions (p > 0.05).

Table 4. Distribution of *Cryptosporidium* spp. infection in fish species

Species	Region	Total No.	Infected	
			No.	%
Cyprinus carpio	Al-Amiria	20	3	15.00
	Al Radwaniyah	20	5	25.00
	Al-Shawaka	20	2	10.00
Silurus triostegus	Al-Amiria	20	0	0.00
	Al Radwaniyah	20	0	0.00
	Al-Shawaka	20	0	0.00
Planiliza abu	Al-Amiria	20	0	0.00
	Al Radwaniyah	20	0	0.00
	Al-Shawaka	20	0	0.00
Total	All Regions	180	10	5.56
p-value = 0.477*				

*** Statistically non-significant (p >0.05)**

Al-Taee (2008) first recorded the *Cryptosporidium* in the *Planiliza abu* raw fish in Iraq by finding the oocysts of the parasite with an infection rate of 28.9%. Even a low percentage must considerably concern, due the ability of transmission the parasite to the humans by contamination with infected intestinal contents during cleaning and preparing fish or when eating undercooked.

In Iraq traditional screening this study total results agree with, Karawan et al., 2012 in the south region of Iraq 6.18% while, Al-Dulaimi et al. (2020) in Babylon and (Hadi and Hussein, 2017) in Thi-Qar governorate recorded an incidence rate of (23.9%), (12.2%) in *Liza abu respectively*). The infection rate was 16.9% in *Cyprius carpio* (carp fish) in Tikrit city. (Mahmood, 2012). Globally, first studies description, epidemiological and histological in cultivated and wild fish of freshwater and marine worldwide (Table 5).

Table 5: Infection of *Cryptosporidium* spp. globally, recorded in wild and cultivated fish

Fish Species	%	References
Carp Cyprinus carpio	14.3	(Pavlásek, 1983)
Cichlid fish (*Oreochromis* spp.	58.8	Landsberg and Paperna, 1986)
Black Nile catfish (*Bagrus bayad)*	10.0	(Hefnawy, 1989)
North African catfish (*Clarias lazera)*	20.0	(Hefnawy, 1989) (Rush *et al.*, 1990)
Nile tilapia (*Tilapia nilotica* syn. *Oreochromis niloticus*	30.0	
Brown trout (*Salmo trutta)*	38.9	
Red drum (*Sciaenops ocellatus)*	21.7	(Camus and López, 1996)
Catfish (*Plecostomus* sp.)	100	(Muench and White, 1997)

This study is close with other studies in Egypt on Tilapia, where the percentage rate which was (20%) with *Cryptospordium nasorum* (Al-Ghaysh and Mahdi, 1998) and was recorded rate of (12%) in Assiut. Mostly, results point out the variability of parasite occurrence and clear the importance of environments and the general health conditions, along with human behaviors (social and traditional events) in relation to the seasonal incidents.

Molecular investigation of fish

The total infection rate with *Cryptosporidium* by nested PCR was relatively low 1% (4/40). Among fish samples only *Cyprinus carpio* (carp) tested positive (28%), (4/14) with statistically significant, at α level < 0.05, shown in Table 6.

Table 6: Total infection rates per fish species detected by nested PCR

Fish species	**Examined Fish**	**Infected Fish**	**Infection Rate (%)**

Cyprinus carpio	14	4	28.5
Silurus triostegus	13	0	0
Planiliza abu	13	0	0
Total	40	4	1
p-value 0.016*			

*** Statistically significant (p<0.05)**

Results of the two DNA fragments from the PCR reaction were ~1325bp and ~ 815bp (Figure 2). This result was lower than overall prevalence detected in cultivated fish with rate of 4.2% in Spain screened by the 18 RNA gene. (Moratal et al., 2022).

