Research Note

Occurrence of *Anisakis* and *Hysterothylacium* nematodes in Atlantic chub mackerels from Libyan coasts

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Article info

Summary

The occurrence of zoonotic parasitic nematodes in Atlantic chub mackerels (*Scomber colias* syn. *Scomber japonicus*) from Libyan waters was investigated, using epizootiological estimations and molecular specific characterization of larvae. Nematodes belonging to *Anisakis* spp., the main etiological agent of anisakiasis in Mediterranean waters, and to *Hysterothylacium* spp. so far considered not pathogenic to humans, were detected. Prevalence values were generally high in visceral cavities (over 40 % for both parasites) while were low for *Anisakis* (around 1 %) and null for *Hysterothylacium* in muscles. Moreover, the level of infections was associated with seasons, a feature potentially useful to plan fishing captures and to elaborate risk mitigation strategies for anisakiasis. Species molecular identification performed on a subsample described the presence of *Hysterothylacium aduncum* as the predominant species, along with *Anisakis pegreffii* and the hybrids (*A. pegreffii* and *A. simplex* sensu stricto), thus posing a concrete zoonotic risk following the consumption of such fish species as a raw preparation.
Keywords: Atlantic chub mackerel; *Anisakis pegreffii*; *Hysterothylacium aduncum*; PCR-RFLP; seasonality; consumers' safety

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Introduction

An adequate intake of fishes would ensure around 50 % of daily requirement of animal protein. Actually, it accounts for the 16 % of the animal proteins consumed by the world's population, and many marine products, such as mackerels, sardines and anchovies are widely consumed in the Mediterranean coastal regions for the high amount of fatty acids and omega-3, which are of significant nutritional value. The last available data on fish consumption reported 20.2 kg in 2015 up to 20.5 kg in 2017 (FAO 2018).
The Atlantic chub mackerel *Scomber colias* Gmelin, 1789 (Family Scombridae) is a pelagic fish largely used along with anchovies and sardines, both for human and animal nutrition. The taxonomic assignment of members of this fish species in the Mediterranean has been intensively studied in recent years, following the evidence that the Pacific and the Atlantic/Mediterranean populations are significantly differentiated and may represent two distinct species (Infante *et al*., 2007). According to these authors, the term *Scomber japonicus* should be retained for chub mackerels from the Pacific and the term *S. colias* for the Atlantic and the Mediterranean. In general, mackerels represent a large amount of all fishes captured worldwide (FAO 2007) and the most caught species in Portugal in 2016 (>26.000t) (DGRM 2017), ranking among the most valuable fish species in Europe (Levsen *et al*., 2018). Atlantic chub mackerels are frequently infected by anisakid nematodes (Costa *et al*., 2003; 2011; Debenedetti *et al*., 2019),

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parasites with a marine life cycle maintained by a prey-predator trophic web among intermediate, paratenic and definitive hosts. The occurrence of nematodes in marine products causes economic losses and medical problems, as larval nematodes belonging to the family Anisakidae may cause a fish-borne zoonotic disease in humans known as anisakidosis, while those belonging to the family Raphidascarididae are commonly considered not zoonotic or of negligible concern for human health (Klimpel & Palm, 2011). In Mediterranean countries, the species *Anisakis pegreffii* is the main etiological agent of anisakiasis, as it is widely spread in paratenic and definitive hosts of Mediterranean waters; similarly, *Hysterothylacium aduncum* is one of the most frequently Raphidascaridid recovered in teleosts, representing mainly an aesthetic problem, potentially constraining fish marketability.

Following the European Authority for Food Safety encouragement to continuous research on aspects related to anisakids and fishes intended for human consumption (EFSA 2010), the aim of the present study was to investigate the presence of *Anisakis* spp. and *Hysterothylacium* spp. in specimens of *Scomber colias* caught off Libyan waters. Although the occurrence of anisakid nematodes in fishes of the western part of the Mediterranean Sea has been largely studied, information on the southern-central part of the Mediterranean are still scarce and worthy of investigation. Only few studies are available on anisakids infecting marine fishes from Libyan coasts but not involving mackerels among the sampled fish species, and in most cases limiting the identification of nematodes at genus level (Sharif & Negm-Eldin, 2013; Kassem & Bowashi, 2015; Eissa *et al.*, 2018). An additional aim was to characterize a subsample of parasitic nematodes at molecular level to identify larvae at species level and to indirectly infer the risk related to fish consumption.

