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Sequencing the Parasitic Nematode *Contracaecum* spp. in Edible Fish (*Planiliza Abu*)

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Abstract: *Contracaecum rudolphii* Hartwich,1964 (Nematoda: Ascaridoidea, Anisakidae) is a typical anisakid recorded globally. Consumption of undercooked seafood, raw or infected, contains the nematode larvae that cause human anisakidosis. Fish (*Planiliza abu*) specimens were obtained from a local market in Karbala, Iraq. This type of fish came from the Razzaza Lake. The prevalence of *Contracaecum* spp. parasites were done over eight months from June 2022 until January 2023, using ITS1, morphological and molecular analysis of the nematodes Contracaecum spp from fish. Of 395 fish, 124 (31.3%) had visceral infections caused by *Contracaecum* spp. larval type (L3). The morphological and genetic identification of *Contracaecum* spp. was validated in the parasitology laboratory of the Veterinary Medicine College at Kerbala University. The result showed that the infection rate in January 2023 was (46.6%) and increased while it was (20%) in September 2022. With the use of ITS1 gene, the molecular analysis for *Contracaecum spp.* was to investigate *Contracaecum* spp. and to confirm it. However, the nematode count, number of infected fish, and length were all substantially different at the P≤0.05. This study detected the isolate=(a1) at the locus=OP787071 and sequenced the parasites. The isolates were confirmed as *Contracaecum rudolphii*, isolate a1 internal transcribed spacer 1, partial sequence. In conclusion, molecular genotyping might be a useful technique for identifying the *Contracaecum* L3 larval species, life-cycle biology, transmission methods, and types of intermediate hosts.

Keywords: *Anisakidosis, Contracaecum rudolphii, Fish, ITS1, Zoonosis.*

Introduction

Anisakiasis is a parasitic infection caused by larval stages of nematodes from the Anisakidae family's genera *Anisakis*, *Pseudoterranova*, and *Contracaecum*. Fish infested by the *Contracaecum* spp. parasites can infect humans as accidental hosts by consuming raw or undercooked infective stage larvae (L3) (Aibinu et al., 2019). The cases of human larvae are incidentally taken by undercooked fish meat or eating raw, and they may lead to anisakidosis, causing fever, stomach pains, vomiting and diarrhea (Decruyenaere et al., 2022; Hirosawa et al., 2020; Shamsi and Butcher, 2011; Shibata et al., 2020; Younis et al., 2017). They are known to have extremely harmful effects on wildlife fish, birds, and marine mammals (Shamsi, 2019). Anisakidosis is a new illness that causes a variety of clinical symptoms in humans and is caused by members of the Anisakidae family (Golden et al., 2022). The detected anisakidae nematodes were in stage 3 (L3), and the mitochondrial gene rRNA was molecularly identified using PCR (Shamsi and Suthar 2016). Both strains (*C. rudolphii* A and B) were found in freshwaters of Crucian carp (*Carassius carassius*), while *C. rudolphii senso lato* was found in the Caspian round goby (*Neogobius melanostomus*) from the Baltic Sea. Only the temporarily strain designated *C. rudolphii* B was identified in Poland. Using (TS-1 and ITS-2) of the ribosomal DNA of nematode from *C. rudolphii* B may be the dominant type in both brackish and freshwater (Szostakowska and Fagerholm, 2007). Additionally, a new location has been added to the parasite species' geographical spread. *Prussian carp*, *Carassius gibelio* were caught in Karataş Lake in Burdur-Turkey. Only *C. rudolphii* was found in one sample (2.63%) (İnnal et al., 2020). However, *Contracaecum Rudolph* samples that were collected from cormorant populations in Italy and Europe revealed two sibling species, *C. rudolph* A, which was more prevalent in brackish water fish, and *C. rudolphii* B, *w*hich was found infecting only freshwater fish identified by sequence analysis of the mtDNA cox2, and ITS region of rDNA gene loci (Mattiucci et al., 2020).

