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Mini-Review on Molecular Markers for the Identification of Fish Nematode Parasites

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Mini Review

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Abstract

A parasite is an organism that survives in or on another species, usually at the expense of its host. Among the fish-infecting parasites, three types of helminth parasites, namely, roundworms (nematodes), flatworms or flukes (trematodes), and tapeworms (cestodes), have the potential to cause zoonotic diseases. A large diversity of parasites that are taxonomically diverse and display a wide range of life cycle methods live on fish as hosts. While many of these parasites are transmitted straight from ultimate host to ultimate host, some must travel through several intermediate hosts before finding a host in (or on) whom they can reach sexual maturity. There are various methods for detecting nematode parasites in various fish species like internal transcribed spacers (ITS), mitochondrial COI, RFLP, AFLP etc. There are various Reports of nematode infection in fish and humans. Accurate identification of fish parasites is crucial to study the host-environment relations and formulate subsequent preventive strategies.

Keywords: Parasite; Molecular Markers; Nematode; RFLP; Fish; Internal Transcribed Spacers (ITS); Mitochondrial COI

Abbreviations: ITS: Internal Transcribed Spacers; WHO: World Health Organization.

Introduction

A parasite is an organism that survives in or on another species, usually at the expense of its host. Parasites depend on their host for food and survival. Various types of parasites can infect animals, plants and fish. Among the fish infecting parasites, three types of helminth parasites, namely, roundworms (nematodes), flatworms or flukes (trematodes), and tapeworms (cestodes), have the potential to cause zoonotic diseases. Around 20,000 species have been described under the phylum Nematoda, ranging from 0.3 mm to over 8 cm [1]. *Dioctophyma renale* (*D. renale*) is a parasitic nematode that belongs to the order Ascaridida, and the family Dioctophymatidae is also known as the giant kidney worm because of larger size [2]. This is a rare human disease cause [3]. *D. renale* has been described in many mammalian species and humans, highlighting this parasite's zoonotic importance [4,5].

Many animals serving as the primary host of *D. renale* are infected by ingesting a second intermediate host (usually fish or frogs) that had, in turn, eaten the first intermediate host (a freshwater earthworm) [6]. In the case of humans, ingested larvae penetrate the human intestine and migrate to the liver. From the liver, the larvae migrate to the kidney (usually unilateral, specifically the right kidney), where they become adults. Eggs laid by the adult worm are excreted through the urine. The infestation of giant adult worms leads to kidney destruction if left untreated. This human disease can occur worldwide [3].

Scientific Classification of Nematodes

Kingdom: Animalia

Subkingdom: Eumetazoa

Clade: ParaHoxozoa

Clade: Bilateria
Clade: Nephrozoa
Super phylum: Ecdysozoa
Clade: Nematoida
Phylum: Nematoda

Taxonomy of *Dioctophyme renale*
Higher classification: Dioctophyme
Scientific name: Dioctophyme renale
Phylum: Nematode
Class: Adenophorea
Order: Rhabditida
Family: Dioctophymidae
Genus: Dioctophyma

Geographic Distribution

D. renale has a broad and likely worldwide distribution in carnivores, although little is known about its occurrence in Africa. Zoonotic infections have been reported in the United States, Iran, India, China, and Indonesia [7]. The first case of a Kidney worm parasite from India was found in a 70-year-old man [5]. It has been suggested that the single report of *D. renale* infection from Australia is a misidentification of Leisegang rings, a specific formation of mineral precipitates in the kidney and other organs that can be mistaken for eggs of *D. renale*.

Morphology

D. renale is the largest nematode to parasitize humans [8]. The size of the adult male worms is 20–40 cm long and 5–6 mm wide, whereas females can grow to 103 cm in length with a width of 10–12 mm. So, female worms are larger than male worms. Both sexes appear bright red in color and taper at the anterior and posterior ends. In the case of male *D. renale* worms have a bursa, which is used to attach to facilitate mating [9]. Eggs are mainly 60–80 micrometers x 39–47 micrometers, contain an embryo, and have characteristic shell sculpturing. The color and shape of the egg are brownish-yellow and oval, respectively. Eggs have a thick shell, and the surface appears pitted except at the poles.

