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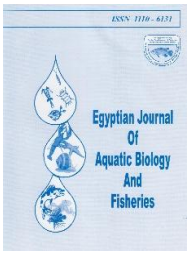

Cryptosporidiosis in *Clarias gariepinus* fish: incidence and molecular aspect

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ABSTRACT

This study scopes out the incidence, histological and molecular detection of cryptosporidiosisin *Clarias gariepinus* freshwater fish obtained from the River Nile and drainage canals in Giza Governorate, Egypt. The detection of *Cryptosporidium* spp. in stomach and intestine scrapings of *Clarias gariepinus* using the modified Ziehl-Neelsen (mZN) technique; the detection of the anti-*Cryptosporidium* antibodies in *Clarias gariepinus* sera using the Enzyme-Linked Immunosorbant Assay (ELISA), histopathological investigation of the stomach and intestine using hematoxylin and eosin (H & E) and molecular identification of isolated *Cryptosporidium* spp. using Polymerase Chain Reaction (PCR), DNA sequencing and phylogenetic analysis. The monthly prevalence of *Cryptosporidium* spp. showed the highest infestation rate in July (87.5%) but the lowest in January (44.4%), while the seasonal prevalence revealed the highest infestation rate during summer and the lowest rate in the winter season (51.20%). ELISA showed a higher prevalence of 69.3% than that prevalence obtained by mZN, 64% for the total examined *Clarias gariepinus* fish. Also, a higher prevalence of *Cryptosporidium* infection (65.5% and 75.8%) was obtained by ELISA than 61.1% and 68.3% by mZN, in both fish groups from the River Nile and drainage canal, respectively. Concerning histological investigation, the detected *Cryptosporidium* oocysts were highly homologous to Cryptosporidium spp. oocysts. Concerning the molecular identification of *Cryptosporidium* spp., PCR analysis revealed the expected positive bands at 1056 bp. DNA sequencing and phylogenetic analysis proved that the isolate *Cryptosporidium* spp. in the present study was *Cryptosporidium molnari*. In conclusion, freshwater fishes (*Clarias gariepinus*) exhibit a high infection rate with *Cryptosporidium* spp. The drainage canals collected fishes showed a higher prevalence than those collected from the River Nile. This indicates an important public health problem and a potential danger to drainage channels in Egypt. ELISA showed a higher prevalence of cryptosporidiosis than mZN, for the total examined *Clarias gariepinus* fish and phylogenetic tests proved a novel species of *Cryptosporidium molnari*.

INTRODUCTION

Cryptosporidium species are protozoan intracellular parasites that infect the microvillus border of the gastrointestinal epithelium of a wide range of vertebrate hosts,

including humans, birds, reptiles, and fish (**Abu El Ezz *et al.* 2011; Ryan *et al.* 2014**). In farmed animals, Cryptosporidiosis is not exclusively an opportunistic illness, resulting in large economic losses, zoonotic implications, and difficulty in control. (**Shaapan *et al.* 2010; Ghazy *et al.* 2016**). Infection with the Cryptosporidium sp. parasite is more common in nations with limited access to safe drinking water; as a result, the infection is more common in developing countries and among children (**Obateru *et al.* 2017; Elfadaly *et al.* 2018**).

Cryptosporidiosis can cause a variety of symptoms in humans and animals, ranging from asymptomatic to vomiting, severe diarrhoea, and death, especially in young people. (**Shaapan *et al.* 2015; Certad *et al*. 2019**). Ingesting environmentally ubiquitous stable oocysts contaminated food or drinking water, which can be acquired through several routes, including person-to-person contact, contact with companion and farm animals, and recreational water, the parasite is transmitted via the faecal-oral contamination route (**Ghazy *et al.* 2015; Shaapan, 2016**). The Cryptosporidiosis, the ensuing disease is typically self-limiting in healthy adult hosts and immune-competent subjects, but it can be fatal in immunocompromised people, such as AIDS patients, malnourished people, and children, especially in underdeveloped nations. Because the environmentally hardy oocysts are resistant to treatment, including chlorine, water is a primary mode of transmission for *Cryptosporidium*. Water is a major method of transmission of *Cryptosporidium*, as the environmentally robust oocysts are resistant to disinfection including chlorine (**Daniels *et al.* 2018; Zahedi and Ryan, 2020**).