To date, *Contracaecum rudolphii* complex currently has five recognized members, those being A, B, C, D, E, and F (D'amelio et al., 2012; Mattiucci et al., 2008; Mattiucci et al., 2020). While all infected fishes by *Contracaecum* larvae in north Iraq's Sulaimani Province represented exactly one species (*C. rudolphii* B) through testing the sequences of ITS1, ITS2, and COX2 **(**Abdullah et al., 2021a**)**. On the other hand, in the al-Sanaf marsh, southern Iraq, the ITS-1 regions of rDNA showed two distinct species; *C. septentrionale* and *C.microcephalus* (Mohammad and Hbaiel, 2019). In this study, molecular studies of Contracaecum larvae in *Planiliza abu* from Razzaza Lake in Kerbala were used.

Materials and Methods

Location: Karbala, also spelled Kerbala, is the administrative center of the Karbala Governorate in central Iraq, approximately (100 km) southwest of Baghdad. It is home to an estimated one million pilgrims who travel there annually. Razzaza or Razaza Lake, is located in western Iraq, west of Karbala (3241N, 4340E). It is Iraq's second-largest freshwater lake, and it used to be an important source of fish. The lake, which covers an area of 1810 square km and is located 40 meters above sea level, has a storage capacity of 26 billion cubic meters of water. Part of the water from Lake Habbaniyah is discharged into Razzaza Lake through a controlled exit route or channel from the Euphrates (Fig. 1).

Figure 1. Map of Iraq, Karbala province, and Razzaza Lake location.

Collection and Examination of Fish: The source of *Planiliza abu* is Razzaza Lake of Karbala, Iraq and samples were purchased from the local market of Karbala. A total (395) samples of fish that belong to one genus *Contracaecum* (Railliet and Henry, 1912) of the Anisakidae family were captured between June 2022 and January 2023. All of the fish were counted, measured, and weighed. Each specimen was traditionally dissected afterwards, and its anisakid larvae content was checked. Each specimen (viscera and flesh) was examined separately and put in Petri dishes. The length and weight were measured to give Prevalence (P), and mean intensity (mI) was calculated by (Shamsi and Suthar 2016). Following a visual inspection, the flesh and then the stereoscopic microscope were used to dissect the viscera, and the number of worms was determined for each sample (Shamsi et al., 2011; Yusni et al., 2022).

Morphological and Examination of *Contracaecum* Larvae: All of the isolated nematodes were examined morphologically. Individual fish larvae were mechanically removed, rinsed in saline solution for 30 minutes, and placed in 70% ethanol alcohol (Pons-Bordas et al., 2020). Lactophenol was used to clear the nematodes so that they could be morphologically evaluated. As suggested by the genus name, these worms' digestive system consists of two ceca that are situated in opposition to one another. The fronts of their bodies also feature an excretory orifice (Martínez et al., 2022), (Fig. 2,3,4, and 5). They should be regarded as the most important morphological traits for differentiating *Contracaecum spp.* from another parasitic anisakid because they endure the longest throughout all stages of growth (Shamsi, 2019).

Figure 2. Fish (*Planiliza abu*) infested by *Contracaecum rudolphii* (CR).

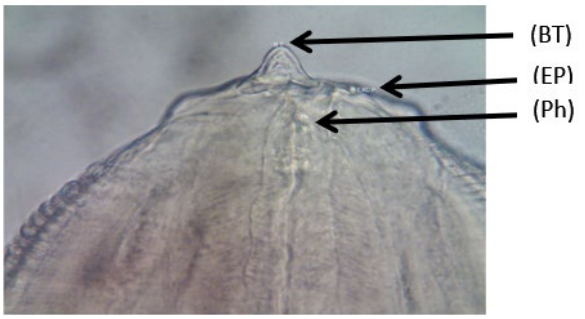

Figure 3. Anterior part of the third-stage larva. Boring tooth (BT), excretory pore (EP), and oesophagus (Ph) (Scale bar= 0.10 mm).

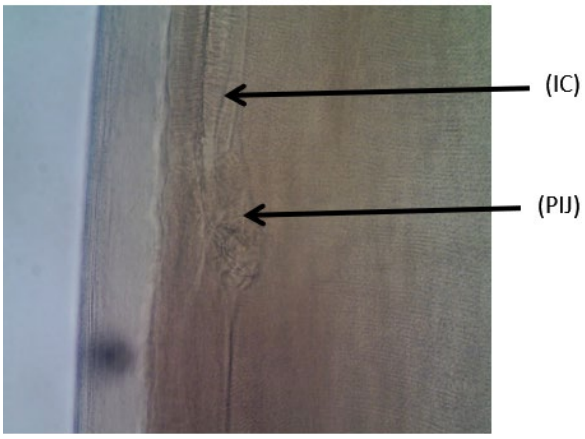

Figure 4. The pharynx-intestine junction area (PIJ), intestinal cecum (IC) (Scale bar = 0.10 mm).

