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Full Research Article

MOLECULAR SURVEY ON THE PARASITIC NEMATODE *CONTRACAECUM* SPP. IN FISH (*PLANILIZA ABU*) DESTINED FOR HUMAN CONSUMPTION

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Abstract

Contracaecum spp. is a parasitic nematode belonging to the Anisakidae family and which has a significant potential zoonotic effects on different hosts. Anisakiasis in humans is caused due to consumption of raw infected or undercooked seafood that contains nematode larvae. In this study, the prevalence of *Contracaecum* spp. nematodes in the fish, *Planiliza abu*, was determined over eight months from June 2022 to January 2023. The source of fish was Razzaza Lake, while the specimens of *Planiliza abu* were purchased from the local market, Karbala city, Iraq. The *Contracaecum* spp. nematodes were examined by morphological and molecular techniques.The total prevalence was 32%, as 133 of 416 fish were infected in the viscera by *Contracaecum* spp. third stage (L3) larvae. Molecular diagnosis of the COX-2 gene in *Contracaecum* spp. from *P. abu* was used to confirm the infection and to study their population biology. In the parasitology laboratory of the Veterinary Medicine College at Kerbala University, we confirmed the nematodes morphologically and molecularly as *Contracaecum* spp. The infection rates in September and November were greater than in October 2022. The results showed a significant positive correlation between the length of the fish and the number of nematodes per fish. This study detected the presence of *Contracaecum* spp. using analysis of mitochondrial cytochrome c oxidase II (COX-2 gene). In conclusion, molecular genotyping might be an effective method for detecting the *Contracaecum* L3 larval

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species, determining the biology of the life-cycles, population structures, transmission methods, and intermediate hosts types.

Keywords: fish, food safety, foodborne pathogens, zoonosis

INTRODUCTION

Human can inadvertently be infected by anisakid nematodes that belong to the Anisakidae family. In that family, aside from the genus *Anisakis*, other nematodes of the genera *Pseudoterranova* and *Contracaecum* are also considered as zoonotic. The zoonotic disease is acquired by human consumption of undercooked, raw or lightly processed fishery products (Mattiucci et al., 2018). Anisakidae infection can cause gastrointestinal and allergic symptoms (Golden *et al.*, 2022). Anisakidae are found worldwide, including in developing and developed regions of Asia and Europe (Aguilar-Marcelino et al., 2022).

Contracaecosis is infection due to consumption of *Contracaecum* spp. (Buchmann and Mehrdana, 2016). *Contracaecum* is an economically important nematode parasite with zoonotic significance (Shamsi *et al.,* 2018). The causative larvae (L3) of adult *Contracaecum* spp. can adhere to the gut or stomach, or they can enter the gut and move through the viscera of different hosts, i.e., marine mammals, piscivorous birds, invertebrates and fish (Shamsi *et al.,* 2018).

Despite the availability of molecular techniques and the low sensitivity and/or specificity of traditional diagnostic techniques, the latter techniques have been used until recently (Chen *et al.,* 2022). At present, the *Contracaecum rudolphii* complex has five recognized members, i.e., A, B, C, D, E and F (Garbin *et al.,* 2011). In north Iraq's Sulaimani Province, all fishes infected with *Contracaecum* larvae harboured just one species and sub-type (*C. rudolphii* B), determined through testing the sequences of ITS1, ITS2, and COX-2 genes (Abdullah *et al.*, 2021a). On the other hand, in the al-Sanaf marsh, southern Iraq, the ITS-1 regions of rDNA showed the fish there contained two distinct species, *Contracaecum septentrionale* and *Contracaecum microcephalus* (Mohammad and Hbaiel, 2019). Therefore, the aim of this study was to use molecular techniques to determine the presence of *Contracaecum* larvae in *Planiliza abu* from Razzaza Lake in Karbala.