The routinely used routine diagnostic procedure of staining Cryptosporidium spp oocysts in faeces or tissue scraping smears with acid-fast staining remains the most specific conventional tool for diagnosis (**Ghazy *et al.* 2016**). Enzyme immunoassays (EIA) that detect parasite antigen are effective approaches, but the sensitivity of these immuno-detection methods is disputed (**Hassanain *et al.* 2016**). None of the laboratory diagnostic procedures, such as acid fast staining and direct or indirect immunofluorescence microscopy, can distinguish between Cryptosporidium species or subtypes, which is crucial for understanding the disease's dynamics and transmission pathways (**Adeyemo *et al.* 2018**), the polymerase chain reaction (PCR), nowadays, is increasingly being utilised as a diagnostic method for detecting Cryptosporidium DNA in tissues and faeces. This technique enables for species identification and subtyping, as well as tracing of the parasite's many transmission routes (**Costa *et al.* 2021**).

In the last decade, a lot of work has gone into studying human and animal cryptosporidiosis, although our understanding of Cryptosporidium infecting fish is still in its infancy (**Karanis, 2018**). Despite the fact that Cryptosporidium spp. has been found in both wild and cultivated freshwater and marine fish in a number of regions throughout the world (**Paparini *et al.* 2017**), the systematics, biology, and epidemiology of Cryptosporidium species found in fish are unknown (Bolland *et al.* 2020). *Cryptosporidium* infections in fish can be asymptomatic, but they can also cause severe

disease and death. To date, more than 29 novel piscine-associated *Cryptosporidium* genotypes have been identified in fish, with the most common three being *Cryptosporidium molnari*, *Cryptosporidium scophthalmi*, and *Cryptosporidium huwi* (**Golomazou *et al.* 2021**).

Cryptosporidiosis is a common waterborne disease, while human species have been shown to survive in both fresh and salt water, the species status of the novel piscine-genotypes described is unknown, so a better understanding of the taxonomy and evolutionary origins of *Cryptosporidium* fish is critical (**Couso-Pérez *et al.* 2019**). Little is known about the frequency and genotypes of Cryptosporidium in Egyptian fish (**Ammar and Arafa 2013**), as a result, the goal of this study is to look at the prevalence and molecular epidemiology of Cryptosporidium species in the freshwater fish *Clarias gariepinus*

MATERIALS AND METHODS

Location

A total of 300 *Clarias gariepinus* fish were taken from two fresh water sources in Giza Governorate, Egypt: the Nile River (180 fish) and drainage canals (120 fish). The fish were immediately transferred alive to the lab for dissection and analysis.

Blood and tissue samples

A three ml syringe was used to collect blood samples from the caudal vein of individual *Clarias gariepinus* fish, as described by **Argungu *et al.* (2017)**. Each fish's stomach and intestines were dissected and divided into three parts (about 2 cm each): the first part was scraped off, stained, and microscopically examined for Cryptosporidium oocysts; the second part was fixed in a 10% formalin solution and kept for histological examination; and the third part was minced and stored at -20°C for DNA extraction and molecular identification (**Yang *et al.* 2016**).

Identification of *Cryptosporidium* spp. oocysts

Fine smears from the stomach and intestine epithelial layers of Clarias gariepinus fish samples were preserved in methanol and stained with a modified Ziehl-Neelsen stain, as described by **Aboelsoued *et al.* (2000)**. Higher-magnification Under a light microscope, a 100X objective lens with a stage micrometre coupled with an eyepiece micrometre can be used to confirm the presence of and measure *Cryptosporidium* spp. oocysts (**Xiao *et al.* 2001**). To compute the mean, use around 20 –50 oocysts with the range in parenthesis as the standard unit of measurement (m = 0.001 mm) (**Ghazy *et al.* 2015**).

Serological assay

Using procedures based on Sheather's flotation, the isolated contaminant-free *Cryptosporidium* oocysts from scraped stomach and intestinal mucosa were utilized for antigen preparation (**Arrowood and Sterling, 1987**). Controlled checkerboard titration was used to identify the optimal antigen, serum, and conjugate concentrations (**Shaapan *et al.* 2021**), and the ELISA test procedures were carried out according to **Hassanain *et al* (2013)**.

