

Prevalence and Molecular Characterization of Anisakidosis in both European (*Merluccius merluccius*) and Lizard Head (*Saurida undosquamis*) Hakes

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Received 13 Nov 2014 Accepted 19 Nov 2014

Abstract -This study focuses on Anisakidosis as a disease caused by a number of Anisakid larvae including *Anisakis simplex*, *Pseudoterranova decipiens*, *Hysterothylacium spp.* and *Contracaecum spp.*, through assessing the clinical signs and the clinical examination of the disease in 170 Lizard head (*Saurida undosquamis*) and 160 European hakes (*Merluccius merluccius*) during the period from April 2011 to July 2012. In addition, identification of the recovered larvae morphologically and using random modified polymorphic DNA (RAPD-PCR) technique. No obvious clinical signs were observed in the examined fish. The post mortem examination revealed the presence of free or encapsulated larvae within the body cavity, on the visceral organs and in the musculature. The seasonal prevalence of Anisakid larvae was the highest in spring and summer and its lowest level was in autumn in both examined fish spp. The highest intensity of larvae was observed in the organs and viscera rather than in the musculature. RAPD technique identified the recovered larvae using four arbitrary primers. It could be concluded that Anisakid larvae can be easily identified using molecular tools by its well characterized genetic finger print and that in turn will help in its diagnosis in parallel with the traditional tools.

Key words; *Anisakid larvae; Hake fish; RAPD-PCR.*

I. INTRODUCTION

Anisakids are nematodes from super family Ascaridoidea (families: Anisakidae and Raphidascarididae). Specially those belong to genera (*Anisakis*, *Pseudoterranova*, *Contracaecum* and *Hysterothylacium*) are of biological and economic importance in the aquatic environment [1]. The life cycle of Anisakid nematodes involves crustaceans (e.g., Krill) as transport hosts, fishes (e.g., herring, hakes, cod) as intermediate hosts and marine mammals (Whale, Sea lions, Dolphin and Seal) as definitive hosts [2]. Although humans are accidental hosts; in which Anisakid larvae do not complete its development but may penetrate the alimentary tract and invade associated organs, causing a range of pathological effects [3, 4]. Moreover, owing to the thermo-stability of *Anisakis simplex* allergens, the ingestion of safely cooked fish containing dead parasites can also be potentially dangerous and can cause severe allergic reactions such as contact dermatitis and asthma [5, 6]. Anisakid larvae can be identified microscopically at genus level based on the morphology of the digestive tract and excretory system [7]. In Egypt, various species of Anisakid larvae have been reported based on the morphology [8, 9, 10]. The main target of this study was to survey the prevalence and abundance of Anisakid larvae from both European (*Merluccius merluccius*) and Lizard head (*Saurida undosquamis*) hakes; depending on both the morphological characters and the molecular genetic finger prints using random modified polymorphic DNA (RAPD-PCR) which is extremely powerful tool for routine identification of Anisakid

larvae and has proved for genetically characterizing different species of Anisakid nematodes and their larval stages [11]. In addition they can estimate their genetic differentiation and relationships [12].

II. MATERIALS AND METHODS

A total of 330 marine fish belonging to hake fishes; 170 Lizard head (*Saurida undosquamis*) and 160 European hakes (*Merluccius merluccius*) were collected from Suez canal and Mediterranean Sea respectively on ice to the lab of Fish Diseases and Management, Benha University, Egypt, since April 2011 to July 2012, where the examination was conducted as soon as possible. Clinical and post mortem examinations were done for all the collected fish species, for determination of any lesions or abnormalities externally and internally following [13]. Each fish was dissected and the abdominal cavity, stomach, serous tissue, spleen, kidney, liver and gonads were examined by naked eyes for Anisakid larvae. Meanwhile, 10 gm of musculature were taken from around the body cavity of each fish and examined under the dissecting microscope. The collected larvae were washed in distilled water, and kept in 70% ethanol for both morphological identification and DNA extraction.

