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ORIGINAL PAPER

Prevalence and Molecular Epidemiology of *Cryptosporidium* Infection in *Clarias gariepinus* Fish in Egypt

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Abstract

Purpose This study investigated the prevalence and molecular detection of *Cryptosporidium* spp. in catfish (*Clarias gariepinus*).

Methods A total of 300 *Carias gariepinus* fish were collected from two freshwater sources: the Nile River (180) and drainage canals (120). The stomach and intestine epithelium of each individual fish sample were screened by modified Ziehl–Neelsen (mZN) staining technique for the detection of *Cryptosporidium* oocysts followed by the serological survey for detection of *Cryptosporidium* antibodies using Enzyme-Linked Immunosorbent Assay (ELISA) and molecular characterization using complemented DNA polymerase chain reaction (cPCR).

Results ELISA showed higher prevalence of 69.3% than that prevalence obtained by mZN, 64% for the total examined *Clarias gariepinus* fish. Also, higher prevalence of *Cryptosporidium* infection 65.5% and 75.8% obtained by ELISA than 61.1% and 68.3% by mZN, in both fish groups from Nile River and Drainage canal, respectively. PCR analysis revealed the expected positive bands at 1056 bp. DNA sequencing and phylogenetic analysis proved that the positive-PCR *Cryptosporidium* isolate identified in the present study was *Cryptosporidium molnari.*

Conclusion Freshwater fishes (*Clarias gariepinus*) are subjected to a high infection rate with *Cryptosporidium* spp.; the drainage canals obtained fishes showed higher prevalence than that collected from Nile River which indicates an important public health problem and a potential risk of drainage canals in Egypt. ELISA showed higher prevalence of cryptosporidiosis than mZN, for the total examined *Clarias gariepinus* fish and phylogenetic analyses confirmed this protozoal organism to be a novel species of *Cryptosporidium molnari*.

Keywords *Clarias gariepinus* · *Cryptosporidium molnari* · Prevalence · Molecular epidemiology

Introduction

Cryptosporidium spp. are intracellular protozoan intracellular parasites that infect the brush border (microvilli) of the gastrointestinal epithelium of many vertebrate hosts, including humans, birds, reptiles, and fish [1]. Cryptosporidiosis among farmed animals is no longer an opportunistic disease and significant economic losses, implications for zoonosis, and difficulty in controlling them are a major concern [2]. The infection with *Cryptosporidium* sp. parasite is generally more prevalent in countries with little access to clean freshwater, therefore, the infection is highly prevalent in developing countries and more common in pediatric populations [3, 4].

Cryptosporidiosis infection in humans varied from asymptomatic to severe vomiting, diarrhea and in young individuals may be fatal [5]. The parasite is transmitted

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through ingestion of contaminated food or drinking water with ubiquitous environmentally resistant oocysts, obtained through several methods, personal contact, companion or farm animal contact and recreational water [6]. Cryptosporidiosis resulting disease is mostly self-limited in adults, healthy hosts, and immunocompetent persons but can be life-threatening in immunocompromised patients, such as AIDS and malnourished patients and children, particularly in developing countries [7]. Water is the main route of transmission of *Cryptosporidium*, as the environmentally firm oocysts are resistant to many disinfectants including chlorine [8].

Improved diagnosis has been expanded for the microscopic, immunological, and molecular detection of *Cryptosporidium* [9]. Haematoxylin and eosin (H&E) staining and light microscopy, is often insufficient to suggest or prove the presence of the organisms [10]. The current routine diagnostic method with acid-fast staining for *Cryptosporidium* spp. oocysts staining in tissue scraping or fecal smears is still the conventional specific tool for diagnosis [2]. Detection of the parasite antigen using the enzyme immunoassays is efficient but is less sensitive as an immune-detection method [11]. None of the diagnostic laboratory techniques, such as acid-fast staining and immunofluorescence microscopy, can identify species or subtypes of *Cryptosporidium*, which is important for understanding the dynamics and pathways of transmission [12], now PCR assay is being a commonly diagnostic tool for *Cryptosporidium* DNA identification in tissues and feces, this technique allows the species and subtyping detection and also tracing of different transmission ways of the parasite [13].