Molecular identification

DNA extraction

Genomic DNA was extracted from washed Cryptosporidium oocysts using the QIAamp® DNA Stool Mini Kit instructions, with some modifications to the manufacturer's protocols, according to the techniques of **Lalonde and Gajadharm (2009)**.

Polymerase Chain Reaction (cPCR)

The Emerald Amp GT PCR mastermix (Takara) Code No. RR310A kit was used in cPCR, and the Oligonucleotide primers Metabion (Germany) were used in cPCR, with specified sequence and to amplify a specific product according to the techniques outlined by **Shaapan, *et al* (2012)**. The agarose gel electrophoreses were carried out and the gel was photographed by a gel documentation system, and the data was analysed through computer software using the emerald Amp GT PCR master mix (Takara) kit, the cycling conditions of the primers during cPCR were at the temperature and time conditions of the two primers using the emerald Amp GT PCR master mix (Takara) kit, the agarose gel electrophoreses were carried out and the gel (**Hassanain *et al.* 2011**).

DNA sequencing

In a commercial sequencing facility, DNA Sequencer, a purified PCR product was sequenced in the forward and/or reverse directions (Fermentas GMBH, Germany). The genotypes/assemblages were aligned with homologous sequences available in the GenBank database using CLUSTAL W, and the sequences were submitted to a BLAST® analysis (Basic Local Alignment Search Tool) (**Thompson *et al.* 1994**).

RESULTS

Morphological detection of *Cryptosporidium* oocysts

The Cryptosporidium spp. oocysts were characterised by a spherical to ovoid form with smooth wall, an incomplete suture line of the oocyst wall, and an acid fast (red-pink) appearance on green back. The diameter of the oocysts ranged from 3.20–4.5 x

3.90–6.05 m, with a mean (3.9 x 5.0) m in diameter and a shape index of 1.4–1.6, which was morphologically comparable to *Cryptosporidium molnari* oocysts (Figure 1).

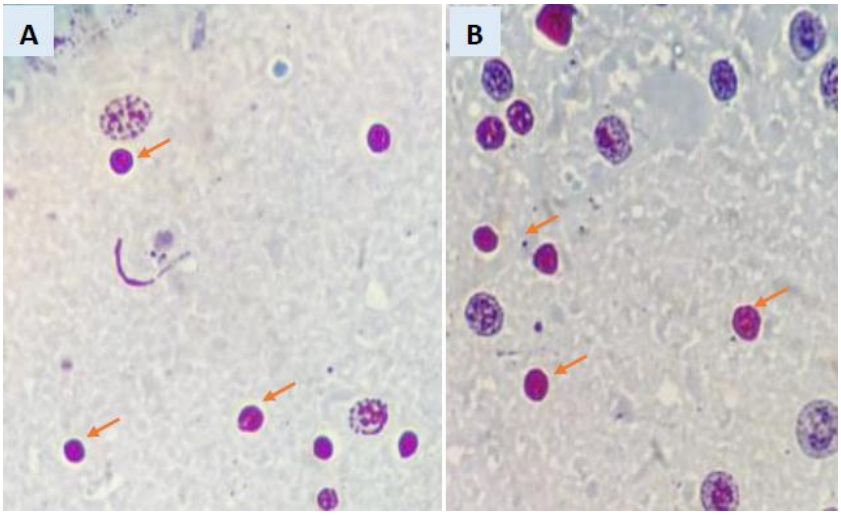

Figure 1: *Cryptosporidium molnari* oocysts in *Clarias gariepinus* stomach (**A**) & intestine (**B**), (Red arrow) stained with Modified Ziehl-Neelsen stain (mZN) (X100).

Prevalence of *Cryptosporidium s*pp. infection among *Clarias gariepinus* using mZN

The monthly prevalence of *Cryptosporidium s*pp. showed the highest infestation rate in July (87.5%), followed by August (77.3), but the lowest in January (44.4%), followed by December (51.8). While the seasonal prevalence revealed the highest infestation rate during summer (80.0 %) and the lowest rate in winter season (51.20%) (Table 1).

