Morphological and molecular characterization of *Anisakis simplex* (Nematoda: Anisakidae) third-stage larvae isolated from retail-marketed smoked herring fish in Assiut, Egypt

Original Article

Shimaa Sameeh[1], Abeer E Mahmoud[2], Enas AM Huseein[2], Mohamed-Elsalahy M Monib[2], Mahmoud Abdelhamid[3]

Departments of Parasitology, Faculty of Veterinary Medicine, Assiut[1] and Aswan[3] Universities, and Medical Parasitology, Faculty of Medicine, Assiut University[2], Egypt

ABSTRACT

Background: *Clupea harengus*, Linnaeus, 1758 is a nutritious pelagic fatty herring, rich in omega-3 fatty acids. Its consumption is associated with human anisakiasis as an intermediate host of *Anisakis* spp.
Objective: To identify the anisakid larval detection rate in smoked *C. harengus* in Assiut Governorate, Egypt and characterize the morphological and molecular features of *Anisakis* spp. 3rd stage larvae.
Material and Methods: The collected anisakid larvae from marketed herring were examined by light and scanning electron microscope (SEM). Characterization of anisakid larvae was performed by PCR as well as sequencing analysis of the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA).
Results: The detection rate of *Anisakis* spp. 3rd stage larvae among the smoked herring was 72% with larval load varying from 2-63 larvae per fish. Macroscopically, they appeared as coiled spiral buttons, yoyo-like, tangled, and free shapes. Larvae were found on the surface of fish gonads, livers, intestinal tracts, mesenteries, and abdominal cavities. The larval body was covered with a cuticular layer and had a marked boring tooth, and mucron. The molecular and phylogenetic analysis, using ITS gene, identified the collected larvae as *A. simplex* and was registered in the GenBank under the accession number OR660093.1.
Conclusion: Detection of *Anisakis* 3rd stage larvae was marked in smoked herring sold in Assiut Governorate, Egypt. Further studies are recommended to investigate the endemicity of *Anisakis* larvae in imported fish.

Keywords: *Anisakis*; Egypt; imported fish; ITS; microscopic; SEM; PCR; zoonosis.

INTRODUCTION

The World Health Organization (WHO) stated in 2012 that the consumption of infected fish and its products is blamed for ~56 million cases of parasitic infections in human[1]. Anisakid are zoonotic fish-borne nematodes that cause human anisakiasis. The traditional cooking methods (salting, air-drying, smoking, and marinating) are ineffective if the treatment time is too short to achieve killing of larvae. Even while the processed fish may not include live anisakid nematodes, the product may nevertheless contain allergenic proteins that could be harmful to consumers[2]. Hence, allergic sensitization is one of the most concerning forms of anisakiasis that potentially results in a wide range of clinical consequences such as urticaria, angioedema, and even anaphylactic shock[3]. The life cycle of *Anisakis* spp. is indirect and occurs in aquatic environments. The definitive hosts are fish-eating birds and marine mammals, while crustaceans act as first intermediate hosts. Thus far, nine species have been recognized within the genus *Anisakis*[4]. Specifically, *A. simplex* and *A. pegreffii* are reported as the most common species that cause

human infections[5]. The prevalence and intensity of *Anisakis* spp. infection in fish varied from one to 100% worldwide based on fishing area, season, fish species, and nematode species[6]. Recently, several reports described the occurrence of Anisakidae larvae in different fish collected from Egypt such as *Caranx sexfasciatus, Cephalopholis miniata, Variola louti*, and *Plectropomus areolatus*[7,8].

Anisakidae family members can infect a broad range of marine fish such as herring (*C. harengus*), chub mackerel, anchovies, horse mackerel, sardines, rainbow trout, salmon, tuna, spotted mackerel, surmullet, Baltic Sea cod, and *Atherina*[9,10]. The recent trend of eating raw food such as sushi and sashimi made from salmon and eating the whole fish body including its viscera as in *Atherina* usually increases infection possibility[2,11]. Since 2009, Egypt boosted its imports of fish to overcome the food gap; most of these imports are whole frozen marine fish[12]. Herring is among the important imported frozen fish species in Egypt and Mediterranean countries[12,13]. The frozen herring is thawed, salted, dried, smoked, and packaged

by Egyptian manufacturers[14]. Smoked herring is a common Egyptian food due to its characteristic aroma and flavor in addition to its nutritional value[15]. Herring is mostly linked to *Anisakis* spp. acting as an intermediate host of these nematodes and could be a source of anisakiasis after consuming its fresh and processed products. Few researches reported the frequency of *Anisakis* spp. larvae in marketed herrings in Egypt[13].