Life Cycle

Non-embryonated eggs are shed with the urine of the definitive host, and after one month in water, L1 larvae develop inside the egg. After being eaten by the invertebrate intermediate host (oligochaete worms), the eggs hatch in the digestive tract and mature into L3 larvae after two moults (usually 2-3 months at 20-30°C). If a paratenic host (fish or frog) eats the intermediate host, the L3 larvae will form cysts in the tissue and will not develop further. Most commonly, the definitive host becomes infected after eating a paratenic host with nested L3 larvae [3]. Consuming intermediate hosts of infected invertebrates can also cause infection but is probably not the main route of infection. After ingestion by the definitive host, the infective larva migrates through the stomach wall to the liver and eventually to the kidney (typically the right kidney). Nymphs become adults about six months after being ingested by the definitive host [10].

This parasite reproduces in the body of the definitive host and is excreted through urine. After that, this parasite enters a new life cycle (Figure 1). Humans can also become infected after eating undercooked paratenic hosts [11]. Although humans can serve as definitive hosts for kidney infections, often, the larvae migrate abnormally, eventually encapsulating in subcutaneous nodules and stopping further development [3].

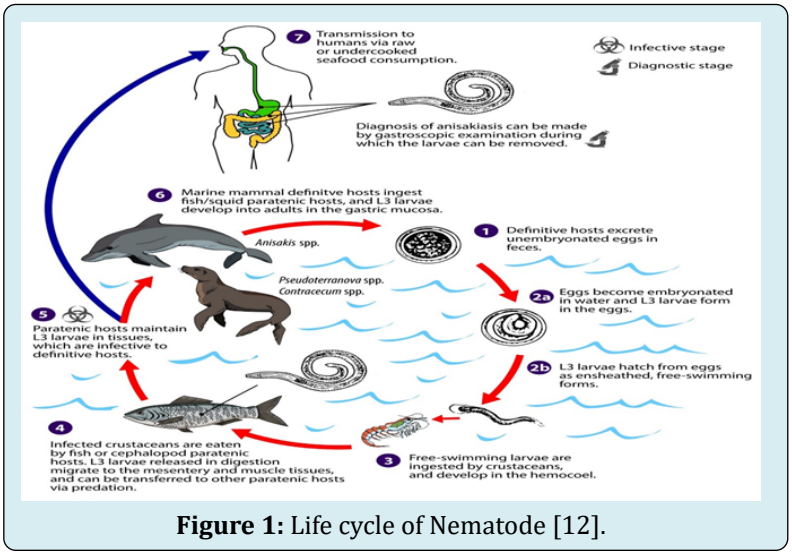

Figure 1: Life cycle of Nematode [12].

Clinical Presentation

Kidney worms can cause hematuria [8], abdominal pain, fever, and eosinophilia. Adult worms have been found in the right kidney. Sometimes L3 larvae are found in migratory, subcutaneous nodules. The most prevalent nematode parasites have been reported from the families Capillariidae (Species of *capillaris*), Camallanidae (Species of *Camallanus*), Ascarididae (Species of *Anisakis, Contraceacum,* and *Terranova*), Spiruroidae (Species of *Ascarophis*), Dracunuloidae (Species of *Philometra, Philomena*).

Capillaria pterophylli is a nematode having a wide range of fish hosts, including cyprinids, gourami, tetras etc. It infects the gastrointestinal tract of cichlids, guppies and swordtails, and other freshwater fish species. However, the nematode species is reported to be found frequently in the intestine of cichlids, namely, angelfish and discus [13]. Usually, the first evidence of *Capillaria* infection is a red, worm-like animal protruding from the anus of a fish. They have indirect life cycles and are also live-bearing nematodes [14]. They are considered ovoviviparous and viviparous, as females incubate the eggs, which hatch into larvae within their bodies. *Contracaecum* species infects freshwater fish and are usually found as adults in fish-eating birds, such as cormorants and pelicans. Larval stages are seen in cyprinids (carp and related species), ictalurids (*channel catfish*), centrarchids (sunfish and bass), tilapia and other cichlids, and perch. The larval stage of the *Contracaecum* species can also infect marine fish, such as whiting, capelin, and cod.

The World Health Organization (WHO) estimated that approximately 56 million parasite infections are associated with consuming fish products. Early detection of nematode parasite life-history phases could help prevent infection spread. Identification of nematode species is difficult as the morphological characters overlap among the species.

