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Short communication

Survey on the presence of *Anisakis* and *Hysterothylacium* larvae in fishes and squids caught in Ligurian Sea

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ABSTRACT

This study aims to investigate the occurrence of Anisakidae larvae (genera *Anisakis* and *Hysterothylacium*) in fishes and squids used for human nutrition and increase the knowledge of the distribution of Anisakidae larvae in this area of Mediterranean Sea. Distribution and correct identification of Anisakidae larvae in fish species is important for the assessment of their relative epidemiological role; especially when the fishes are of high commercial value and largely used in human nutrition. The study investigated the occurrence of Anisakidae larvae (genera *Anisakis* and *Hysterothylacium*) in 195 fishes owing to 22 different species and 60 squids (*Illex coindetii*) sampled in northern Ligurian Sea. A combination of morphological and PCR-RFLP methods have been used. A total of 177 anisakid larvae were isolated in 42/195 (21.5%) fish of 11 species and only one larva in one squid. These larvae were identified morphologically as belonging to the genera *Anisakis* (36/177) or *Hysterothylacium* (142/177). All *Anisakis* larvae were isolated from *Trachurus trachurus* and were identified at species level by PCR-RFLP as belonging to *Anisakis pegreffii*. *Hysterothylacium* sp. in short, larvae presence occurred in most of examined fish species with a higher density in *Mullus barbatus* (mean intensity 5.6 larvae) and *Serranus scriba* (MI 5.3).

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1. Introduction

Anisakidosis is an important fish-borne zoonosis caused by larval stages of nematodes of the family Anisakidae, genera *Anisakis*, *Pseudoterranova* and *Contracaecum* (Hochberg and Hamer, 2010). Digestive disorders and/or allergies (Valero et al., 2003; Audicana and Kennedy, 2008) in human can occur as a consequence of accidental ingestion of raw, undercooked or improperly processed fishes and/or cephalopods parasitized by third-stage larvae (L3). As a consequence, scientific attention to the Anisakidae

nematodes and especially to the two genera certainly zoonotic – *Anisakis* and *Pseudoterranova* – continues to remain high due to the large number of marine fish and cephalopod species associated with the transmission of mentioned disease (Mattiucci and Nascetti, 2008; Santana and Cives, 2009) and the wide geographical distribution throughout the year of Anisakidae larvae. Moreover the increasing global demand of seafood, and a growing preference for raw or lightly cooked food in several Western countries can increase the risk of parasite exposure (Broglia and Kapel, 2011). Despite during the last 20 years the knowledge of these parasites was improved, the European Food Safety Authority (EFSA) recommends that research in this topic should be continued: the collection of systematic data on the complete life cycle, geographical and

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seasonal distribution, prevalence, intensity, and anatomical location of parasites of public health importance in wild caught fishery products (EFSA, 2010). Although the existence of various reports on the circulation of these zoonotic parasites in fishes of high commercial value and used for human consumption in the Mediterranean Sea (Farjallah et al., 2008; Angelucci et al., 2011; Chaligiannis et al., 2012; Meloni et al., 2011; Cavallero et al., 2012) the amount of information concerning Italian costs and in particular North western area are still poor and requires further investigations (Manfredi et al., 2000; Mattiucci et al., 2004). In order to increase these specific knowledge we investigated the occurrence of Anisakidae larvae, in particular the two genera *Anisakis* and *Hysterothylacium* in fishes and squids caught in northern Ligurian Sea, using a combination of morphological and molecular analyses to identify the larvae at the species level.

2. Materials and methods

2.1. Sampling hosts

A total of 195 fish specimens belonging to 22 different teleost species and 60 cephalopods owing to the species *Illex coindetii*, were collected from different fish market in La Spezia from October 2010 to September 2011. The fish markets were selected for area of fish capture (43°53' N, 08°57' E; 43°57' N, 09°44' E) in order to cover the north-eastern part of Ligurian Sea. Samples were then transferred in refrigerated condition to laboratory and immediately examined for Anisakid larvae (Table 1).

2.2. Parasite detection

Each fish and squid was identified at species level, measured and weighed, then analyzed for the presence of larvae of Anisakidae nematodes by visual examination of body cavity and subsequent evisceration. Viscera and internal organs were left 30 min under a heat source in physiological saline solution (pH 7.3) to allow the escape/mobilization of the larvae when present. Then visceral surfaces were scanned by eyes and under a microscope for the detection of nematode larvae. The muscular portion of fishes has been sectioned in less than 5 mm thick fillets and observed by transillumination. All the detected larvae were isolated, counted, observed under a stereomicroscope (Nikon SMZ 800) for identification at genus level, using morphological characters, then fixed in 70% ethanol (Koyama et al., 1969; Thomas and Lawrence, 1995; Shih, 2004; Zhang et al., 2007; Quiazon et al., 2008). The larvae morphologically identified as belonging to the genus *Anisakis* were subjected to further molecular characterization by PCR-RFLP to identify the species.

2.3. DNA extraction

DNA was extracted from 30 mg of each larva using a commercial kit (PureLink Genomic DNA Kits, Invitrogen) and eluted in a final volume of 100 µl, according to protocol supplied. The kit is based on the selective binding of nucleic

acids to silica membranes and subsequent elution. Nucleic acids were stored at –20 °C until use.