Researchers can gain more insight into the ecology and epidemiology of anisakids, as well as assist with diagnosis and control, by accurately identifying these nematodes[16]. Hence, screening these species for *Anisakis* spp. larvae became of great concern as a food-borne hazard. Because of their low level of organ development and lack of distinguishing morphological characteristics, anisakid L3 larvae are difficult to identify[17]. Their identification is usually carried out using molecular techniques such as DNA sequencing[8]. Thus, the aim of the present study is to clarify the anisakid larval infection rate in smoked herring fish (*C. harengus*) in Assiut Governorate, Egypt. Detailed morphological characteristics of *Anisakis* spp. larvae were conducted using light microscopy and SEM followed by gene sequencing of their (ITS) region in the nuclear ribosomal DNA.

MATERIAL AND METHODS

This descriptive analytical study was conducted at Medical Parasitology Department, Faculty of Veterinary Medicine, Assuit University during the period from August 2022 to December 2022.

Study design: The study was conducted as a brief survey based on marketed smoked herring fish as a popular meal in Assiut Governorate, Egypt. For the investigation, fish samples were collected from local markets and carefully examined grossly. Any Anisakidae larvae were harvested and characterized morphologically and molecularly.

Fish samples collection: Fifty smoked *C. harengus* herrings were purchased from local markets in Assiut Governorate, Egypt, transferred in an insulated icebox, and identified[18]. Batch labels of the collected fish indicated that they originated from the European Union. The weight and length of the collected samples

ranged from 280 to 320 g and 29 to 31 cm, respectively (Fig. 1a).

Parasitological examination: Fish were dissected from the vent to the gills using clean scissors and scalpels as previously described[7]. The viscera (gonads, livers, intestinal tracts, and mesenteries), muscles, and body cavities were precisely examined for the existence of *Anisakis* spp. larvae by the naked eye. The detected larvae were pulled out manually from the infected fish. The larval number per fish was counted. The frequency of infection among examined fish was calculated as follows: [(Number of infected fish/Total number of examined fish) × 100][19]. The mean intensity of larvae was evaluated according to Mostafa *et al.*[20]. using the equation: [Total isolated larvae per species of fish/Total number of fish infected]. Any detected encapsulated larvae were released by a needle[21]. All larvae were washed thoroughly in phosphate-buffered saline (PBS) (Paneco-Ltd, Russia) for 30 min.

Morphological identification of *Anisakis* spp. larvae
• **Light microscopy:** Using light microscopy, an initial identification was established to distinguish *Anisakis* spp. L3 from any other related nematodes based on size and the excretory and digestive systems. The larvae were sectioned into three separate pieces: the anterior and posterior parts were allocated for integrative morphological examination by light microscope, and the middle parts were immediately frozen in 70% ethanol at -20℃ for molecular identification[22,23].

Both anterior and posterior extremities of the collected larvae were cleared in Amann's lactophenol[24] and examined at x10, and x20 objective lenses using an Olympus BX43, Olympus Optical Co., Ltd., Tokyo, Japan. The larvae were photographed using a light microscope conjugated with an Olympus Soft Imaging Solution GmbH Digital Camera (Johann-Krane-Weg 39, 48149 Münster, Germany). The size was measured using EPview™ software and expressed as the range in millimeters followed by the mean ± standard error (SE) in brackets. The larvae were discriminated at the genus level using the taxonomic keys[25].