MATERIALS AND METHODS

Location: Karbala, also spelled Kerbala, is the administrative centre of the Karbala Governorate in central Iraq, approximately (100 km) southwest of Baghdad. Razzaza (also spelled Razaza) Lake is located in western Iraq, west of Karbala (3241N, 4340E). It is Iraq's second-largest freshwater lake and used to be an important source of fish. The lake, which covers an area of 1810 km2 and is located 40 m above sea level, has a storage capacity of 26 billion m3 of water. Part of the water from Habbaniyah Lake is discharged into Razzaza Lake through a controlled exit route or channel from the Euphrates.

Fish collection and inspection

The whole fish sampled (*Planiliza abu; n* = 416) were purchased from the local market in Karbala. The fish all contained genus *Contracaecum* (Railliet & Henry, 1912) anisakid nematodes and had been captured from Razzaza Lake between June 2022 to January 2023. All the fish were counted, measured and weighed. Each fish was traditionally dissected and then its anisakid larvae content was examined. Each fish specimen (viscera and flesh) was examined separately. The length and weight were measured to give the prevalence (P) of the parasite in the studied fish, and the mean intensity (mI) of infection was calculated. Following a visual inspection, a stereoscopic microscope was used to dissect the viscera and the number of parasites was determined for each fish (Shamsi and Suthar, 2016).

Morphological and parasitological examination

All isolated nematodes were examined morphologically (Shamsi, 2019). Individual fish larvae were mechanically removed, rinsed in saline solution for thirty minutes, and placed in 70% ethanol (Pons-Bordas *et al.,* 2020). Lactophenol was used to clear the nematodes so they could be morphologically evaluated. As suggested by the genus name, these worms' digestive systems consist of two caeca that are situated in opposition to one another. The front of their bodies also features an excretory orifice (Figures 1, 2 and 3). These features, due to their maintenance throughout all stages of parasite growth, are considered as the most important morphological traits for differentiating *Contracaecum* spp. from the other parasitic anisakids (Shamsi, 2019). Statistical analysis of results was conducted by the Chi-square test. SPSS statistical software (version 24) was used to analyse data. The Pearson correlation coefficient was used to analyse differences between factors (Peck *et al.,* 2015).

Molecular analysis of *Contracaecum* larvae

Molecular analysis was conducted on eight *Contracaecum* larvae. One larva was selected randomly for each month of the study. Total DNA was extracted, using a commercial kit (Ginaid, Korea), from the centre region of the larvae. PCR was performed, focused on the mitochondrial cytochrome c oxidase II (COX-2) gene, amplified using 210 (5'-CACCAA CTC TTA AAA TTA TC-3') and 211 (5'-TTT TCTAGT TAT ATA GAT TGR TTT YAT-3') (Nadler and Hudspeth 2000). The amplification volume was prepared as 25 μL, which included 5 μL of an extract containing DNA, 1.5 μM of forward primer, 1.5 μM of reverse primer and 25 μL of double-distilled water as the final volume in the mastermix kit (Promega, USA). Initial denaturation of the reaction was carried out for 5 min at 94 ^{O}C, followed by 35 cycles at 94 ^{O}C for 30 s (denaturation), 50 ^{O}C for 60 s (annealing), and 72 ^{O}C for 1 min (extension), with a final extension step at 72ºC for 7 min to ensure all amplification reactions had reached completion. PCR parameters were applied in accordance with D'amelio *et al.* (2007). Analysis of the PCR results was done by Safe-Red™ agarose gel electrophoresis. UV transillumination was used and amplified rRNA products were visualized on 1.5% agarose.

Ethical Statement

The ethics standard required was approved under No UOK.VET. MI.2022.059, issued by the Ethical Committee of the University of Kerbala, College of Veterinary Medicine.

RESULTS

A morphology and consistent molecular genetic approach was used in the current study to examine the nematodes and identify them as the third stage (L3) larvae of *Contracaecum* spp. The majority of these nematodes were determined to be *Contracaecum* spp., according to the unique features of this parasite and using light microscopic examination (Figures 1-3). The overall prevalence was 32% (133 of 416 fish were confirmed as harbouring *Contracaecum* larvae; Table 1). The total mean intensity of infection (number of parasites per fish) was 1.22, with a range of 1-4 worms per fish.