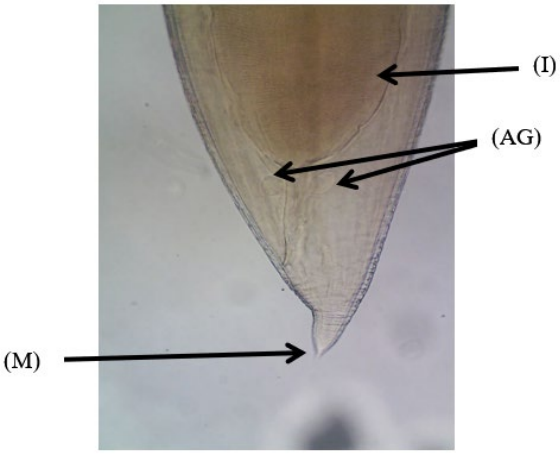

Figure 5. Posterior part of contracaecum: intestine (I), anal glands (AG), and mucron (M) (Scale-bar= 0.10 mm).

Statistical Analysis: The results data were analyzed by Chi-square. The SPSS statistical software (version 24) was used to analyze data. The Pearson correlation coefficient was analyzed between factors (Peck et al., 2015).

Molecular Analysis: A total of molecular were detected by the morphological examination used to detect eight third-stage larvae belonging to the *Contracaecum* genus of anisakid larvae. Then, eight *Contracaecum spp.* larvae were subjected to a molecular approach. DNA extraction from middle parts of *Contracaecum* larvae.

The molecular analysis was conducted on eight *Contracaecum* larvae. The larvae were selected randomly for each month. DNA extraction kit (Geneaid Biotech, Korea) was used. The total DNA was recovered from the center region of the larvae (Fig. 6). Amplifications focused on the ITS regions that were amplified using the primers NC5 (5'-GTA GGT GAACCT GCG GAA GGA TCA TT-3') and NC2 (5'-TTA GTTTCT TTT CCT CCG CT-3') (Zhu et al.,1998).

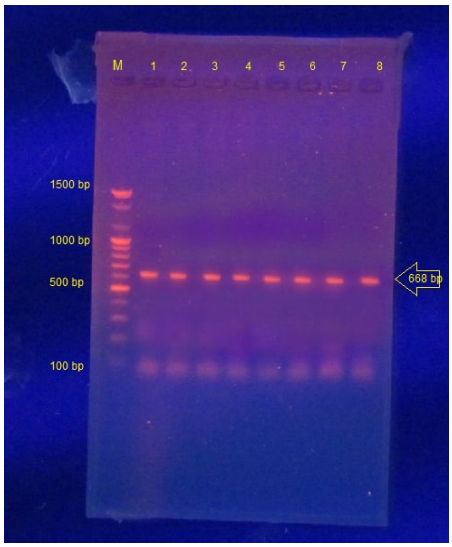

Figure 6. Electrophoresis of PCR product of *Contracaecum* of ITS1a gen for *Contracaecum* larvae at 668 bp according to DNA Ladder (100 -1500 bp), Lines: 1–8: positive all *Contracaecum* samples.

PCR conditions followed the protocol described by Pekmezci et al. (2014). Briefly, the Amplification of DNA fragments of interest from genomic DNA was performed using the polymerase chain reaction (PCR). The reaction volume was prepared as 25 µl which included 5 µl of a sample containing DNA, 1.5 µM forward primer, 1.5 µM of reverse primer, and double-distilled water to a final volume of 25 µl in the master mix kit (Promega, USA). The reaction was performed with an initial denaturation step at 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 seconds. (denaturation), 65 °C for 60 s (annealing) and 72 °C for 1 min (extension), with a final extension step at 72 °C for 7 min to ensure all amplification reactions had reached completion. The PCR products were analyzed by Safe-Red™ -agarose gel electrophoresis. PCR conditions were also used according to (D'amelio et al., 2007). UV transillumination was used and visualized on 1.5% agarose of the amplified rRNA products. The sequences of nucleotide acquired in the current study

were listed in GenBank under accession the number of OP787071.