Molecular markers for the identification of nematode species

The recent development of molecular techniques has provided an alternative and, in some cases, more accurate diagnostic tools. The methods include PCR-RFLP (Restriction Fragment Length Polymorphism) sequencing of rDNA, ITS (Internal transcribed spacers), and mitochondrial DNA markers [15,16]. The utility of 18S rRNA in delineating identification and phylogenetic inference is well elucidated in different forms of organisms like nematodes, digeneans, and cestodes [17]. Previous studies used the PCR-RFLP method to differentiate closely related nematode species by using this PCR-RFLP method. They identify *A. simplex, C. osculatum, P. decipiens and H. aduncum* from harbour porpoises (*Phocoena phocoena*) [18]. Further, mitochondrial DNA sequences are valuable for differentiation between sibling species within *Contracaecum* ogmorhini [19]. Paggi L, et al. [20] used allozymes to resolve the species complex of *Pseudoterranova* and reported species *P. decipiens (sensu stricto), P. krabbei, P. bulbosa and P. azarasi*. Similar techniques (Allozyme) were used by Mattiucci S, et al. [19] for differentiation between *Anisakis typica*, *A. simplex* s.s, *A. pegreffii*, and *A. simplex* C. Zimik P, et al. [21] characterised *Clinostomum metacercariae* from an ornamental fish, *Trichogaster fasciata* using morphological and molecular markers. The study used two molecular markers, the nuclear ribosomal DNA & internal transcribed spacer 2 (rDNA-ITS2) and the mitochondrial cytochrome c oxidase subunit 1(mtCO1). Jabbar A, et al. [22] documented anisakid nematodes in estuarine and near-shore fish species from Southern Western Australia. This study examined 108 fish representing 13 species of anisakid larvae. The ITS region was used to characterise the parasites, and the results showed the occurrence of the anisakid parasite in 11 fish species. Abe N, et al. [23] reported phenotype plasticity in *Anisakis simplex* and revealed the existence of three sibling species, *A. simplex*, *A. pegreffii* and *A. simplex* C, in this group. They use PCR-RFLP and direct sequencing of the ITS region of rDNA. Among the 26 isolates, 24 were identified as *A. simplex sensu stricto*, and the other two as *A. pegreffii*. This is the first case which confirms the distribution of *A. pegreffii* in Japan and detects. *pegreffii* larvae in Pacific cod.

Pontes, et al. identified anisakid parasites from *Aphanopus carbo, Scomber japonicus,* and *Trachurus picturatus* caught in Madeiran waters using by PCR-RFLP method. They amplify the rDNA region (spanning the ITS-1, ITS-2, and the 5.8S subunit). Three distinct species were identified in *A. carbo*, namely *Anisakis simplex sensu stricto*, *Anisakis pegreffii*, and *Anisakis ziphidarum*; 5 in *S. japonicus*, i.e., *A. simplex* s.s., *A. pegreffii*, *Anisakis physeteris*, *Anisakis typica*, and *A. ziphidarum*; and 3 in *T. picturatus*, i.e., *A. simplex* s.s., *A. pegreffii*, and *A. typical*.

Molina FI, et al. [24] provided a molecular basis for classifying Saprolegnia using RFLP of rDNA. RFLP profile of the ribosomal DNA (18S-ITS-5.8S rDNA) was generated from 33 strains representing 18 species of Saprolegnia. They use the Polymerase Chain Reaction (PCR) to amplify the 18S rDNA and the region spanning the two internal transcribed spacers (ITS) and the 5.8S ribosomal RNA gene. After that, the amplified products were subjected to restriction endonucleases to generate various fingerprints. Restriction polymorphisms in PCR-amplified rDNA provided a molecular basis for classifying Saprolegnia. Aibinu IE, et al. [25] updated current knowledge on Anisakis as a food-borne parasite with particular focus on the increasingly reported diversity of fish and crustacean hosts, allergens and immunological cross-reactivity with invertebrate proteins rendering this parasite a significant public health issue. Paladini, et al. reported that