• **Scanning electron microscopy (SEM):** For SEM, larval specimens were fixed for 24 h in 5% fresh glutaraldehyde, washed in PBS and post-fixed for one hour in 2% osmium tetroxide in sodium cacodylate
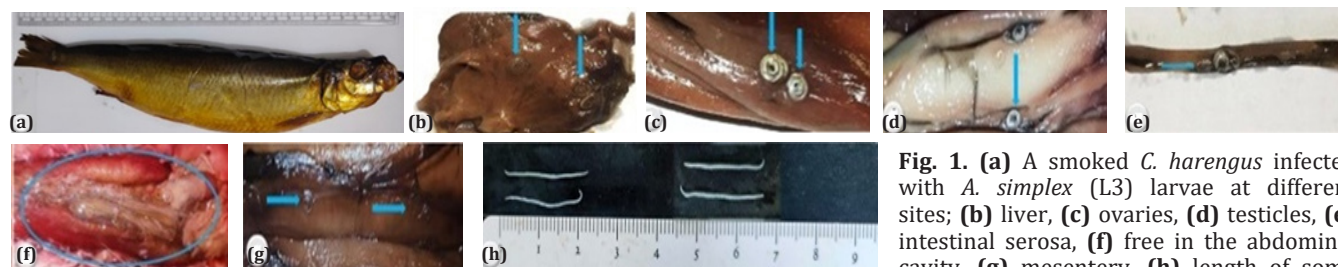

Fig. 1. (a) A smoked *C. harengus* infected with *A. simplex* (L3) larvae at different sites; **(b)** liver, **(c)** ovaries, **(d)** testicles, **(e)** intestinal serosa, **(f)** free in the abdominal cavity, **(g)** mesentery, **(h)** length of some isolated larvae.

buffer, then washed in the buffer. Following the dehydration process with an increasing graded series of alcohol (30%, 50%, 70%, 90%, and 100%; a half hour each), the samples were dried, put on special holders, and coated in gold[24]. The larvae were inspected and captured by camera in the Electron Microscopy Unit at Assiut University using a JEOL Ltd. SEM running at 15 KV.

Molecular identification of *Anisakis* spp. larvae using PCR: The detected *Anisakis* spp. larvae were molecularly ascertained according to Cavallero *et al.*[26]. In brief, the middle parts of the larvae were grinded in a mortar and homogenized using a metal-bar homogenizer (Wisetis, HG-15D, Wertheim, Germany). *Anisakis* spp. DNA was extracted using the QIAamp® DNA kit (Catalogue no.51504) according to the manufacturer's protocol. The DNA was amplified by PCR using the *Anisakis* species-specific primer set; ITS-NC2 (F): TTAGTTTCTTTTCCTCCGCT and ITS-NC5 (R): GTAGGTGAACCTGCGGAAGGATCATT, and Go Taq® Green Master mix (Promega). The reactions were subjected to an initial start at 95°C for 5 min followed by 40 cycles each consisting of a denaturation step at 95°C for 35 sec, annealing at 53°C for 35 sec, and extension at 72°C for 50 sec, and the final step over-extension at 72°C for 10 min in a Veriti 96-well thermocycler (Applied Biosystems, Germany). The amplified products (10 μl) were mixed with loading dye (2 μl) and observed on 1.5% ethidium bromide agarose gel. A UV transilluminator (JY02, Cleaver, UK) was used to detect the positive bands compared to the molecular size of 100-3000 bp DNA ladder (RTU, Cat. No. DM001.R500). The images were processed and analyzed utilizing a documentation system and gel imager (Compact M, Biometra, Germany).

DNA sequencing: For sequencing, the amplified PCR products were purified from gels by using the EnzSAPTM PCR Clean-up Reagant (Edge BioSystems) and then sequenced by SolGent Company Limited (Daejeon, South Korea) in the presence of the amplification primer set. The ITS sequence was assembled using DNASTAR (version 5.05) and assessed through nucleotide BLAST against closely comparable sequences from GenBank. The BLASTed ITS gene sequence was uploaded to GenBank to obtain an accession number.

Alignment and phylogenetic analysis: Using BLAST analysis (Basic Local Alignment Search Tool), the gene sequence identity was checked with GenBank. The MegAlign module of Laser gene DNA Star version 12.1 was used to create the phylogenetic tree. Phylogenetic analyses were performed in MEGA6 using greatest likelihood, neighbor-joining, and maximum parsimony[27].

Statistical analysis: Parasite occurrence was expressed in terms of infection rate (% infected) and infection mean intensity (mean number of larvae per fish ± standard error) using SPSS 20.0 software.

Ethical considerations: The authors abided by all accepted ethical standards for handling and sampling fish as approved by the Faculty of Veterinary Medicine at Assiut University under the number 06/2023/0125.