Material and Methods

Sampling and infection parameters
A total of 360 individuals of *Scomber colias,* measuring 20.1 – 26.3 cm and weighting 70.3 – 158.2 g, were purchased from the fish market in Tripoli, Libya, from September 2013 to August 2014, sampling 90 specimens for each season. Stored in ice, the fish were directly transferred to the Laboratory of Quality and Diseases of Marine Organisms at the Marine Biology Research Center. Visceral cavities were visually inspected and the remaining parts were filleted. All material was checked for the presence of larval nematodes using a stereomicroscope and with light candling method according to EFSA (2010).

The recovered nematodes, excluding those randomly selected for molecular identification, were fixed, clarified and preserved as previously described (Eiras *et al.* 2006; Lasee 2004; Navone *et al.* 1998 and Roberts 1989). The larvae were morphologically identified at genus level according to available diagnostic keys (Berland 1961; Davey 1971).

Epizootiological parameters as prevalence, mean abundance and intensity of infection as defined by Bush *et al.* (1997), and ecologically relevant parameter as aggregation index, were calculated using Quantitative Parasitology (Reiczigel *et al.*, 2013). Parameters were calculated as indices of overall infections in relation to parasitic genus, to seasons (trimesters) and to anatomic site of larvae recovery (body cavity vs fillets). Statistical significant differences among prevalence values, with regard to seasons, were evaluated by using the chi-square test or Fisher's test.

Species identification with molecular diagnostic keys
A subsample of 100 larvae, randomly selected among different capture batches, were characterized at genetic level using a molecular approach based on PCR–RFLP of the nuclear ribosomal internal transcribed spacer (ITS) region, since it is informative for taxonomic/diagnostic purposes for the genera *Anisakis* and *Hysterothylacium* (D'Amelio *et al.*, 2000; De Liberato *et al.*, 2013). In details: genomic DNA was isolated from entire larvae using the Wizard Genomic DNA purification kit (Promega, Madison, WI), according to the manufacturer's protocol. In brief, after removing the anterior and posterior ends for morphological studies, the remaining part of individual nematodes were each placed in 600 µl of a mixture containing 0.5 Methylenediaminetetra-acetic acid (EDTA) plus Nucleilysis solution and then crushed employing a sterile pestle. Proteinase K (17.5 µl at 20 mg/ml) was added to each tube, which was incubated at 55°C for 3 h. RNase solution (3 µl at 4 mg/ml) was added, and the tubes were incubated at 37°C for 30 min. Subsequently, protein precipitation solution (200 µl) was added, the tubes were vortexed and chilled on ice for 5 min, and the DNA was precipitated with ethanol. Each DNA pellet was air-dried for 20 min and dissolved in 100 µl of DNA rehydration solution.

The entire ITS region (ITS-1, 5.8S, ITS-2), of around 1000 base pairs, was amplified using 20ng of template DNA, 10 mM Tris–HCl (pH 8.3), 1.5 mM MgCl2 (Bioline), 40 mM of nucleotide mix (Promega), 50 pmol/µl of the primer forward NC5 (5-GTAGGTGAACCTGCGGAAGGATCAT-3) and the reverse primer NC2 (5-TTAGTTTCTTCCTCCGCT-3) (Zhu *et al.*, 2000), and 1.0 U of BIOTAQ DNA Polymerase (Bioline) in a final volume of 50 µl. PCR were carried out using the following parameters: 10 min at 95°C, thirty cycles of 30 s at 95°C, 40 s at 52°C and 75 s at 72°C, with a final extension of 7 min at 72°C. A negative control was included in each amplification. Aliquots of individual PCR products were separated by electrophoresis using agarose gels (1 – 1.5 %), stained with GelRed (25 µg/ml) and detected by the use of ultraviolet transillumination. Gel images were captured electronically and analysed using Bio-Rad's Image Lab software.

The endonucleases *Hin*fI and *Hha*I were used to digest positive amplicons in order to identify larval nematodes at species level. Digestions were performed with incubations of four hours at 37°C. Amplification fragments obtained were separated by 2 % agarose gel electrophoresis and their sizes were determined by comparison with a 100 bp DNA ladder marker (Promega, Madison, WI).