In the last decade, much effort has been concentrated to study the human and animal cryptosporidiosis, while by comparison, the knowledge of Cryptosporidium infecting fish is still in its early stage [14]. Although many records of *Cryptosporidium* spp. in both cultured and wild fresh water and marine fish are detected in numerous countries worldwide [15], the systematics, epidemiology, and biology of fish *Cryptosporidium* species are imperfectly understood [16]. To date, more than 29 novel genotypes of piscine *Cryptosporidium* are recognized in fish the most common 3 are: *Cryptosporidium scophthalmi*, *Cryptosporidium molnari* and *Cryptosporidium huwi* based on molecular studies [17].

Cryptosporidiosis is a typical waterborne disease, and however, the survival of human species in fresh and seawater has been demonstrated, but the described species status of these new fish genotypes is unclear, so it is important to better understand the evolutionary origins and the taxonomy of piscine—*Cryptosporidium* [18]. In Egypt, relatively little is known about the genotypes and the prevalence of *Cryptosporidium* parasites in fish [19], therefore, the present work objective is to explore *Clarias gariepinus* freshwater fish infection with *Cryptosporidium* especially the prevalence and molecular epidemiology.

Materials and Methods

Fish Sample Collection and Preparation

Fish and Location

Clarias gariepinus was selected in this study because it is easily infected by many medically important and zoonotic parasites in the wild and cultivated environment due to its ability to live in contaminated polluted water areas and its predatory feeding nature. A total number of 300 *Clarias gariepinus* fish were collected from two freshwater sources: Nile River (180 fish) and drainage canals (120 fish) at Giza Governorates, Egypt. Fish were transported immediately alive to the laboratory for dissection and examination.

Blood Samples

The fish blood samples were obtained from the caudal vein of individual *Clarias gariepinus* fish by a 3 ml syringe, according to methods described by Argungu *et al*. [20]. The obtained blood samples were kept in clean glass tubes at room temperature for two hours, centrifuged for 15 min at 3000 rpm to separate the serum. Serum samples were separated, collected in 1.5 ml Eppendorf tubes, serial-numbered, labeled, and kept at -20 ^{0}C until used for the serological assay.

Tissue Samples

Stomach and intestines were dissected out from each fish, and each was divided into three parts (about 2 cm): the 1st part of each was scraped off and the scraped epithelial layer smeared on glass slides, stained, and microscopically examined for detection of *Cryptosporidium* oocysts, the 2nd part was fixed in a 10% formalin solution and kept for histological examination, while the 3rd part was minced with sterile blades, stored in Eppendorf tubes (1.5 mL) and kept freeze at – 20 °C for extraction of DNA [21].

Detection of *Cryptosporidium* spp. oocysts

Staining of *Cryptosporidium* oocysts

Fine smears from the stomach and the intestine epithelial layer of collected *Clarias gariepinus* fish samples were methanol-fixed and Ziehl–Neelsen stained as the technique cited by Henriksen and Pohlenz [22]. This technique was modified to become a simple and efficient method for

staining *Cryptosporidium* spp. oocysts, appear as bright red, green background and green–blue fecal debris, yeasts, or tissues [23].

Measurement and Identification of *Cryptosporidium* spp. oocysts

Under the light microscope, objective lens of 100X higher magnification was used with help of stage micrometer conjugated micrometer eyepiece to identify and measure the detected *Cryptosporidium* spp. oocysts [24]. Microns (μm = 0.001 mm) are the standard measuring units, and about 20–50 oocysts were used for the mean calculations [25].