A. Morphological examination

The recovered larvae were cleared in lactophenol and permanently mounted in glycerin-gelatin. The slides were left to dry for 24 hours and examined microscopically [14]. The following morphological characteristics were measured: body width, esophagus length, ventriculus length, tail length, body length/body width, body length/esophagus length, body length/ventriculus length and body length/tail length. All measurements were made directly with an eyepiece micrometer and were given in millimeters. The larvae were then identified according to their morphological characteristic features following [15, 16, 27].

The prevalence and the intensity of anisakid larvae infestations were calculated as defined by [17].

B. DNA extraction

DNA extraction from Anisakid larvae was carried out using SDS method of [18] with few modifications. Two to five anisakid larvae (irrespective their type) were placed in an Eppendorf tube and kept in liquid nitrogen for few seconds to facilitate the rupture of cell membranes. The tissue was crushed by a pestle in lysis buffer (10 μ l of SCE (sorbitol 1M, sodium citrate 0.1 M, EDTA 0.06 M), 30 μ l of SDS-EDTA (SDS 1%, EDTA 0.15 M) and 10 μ l of proteinase K 1%) and then incubated at 42 °C over night. Subsequently, the DNA was purified with one phenol-chloroform-isoamyl alcohol extraction, followed by one chloroform-isoamyl alcohol extraction and then an ethanol precipitation. The precipitated pellet was re-suspended in 50 μ l distilled water and kept at -20 °C until use.

C. Polymerase chain reaction (PCR) conditions

Ten μ l from DNA aliquot of each sample was mixed well to prepare the bulked DNA sample representing Anisakid larvae. This generated bulked DNA sample was used for RAPD-PCR fingerprint of Anisakids using four arbitrary primers "Table.2". PCR was performed in 25 μ l volumes tubes containing; 2.5 μ l DNTPs (0.5mM), 1.5 μ l MgCl₂ (1.5mM), 2.5 μ l Buffer (10X), 2.5 μ l Primer (10 p.mol), 0.35 μ l Taq DNA polymerase (250 U), 2 μ l Template DNA (25ng) and 13.65 μ l H₂O (DW). Amplification reactions were carried out in (Perkin Elmer Gene Amp PCR Thermocycler 2400), with the following conditions; 94°C for 2.5 min followed by 40 cycles including a denaturation step at 94°C for 45 sec., an annealing step at 37 °C for 30 sec. and polymerization step at 72°C for 2 min. with a final extension step at 72°C for 12 min.

D. PCR product electrophoresis

A volume of 15 μ l of the amplified RAPD products was loaded in each well in sub marine mini gel agarose electrophoresis apparatus (BIORAD) and standards DNA (100 bp +1.5 kb ladder DNA yielding 1500, 1000, 900, 800, 700, 600, 500,400,300,200 and 100 base pair (bp) bands as well as 50 bp ladder producing bands of 500, 450, 400, 350, 300, 250, 200, 150, 100 and 50 bp) were loaded onto a 1.2% agarose gel and separated by electrophoresis. Gel was stained with ethidium bromide (0.2 μ g/ml) and then the products were visualized by UV light. PCR products were photographed by gel documentation system (Gel Doc. BIORAD 2000) under UV trans-illuminator.

III. RESULTS

A. Clinical and postmortem examination

The examined fish showed no clinical signs. Most of Anisakid larvae were found attached to the visceral organs specially stomach and liver surfaces of the examined fish, also in the mesentery or free in the body cavity. Sometimes larvae were seen either free or encapsulated in the musculature. The infested liver showed paleness with hemorrhagic area around the encapsulated larvae "Plate. 1".

B. Description of the detected Anisakid larvae

Microscopic examination revealed larval type consistent with *Anisakis simplex*, *Pseudoterranova decipiens*, *Hysterothylacium* spp and *Contracaecum* spp." Plate. 2"

Anisakis simplex, (Rudolphi 1809): Description based on 30 third larval stages, it is 5.2-20 mm long X 0.25 mm wide. There is a boring tooth at the anterior end and excretory pore near it. The esophagus has an anterior muscular part (2-2.5 mm long) and a posterior ventriculus (0.5- 1.1 mm long). Both the intestinal cecum and the ventricular appendix are absent. The tail is short 0.1 -0.22mm with a mucron at its tip.