Table 1. Epizootiological parameters obtained for chub mackerels infected by *Anisakis* spp. and *Hysterothylacium* spp. analysed in the present study (CI: confidence interval; NIF: number of infected fish; P %: prevalence indicated as percentage; ml: mean intensity; mA: mean abundance; K: aggregation index).

	NIF	P %	ml	mA	K
Anisakis spp.	151	41.9	5.76	2.42	0.2
(CI)		(36.8 – 47.2)	(4.32 – 8.55)	(1.78 – 3.68)	
Hysterothylacium spp.	158	43.9	2.25	0.98	0.53
(CI)		(38.7 – 49.2)	(1.96 – 2.66)	(0.82 – 1.2)	

Results

On a total of 360 fish examined, a number of 1317 larvae were recovered and morphologically identified as belonging to two genera: *Anisakis* (n= 969) and *Hysterothylacium* (n=348). Specimens belonging to *Anisakis* genus showed morphological features of type I larvae.

The subsample of nematodes analysed using diagnostic molecular keys (n=100) gave positive amplification for 35 specimens, probably due to spoilage of material during the transfer to Rome. All the 35 larvae were found in the viscera. Restriction fragments analyses revealed three distinct banding patterns corresponding to three nematode taxa: *Anisakis pegreffii* (n=16), the hybrid genotype of *A. pegreffii* and its sibling species *Anisakis simplex* sensu stricto (n=1), and *Hysterothylacium aduncum* (n=18). Among the taxa recovered, at least two are considered pathogenic for humans, namely *A. pegreffii* and the hybrid genotype. Two larvae of *A. pegreffii* and the hybrid were found in the same fish specimen, while all other *Anisakis* were from different fish specimens. Regarding *Hysterothylacium*, two larvae were from the same fish specimen and the remaining from different fish specimens.

Regarding the parameters of infection (Table 1), most of larvae were recovered in the visceral cavity, with the exception of nine *Anisakis* spp. larvae recovered in the muscular tissue of three individual hosts (prevalence 1.1 %, CI 0.3 – 2.8). Moreover, the distribution of nematode larvae in the host population resulted strongly aggregated in *Anisakis* (k=0.20) or moderately aggregated in *Hysterothylacium* (k=0.53). Considering prevalence estimations according to the fishing period (Table 2), the highest values

of prevalence obtained for *Hysterothylacium* infection was during spring, while *Anisakis* maximum infection level occurred in summer months. The overall differences between infected and non-infected hosts according to seasons were all highly significant for both parasitic species (p≤ 0.00001). When seasonal differences in infection level are evaluated as pairwise comparisons, all combinations showed very significant statistical *p*-values (ranging from 0.0000001 to 0.00027) with the exception of the differences for *Hysterothylacium* in autumn vs. winter (*p* = 0.0985), and in summer vs. spring (*p* = 0.0985)

Discussion

The recovery of two pathogenic *Anisakis* species, namely *A. pegreffii* and the *A. pegreffii/A. simplex* s.s. hybrids, in fish intended for human consumption, represents a public health hazard and an economic issue. Previous surveys on the presence of anisakids in fishes collected from the Mediterranean basin have reported high values of infection by *A. pegreffii* in Atlantic chub mackerels from Spain and Sardinia (Casti *et al*., 2017; Madrid *et al*., 2016; Piras *et al*., 2014). Other investigations carried out on *Scomber japonicus* and on *Scomber scombrus* reported the occurrence of *A. simplex* sensu stricto and hybrids in the Western Mediterranean waters (Farjallah *et al*., 2008; Ferrantelli *et al*., 2014). *A. pegreffii* and hybrid forms were also recovered in horse mackerels (*Trachurus trachurus*) caught from two localities off Libyan coasts (Eissa *et al*., 2018). Additional *Anisakis* species, i.e. *Anisakis physeteris* (Goffredo *et al*., 2019) and *Anisakis typica* (Farjallah *et al*., 2008), were observed in the Easternmost Mediterranean waters.