Serological Assay

For detection of *Cryptosporidium* antibodies in the sera of *Clarias gariepinus* fish, Enzyme-Linked Immunosorbent Assay (ELISA) was used. Negative and positive control sera were obtained from positively and negatively confirmed fish with previously used staining technique of modified Ziehl–Neelsen for *Cryptosporidium* oocysts. Isolated contaminant-free *Cryptosporidium* oocysts from the scraped stomach and intestinal mucosa were used for antigen preparation via methods based on Sheather's flotation as substantive by Arrowood and Sterling [26]. Antigen, serum, and conjugate optimum concentrations were identified by controlled checker-board titration [27] and the ELISA test method was adopted in accordance with procedures of Hassanain *et al*. [28].

Molecular Identification

Cryptosporidium spp. oocysts PCR Sample Preparation

Cryptosporidium spp. oocysts were purified prior to the DNA extraction and inoculation, *Cryptosporidium* spp. oocysts were purified from confirmed mZN-positive scraped stomach and intestinal mucosa of *Clarias gariepinus* fish using the sucrose Sheather's solution and Percoll flotation, then the purified *Cryptosporidium* spp. oocysts were washed 4 times with distilled water and kept in PBS solution at − 20 °C in a 1.5 mL Eppendorf tube until used for DNA extraction [29].

DNA Extraction

From the washed *Cryptosporidium* oocysts, the extraction of genomic DNA was carried out using the Mini Kit QIAamp® DNA Stool instructions with modifications to the manufacturer's protocols according to procedures described with Lalonde and Gajadhar [30].

Polymerase Chain Reaction (cPCR)

Preparation of the PCR Master Mix was adopted according to Emerald Amp GT PCR master mix (Takara) of kit Code No. RR310A and the primers of Oligonucleotide Metabion (Germany), were used in cPCR, with specified sequence and product amplification according to method described by Jellison *et al*. [31] (Table 1). The two primers used during cPCR underwent cycling conditions of time and temperature using master mix (emerald Amp GT PCR) kit, electrophoresed using agarose gel, photographed with a gel-documentation system and through computer software the data analyzed [12].

DNA Sequencing

Forward and reverse direction sequencing of the purified PCR product was done commercially in the laboratory, DNA Sequencer (Fermentas GMBH, Germany). The obtained sequences were displayed and analyzed with BLAST® (Basic Local Alignment Search Tool), the fragment's genotypes were aligned with GenBank database available homologous sequences using CLUSTAL W with manual adjustments to establish sequence identity to GenBank accessions no. [32].

Statistical Analysis

SPSS (version 20) statistical program (SPSS Inc., Chicago, IL) was used to carry out a one-way analysis of variance (ANOVA).

Results

Morphological Identification of *Clarias gariepinus Cryptosporidium* oocysts

The obtained mZN-stained oocysts from the stomach and intestinal scraped mucosal samples were similar in the morphological characteristics of the *Cryptosporidium* spp. oocysts showed smooth wall, ovoid to spherical shaped oocysts, and occasionally may show an incomplete suture line at the oocysts wall and presented as red–pink (acid-fast)

Table 1 Oligonucleotide primers sequences source: (Metabion, Germany)

Gene	Primer	Sequence	Amplified product
18S rRNA	*KLJ1*	CCACATCTAAGGAAG GCAGC	1056 bp
	KLJ2	ATGGATGCATCAGTG TAGCG	