adult nematodes of Philometra from fish tissue could infect humans through open wounds in the skin and elicit diseases. Davidovich N, et al. [26] found that the hybrid tilapia (*Oreochromis aureus x Oreochromis niloticus*) and red drum (*Sciaenops ocellatus*), farmed in polyculture were found to be heavily infected with nematodes referable to *Contracaecum* larvae. In the case of hybrid tilapia, the prevalence of infection was 53.8%, whereas, in the red drum, the disease prevalence was 40.9%. They studied this nematode using both morphological and molecular approaches and found that the same species of *Contracaecum* parasitised both the infected fish species. Further, it was observed that the larvae localised in the pericardial cavity of hybrid tilapia, while in the case of the red drum, it was found in the abdominal cavity. Genetic analysis of internal transcribed spacer rDNA and cox2 mtDNA (Mitochondrial DNA) showed high similarity to the unidentified *Contracaecum* larvae detected in several fish species in Ethiopia, Egypt and Kenya. Pekmezci GZ, et al. [27] characterised *Eustrongylides* sp. larvae using ITS regions, SSU rRNA, and COI markers. They collect fish samples of pike-perch Sander lucioperca (L.) from Northern Turkey. Based on ITS sequence analysis, they identify that the fourth stage of Eustrongylides spp. was genetically identified as *E. excisus* species. They did molecular characterisation of *E. excisus* for the first time in Turkey [28].

Reports of nematode parasite infection in fish and humans

Norouzi R, et al. [4] reported a case of human infection with *Dioctophyma renale* from Iran. The nematode infection was observed in a 75-year-old man from Kurdistan province, the western part of Iran. Ultrasound and computed tomography revealed parasitic helminth, consistent with *D. renale*, 30 cm long and 1.2 cm in diameter, in the right kidney. Hajialilo E, et al. [29] detected *D. renale* in the case of male Vulpes in the Caspian littoral of Iran. *D. renale* is a food-borne helminthic infection humans can acquire while consuming aquatic parasitised edible items. Sadighian A, et al. [2] reported that the prevalence of disease in stray dogs and jackals in north Iran was reported at 13% and 35%, respectively. Cheng identified these worms based on characteristic morphologic and morphometric parameters introduced by pioneer parasitologists. De Noia M, et al. [30] developed a rapid non-lethal, non-invasive environmental DNA method to detect the parasite's presence in the eel swim bladder. They identify an invasive nematode parasite, *Anguillicoloides Crassus*. This nematode parasite causes a massive decline in the eel population and impacts the eel species' physiology and life history. So, the early detection of this parasite is critical to protect the eel population from this nematode [31]. They screen about 131 samples of wild eels from Ireland and the UK during 2017 and 2019. Ampili M, et al. [32] identified 15 parasites from the alimentary canal of three indigenous fish species, *Anabas testudineus*, *Heteropneustes fossilis*, and *Mystus gulio*, from six different regions of Vembanad Lake in Kerala. Out of 15 parasites, they observed five species of trematode worms; six comprised nematode worms, one acanthocephalan, one Ciliophora, one crustacean and one *Myxozoa*. They observed most of the parasites in the intestine of host fish, while the crustacean *Noto diaptomus* and nematode *Huffmanella sp* were found in both the stomach and intestine. *Anabas testudineus* were infected with *Camallanus anabantis* and *Capillaria sp*. All of *Heteropneustes fossilis* showed infestation of *Capillaria sp*. But entire *Mystus gulio* was infected with *Myxozoa sp*. Acanthocephalan Pallisentis sp. was present in all three host species. *Myxozoa sp.* exhibited the highest mean abundance in *M. gulio*. *Parascarophis sp*. indicated the most incredible mean intensity in the case of *A. testudineus*.