Pseudoterranova decipiens (Krabbe,1878): Description based on 30 third larval stages, it is 25.2-28.5 mm long X, 0.74-0.82 mm wide. There is a boring tooth below the oral aperture and the excretory pore below the oral tooth. The esophagus is slender 3.5 mm long, ventriculus is elongate (0.9-1.1mm long), the ventricular appendix is absent and the intestinal cecum is 0.4-0.7 mm in length. The tail is (0.54mm long) and tipped with a mucron.

Hysterothylacium spp. (Ward and Magath, 1917): Description based on 15 third larva stage, it is 11.33- 12.4mm long X 0.14 -0.16mm wide. There is a boring tooth at the anterior end. The excretory pore is below the nerve ring. The length of the esophagus is 1.7-2.2 mm, the ventriculus is spherical in shape with a length of 0.1-0.15 mm. The intestinal cecum is short anteriorly projected (0.34-0.5 mm long) and the ventricular appendix is long (2.9- 3.8 mm). The tail is 0.7mm long and without a mucron.

Contracaecum spp. (Raillet and Henry, 1912): Description based on 10 third larval stages, it is 12.92-23.85 mm long X 0.52-0.82 mm wide. There is no anterior boring tooth. The excretory pore is near oral aperture. The length of the esophagus is 1.53-2.28 mm. The ventriculus is short subglobular (0.08-0.29 mm), ventricular appendix (0.5-1.3 mm long) is much shorter than intestinal cecum (1.08-1.89 mm long). The tail is conical (0.23mm long) and it has not a mucron.

C. Prevalence of Anisakid larvae among examined hakes

The seasonal prevalence of Anisakid larvae in both lizard head and European hake was the highest in spring followed by summer and winter and the lowest in autumn "Fig. 1; a and b". The larvae were observed mostly within the body cavity and attached to the organs or in the musculature and its intensity ranged from 2-10 and 4-23 parasites per fish in the musculature and visceral organs (viscera and organs) of lizard head hakes respectively, while the range of Anisakid larvae intensity in European hakes was 1-4 and 3-25 parasites in the musculature and visceral organs respectively and the mean abundance of Anisakid larvae in the musculature and organs of lizard head was higher than that observed in European hake musculature and viscera "Table.1".

D. Molecular identification of the Anisakid larvae

Data of RAPD-PCR analysis using the four arbitrary primers were shown in "Fig. 2" and Table. 3. All the analyzed RAPD primers were able to amplify the bulked DNA sample of Anisakid larvae. The range of RAPD-PCR band products was between three for ILO004 and five for ILO868, ILO524 and M13 primers. The molecular weights of the resultant bands ranged from 150 bp to 660 bp.

The PCR products of the bulked DNA sample generated by primer ILO868 revealed a maximum number of 5 bands with molecular weights ranging from 300 to 650 bp. The maximum number of generated clear bands by ILO524 were five with a molecular weight spectrum of 250 - 510 bp. The RAPD fingerprint of Anisakid using ILO004 had three distinct bands of 150, 390 and 550 bp. The RAPD identification of Anisakid larvae utilizing M13 was distinguished by five obvious PCR bands (300, 340, 440, 460 and 650 bp).

IV. DISCUSSION

European (*Merluccius merluccius*) and Lizard head (*Saurida undosquamis*) hakes are considered as a common and commercially important fishes caught from Mediterranean Sea and gulf of Suez in Egypt respectively [19, 20] and distributed to all Egyptian governorates.

In the present study, the distribution of Anisakid larvae in the examined fish was highly related to the organs specially stomach and liver surfaces, mesentery, body cavity and musculatures. Most of larvae were attached to the viscera (encapsulated) or free in the body cavity of infested fish. These results were matched with those observed by [21, 22]. Also, the pathological findings observed in the

organs were also nearly the same mentioned by the same authors [21, 22].