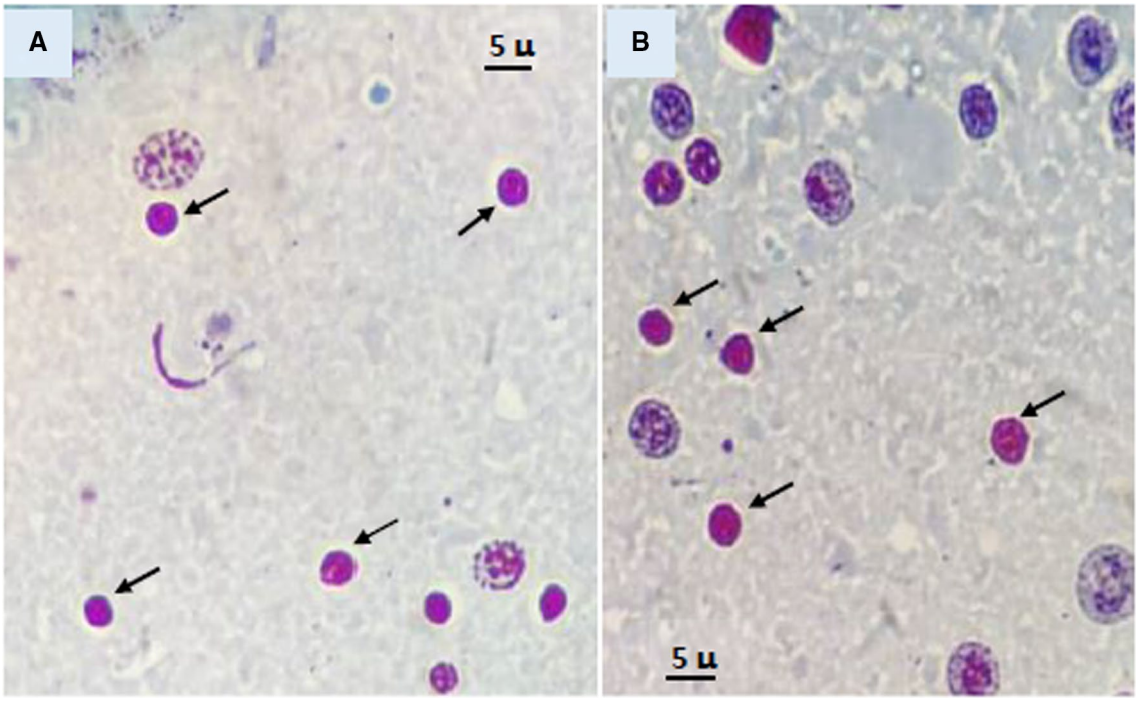

Fig. 1 *Cryptosporidium molnari* oocysts in *Clarias gariepinus* stomach (**A**) and intestine (**B**), stained with Modified Ziehl–Neelsen stain (mZN) (×100)

Table 2 Dimensions of *Cryptosporidium molnari* oocysts detected in *Clarias gariepinus* fishes

	Dimensions of the *Cryptosporidium molnari* oocysts oocysts (µm)
Length × width	3.20–4.5 × 3.90–6.05
Mean	3.9 × 5.0
Shape index (SI) L/W	1.4–1.6 (1.5)

with green–black (Fig. 1). The about 50 detected measurements of *Cryptosporidium* spp. oocysts varied from 3.20–4.5 × 3.90–6.05 µm of mean (3.9 × 5.0) µm in diameter and its shape index is 1.4–1.6 of mean (1.5) which is morphologically similar to *Cryptosporidium molnari* oocysts (Table 2).

Prevalence of *Cryptosporidium* spp. Infection Among *Clarias gariepinus* Fish Using mZN and ELISA

The overall *Cryptosporidium* infection prevalence of the total examined *Clarias gariepinus* fishes (300): 180 from Nile River and 120 from drainage canals was 64.0%. A higher prevalence of 68.3% was recorded in drainage canals fishes while lower in fishes collected from Nile River (61.1%) using mZN staining technique (Table 3). ELISA assay revealed that 69.3% of total examined fishes had

Table 3 Prevalence of *Cryptosporidium* spp. infection in *Clarias gariepinus* fishes using modified Ziehl–Neelsen staining technique

	Fish no	Organ	+ve No	+ve %	Total +ve (%)
River Nile fish	180	Stomach	92	51%	110 (61.1%) (in both stomach and intestine)
		Intestine	64	35.5%	
Drainage canal fish	120	Stomach	72	60%	82 (68.3%) (in both stomach and intestine)
		Intestine	45	37.5%	
Total	300				192 (64%)

antibodies against *Cryptosporidium* spp. and also, drainage canals fishes showed the higher infection rate (75.0%) than Nile River fishes (65.5%) (Table 4).