Table 1. Number of *Planiliza abu* fish infected with *Contracaecum*, fish length, sex, and infection rate (monthly from June 2022 to January 2023).

Month when fish were captured	Sex of infected fish		Total no. of fish examined	No. of infected fish	% of infected fish	Length (cm)		No. of nematodes	R correlation (No.of nematodes and No.of infected fish)
	Male	Female				Fish with infection	Uninfected fish		
Jun	1	11	59	12	20.3	10.41	9.57	48	P value =0.05
July	4	11	56	15	26.7	10.23	9.32	60	
August	4	9	30	13	43.3	9.26	9.44	52	
September	5	13	70	18	25.7	11	10.16	72	
October	6	13	53	19	35.8	9.56	9.5	44	
November	3	14	47	17	36.1	9.58	9.53	68	
December	3	17	52	20	38.5	10.4	9.75	80	
January	2	17	49	19	38.7	10.15	10.26	76	
Total			416	133	32.0			508	

The results showed a significant positive correlation between the length of the fish and the number of nematodes per fish at a significance level of $P \leq 0.05$.

Contracaecum spp. prevalences were highest in *P. abu* captured during August, December and January, and lowest in June**.** The relationship between fish length, number of nematodes, and number of infected fish was significant at the level of P≤0.05 according to (R) correlation**.**

Among the 133 *Contracaecum* spp. larvae, eight were subjected to a molecular approach, with DNA extracted from the centre regions of the larvae. Amplification of DNA fragments of the COX-2 gene of interest showed all eight *Contracaecum* spp. larvae produced a DNA fragment of 558 bp (Fig. 4).

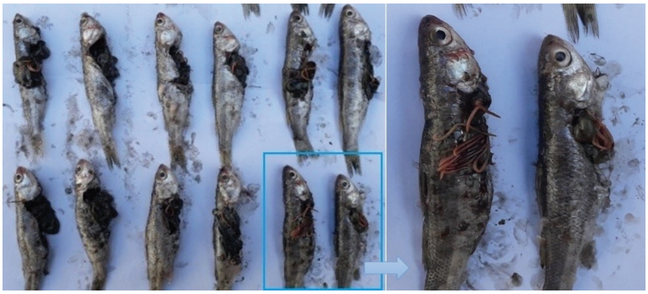

Figure 1. The third stage of *Contracaecum* spp. larvae in the viscera

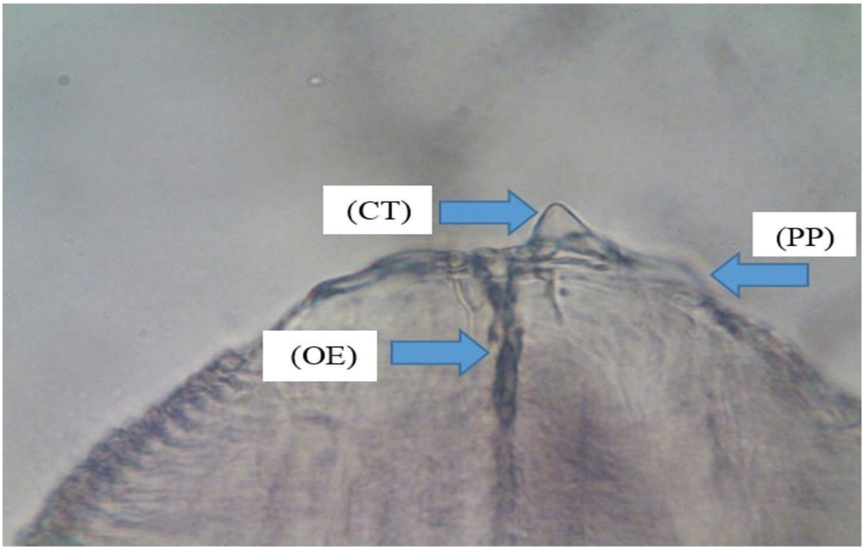

Figure 2. Anterior part of *Contracaecum* third stage larva with ventral cephalic tooth (boring tooth) (CT), papilla part (PP) and oesophagus (OE) (arrow scale bar = 0.10 mm)