RESULTS

Macroscopic examination revealed that the infection rate of Anisakidae third-stage larvae among the herring samples was 72% (36/50). A total of 400 *Anisakis* spp. larvae were collected from all infected fish with an average of 2-63 larvae/fish. The mean intensity of *Anisakis* spp. larvae in the infected fish was 11.1 (400/36). The detected larvae were coiled and encapsulated in a transparent capsule within the gonads, livers, intestines, and mesenteries. No larvae were observed in muscles. They shared common gross features, being slender, transparent white, measuring 11-22 mm (mean 16.65 ± 0.58) in length by 0.473-0.618 mm (mean 0.546 ± 0.013) in width (Fig. 1b-h). Some larvae appeared tangled, while others appeared as "spiral buttons", or coiled with free-end "yoyo shapes" (Fig. 2).
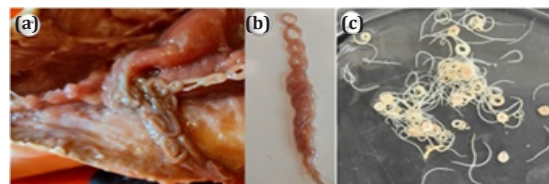

Fig. 2. Various shapes of *A. simplex* L3 recovered from *C. harengus*: **(a)** and **(b)** plexus appearance, **(c)** completely free, Yoyo shapes, and spiral buttons.

Light microscopy: By ordinary light microscopy all larvae revealed characteristic features of *Anisakis* spp. larval type (I). The body was covered with a cuticular layer, had a marked boring tooth dorsally on the head, and a mucron on the tip of the tail. The excretory duct was running from the excretory pore, which was situated ventrally below the larval tooth. The nerve ring surrounded a muscular esophagus (Fig. 3a). The ventriculus was long and connected obliquely. There were no intestinal diverticula or caeca (Fig. 3c). The rectum appeared as a short oblique canal terminated by the anus and encircled by rectal glands. The tail was rounded with a tapered terminal mucron whose length ranged from 10–30 μm (20.1±1.19) (Fig. 3b).

SEM examination: By SEM examination the bodies of the recovered larvae were cylindrical, (Fig. 4a). The larval cuticle had transverse and longitudinal striations posterior to the papillae (Fig. 4b). The anterior extremity was flattened and had a three-sided anteroventral mouth surrounded by three interlocked lips; a dorsal lip and two subventrals. Ventral to
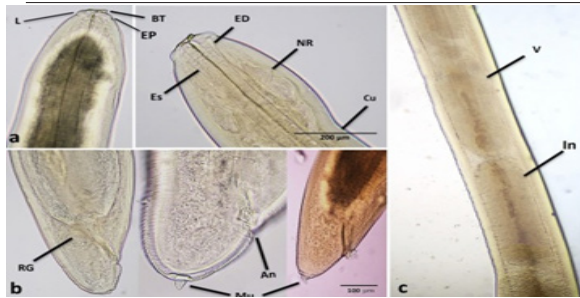
Fig. 3. Light micrographs of *A. simplex* L3. **(a)** anterior end showing lips **(L)**, boring tooth **(BT)**, excretory pore **(EP)**, excretory duct **(ED)**, esophagus **(Es)**, nerve ring **(NR)**, cuticle **(Cu)**; **(b)** posterior end showing rectal gland **(RG)**, anus **(An)**, mucron **(Mu)**; **(c)** middle part showing ventriculus **(V)**, intestine **(In)**.

the mouth, there was a triangular boring tooth that projected upward dorsally. An excretory pore appeared as a transverse slit between the ventrolateral lips opening below the boring tooth (Fig. 4 c,d). Four tiny papillae (two dorsolateral and two ventrolateral) encircled the mouth opening. The papillae were round, flat, and well-defined. The posterior end was rounded with a prominent conical mucron (Fig. 4e).