Comparison Between the Prevalence of *Cryptosporidium* spp. Infection Among *Clarias gariepinus* Using mZN and ELISA

ELISA serological test showed a higher prevalence (69.3%) than that prevalence obtained by mZN (64%) for the total examined *Clarias gariepinus* fish. Also, ELISA test revealed

Table 4 Prevalence of *Cryptosporidium* spp. infection in *Clarias gariepinus* fishes using ELISA

	Fish no	+ ve no	+ ve %
River Nile fish	180	118	65.5%
Drainage canal fish	120	90	75.0%
Total	300	208	69.3%

a higher prevalence of *Cryptosporidium* infection, 65.5% and 75.0% than 61.1% and 68.3% obtained by mZN, in both *Clarias gariepinus* fish groups from Nile River and Drainage canal, respectively (Fig. 2).

Molecular Detection of *Cryptosporidium* spp.

Conventional PCR Analysis (cPCR)

Conventional polymerase chain reaction (cPCR) analysis was used for examination of one negative and 2 positive prepared PCR samples of *Clarias gariepinus* scraped stomach and intestinal mucosa which were previously confirmedly mZN-positive for the Cryptosporidium oocysts; revealed the positive expected bands at 1056 bp for the two mZN + ve tested fish samples (lane 2 to 3), while the other mZN -ve tested fish sample (lane 1) showed negative PCR for *Cryptosporidium* spp. (Fig. 3).

DNA Sequencing and Phylogenetic Analysis

Molecular discrimination of *Cryptosporidium* oocysts was done using PCR amplification and the partial nucleotide sequencing which was isolated from Egyptian *Clarias gariepinus* fish revealed 1st (1325 bp) and the 2nd (825 bp) expected PCR products obtained from the all examined Egyptian isolates. The BLAST search analysis and DNA sequencing alignments of isolated *Cryptosporidium*

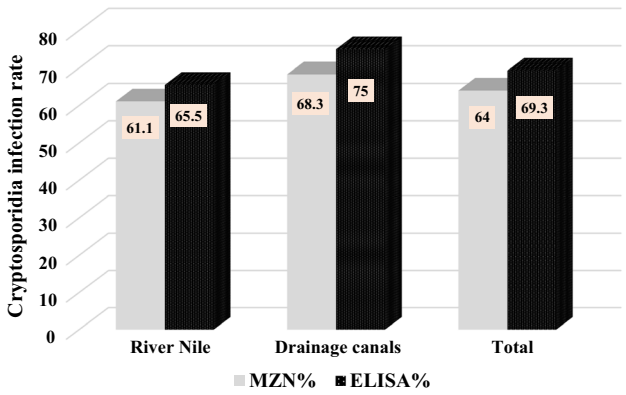

Fig. 2 Comparison between the prevalence of *Cryptosporidium* spp. infection among *Clarias gariepinus* using mZN and ELISA

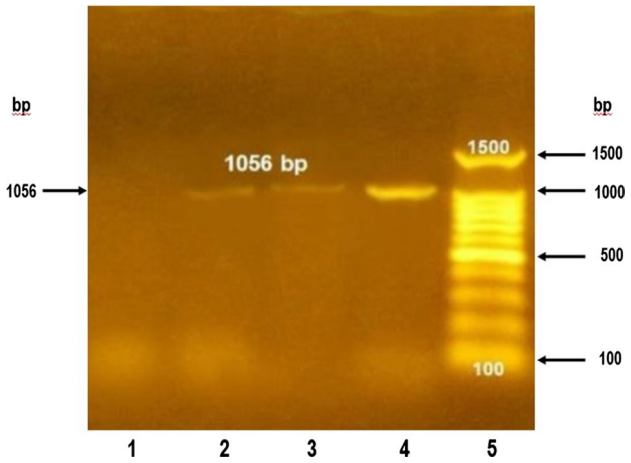

Fig. 3 PCR analysis for *Cryptosporidium* spp. from *Clarias gariepinus* fish samples: negative control sample (lane 1), + ve tested fish samples (lane 2 and 3), positive control sample (lanes 4) and DNA markers (lane 5)