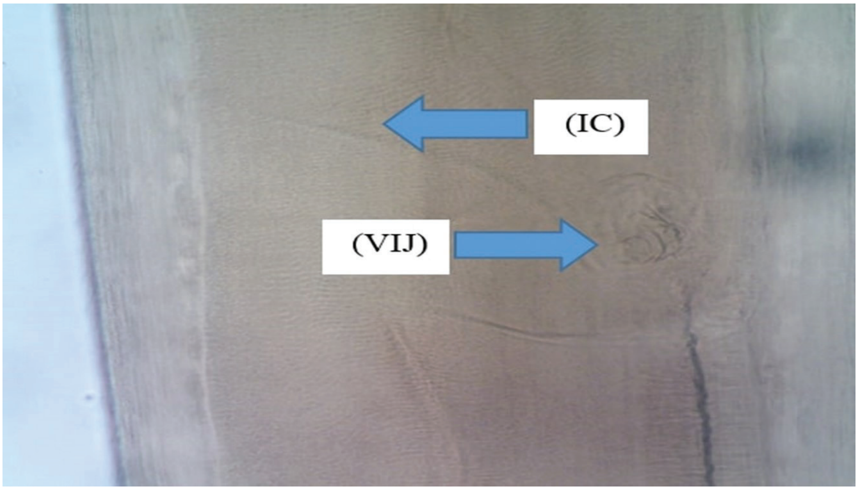

Figure 3. The ventricle-intestine junction (VIJ) in the central region of a larva, showing intestinal caecum (IC) (Scale bar = 0.10 mm)

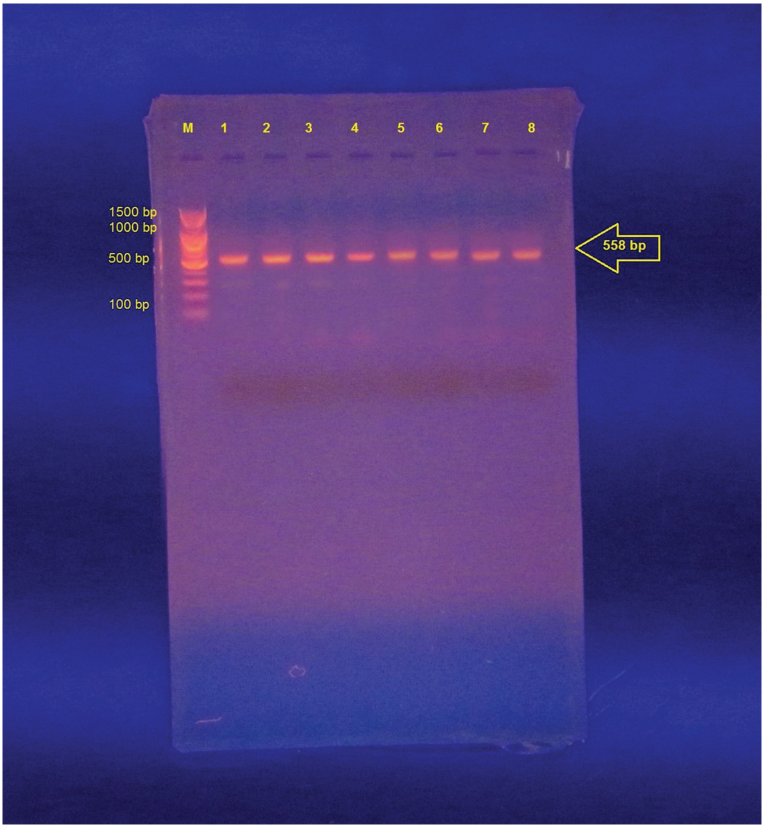

Figure 4. Gel electrophoresis for COX-2 gene in *Contracaecum* spp. M: 1500-bp DNA marker, Lanes: 1–8 are positive for a 558 bp fragment, confirming the gene in *Contracaecum* larvae

DISCUSSION

We studied the nematodes that parasitize Razzaza Lake's *P. abu*, and found the overall prevalence of *Contracaecum* infection in the fish was 32%. This prevalence reported herein accords with the 30% prevalence of *Contracaecum* spp. larvae detected in fish species from Lake Nasser, Egypt (Hamouda and Younis, 2022).