To detect sequence similarities, NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) ran BLAST searches. For analysis, GenBank sequences of Contracaecum species were retrieved. The analysis included 28 nucleotide sequences, including one from Contracaecum Rudolph (OP787071). The similarity was determined using the Maximum Likelihood approach in the MEGA program (11.0.13). The search tree was built automatically using Neighbor-Join and the Maximum Composite Likelihood approach, and the topology with the greatest log-likelihood value was picked (Fig. 7).

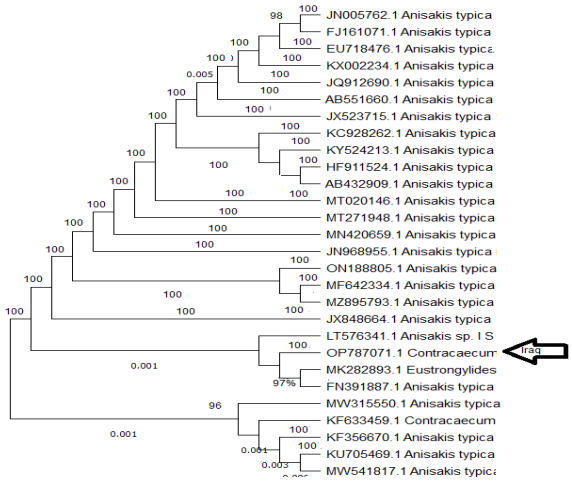

Figure 7. Phylogenetic analysis of ITS1 homologs from different Anskidae. Neighbor-joining reconstruction between the sequence of *Contracaecum rudolphii* obtained in this study and sequences of *Eustrongylides* and Anisakis typical species, recorded in GenBank and Phylogenetic analysis from the NCBI BLAST database. The MEGA (11.0.13) tool was used to compare the sequences to those performed independently for each gene fragment.

Ethical Approval: Ethics required are approved by the Ethical Committee of the University of Kerbala / College of Veterinary Medicine. Date: 23/02/2023, Ref: UOK.VET. PA.2022.05.

Results

The nematodes were examined and identified as Contracaecum third larval stage in the current study using a morphology consistency molecular genetic approach and phylogenetic analysis. Generally, all the nematodes have been *Contracaecum spp.* using light microscopic inspection depending on the special characteristics feature of this parasite (Figs. 2-7). This survey was completed during a period, from Jun 2022 to January 2023 to study nematode parasites of *Planiliza abu* of Razzaz Lake. Primer sequences of *C. rudolphii* isolate internal transcribed spacer 1(ITS1) (Table 1). The total prevalence of 395 fish was 124(31.3%). Infection rates were greatest in September and November and lowest from October to 2022 (Table 2). The relation among length, No. of nematode, and No. of infected fish have been signed at the level P≤0.05 according to (R) correlation (Table 3).

Discussion

The genus *Contracaecum spp.* includes more than 100 species, which are distributed globally from different hosts (Shamsi et al., 2009). Anisakiasis is widely distributed with their larvae recorded in various fish species from different countries, resulting *Anisakis* nematodes but it remains a neglected zoonotic disease (Aguilar-Marcelino et al., 2022; Shamsi and Barton, 2023). Because the zoonotic nematode Anisakidae family poses a risk to human health, it is crucial to identify fish (Buchmann and Mehrdana, 2016). Anisakidae is a family of nematode parasites, and one of the most significant fish-borne zoonoses in Europe is anisakidosis. It results from consuming the infectious larvae in their third stage causing the subacute abdomen and masquerading as an intraperitoneal malignancy (Dinas et al., 2024).