Figure 2: Agarose gel 1.5% electrophoresis image that showed the Nested PCR product analysis of conserved region in *SSU ribosomal RNA* gene in *Cryptosporidium* species found in fish samples. Where M: marker (2000-100bp). Lanes (upper lines) showed first PCR amplification that showed some positive samples at (1325bp) PCR product. The Lanes (downline) showed second Nested reactions that showed some positive amplification at (815bp) Nested PCR product

The homology sequence between local *Cryptosporidium* sp. Fish, Number 1 to Number 5 isolates and other nine NCBI-Genbank correspondent isolates. By (BLAST), results similarities revealed *C. parvum* and other species showed different genetic homology sequence identity with high similarity, 100% with isolate from Iran to lower percentage by 98.10% with USA, while, distinct by 94.70% with *C. baileyi* from Iraq as shown in **Table 7**. However, DNA fragment Sanger sequencing may clear some alien data about parasites isolates, taxonomy, transmission, and geographical distribution. However, gaps remain, including lack of genotyping for many Cryptosporidium that are important in both human and veterinary health.

The *Cryptosporidium parvum* fish isolates deposited in NCBI Genbank, identified by accession numbers (PP979853, PP979854, PP979855, PP979856, PP979857) were obtained.

Table 7: 18S rRNA Sequence identity between local fish *C. parvum* isolate (PP979853 - PP979857) and others have recorded in the GenBank

Local isolate		**NCBI-BLAST *Cryptosporidium* isolate**		
No.	**Access No.**	Species / Country	Access No.	%
Cryptosporidium sp. fish No.1	PP979853.1	*C. parvum* / Iran	KU200953.1	100
Cryptosporidium sp. fish No.2	PP979854.1	*C. parvum* /Iraq	OP420775.1	99.86
Cryptosporidium sp. fish No.3	PP979855.1	C. parvum/USA	AF093492.1	99.60

Cryptosporidium sp. fish No.4	PP979856.1	*C. parvum* / USA	AF093491.1	99.21
Cryptosporidium sp. fish No.5	PP979857.1	*C. parvum* /UK	L16997.1	99.20
Cryptosporidium sp. fish No.4	PP979856.1	*C. baileyi*/	KT151551.1	99.06
Cryptosporidium sp. fish No.1	PP979853.1	*C. muris* / Czech Republic	KJ469983.1	99.02
Cryptosporidium sp. fish No.2	PP979854.1	*C. parvum* / USA	AF115377.1	98.10
Cryptosporidium sp. fish No.2	PP979854.1	*C. baileyi* / China	MN410720.1	95.20
Cryptosporidium sp. fish No.1	PP979853.1	*C. baileyi* / Iraq	OP420782.1	94.70

Phylogenetic tree

The DNA sequencing method was carried out to species level analysis rRNA gene in current study fish *Cryptosporidium,* 1-5 isolates and Genbank database country sequences (**Figure 3**).