Table 1: Monthly and seasonal prevalence of *Cryptosporidium* in *Clarias gariepinus*

Fish		Total				Total	
Months	**No. of exam**	**No. of inf.**	**% of inf.**	**Season**	**No. of exam**	**No. of inf.**	**% of inf.**
Dec	27	14	51.8				
Jan.	27	12	**44.4**				
Feb.	28	16	57.1	**Winter**	82	42	**51.20**
March	25	16	64.0				
April	26	17	65.4	**Spring**			
May	26	19	73.0		77	52	67.50
June	24	18	75.0				
July	24	21	**87.5**	**Summer**	70	56	**80.00**
Aug	22	17	77.3				
Sep	24	16	66. 6				
Oct	22	13	56.5	**Autumn**	71	42	59.10
Nov	25	13	52.0				
Total	**300**	**192**	**64 %**	**Total**	**300**	**192**	**64 %**

Comparison between the prevalence of *Cryptosporidium* spp. infection among *Clarias gariepinus* using mZN & ELISA

For the total Clarias gariepinus fish studied, the ELISA serological test revealed a greater prevalence (69.3%) than the mZN (64%) prevalence. In addition, ELISA tests demonstrated a greater prevalence of *Cryptosporidium* infection in both *Clarias gariepinus* fish groups from the River Nile and the Drainage Canal, with 65.5 % and 75.8 %, respectively, compared to 61.1 % and 68.3 % acquired by mZN (Table 2).

Table 2: Comparative prevalence of *Cryptosporidium* spp. infection among *Clarias gariepinus* using mZN & ELISA

Fish	Fish no.	mZN	ELIZA
		+ve no (%)	+ve no. (%)
River Nile fish	180	110 (61.1%)	118 (**65.5 %**)
Drainage canal fish	120	82 (68.3%)	90 (**75.0 %**)
Total	**300**	**192 (64%)**	**208 (69.3 %)**

Molecular detection of *Cryptosporidium* spp.

The expected positive bands at 1056 bp were found in positive prepared PCR samples of *Clarias gariepinus* scraped stomach and intestinal mucosa, which had previously been verified with the mZN technique for Cryptosporidium oocysts. *PCR amplification and partial nucleotide sequence analysis* were performed on Cryptosporidium oocysts isolated from Egyptian Clarias gariepinus fish. All of the Egyptian isolates tested produced the expected first (1325 bp) and second (825 bp) PCR products. The positive PCR Cryptosporidium isolate found in this investigation was *Cryptosporidium molnari*, according to phylogenetic analysis (Fig. 2).

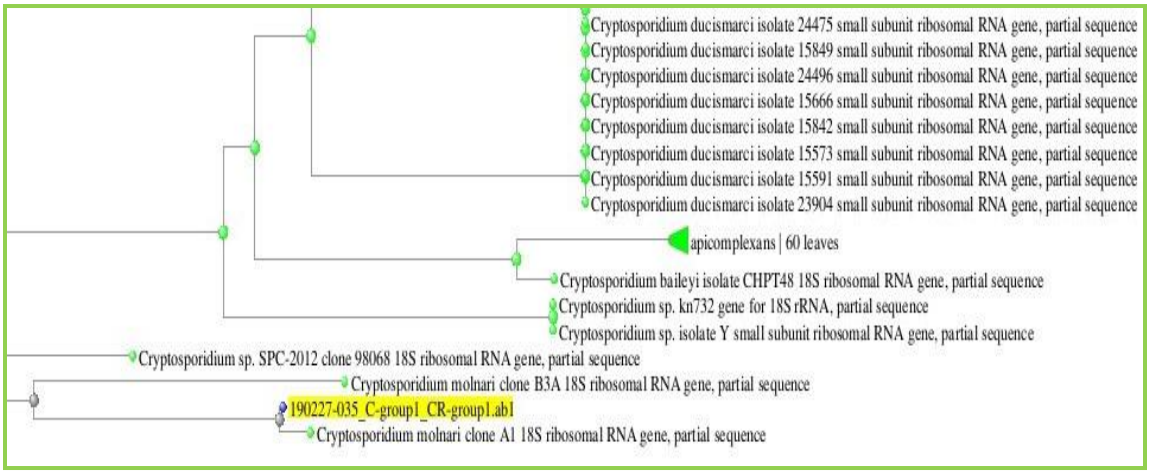

Figure 2: Phylogenetic tree on *Cryptosporidium* Egyptian isolate from *Clarias gariepinus* fish have high homology with *Cryptosporidium molnari* isolate

DISCUSSION

After staining with modified Zeihl-Neelsen, the morphological appearance of detected Cryptosporidium oocysts obtained from the stomach and intestinal scraped mucosa of Clarias gariepinus fish in our study were bright red, spherical smooth wall, oocysts with an incomplete suture line, and with a mean diameter of 3.9 x 5.0 m. In addition, these oocysts were almost identical to those described by **Xiao *et al.* (2004)** in prior research, and they agree with Cryptosporidium molnari, which was first discovered in gilthead sea bream (**Alvarez-Pellitero and Sitja-Bobadilla, 2002**).