In this study, the nematodes were investigated and identified as Contracaecum third larval stage utilizing a morphological consistency molecular genetic technique. This survey was made from June 2022 to January 2023, and it studied the nematodes that parasitize the viscera the *P. abu* from Razzaza Lake. Generally, all the nematodes have been confirmed as *Contracaecum spp.* using morphological examination depending on the specific features of this parasitic nematode (Figs. 2-5). The total prevalence of 395 fish was 124 (31.3%) (Table 2). According to morphological examination, all of the fish were of the larval type Contracaecum. This result agrees with (Jawad et al., 2022), who confirmed nematodes in fish (*P. abu*) as *Contracaecum* spp. by Kerbala University's Veterinary Medicine College in the parasitology lab using morphological examination. The monthly infection in September and November had high infection rates, while in October, it had low infection rates of 32.0%, 31.5%, and 0.1%, respectively. In this study, the infection rates were the greatest in January 2023 (46.6%), but they were the lowest in September 2022 (20%), This may be climatic condition changes, founding or increased intermediate hosts and less fishering leading to favorable to increase infection (Table 2). Another study found 30% of *Contracaecum spp.* larvae in fish species from Lake Nasser, Egypt (Hamouda and Younis, 2022). In natural settings, the parasite despises the capacity to kill the intermediate host and prefers to finish the life cycle on the ultimate host. These factors may decrease the possibility of *Contracaecum* L3 larvae being transmitted to their ultimate host, resulting in a decreased total infection (Barson, 2004). In the current study, however, the gender length and infection of fish have not been significant within months (*P*>0.05). While with weight, none of the infected fish have been signed at the level P>0,05. The gender with length at level P≤0.01 and gender with weight at the level P≤0.05 according to Pearson correlation (Table 3). These characteristics were non-significant with host size, prevalence, infection severity, and body condition in *Clarias gariepinus* from Lake Chivero, Zimbabwe (Barson, 2004).

Table 1. Primer sequences of *C. rudolphii* isolate internal transcribed spacer 1(ITS1).

Primer	Sequence (5' – 3')	Amplicon size
ITS1(F)	GTA GGT GAACCT GCG GAA GGA TCA TT	
ITS1(R)	TTA GTTTCT TTT CCT CCG CT	**668 bp**

Table 2. Prevalence, length, gender and infection in the *Planiliza abu*.

Months of study	Length		Gender		Total no. of fish	No. of infected fish	%	No. of nematodes in fish
	infection	No infection	infected Male	Female infected				
Jun	10	9	2	13	58	15	25.8	48
2022 July	11	10	3	10	55	13	23.6	60
August	8	9	5	6	31	11	35.4	52
September	12	10	4	8	60	12	20	72
October	8.5	10.5	7	11	50	18	36	44
November	11	10.5	4	11	45	15	33.3	68
December	12	11.5	4	15	51	19	37.2	80
2023 January	13	12.5	5	16	45	21	46.6	76
					395	124	31.3	508

Table 3. Pearson correlation of infection in the *Planiliza abu*.

	Pearson correlation					
	Length	**Weight**	**Gender**	**No of infected fish**	**health fish**	**No of fish**
Length	1	0.789*	0.879**	0.686*	0.252	0.467
Weight		1	0.794*	0.66	0.143	0.475
Gender			1	0.515	0.209	0.509
No of infected fish				1	-0.177	-0.018
Health fish					1	0.899**
No of fish						1

* Correlation is significant at the 0.05 level (2-tailed).
** Correlation is significant at the 0.01 level (2-tailed).

Currently, the overall mean intensity was 4 and 1-19 worms per fish degree of infection, which is consistent with the findings (Al-Zubaidy, 2009) and disagreement with (Barson, 2004) who discovered (1-7) worms per fish and mean intensity (2.2). This variation might be due to ecological ambient variables, location, sample size, and intermediate host characteristics. In some of the studies, a survey of parasites of *P. abu* of Razzaza Lake was conducted in Karbala, Iraq (Al-Saadi et al., 2010; Al-Zubaidy, 2009; Jawad et al., 2022) (25.9%, 0.8%; in 2019, 48.73% of 148 fish were caught, 65.08% of 277 in 2020, and 9.6% of 577 in 2021).

Iraq's southern al-Sanaf swamp Sequence compares between Contracaecum larvae and the ITS-1 sections of rDNA amplified using PCR and proved that *Nycticorax nycticorax* had two different species. The first *C. septentrionale* and second *C. microcephalum*, both initially identified in Iraq, entered into GenBank with the entry numbers of MK424799.1 and MK424795, respectively Mohammad and Hbaiel (2019).

In Sulaimani Province, Iraq, contra caecum has been identified in 13 different species of freshwater fish, one from each of the following families: Bagridae, Heteropneustidae, Mastacembelidae, Mugilidae, Siluridae, and Sisoridae. All *Contracaecum* larvae were determined by morphological and genomic (ITS1, ITS2, and COX2) analysis to be members of the same species (*C. rudolphii* B), with infection rates ranging from 0.92% to 19.35% (Abdullah et al., 2021a). ITS-1, ITS-2 and COX-2 showed that all infected fish species representing one species (*C. rudolphii* B), are gathered in five Cyprinid fish species in this location, and these findings were published at the same region utilizing modern study techniques (Abdullah et al., 2021b).