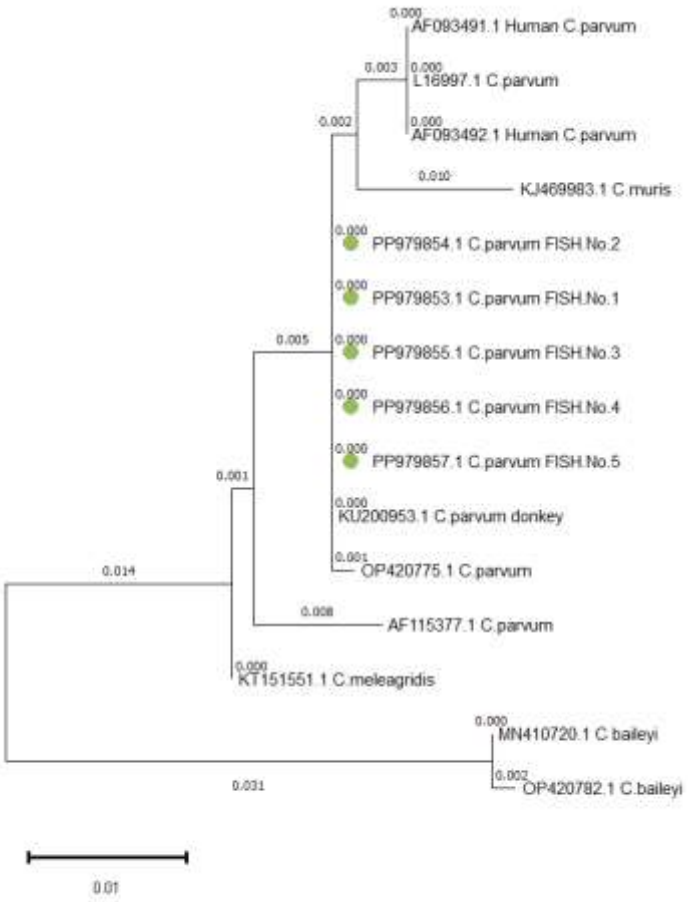

Figure 3: Phylogenetic tree analysis based small subunit ribosomal RNA gene partial sequence in local *Cryptosporidium* sp. IQ. Fish isolates that used for genetic species analysis. The phylogenetic tree was constructed using the Neighbor-Joining method in (MEGA 11.0 version). The *Cryptosporidium* sp. IQ.Fish No.1-No.5 isolate showed closed related to NCBI-BLAST Cryptosporidium parvum (KU200953.1) at total genetic changes (0.05-0.01%)

The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 15 nucleotide sequences. There were a total of 1572 positions in the final dataset. Evolutionary analyses were conducted by (MEGA 11) (Tammura et al., 2021). The isolates of *Cryptosporidium* spp. show's a 100% identity with each other and close relation to *C. parvum* (OP420775.1) from human/ Iraq and KU200953.1(donkey, China)) at total genetic changes (0.05-0.01%). The *C. parvum* formed subclade belong to major clade in the second tree bifurcation. Two

isolate of *C. baileyi* from China and Iraq worked as an tree out group. The *C. parvum* fish isolates were deposited in NCBI Genbank and identified by accession numbers (PP979853, PP979854, PP979855, PP979856, PP979857) were obtained.

These phylogenetic included 15 DNA fragment sequences. Codon positions included 1st ,2nd and 3rd +Noncoding. A total of 735 positions in the final dataset. The current molecular results presented similar parasite species with the previous studies in different hosts. For example, a studies, reported that *C. parvum* in poultry and cattle, with a very low percentage, found in 1.0% (2/197) and suggest that can be a source of human infection (Maysoon and Al-Zubaidi, 2018; Hazzaz *et al.,* 2020). In Egyptian study, identified *C. parvum* in 6 %, (3/50) of pigeon (Abou Elez RMM *et al.,* 2023). A survey study in a hospital in Wasit Province in Iraq found a high prevalence, with *C. parvum* 60% (48/80) in the admitted patients (Rahi and Ali, 2022). It is important for acknowledge that *C. parvum* has been detected in various species including pigeons, turkeys, broiler flocks, and layer flocks which present a serious one health risk and general public health concern (Helmy et al., 2017).

Histopathological findings

In the positive sample the cross examination on postmortem appears that the intestinal system have enteritis appearances (Figure **4**). The presence examination suggested the number of *Cryptosporidium* sp. at the gastric mucosa with appearance of oocysts' life stages. Inflammatory cells including eosinophil infiltrated the lamina propria and spread to the submucosa **(Figure 5).** Sometime merozoites are found in the crypts, reddish filling the goblet cells between villous epithelialcells and the cryptmucosal epithelium. (Ryan *et al.,* 2015; Bolland *et al.,* 2020).