The morphological characteristics of *Anisakis simplex*, *pseudoterranova decipiens* and *contracaecum* spp. larvae recorded in this study were in accordance with that described by [15, 23, 24, 25, 26, 27]. The morphological characters of *Hysterothylacium* larvae in the present study were similar to *Hysterothylacium* spp. type KE described by [27] in *Caranx malabaricus*, *Lutjanus coccineus*, *Saurida undosquamis*, *Trichlurus lepturus* and *Argyrops spinifer* in Kuwait. The detected species in the current study was different from that recorded by [10] in lizard head hakes in Sharkia province except for *pseudoterranova decipiens*, this may be ascribed to the variation of the examined fish species. The infestation by *Hysterothylacium* and *Contracaecum* was less frequent than the other detected species, this may come in consistent with [28] who found that the Infestation with larvae of *Anisakis* occurs relatively frequently and [29, 30] who reported that the infestation with *Hysterothylacium* and *Contracaecum* is less common. It seems that the detected species of *Contracaecum* in the present study is *C. multipapillatum*. To our knowledge, both *C. multipapillatum* and *Hysterothylacium* spp. type KE were not previously recorded in this type of fish in Egypt, so, further genetic analysis is needed for a more accurate species identification. .

Anisakid nematodes are widely distributed, and their prevalence among a variety of marine fish species had been previously identified [8, 9, 21, 31, 32]. In the current study the peak of the seasonal prevalence of *Anisakid* larvae was observed in spring and summer, nearly the same results was recorded by [22]. This may be attributed to the temperature factor which enhance the life cycle of the parasite [33] and the abundance of natural food specially crustacean which is the main food taken by hake fishes in spring and summer [34] and play role in *Anisakid* life cycle. The intensity of *Anisakid* larvae was the highest in the viscera and organs in both examined spp. rather than in the musculature. Although a low number of the larvae was detected in the musculature, it is an indication that the larvae migrate to muscles after capture [35] and would increase the risk of human anisakidosis, furthermore there is a degree of cross reactivity between *Anisakis* allergens and other anisakids which render individuals sensitive to the allergens of other species [36]. This article presents a molecular genetic characterization using RAPD-PCR of the extracted *Anisakid* larvae populations. The four primers have been successfully used to determine the molecular genetic fingerprint of found *Anisakids* (*Anisakis simplex*, *pseudoterranova decipiens*, *Hysterothylacium* sp. type KE and *contracaecum* spp.) in respect to RAPD technique.

The limited number of the generated markers (3-5 PCR bands) provides for the first time a preliminary well-recognized genetic fingerprint of *Anisakids* infesting Egyptian coasts. Further studies are needed for assessment of genetic diversity, differentiation and phylogeny of *Anisakids* found in Egypt. Therefore, it is difficult to compare current study markers identifying fingerprint of one bulked *Anisakid* sample to other studies using more number of individual larval specimens. For instance, in a genetic diversity study by [37]; 143 RAPD-PCR bands were identified in 42 individual larval specimens of *A. simplex* infecting Spanish coasts using the same primers. Since the genetic fingerprint study can be done using one bulked DNA specimen.

V. CONCLUSION

The high prevalence of *Anisakid* larvae among examined lizard fish, which considered an important low priced meal for poor people in Egypt refers the possibility of human infection occurs after eating raw, under cooked or improperly processed fish which has adverse health effects in humans. In addition the allergens which is produced by either live or dead *anisakis* may lead to hypersensitivity to human, furthermore the cross reactivity between *Anisakis* allergens and other anisakids renders individuals sensitive to the allergens of other species. Moreover, this study provides well-identified genetic fingerprint for identification of *anisakid* larvae (*Anisakis simplex*, *pseudoterranova decipiens*, *Hysterothylacium* spp. and *contracaecum* spp.) and diagnosis of fish *Anisakidosis*. We argue further studying the genetic diversity and phylogenetic classification of *Anisakids* infecting Egyptian coasts.

ACKNOWLEDGEMENT

We appreciate Prof. Dr. Adel A. Shaheen, Prof of Fish Diseases & Management for his helpful advices and useful information.