Molecular identification of *Anisakis* spp. larvae using PCR: Results showed the typical 1000 bp band of the *Anisakis* species-specific gene (Fig. 5).
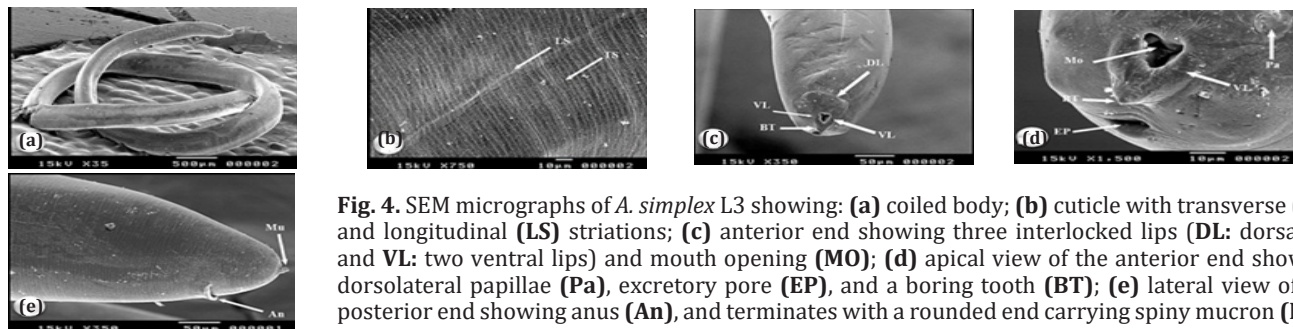

Fig. 4. SEM micrographs of *A. simplex* L3 showing: **(a)** coiled body; **(b)** cuticle with transverse **(TS)** and longitudinal **(LS)** striations; **(c)** anterior end showing three interlocked lips (**DL:** dorsal lip and **VL:** two ventral lips) and mouth opening **(MO)**; **(d)** apical view of the anterior end showing dorsolateral papillae **(Pa)**, excretory pore **(EP)**, and a boring tooth **(BT)**; **(e)** lateral view of the posterior end showing anus **(An)**, and terminates with a rounded end carrying spiny mucron **(Mu)**.
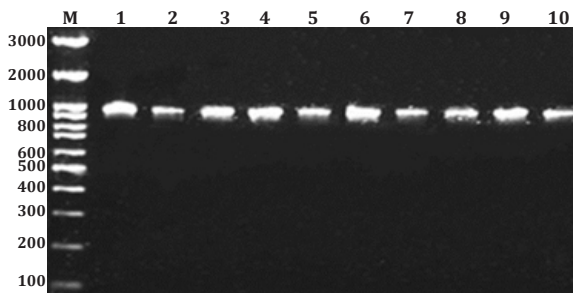

Fig. 5. Agarose gel electrophoresis of PCR amplification products with ITS-NC primer set showing the typical 1000 bp band of the *Anisakis* species-specific gene, 1.5% agarose, 9 V/cm for 90 min. **Lane M:** DNA size marker 100-3000 bp, **Lane 1-10:** numbers of the samples.

The nucleotide sequences of the replicated ITS region in individual *Anisakis* spp. isolated from *C. harengus* were deposited in the GenBank database (SUB13886900) under the accession number OR660093.1. PCR products of this region consisted of 1000 bp. The alignment of ITS sequences of our identified strain was mostly identical (99.27 %) to those of *A. simplex* previously described in Spain with accession number OR717508.1 (Fig. 6). The phylogenetic arbor was created based on the ultimate eventuality procedure involving the Anisakidae family as sister Taxa. There were two primary clades on the phylogenetic tree (A & B) with the family Anisakidae as an independent subclade, which is further clustered into two subclades. The first clade (A), clustered in 2 subclades (A1 & A2), includes *A. pegreffii, A. typica*, and *A. simplex* isolated from many different geographical areas. The second clade (B) is clustered into 2 subclades (B1 & B2); subclade B1 contains the isolated sample of

our study, which was clustered on the same clade with high identity (99.27 %) with *A. simplex* (OR717508.1), isolated from the European anchovy (*Engraulis encrasicholus*) host in Bay of Biscay, Spain[28].