In this work, the prevalence of Cryptosporidium spp. infection in Clarias gariepinus fishes was determined using an ELISA serological test, which revealed a greater prevalence (69.3%) than the mZN test (64%). Using a modified Ziehl-Neelsen staining technique, drainage canal fishes had a greater prevalence (68.3%) than River Nile fishes (61.1%), and drainage canal fishes had a higher infection rate (75.8%) than River Nile fishes (65.5%) when using an enzyme linked immunosorbant assay. The ELISA's sensitivity was much higher than mZN's, which was unsurprising because the ELISA detects pathogen antigens from past infections and/or active infections (**Omoruyi *et al.* 2014**). Using both ELISA and mZN assays, drainage canal fishes had a higher *Cryptosporidium* infection rate than River Nile fishes in this study. This could be due to the sanitary condition of the area, the location of the drainage canals from living areas, the number and class of people visiting the canal and its purpose, or biological pollution (**Ammar and Arafa 2013**). Furthermore, chronic exposure to pollutants or environmental stress, which is more common in drainage canals fishes than in River Nile fishes, leads to immunosuppression via the release of corticosteroids, making the fish more susceptible to pathogenic organisms, which is thought to be the main cause of high parasitic disease rates in fish (**Mallik *et al.* 2021**).

In this study, by ELISA and mZN assay, the overall prevalence of Cryptosporidium in Clarias gariepinus fish was 69.3% and 64% percent, respectively. Previous research has found a wide range of prevalences, ranging from 0.8 to 100% (**Zanguee *et al.* 2010; Yang *et al.* 2016**). *Cryptosporidium* infection was far more common, especially among immature fish, whereas the juvenile turbot were heavily parasitized by *C. scophthalmi*, with infection rates of up to 100% (**Alvarez-Pellitero *et al.* 2004**). *C. molnari*, on the other hand, was found in gilthead bream and European sea bass fish weighing 30 to 100 grammes, but no infections were found in fish weighing more than 300 grammes (**Sitja-Bobadilla *et al.* 2005**). Correspondingly, *Cryptosporidium* infection was found in hatchery-reared *Oreochromis niloticus* fry and fingerlings but not adults (**Reid *et al.* 2010**).

Some experts believe there is insufficient data to generate acceptable names for parasites that resemble *Cryptosporidia* in fish until more molecular data is available for the fish species *Cryptosporidia* (**Fayer, 2010**). The positive cPCR *Cryptosporidium*

isolate found in this investigation was *Cryptosporidium molnari*, according to DNA sequencing and phylogenetic analysis. The acquired results confirm the well-known fact that shorter amplicons amplify considerably more efficiently by the cPCR than longer amplicons, and that cPCR positives are owing to non-specific amplification; however, the assay has been well verified (**Yang *et al.* 2016**). The *C. molnari* SSU rDNA sequence derived from *Clarias gariepinus* fish identified in this investigation was identical to that of C. molnari found in gilthead sea bream. (**Palenzuela *et al.* 2010**) and identical to that of *C. molnari* in the butter bream from Spain (**Zanguee *et al.* 2010**). Many fish species, including a madder sea-perch, an upside-down catfish, and a wedgetailed blue tang, have previously been molecularly identified as having *Cryptosporidium molnari*-like genotypes (**Certad *et al.* 2019**).

CONCLUSION

The high incidence of *Cryptosporidium in Clarias gariepinus* freshwater fishe in this study shows that more research is needed to better understand the public health implications. Furthermore, the increased incidence of drainage canal fishes compared to River Nile fishes implies a significant public health issue and a potential risk of drainage canals in Egypt. The "gold standard" will be a combination of the mZN staining approach with any of the ELISA and PCR tests, with high specificity and sensitivity, ensuring that undiagnosed *Cryptosporidium* infection does not occur. This study's phylogenetic analysis indicated that this protozoal organism was a new *Cryptosporidium molnari* detected in *Clarias gariepinus* fish.

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