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APPENDIX

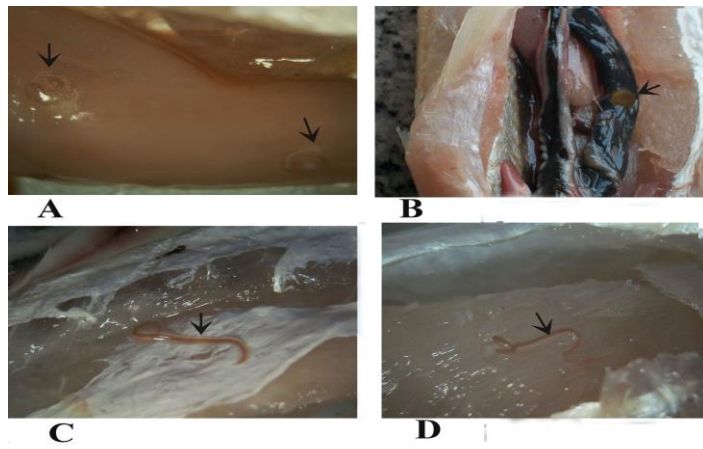


Plate .1 : Anisakid larvae (arrows); A) encapsulated within lizard head hake stomach wall, B) encapsulated on the viscera of European hake , C) free on the viscera of lizard head hake and D) free within the musculature of Lizard head hake.

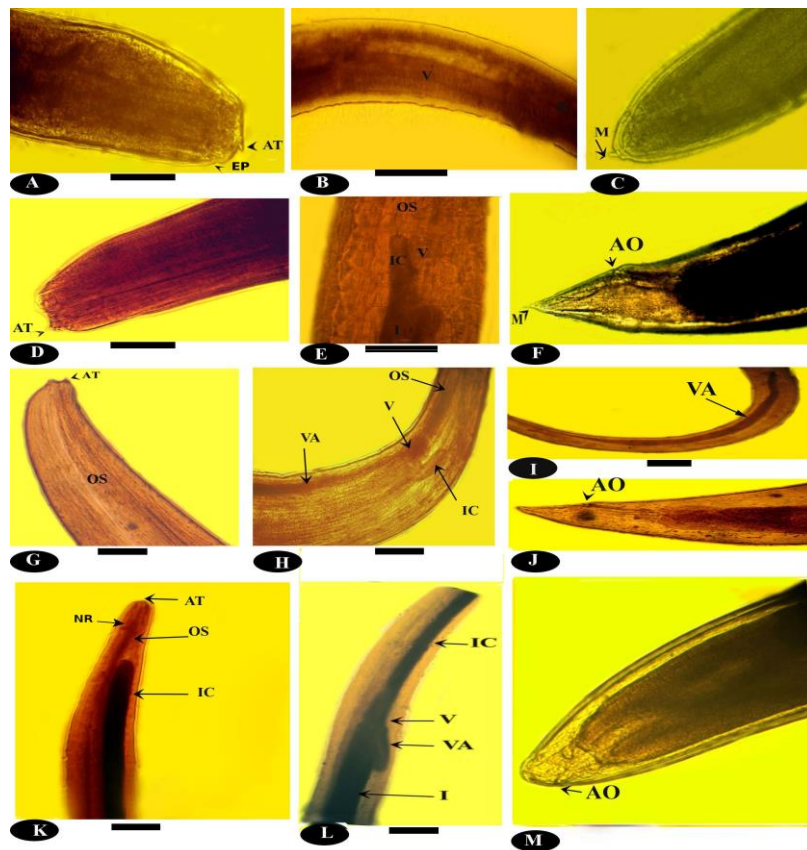


Plate. 2: Figure A -M Larval stages of anisakid larvae. *Anisakis Simplex*. Fig. A. Anterior end. Fig. B. Ventricular region. Fig. C. Posterior end, *Pseudoterrova decipiens*. Fig. D. anterior end. Fig. E. Ventricular region. Fig. F. Posterior end. *Hysterothylacium* spp.. Fig.G. Anterior end. Fig. H. Ventricular region . Fig. I and J. Posterior end. *Contracaecum* spp. Fig. K. Anterior end. Fig.L. Ventricular region. Fig. M. Posterior end. Scale bar 0.5mm. Abbreviation: AT: anterior boring tooth, EP: excretory pore, V: ventriculus , M: mucron , OS: oesphagus, VA: Ventricular appendix, I: intestine, IC: Intestinal cecum, NR: nerve ring, AO: anal opening.