DISCUSSION

Eating smoked herring that contains *Anisakis* spp. L3 can cause anisakiasis[29]. Living larvae can penetrate the gastric mucosa causing abdominal anisakiasis[30,31]. Although smoking and other cooking processes of herring may kill Anisakidae L3[5], dead larvae could induce an allergic reaction due to their thermal- and pepsin-resistant properties. The protein of dead larvae is a powerful potential allergen that might cause anaphylaxis[3]. Furthermore, fish processing or storage can facilitate the larvae's transfer from viscera to muscles[32]. As previously reported, there are currently few investigations on the detection rates of
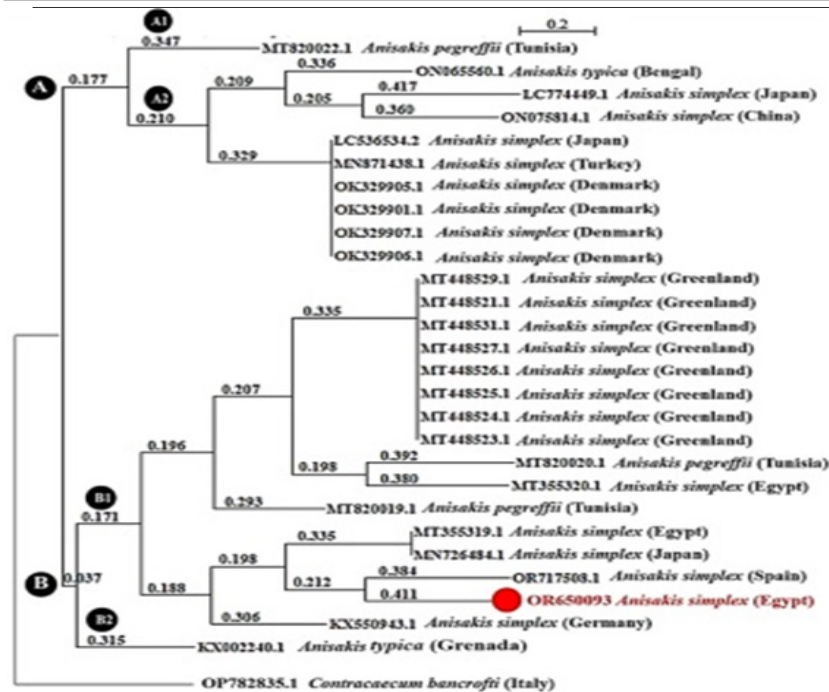
Fig. 6. Phylogenetic analysis of *A. simplex* L3 using the neighbor-joining method with p-distance and 1000 bootstrap replications based on the entire ITS rDNA region. Sequences exploring the relationships among *Anisakis* spp., including the nucleotide sequences obtained in this investigation (shown in red). The tree was rooted to *Contracaecum bancrofti* isolate as the out-group.

these nematodes in Egyptian-marketed herring fish[20]. The current study tackled a significant issue about the infection of *C. harengus* with *A. simplex* larvae. The identification of these larvae could help in the control and prevention of anisakiasis.

The overall detection rate of *A. simplex* larvae in our study was 72% (36/50). In a similar study with the same sample size, Baher *et al*.[13] detected a total 70% (35/50) infection rate of herring fish with *Anisakis* larvae in various Egyptian localities; Damietta (18%), Alexandria (16%), Port-Said (14%), Ismailia (12%), and Suez (10%). An estimated detection rate of 19.05% was reported in smoked herring by Arafa *et al*.[14] in various Egyptian localities. A large-scale study conducted by El Meghanawy *et al*.[29] revealed that the detection of *A. simplex* among imported smoked herring fish originating from Russia varied from a high percentage in Sohag (60.43%, 139/230) and Menofia (57.20%, 123/215) to low in Alexandria (35%, 48/137), Qaliobya (34.12%, 43/126), and Assiut (28.97%, 31/107) with a whole percentage of 42.7%. In El-Sharkia Province, Mostafa *et al*.[20] recorded 87.1% (122/140) *A. simplex* detection rate in herring fish.

In Egypt, herring fish is either imported already smoked or is frozen, then subjected to smoking by Egyptian manufacturers[14]. The variation in the detection rate is probably linked to differences in detection method, fish origin, and sample size[20,33]. In our study, the origin of the examined herring was the European Union. Debenedetti *et al*.[34] recorded a clear variation in the infection rate of *Anisakis* spp. larvae in different fish species collected from the Atlantic Ocean than from the same fish species collected from the Mediterranean Sea.

The intensity of *Anisakis* spp. infestation plays a crucial role in defining the level of risk that customers face[35]. The majority of research on *Anisakis* spp. larvae isolated from fish in Egypt did not specify their intensity[10]. In our study, the infected smoked herring harbored from 2-63 larvae/fish with a mean intensity of 11.1 larvae/infected fish, increasing the possible risk to consumers[21]. In line with our study, Mostafa *et al*.[20] reported a mean intensity of 11 larvae/infected fish. These estimated values are higher than that reported by Arafa *et al*.[14] and Hussien *et al*.[15] who calculated mean intensities of 4.71, and 6.83 larvae/infected fish, respectively.