Egyptian isolates proved 100% similarity between the amplified fragment, 825 bp of Egyptian isolates which compared with the equivalent of the 18S rRNA sequences of *Cryptosporidium molnari* and *C. molnari*-like organism published sequences deposited in Gene bank and with Phylogenetic analysis which provided that the positive-PCR *Cryptosporidium* isolate identified in the present study was *Cryptosporidium molnari* (Fig. 4). (GenBank accession no. MK791220.1).

Discussion

The microscopic identification of *Cryptosporidium* spp. was based on standards, such as oocyte measurements and morphology, and this suggestion was consistent with Xiao *et al.* [24], who cited the oocysts morphometric measurements as the master factor for classifying *Cryptosporidium* spp. which is important and prerequisites for identifying a new species. The morphological appearance of detected *Cryptosporidium* oocysts obtained from the stomach and intestinal scraped mucosa of *Clarias gariepinus* fish in our study after mZN staining was bright-red, spherical smooth wall. Oocysts occasionally showed an incomplete suture line and with a mean diameter of 3.9 × 5.0 µm. These detected oocysts were nearly similar to 4.4 × 5.2 µm which were descripted in previous studies by O'Donoghue [33] and Xiao *et al.* [34], and that agree with originally described *Cryptosporidium molnari* from gilthead sea-bream [35], and also perfectly look like *C. molnari* oocysts isolated from stomach of Murray cod fish described by Barugahare *et al.* [36], which were semi-spherical and varied from 4 to 5 µm in diameter with an incomplete suture line of the oocysts wall.