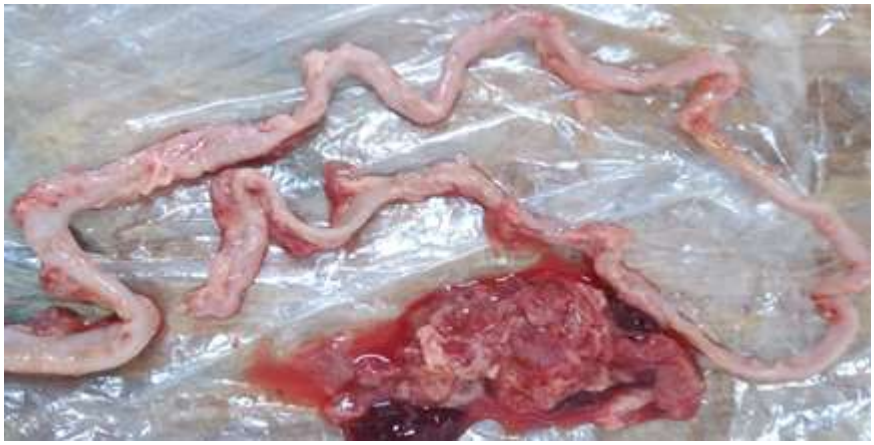

Figure 4: Histological examination of infected carp fish (intestinal epithelium) stained H&E stain 40X. with appearance of *Cryptosporidium* oocysts' life stages

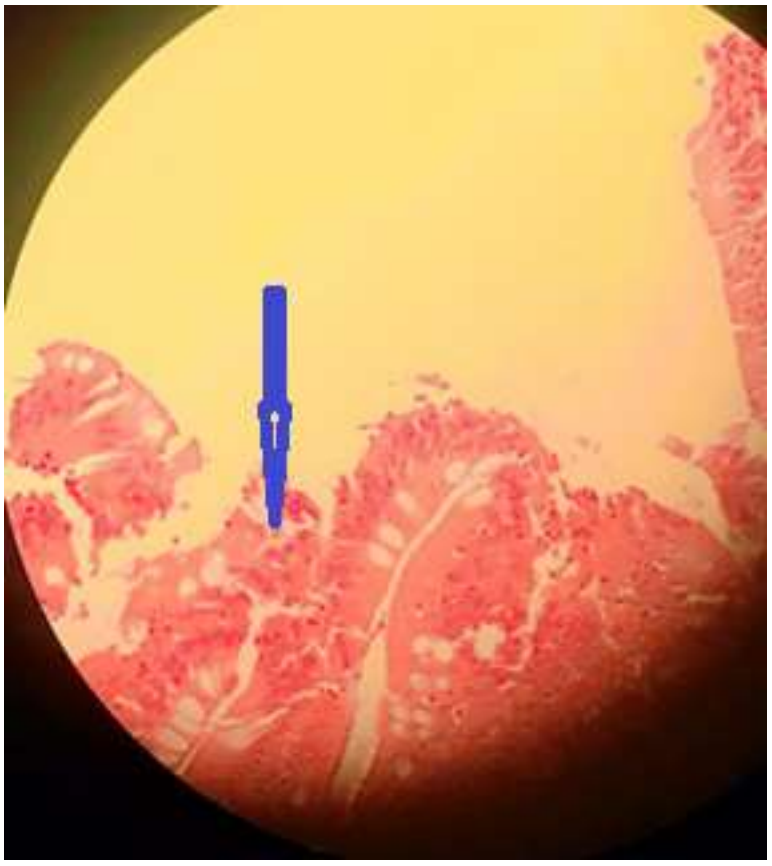

Figure 4: Histological examination of infected carp fish (Intestinal epithelium) stained H&E stain X40 with appearance of *Cryptosporidium* oocysts' life stages (blue arrow)

4. Conclusion and future scope

The study presents low occurrence of Cryptosporidium in fish in different areas of Baghdad. The results emphasize some molecular conformation underlying the local and regional Cryptosporidium isolates. However, DNA fragment Sanger sequencing may clear some alien data about parasites, much information is still unclear. Fish infection with types and genotyping suggests a risk for zoonotic transmission concerning humans.

Abbreviations

nPCR; Nested Polymerase Chain Reaction, NCBI; National Center for Biotechnology information, SSU; Small Subunit, µl;microleters, miute; min, second; sec., modified Ziehl-Neelsen stain; mZNabbreviations

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Authors' contribution

NMO formed of the research, evaluated and raw the data, and wrote the outline of the paper. ARA, aided in accomplishment writing this manuscript, its conclusion and suggestion.

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