The larvae in our study were encountered on the surface of one or more organs of the fish viscera (the gonads, livers, intestinal tracts, and mesenteries) and abdominal cavities. This observation agreed with Hussien *et al*.[15] and Baher *et al*.[13] who recorded that the *Anisakis* spp. larvae infection is only found in the herring viscera. Moreover, Liu *et al*.[36] found that the infection rate of *Anisakis* spp. in muscle was lower (7.8%) than in other fish organs, while Cavallero *et al*.[26] described that most of *Anisakis* spp. larvae were isolated from the visceral cavity, except for nine larvae that were recovered in the muscular tissue of three individual fish samples. Due to different fish hosts and/ or location-associated abundance of certain nutrients, anisakid larvae have distinct tissue preferences. They can parasitize the viscera more than other marine fish organs[10]. The main presence of larvae in viscera indicates that applying basic sanitary measures and the right evisceration of the fish can reduce the public health risks that result from heavy larval consumption by mistake[20]. The results of the current study found that the detected larvae were coiled inside transparent

capsules or free living on the surface of visceral organs of fish. The capsulated larvae were tightly attached to the fish tissue so that their removal is difficult requiring more attention and experience during fish cleaning[37].

Generally, there are distinct generic differences amongst larval anisakids, highlighting the importance of the exterior fine morphology in their identification[38]. Larvae of *Anisakis* spp. are classified into *Anisakis* types (I) and (II) according to the length of the ventriculus and whether or not the tip of the tail has a mucron[25]. All examined larvae in our study had a long ventriculus and round posterior ends with mucron, indicating that they belong to *Anisakis* type (I). Our recorded morphological findings are supported by and agree with previous reports by Cheypanya *et al.*[39] on cobia (*Rachycentron canadum*), Setyobudi *et al.*[25] on chum salmon (*Oncorhynchus keta*), and Mostafa *et al.*[20] in Atlantic herring (*C. harengus*) and Mediterranean horse mackerel (*Trachurus trachurus*). The mucron length can be a distinguishing morphological character for differentiating *A. simplex* from other type (I) anisakidae[20,22]. In this context, we found that its length ranged from 10-30 μm and these measurements overlapped with *A. simplex*. Mostafa *et al.*[20] and Setyobudi *et al.*[25] recorded similar mucron lengths (18-30, and 12-31 μm, respectively).

In the current study, SEM confirmed that the recorded features are characteristic of *Anisakis* type I. The current SEM data matched with several other studies[25,29,33]. However, Hassan[40] described papillae-like structures arranged in the tail region that did not appear in the present SEM study. While morphological features could provide the initial clue for *Anisakis* spp., molecular and sequencing could provide their decisive identification. In the current study, PCR assay and sequencing minimizes the possible misdetection with other anisakids that have similar morphological features[14,29].

The ribosomal RNA ITS-encoding genomic DNA proved helpful in validating species identification[41]. Sequencing of the ITS allows accurate judgment improving taxonomic settlement and discriminating related species[36,41] because some *Anisakis* spp. with similar morphology vary genetically. A high similarity was observed between the sequence of our *A. simplex* isolate (OR660093.1) from imported herring and that isolated from Biscay Sea anchovy fish (*Engraulis encrasicolus*) in Spain, previously deposited in the GeneBank with the accession number (OR717508.1).

This agreed with several other studies[14,25,42] that used the sequencing of ITS regions to identify *A. simplex*, recovered from different infected fish (*Oncorhynchus keta*, *C. harengus*, and *Scomber Japonicus*). Mostafa *et al.*[20] isolated two types of *Anisakis* spp. larvae (*A. simplex* and *A. typica*) from herring fish and Mackerel, respectively, and found that they belonged to clade

I. This data confirms the high level of infection in imported herring collected from local markets in Assiut with the zoonotic *Anisakis* spp. larvae.

In conclusion, *A. simplex* morphological features were characterized, and molecularly confirmed by gene sequencing. *Anisakis* 3rd stage larvae were highly detected in smoked herring marketed in Assiut Governorate, Egypt. This is worrisome because of possible endemicity of *Anisakis* larvae and its originated allergy which calls for necessary accurate examination of the imported fish. Accordingly, initiating active surveillance of imported fish is recommended.

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