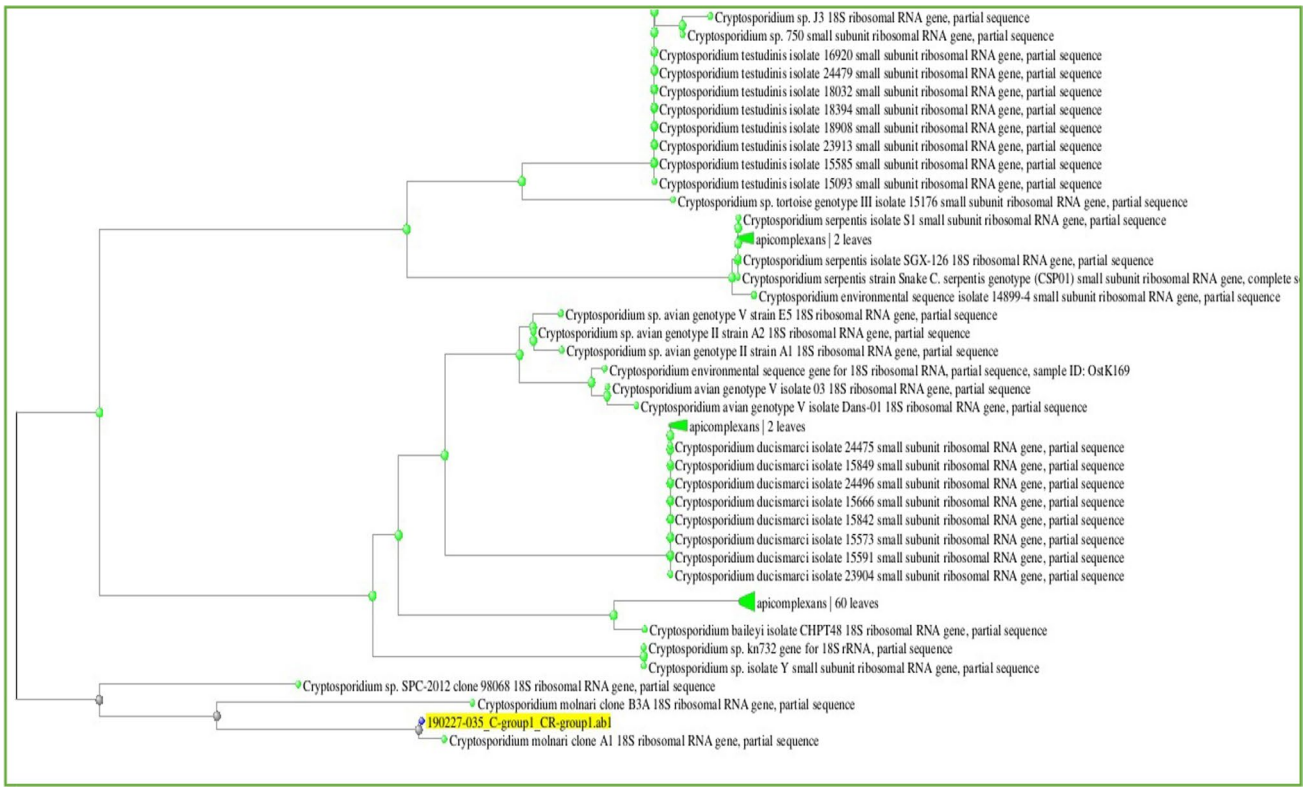

Fig. 4 Phylogenetic tree on *Cryptosporidium* Egyptian isolate from *Clarias gariepinus* fish have high homology with *Cryptosporidium molnari* isolate

The screening of *Cryptosporidium s*pp. infection among *Clarias gariepinus* fishes in this study revealed an overall prevalence of ELISA serological test showed higher prevalence (69.3%) than that prevalence obtained by mZN (64%). Using modified Ziehl–Neelsen staining technique, higher prevalence (68.3%) was recorded in drainage canals fishes while lower in fishes collected from Nile River (61.1%), and also, drainage canals fishes showed highest infection rate (75.8%) than Nile River fishes (65.5%) using ELISA. The greatly increase in the sensitivity by ELISA than mZN was comprehensible because the ELISA detects pathogen antigens which may have been from an active or previous infection as active mature oocysts mostly detected is higher specificity with the mZN technique [37]. Drainage canal fishes showed a higher *Cryptosporidium* infection rate than Nile River fishes using both ELISA and mZN assay in this study, this may be referred to the bad sanitary characteristics of the place, the drainage canal location from living place, the number and category of people visiting the canal and its purpose, biological pollution [19]. Also, the chronic exposure to pollutants or environmental stress for drainage canal fishes more than Nile River fishes, initiated immune suppression through corticosteroids releasing, which lead to the fish more susceptible for many pathogenic organisms and this is considered as the main responsible way of high fish parasitic disease infection [38].

The overall prevalence of *Cryptosporidium* in *Clarias gariepinus* fish by ELISA and mZN assay in the present study was 69.3%, 64%, respectively. Lower prevalence 30.1%, 24.4%, and 30.8% recorded in freshwater goldfish from a local aquarium, local fish farm, and local bait shop respectively [39]. A large discrepancy in previous studies reported ranged from 0.8 to 100% [21, 29, 40–42]. The prevalence in farmed fish is generally higher than wild-caught fish, presumably due to the over-crowdedness and low exchange rates of water, and hence, high exposure opportunities to infection [41]; for example, wild marine fish, observed a prevalence of only 2.4% [43], whereas fresh water aquarium fish showed a prevalence of 10.5% [29] and another study in six fresh water fish species infected with of *Cryptosporidium* from Lake Geneva (Lac Leman) revealed an overall prevalence of 36.6% [44]. The prevalence of infection with *Cryptosporidium* is much higher, mostly among juvenile fish, whereas Turbot was intensively parasitized with *C. scophthalmi* infection rates as high as 100% [45]. On the other hand, *C. molnari* in European sea bass and gilthead bream fish were identified as 30 to 100 g weight class, while in fish weighing over 300 g, there are no infections observed [40]. Similarly, hatchery-reared *Oreochromis niloticus* fry,

and fingerlings are the only were infected, not adults with C*ryptosporidium* [43].