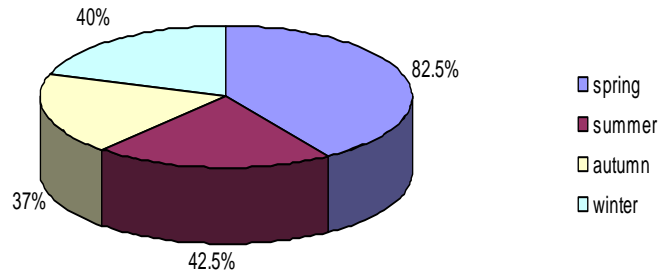


Fig (1a):- The seasonal prevalence of Anisakid larvae in *Saurida undosquamis*

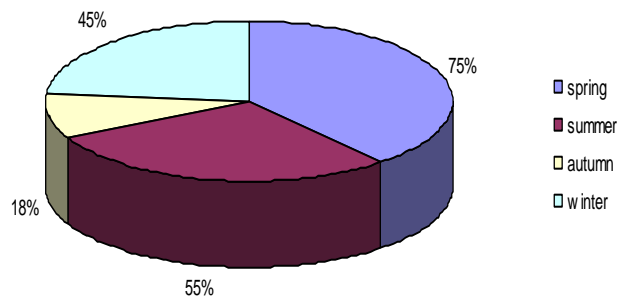


Fig (1b) The seasonal prevalence of Anisakid in *Merluccius merluccius*

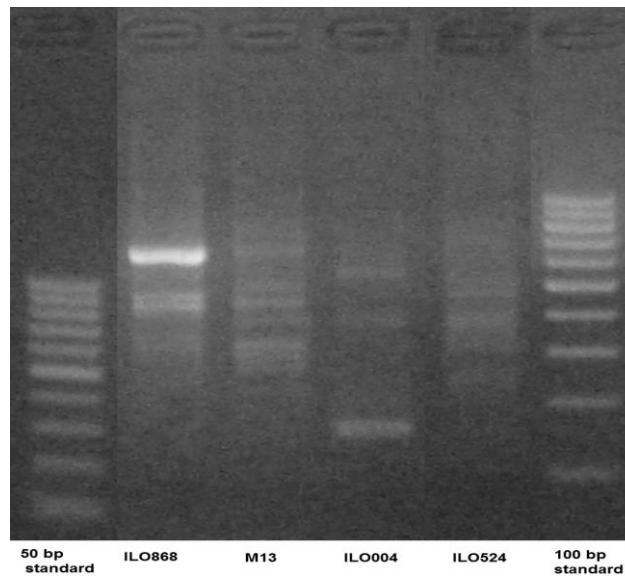


Figure 2. RAPD-PCR fingerprints of the bulked Anisakid larval DNA sample using four primers ILO868, M13, ILO004 and ILO524

Table 1. The total prevalence, mean intensity and mean abundance of anisakid larvae retrieved from both European and lizard head hakes.

	<i>Merluccius merluccius</i>		<i>Sarurida undosquamis</i>	
	Musculature	(Viscera+organs)	Musculature	(Viscera+organs)
Mean intensity	0.032	0.136	0.05	0.15
(range)	(1-4)	(3-25)	(2-10)	(4-23)
Mean abundance	0.0156	0.065	0.025	0.076

Mean intensity = number of parasites/number of infected fish, mean abundance = number of parasites/number of examined fish.

Table 2. List of primers names and their nucleotide sequences

Arbitrary primer name	Arbitrary primer sequence
ILO-004	GGAATTTTCGGCTTCACTAC
ILO-524	CGCGCCCGC
ILO-868	CAGCCTCGGC
M13	TGACCGGCAGCAAAATG

MW: molecular weight of the observed bands in base pairs; +: present; -: absent

Table 3. RAPD-PCR fingerprints of the bulked Anisakid larval DNA sample using four primers ILO868, M13, ILO004 and ILO524

MW	ILO868	M13	ILO004	ILO524
660	-	+	-	-
650	+	-	-	-
550	-	-	+	-
510	-	-	-	+
500	-	+	-	-
490	-	-	-	+
460	+	-	-	-
450	-	+	-	-
440	+	-	-	-
400	-	-	-	+
390	-	+	+	-
340	+	-	-	-
300	+	+	-	+
250	-	-	-	+
150	-	-	+	-