2.4. PCR-RFLP analysis

The nuclear rDNA region containing ITS-1, 5.8 rRNA gene and ITS-2, were amplified by PCR using the primers NC5 (5'-GTAGGTGAACCTGCGGAAGGATCATT-3') and NC2 (5'-TTAGTTTCTTTCTCCGCT-3') (Zhu et al., 1998). PCR was performed using a mixture containing 1 × PCR buffer, 2.5 mM MgCl₂, 0.2 mM dNTPs each, 0.5 µM of each primers, 1.25 U of FastStart Taq DNA Polymerase (Roche, Mannheim, Germany), 5 µl of DNA sample and water to a final volume of 50 µl. The reaction was carried out with an automated thermocycler (GeneAmp2720; Applied Biosystems, Foster City, CA) as follows: 4 min at 95 °C, then 35 cycles of 30 s at 95 °C, 30 s at 58 °C and 90 s at 72 °C followed by a final extension of 15 min at 72 °C. Negative and positive controls were included in all amplifications. PCR products were separated by electrophoresis on 2% agarose gel, stained with 10,000× Gel Green (Biotium, Hayward, USA) and visualized by UV. The amplified products were analyzed by RFLP technique using the restriction enzymes: *HhaI*, *HinfI* and *TaqI* (Invitrogen) that allows the identification of the members of *Anisakis* genus according to the genetic markers described by D'Amelio et al. (2000) and implemented by Pontes et al. (2005). Manufacturer's recommendations were followed with regard to the digestion of PCR products. Finally digested products were analyzed by electrophoresis on a 2% agarose gel stained with 10,000× Gel Green (Biotium, Hayward, USA) and the bands visualized with UV transilluminator (Gel Doc; Bio-Rad S.A., Ivry Sur Seine, France). The size of restriction fragments was compared with molecular size marker (AmpliSize Molecular Ruler 50–2000 bp Ladder, Bio-Rad).

2.5. Sequencing

Some larvae identified as *Anisakis pegreffii* using PCR-RFLP analysis were sequenced for further confirmation. Sequencing was carried out directly on purified fragments with ABI 310 Genetic Analyser (Applied Biosystems), using ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit, version 1.1 (Applied Biosystems). The nucleotide sequences were submitted to BLASTN sequence similarity search at the NCBI database and were aligned with the gene sequences available in GenBank database.

2.6. Statistical analysis

The fish length and parasite abundance association was analyzed using Pearson's correlation coefficient *r*. A *P*-value of less than 0.05 was considered statistically significant.

3. Results

The 195 analyzed fish belonging to 22 species, 21.5% (42/195) harboured in both body cavity and viscera a total of 177 Anisakidae larvae while no larva was isolated from muscle portions (Table 1). From these only 20.3% (36/177) larvae were identified morphologically as belonging to the

Table 1

Data on fishes and cephalopods species from northern Ligurian Sea tested for Anisakidae larvae and their level of infection.

Species	N	Mean length (range, cm)	Mean weight (range, g)	No. of specimens infected/tested (%)	Number of collected larvae	Mean intensity of infection (MI) $\pm$ SE ^b
<i>Mullus barbatus</i>	67	11.5–20	22–101	17/67 (25.4)	96	5.64 $\pm$ 0.58
<i>Illex coindetii</i>	60	21–36.5	17–162	1/60 (1.7)	1	1 $\pm$ 0.24
<i>Trachurus trachurus</i>	32	19.3–26	58–220	5/32 (15.6)	43	8.6 $\pm$ 0.71
<i>Pagellus erythrinus</i>	32	14–22	38–125	5/32 (15.6)	5	1 $\pm$ 0.24
<i>Boops boops</i>	18	16.5–23	40–94	6/18 (33.3)	10	1.7 $\pm$ 0.32
<i>Lithognathus mormyrus</i>	11	16.5–25	58–195	1/11 (9.1)	1	1 $\pm$ 0.24
<i>Chelidonichthys obscurus</i>	6	14.8–20	34–73	1/6 (16.7 ^a)	1	1 $\pm$ 0.24
<i>Merluccius merluccius</i>	4	23–26	83–105	0/4	0	0
<i>Serranus scriba</i>	3	15.2–19.2	44–77	3/3 (100 ^a)	16	5.33 $\pm$ 0.56
<i>Diplodus annularis</i>	3	16.5–19	72–123	1/3 (33.3 ^a)	2	2 $\pm$ 0.34
<i>Raja asterias</i>	3	26.5–32	102–200	0/3	0	0
<i>Pagellus acame</i>	3	16–19.5	51–83	0/3	0	0
<i>Conger conger</i>	2	40–44	65–119	1/2 (50 ^a)	1	1 $\pm$ 0.24
<i>Diplodus sargus</i>	2	16.8–18	76–111	0/2	0	0
<i>Scorpaena scrofa</i>	1	12	34	1/1 (100 ^a)	1	1 $\pm$ 0.24
<i>Spondyliosoma cantharus</i>	1	16	63	1/1 (100 ^a)	1	1 $\pm$ 0.24
<i>Arnoglossus laterna</i>	1	9.5	7	0/1	0	0
<i>Helicolenus dactylopterus</i>	1	18	94	0/1	0	0
<i>Lophius piscatorius</i>	1	22	181	0/1	0	0
<i>Aulopus filamentosus</i>	1	22.5	99	0/1	0	0
<i>Trachinus draco</i>	1	14.5	22	0/1	0	0
<i>Trigla lyra</i>	1	21	76	0/1	0	0
<i>Uranoscopus faber</i>	1	15	60	0/1	0	0
Total	255	–	–	43/255 (16.8%)	178	3.3 $\pm$ 0.44