Taxonomists have argued the need for an integrated approach using standard morphological, biological and advanced molecular methods to describe C*ryptosporidium* types [46]. Currently, *C. molnari* is the unique recognized species that parasitizes fish accepted as valid species of *Cryptosporidium* [47]. Until more, molecular data for Cryptosporidia fish species are available; some researchers feel that there are not enough data to determine valid names for those Cryptosporidia-like parasites in fish [48]. In the current study, PCR analysis of two positive samples of *Clarias gariepinus* scraped stomach and intestinal mucosa confirmed with mZN for infection with *Cryptosporidium* oocysts exhibited the positive expected bands at 1056 bp. DNA sequencing and phylogenetic analysis proved that the positive-cPCR *Cryptosporidium* isolate identified in the present study was *Cryptosporidium molnari*. The obtained results verify proof of widely known that the shorter amplicons amplified by the cPCR more efficiently than those longer amplicons and the cPCR-positives were due to non-specific amplification; however, the assay has been extensively validated [21]. The obtained sequence of the nucleotide from *C. molnari* SSU rDNA of *Clarias gariepinus* fish identified in present study was identical to that of *C. molnari* detected in the gilthead sea-bream [49] and very similar to that of *C. molnari* in butter bream fish from Spain [29]. *Cryptosporidium molnari*-like genotypes also, were previously molecularly identified in many fish species, such as a golden algae eater, a green chromes, a butter bream, a madder sea-perch, an upside-down cat-fish and a wedge-tailed blue tang [50].

Conclusion

The high infection rate in *Clarias gariepinus* freshwater fishes with *Cryptosporidium* in this study suggests that the need for future research to gain a better understanding of public health effects is warranted and may also be a good steward of environmental pollution or ecosystem health. Also, the higher prevalence in drainage canals obtained fishes than that collected from Nile River indicates the important public health worry and a potential danger to drainage channels in Egypt. ELISA showed higher prevalence of cryptosporidiosis than mZN, for the total examined *Clarias gariepinus* fish, and combination of mZN staining technique with one of the ELISA and/or PCR assays will be the “gold standard” and increased the specificity and sensitivity, ensuring that undiagnosed *Cryptosporidium* infection is not obtained. The phylogenetic analyses in this study prove and confirm novel detection of this protozoal organism of *Cryptosporidium molnari* in *Clarias gariepinus* fish.

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Author contributions Dr. RMS: planned and design the study, serological, molecular investigation, and drafting the paper. Dr. FAA: sharing in the idea and study design, identification of *Cryptosporidium* sp. and participated in drafting the manuscript. Dr. KGV: sharing in study conception, laboratory work, interpreted the data results, and helped in manuscript preparation and Dr. GIS: involved in samples collection and preparation, sharing serological and molecular tests. All authors read and approved the final manuscript.

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Declarations

Conflict of interest The authors of the current work declare that they have no conflicts of interest in this study.

Ethical approval The study was ethically cleared and approved by Medical Research Ethical Committee Research, National Statement on Ethical Conduct in Human and animals Research at National Research Centre, Egypt under registration number # 21-054#.

Significance The freshwater fishes (*Clarias gariepinus*) are subjected to high infection rate with *Cryptosporidium* sp.; the drainage canals obtained fishes showed higher *Cryptosporidium* infection prevalence than that collected from River Nile which indicates an important public health problem and a potential risk of drainage canals in Egypt. ELISA showed higher prevalence of cryptosporidiosis than mZN for the total examined *Clarias gariepinus* fish. ELISA and PCR will be the "gold standard" where specificity and sensitivity will be very high, ensuring that undiagnosed *Cryptosporidium* infection does not occur. The phylogenetic analyses in this study confirmed this protozoal organism to be a novel detection of *Cryptosporidium molnari* in *Clarias gariepinus* fish.

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