The number of infected female fish in our study was higher than the number of infected males because we used non-targeted, random sampling, without targeting of one or other sex. In all the studied fish, only the larval form of *Contracaecum* was detected, so we depended on identifying characteristic features in the larvae, with confirmation using amplification of the mitochondrial cytochrome C oxidase II gene, COX-2. This approach agrees with Jawad *et al.* (2022) in the same region and also another study (Al-Saadi *et al.*, 2010). Since piscivorous birds, which serve as the parasite's final hosts, can be killed or captured, thus disrupting the infection cycle, there could have been a low parasite prevalence (Barson, 2004) at times during our study. If an appropriate, specific host connected to the aquatic environment eats an infected fish, the *Contracaecum* life cycle can be completed, as occurs in cormorants (Al-Warid *et al.*, 2021).

Since a single larva can spread infection, the correlation between the presence of anisakids in the flesh and the time between capture and sale is particularly significant. The amount of time between catching and eating increases the risk of human infection and is considered the biggest risk for consumers (Madrid *et al.*, 2012). High prevalences of *Contracaecum* occurred in Razzaza Lake's *P. abu* during August, December and January (43.3%, 38.46% and 38.7%, respectively), while a low prevalence (20.3%) occurred in June. These variations could be attributed to factors or parameters that influence parasite assemblages in fish hosts. These variables are either biotic or abiotic in nature. The host's nutrition, specificity, age, sex and size and the parasite size are all factors to consider (Iyaji *et al.*, 2009).

The longest fish (17cm) was captured in January, the shortest (12cm) in December. At the $P \leq 0.05$ level, the length of the *P. abu* studied had a significant effect on the number of infected fish and the number of nematodes. This is in contrast to another study (Barson, 2004) on *Clarias gariepinus* from Lake Chivero, Zimbabwe, in which host size was unrelated to parasite prevalence, infection intensity, and body condition. Vuić *et al.* (2022) found 20/199 (10.1%) of Prussian carp (*Carassius gibelio*) from Lake Sakadaš, Croatia, harboured *Contracaecum* larvae, and their effect on their host's health was measured by presence of number parasites, and fish biomass, weight, and length.

In our study, the overall intensity of infection was 1.22 (range 1-4) parasites per fish, in agreement with (Al-Zubaidy, 2009), reported in different localities in Iraq, where the intensity was 1.1 and 3.5 parasites per fish in winter and summer, respectively. Our current results do not agree with Barson (2004), who detected 1-7 parasites per fish, or the mean intensity of 2.2 reported in Chile (Garbin *et al.*, 2011). The differences could be related to the specific intermediate hosts, fish population species, ecology, and the aquatic environment.

The host length played an important role in infection with *Contracaecum* spp. larvae, numbers of which were positively associated with fish length, as also reported previously (Al-Zubaidy, 2009). Another investigation into parasites in market fish in Iraq was conducted from November 2019 to December 2021 (Jawad *et al.*, 2022). In the same water body (Razzaza Lake) and host (*Planiliza abu*), that study reported parasite prevalence in the last two months of 2019 was 48.73% in 148 fish, 65.08% in 277 fish in November 2019 to December 2021, and 9.6% in 577 fish in 2021. The current study is also in agreement with Al-Zubaidy (2009). Monthly, of 158 mugilid fish analysed for *Contracaecum* spp. larvae, on average, 41 (25.9%) were infected (Al-Zubaidy, 2009). Al-Saadi *et al.* (2010) describe *P. abu* endoparasites in Al-Husainia Creek, Karbala Province, central Iraq, and found 0.8% of fish harboured *Contracaecum* spp. L3 larvae.