The differential occurrence of anisakid species in distinct areas

Table 2. Prevalence parameter estimated according to seasons (autumn: September, October, November; winter: December, January, February; spring: March, April, May; summer: June, July, August) obtained for Atlantic chub mackerels infected by *Anisakis* spp. and *Hysterothylacium* spp. analysed in the present study (CI: confidence interval; P%: prevalence indicated as percentage; *p*: p value).

	Anisakis spp.		*Hysterothylacium* spp.	
	P%	CI	P%	CI
Autumn	37.80	27.8 – 48.6	24.40	16.0 – 34.6
Winter	17.80	10.5 – 27.3	33.30	23.7 – 44.1
Spring	50.00	39.3 – 60.7	63.30	52.5 – 73.2
Summer	62.20	51.4 – 72.2	54.40	43.6 – 65.0
p	< 0.00001		< 0.00001	

of the Mediterranean basin may be helpful for the successful use of *Anisakis* larvae for tagging *Scomber* spp. fish stocks, for fisheries management purposes and for the evaluation of food safety in relation to consumers' habits, which could benefit also from the information on the maximum and minimum levels of *Anisakis* infection. Although molecular identification on a larger number of larvae appears needed, the lack of *A.simplex* s.s. records in the area may suggest that the mackerels sampled could belong to a local Mediterranean stock. Conversely, previous findings of this anisakid species in *Scomber scombrus* from Tunisian coasts may be related to mackerel stocks migrating from the Atlantic Ocean through the Gibraltar strait (Farjallah *et al*., 2008).

Parameters of parasitic infection here observed are in agreement with those reported in previous studies carried out in members of the genus *Scomber* (Abattouy *et al*., 2011; Levsen *et al*., 2018). Accordingly, *A. pegreffii* is the prevalent anisakid species reported in mackerels (Abattouy *et al*., 2011; Casti *et al*., 2017; Goffredo *et al*., 2019; Mladineo & Poljak 2014; Piras *et al*., 2014), with the majority of larvae commonly recovered in the visceral cavity. The low number of larvae found in muscles in the present study may represent a feature of potential interest for public health issues, as fillets are the edible part of the fish, thus suggesting a reduced probability of human infection. Mackerels are commonly consumed cooked in Libya, with the exception of the most common traditional Libyan dish based on salted-dry fillets mackerels. Moreover, evidences related to potential human sensitizations for the presence of *Anisakis* antigens also in cooked or salted fish are completely missing for the area under study.

The distribution of nematode larvae in the host population resulted strongly aggregated in *Anisakis* spp. or moderately aggregated in *Hysterothylacium* spp. This is highly common in parasite ecology and may be of interest for control organisms given the potential implication in setting sampling size assessment for routine monitoring and control programs (Shvydka *et al*., 2018).

Regarding the relationships between infection level and seasonal trend observed in *Anisakis*, prevalence values were found higher in spring and summer months rather than in autumn and winter. These results are consistent with those of Akmirza (2003), who reported a higher prevalence of infection by *A. simplex* s.l. in mackerels during spring from two areas of Turkey, and with those of Gutiérrez-Galindo *et al*. (2010) which observed a higher prevalence in summer from Tarragona waters (NE Spain). This information may help both consumers, for the choice of fish species to consume, and policy makers and producers' categories to plan a seasonal fishing strategy aimed at mitigate the health risks related to *Anisakis*, as recently suggested also by Cammilleri *et al*. (2019).

A similar study carried out on *Merluccius merluccius* collected from Libya and on the zoonotic potential in the area advocated that the lack of reported cases of human infection was most likely related to the infrequent habits of consuming raw fish. However, the existence of human infections cannot be excluded given the paucity of awareness for anisakiasis among Libyan physicians (Sharif & Negm-Eldin, 2013).

Conclusions

The continuous monitoring of fish and fish products intended for human consumption is highly recommended by the European Food Safety Authority, with particular attention to *Anisakis* spp., being the only fish-borne parasite able to trigger an allergic response in humans (EFSA 2010). In the present study, Atlantic chub mackerels from south Mediterranean basin resulted infected by three parasitic nematode taxa, and at least two species recovered are considered pathogenic for humans, namely *A. pegreffii* and the hybrid genotype. Levels of infection suggest a residual, but still existing, zoonotic risk for consumers, as a small amount of larvae belonging to pathogenic species was recovered from fish fillets.

Conflict of interest

Authors state no conflict of interest

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