Fernando SS, et al. [33] identified the eggs of *Dioctophyma renale* in the case of a 47-year-old, previously healthy farmer from Grafton, N.S.W. in Australia, who suffered from minor trauma to his loin. After two months, he developed loin pain and hematuria. After ultrasound and arteriography, they found the renal cyst in the fibrous cyst wall and the surrounding fat. The shape of the cyst was ring-like, and the size of the cyst was 70 X 45 mu. Soulsby EJL, et al. [34] identified the porcine kidney worm, *Stephanurus dentatus*, from a federal pig slaughtered on August 27, 1967. This parasite is commonly observed in domestic pigs in tropical and subtropical countries. The affected pig came from a feral swine herd maintained on Robert's Island about 12 miles east of Yarmouth, Nova Scotia. Pinky, et al. [35] studied the effect and prevalence of Dioctophymotidae nematode (*Eustrongylides sp*.) in *Xenentodon cancila*. They found red-coloured nematodes in the host fish' swim bladder, liver, muscles and ovaries. They also observed histopathological changes like necrosis, degeneration of oocytes, and Artesia and a reduction in the number and size of oocytes. They observed prevalence during pre-spaw post-spawning Rosser TG, et al. [28]. It was noticed that only the female hosts were infected while the male individuals remained unaffected. The calculated value of GSI (Gonadosomatic index) (6.52±0.87) and fecundity (146.36±29.58) in infected fish were observed to be low as compared to the GSI (11.84±1.53) and fecundity (209.6±23.68) of the uninfected ones. Zhang S, et al. [36] identified two nematode parasites, i.e., *Gnathostoma spinigerum* and *Eustrongylides sp*, in eel using both morphological and molecular approaches. The molecular approach used the internal transcribed spacer (ITS) and the mitochondrial COI to identify the nematodes De Noia M, et al. [30]. One hundred twenty samples of *Monopterus albus* were screened, and 78 nematode larvae were isolated and then identified using the molecular markers tools. In this study, they observed that the transmission rate of nematode infection to humans is increasing in China due

to the consumption of fish species infected with nematode parasites. Boomker J, et al. [37] observed the occurrence of nematode parasite *Eustrongylides* sp. in the stomach of Nile crocodiles in Botswana. During 2003 and 2005, they conduct a survey on the diet of Nile crocodiles and two young nematodes in the stomach of Nile crocodiles. They find that the piscivorous birds may be the final host of *Eustrongylides* species [38].

Yimer E, et al. [39] identify the various common nematode fish parasites in lake Chamo to collect information on the pathology of fish found in Ethiopia. During 1997-1998 they collected 583 different species of fish. Upon observation, they identified the *contracaecum* species in the mesentery of *Oreochromis niloticus, Clarius gariepinus, Oreochromis niloticus, Hydrocynus forskalii, Bagrus docmac,* and *Lates niloticus*. And they also identify *clinostomum* species in the branchial cavity of *Oreochromis niloticus* species. In the case of *Clarias gariepinus*, they also identify the Amplicaecum parasite. They also report the larval nematodes of Porrocaecum and *Eustrongylides* in Ethiopia. They also observe the cestodes are mainly found in the abdominal tissues, liver, intestine, and stomach in the case of *Synodontis schall* fish species. Angelou A, et al. [40] reports a fish case of *Dioctophyma renale* in the case of a dog in Greece. They observe that this nematode parasitise mainly infects the right kidney of dogs. They observed that the 6-year-old female crossbreed dog suffered from tachypnea, tachycardia and severe hematuria. Still, after a few days, the dog died from this parasite. Upon post-mortem, they saw the parasite in the right part of the kidney, but the left kidney was normal without any infection.

Kauffman JC, et al. [41] identified three adult giant kidney worms (*Dioctophyma renale*) in the right kidney of *Mustela frenata* from Pike County, Pennsylvania; both weasels showed no clinical signs of decreased fitness at necropsy. But they observed that the right kidneys of both animals were enlarged and contained nematode parasitise. This is the first report that described the infection of long-tailed weasels by giant kidney worms in Pennsylvania. Batte EG, et al. [42] identified the swine kidney worm (*Stephanurus dentatus*) parasite in wild swine populations in warm climates. They observed that this swine kidney worm would affect growth and productivity. The swine kidney worm, *Stephanurus dentatus*, has been identified in the wild swine populations in warm climates by Battle, et al. [42] The parasite has been found to affect the growth and productivity of the swine, and it can be diagnosed by examining worms' eggs in the pig's urine or the post mortem studies.

The first report on the complete mitochondrial genome of *Stephanurus dentatus* species was made by Deng YP, et al. [43] Sequencing of the Mt genome was performed by Illumina Hiseq 6000 platform, revealed that the genome size of this species is about 13,735 bp, containing 36 genes [44-47]. Phylogenetic analysis using the amino acid sequences of 12 protein-coding genes supported the hypothesis that *S.dentatus* is closely related to the family Chabertiidae which provided insights into the phylogenetic relationship of the family Syngamidae within the superfamily Strongyloidea. The findings of this work can be used to develop molecular markers for the identification of *S. dentatus*.

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