In addition to microscopical examination and molecular analysis by PCR, sequencing of five COX-2 variants in *Contracaecum* larvae from 17 Cyprinidae freshwater fish in Sulaimani Province, Kurdistan Region, Iraq, revealed *Contracaecum rudolphii* B in all infected fish species (Abdullah *et al.*, 2021b). Although, the diagnosis of nematodes depends on specific characteristic features of *Contracaecum* spp., such molecular assays allow better consideration of the parasites. Among the molecular tests, the mitochondrial COX-2 gene has been used for detection and confirmation of *Contracaecum* infection in different hosts. In the current study, eight nematodes were selected randomly, i.e., one from each month of the study, and the COX-2 gene analysed to show the characteristic 558 bp fragment was present in all specimens. Many studies have used the COX-2 gene as a tool for detection and species differentiation, including Pons-Bordas *et al.* (2020), who sequenced the COX-2 gene in 18 parasites recovered from three species of cetaceans. Single or combined infestations of twelve individual cetaceans were caused by *Anisakis simplex* sensu stricto and six by *Anisakis pegreffii*, with fragments generated ranging from 432-632 bp in length. Another study, based on characteristic morphological features, sequence analyses of multiple COX-2 loci and on the small subunit rRNA, supported a host-parasite association between *C. rudolphii* A, B and C in *Clarias gariepinus* (Garbin *et al.*, 2011). The different host-parasite associations could be related to ecological environmental factors, location, sample size and factors related to the intermediate hosts. Nematode COX-2 genes were molecularly characterized in mullet (*Mugil curema*) from the Chautengo Lagoon, Guerrero, Mexico, and 283 of the 460 studied nematodes were L3 *Contracaecum* spp. (Martínez *et al.*, 2022). Some fish, like carp, play important roles in distributing zoonotic parasites not only within a fresh-water catchment region but also from marine to freshwater systems (Shamsi *et al.*, 2018).

Contracaecum, the most important zoonotic nematode in the Anisakidae family, has a significant negative impact on human health, and this important parasite was found in the abdominal cavity of *P. abu* obtained from the local fish market. Globally, *Contracaecum* are widely dispersed, recorded in numerous fish species from various nations (Shamsi, 2019; Barson, 2004). In some cultures, the small fish consumed can harbour infective parasites or carcinogenic heavy metals (Al-Warid *et al.*, 2021). *Contracaecum* spp. can cause significant effects in infected individuals, such as allergy, nausea, vomiting and gastric pain. In fact, the illness is a severe, emerging human health problem that has a negative impact consumer confidence (Bao *et al.*, 2018). At least, two thirds of consumers would stop purchasing/consuming fish if parasites are present in the fish (Bao *et al.*, 2018). Generally, people are deterred from buying contaminated goods when anisakid larvae are found in fish (Mostafa *et al.*, 2020). Because the Anisakidae family poses a risk to human health, it is crucial to identify fish containing these parasites (Jawad *et al.*, 2022; Buchmann and Mehrdana, 2016).

Parasitic nematode transmission is not limited to just a few fish species, and nematode parasites of different species can cause human infection. Eating raw fish in many traditional preparations was characterized as a culinary preference in Chile (Muñoz-Caro *et al.*, 2022). In that study, the molecular method used (mitochondrial DNA COX-1) was used to detect and identify collected anisakid L3 larvae of *Pseudoterranova cattani* and *Anisakis pegreffii* species from fish destined for human consumption. The 180 fish sampled were from three separate fish species, sea bream (*Brama australis*), snoek (*Thyrsites at*), and Chilean hake (*Merluccius gayi*) (Muñoz-Caro *et al.*, 2022). Pekmezci and Yardimci (2019) used molecular methods to study the COX-2 gene in L3 larvae from fish in Turkish waters. Three species were identified, *Anisakis typica*, *A. pegreffii* and *A. simplex* sensu stricto (s.str.)/*A. pegreffii* hybrid genotype. Turkish populations living in the Black Sea area consume fish that are possibly pose less risk with respect to allergies or anisakiasis in humans, while people in the Aegean and Mediterranean Sea regions are exposed to *A. pegreffii* as a zoonotic parasite, suggesting the Turkish communities who eat seafood from these areas are exposed to a high threat of anisakiasis or allergies. Abdullah *et al.* (2021b) reported a study from Iraq using ultra-morphology and molecular examination (including COX-2 analysis), and revealed all of the five infected cyprinid fish species harboured just one *Contracaecum* species and subtype (*C. rudolphii* B). Verma *et al.* (2022) studied *Contracaecum* in hillstream loaches from local markets, and which were destined for human consumption in the Western Ghats, India. Electron microscopy and COX-2 examination revealed *Contracaecum* genus. Finally, in Assiut Governorate, Egypt, Thabit and Abdallah (2022) described *Contracaecum* larvae in Nile Perch using light and electron microscopy and gene analysis to determine the larva species.