In this work, the phylogenetic connections between *C. rudolphii* and other genera, such as *Anisakis typica* and *Eustrongyloides spp.* were determined using maximum likelihood using the ITS-1 gene. MEGA (11.0.13) was used to create the phylogenetic tree. *Eustrongyloides spp.* (MK282893.1) and *Anisakis typica* (FN391887.1) were highly identical to *C. rudolphii* 1 with 97% similarity and formed a clade with them, (Table 1) (Figs. 6 and 7).

This study utilizes PCR and sequencing of eight Contracaecum larvae from 200 samples over eight months, comparable to another study from Shadegan Wetland, Iran. All of the discoveries were proven to be *Contracaecum* spp. based on the phylogenetic tree and genetic distance, which identified *A. pegreffii* and *C. rudolphii* as the species of all nematodes. Primers NC5-NC2 were used to amplify an ITS segment from the worms *Barbus grypus* and *Mesopotamichthys sharpeyi,* which were subsequently subjected to *Contracaecum speciosus* and *Anisakis* infections (Mohammadi et al., 2021).

The ITS ribosomal gene was identified using PCR, and the mitochondrial genes COX2 and rrnS were molecularly

characterized in Guerrero mullet fish (*Mugil curema*). *Contracaecum sp.* was discovered with a frequency of 283 (61.5%) of 460 nematodes in stage 3 (L3) (Martínez et al., 2022). In South Wales, Australia, the ITS-2 region of rDNA, found in the intestinal tissue of carp from Coonancoocabil Lagoon, was used to identify a unique *Contracaecum bancrofti* (type IV) (Shamsi et al., 2018). The sequences of the first and second internal transcribed spacers (ITS-1 and ITS-2, respectively) of each morphospecies' nuclear ribosomal DNA are examined. The comparison of ITS-1 and ITS-2 sequencing data for individuals of *C. ogmorhini* sensu lato from pinnipeds with other species revealed that ITS-2 can be used for differentiation among Contracaecum species based on morphological data and was useful in confirming the taxonomic status of individual species in Australia (Shamsi et al., 2009).

In Ethiopia along Lake Tana Based on ITS1 analysis, two separate Contracaecum species were identified: *Contracaecum sp.* 1 and *Contracaecum sp.* 2, which shared 99% and 98% of their characteristics with *Contracaecum sp.* While *Contracaecum* sp. 1 and *Contracaecum* sp. 2 revealed 91% and 89% similarity with *Contracaecum multipapillatum* in their rrns, respectively (Kibet et al., 2021). This work highlights the need to integrate morphological and molecular techniques with (ITS1) to identify *Contracaecum rudolphii* larval stages, particularly those that occur in fish (*P. abu*).

Conclusion

In conclusion, the present study is the first molecular sequencing in fish (*P. Abu*), that has not been previously recorded from a local market in Razzaza Lake in Karbala, Iraq. The results have been confirmed that *C. rudolphii* depends on (ITS1) analysis. Further studies are needed to extend the knowledge of *Contracaecum* species distributed in a local market in Razzaza Lake in Karbala, Iraq. A good finding as a molecular genotyping might be a useful technique for identifying the Contracaecum L3 larval species., life-cycle biology. There is very little risk from zoonotic anisakids, such as *C. rudolphii*, in the area under study. Therefore, it is crucial to use genetic and molecular methods when learning about one species of fish, and should be expanded to other species. To reduce the risk of human infections, molecular searches for *Contracaecum* larvae in eaten seafood, particularly fish hosts, are required to support food safety.

Conflict of Interest

The authors declared no conflicts of interest regarding this manuscript's publication.

Ethical Approval

Ethics required are approved, by the Ethical Committee of the University of Kerbala/ College of Veterinary Medicine. Date: 23/02/2023, Ref: UOK.VET. PA.2022.05.

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Similarity Rate

We declare that the similarity rate of the article is 4% as stated in the report uploaded to the system.

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Author Contributions

The authors are equall in Motivation, Concept, Design, Control/Supervision, Data Collection, Analysis, Literature Review, Writing the Article and Critical Review.

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