CONCLUSIONS

The classic parasitological examination for Anisakidae is still the primary diagnostic method due to speed and cost, while molecular methods are also important, especially in the identification of species and strains, as shown in various studies. The control of fish-borne parasites is a notable part of food safety, as some fishborne parasites, including Anisakidae, are zoonotic and so are extremely important for public health. In seafood, and especially in fish hosts that are destined for human consumption, molecular surveys for *Contracaecum* larvae are necessary in order to decrease the risk of human infection. Fish industries (production, processing) must employ suitable visual inspection before fish are presented for sale in markets. The routine method of artificial digestion of fish tissues should be continuously used. More research studies should be focus on genera/species of zoonotic parasites in fish, and for C. *rudolphii*, the subtype (A, B, C, D, E and F) should be confirmed.

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Authors' contributions

Firas Alali, Muayad A. Hussein, and Marwa Jawad: sampling; analysis of results; PCR; writing the manuscript; and editing the final version of manuscript.

Competing interests

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MOLEKULARNO ISTRAŽIVANJE PARAZITSKE NEMATODE *CONTRACAECUM* SPP. U RIBI (*PLANILIZA ABU*) NAMENJENOJ ISHRANI LJUDI

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Kratak sadržaj

Contracaecum spp. je parazitska nematoda koja pripada familiji Anisakidae i koja ima značajan zoonozni potencijal na različite domaćine. Anisakijaza ljudi nastaje usled konzumiranja sirove inficirane ili nedovoljno termički tretiranih plodova mora koji sadrže larve nematoda. U ovoj studiji, prevalencija *Contracaecum* spp. nematoda u ribi, *Planiliza abu*, utvrđena je tokom osam meseci od juna 2022. do januara 2023. godine. Poreklo ribe je bilo iz jezera Razaza, dok su primerci *Planiliza abu* kupljeni na lokalnoj pijaci, grad Karbala, Irak. The *Contracaecum* spp. nematode su ispitivane morfološkim i molekularnim tehnikama. Ukupna prevalencija iznosila je 32%, pošto je 133 od 416 riba bilo inficirano parazitom – larve treće faze (L3). Molekularna dijagnoza COX-2 gena *Contracaecum* spp. iz *P. abu* je korišćen za potvrdu infekcije i proučavanje njihove populacione biologije. U laboratoriji za parazitologiju Veterinarskog fakulteta Univerziteta Kerbala potvrđeno je da su nematode morfološki i molekularno *Contracaecum* spp. Utvrđen je veći stepen infekcije u septembru i novembru nego u oktobru 2022. godine. Rezultati su pokazali značajnu pozitivnu korelaciju između dužine ribe i broja nematoda po ribi. Ova studija je otkrila prisustvo *Contracaecum* spp. korišćenjem analize mitohondrijalne citohrom c oksidaze II (COX-2 gen). U zaključku, molekularna genotipizacija može biti efikasan metod za detekciju oblika larve *Contracaecum* L3, određivanje biologije životnog ciklusa, strukture populacije, metoda prenosa i tipova prelaznih domaćina.

Ključne reči: riba, bezbednost hrane, patogeni organizmi koji se prenose hranom, zoonoze