^a These relative percentage may be affected by the small sample size.^b SE: standard error.

genera *Anisakis* while 80.2% (142/177) to *Hysterothylacium*. In particular the 9.3% (5/42) of infected fishes were positive for *Anisakis* and 95.2% (40/42) for *Hysterothylacium* while in 7.14% (3/42) of fishes were present both genera. The only fish species found to be infested with *Anisakis* was the horse mackerel (*Trachurus trachurus*) with an average of 7.2 larvae for each subject. *Anisakis* sp. larvae were absent in winter and spring, with lowest prevalence and mean intensity (MI) in autumn (1.9% and 1.5 respectively) and highest in summer (5% and 11 respectively). *Hysterothylacium* sp. larvae were present in most of fish species examined with a higher infestation in *Mullus barbatus* (MI of 5.6 larvae for each subject), *Serranus scriba* (MI 5.3) and *T. trachurus* (MI 2.3) followed by *Boops boops*

(MI 1.6), *Diplodus annularis* (MI 2), *Pagellus erythrinus* (MI 1) and other (Table 2). In general *Hysterothylacium* sp. infection occurred through all season, with lowest prevalence in spring (3.33%) and highest in winter (35%) (Fig. 1). *Anisakis* larvae were subjected to species identification by biomolecular analysis. All larvae morphologically referred to genus *Anisakis* were identified by PCR-RFLP as belonging to the species *A. pegreffii*. The sequenced larvae were confirmed as *A. pegreffii* (accession no. EU718479.1). Concerning the 60 short-finned squid examined, only one larva (1.6%) belonging to genus *Hysterothylacium* was isolated. No significant correlation was found between fish host size and larval abundance in all fish species investigated.

Table 2Prevalence and mean intensity of *Hysterothylacium* larvae identified from fishes and cephalopods by morphological methods.

Species	No. of specimens	No. of infected specimens	No. of infected specimens/tot specimens examined (%)	No. of larvae	Mean intensity of infection (MI) $\pm$ SE ^b
<i>Mullus barbatus</i>	67	17	25.4	96	5.64 $\pm$ 0.58
<i>Illex coindetii</i>	60	1	1.67	1	1 $\pm$ 0.24
<i>Pagellus erythrinus</i>	32	5	15.6	5	1 $\pm$ 0.24
<i>Boops boops</i>	18	6	33.3	10	1.67 $\pm$ 0.31
<i>Chelidonichthys obscurus</i>	6	1	16.7	1	1 $\pm$ 0.24
<i>Lithognathus mormyrus</i>	11	1	9.1	1	1 $\pm$ 0.24
<i>Serranus scriba</i>	3	3	100 ^a	16	5.33 $\pm$ 0.56
<i>Diplodus annularis</i>	3	1	33.3 ^a	2	2 $\pm$ 0.34
<i>Trachurus trachurus</i>	32	3	9.3	7	2.3 $\pm$ 0.37
<i>Conger conger</i>	2	1	50 ^a	1	1 $\pm$ 0.24
<i>Scorpaena scrofa</i>	1	1	100 ^a	1	1 $\pm$ 0.24
<i>Spondyliosoma cantharus</i>	1	1	100 ^a	1	1 $\pm$ 0.24
Total	236	41	17.4	142	3.3 $\pm$ 0.44

^a These relative percentage may be affected by the small sample size.^b SE: standard error.

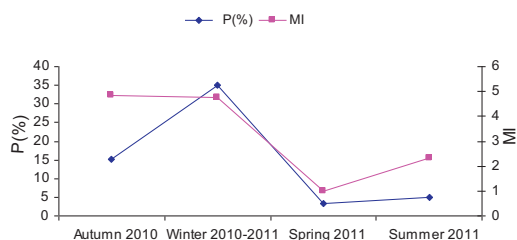


Fig. 1. Seasonal distribution in prevalence (P) and mean intensity (MI) of *Hysterothylacium* sp. throughout the sampling period.

4. Discussion

The results show that *Hysterothylacium* larvae occurs most frequently than *Anisakis* ones in all tested species caught in the northern Ligurian Sea with the exception of *T. trachurus*, the only fish species in which we found *Anisakis* larvae. Coinfection of these two genera in horse mackerel was reported also by other authors and also in these studies the larvae of *Anisakis* were present in greater number (Fioravanti et al., 2004; Angelucci et al., 2011; Chaligiannis et al., 2012). However few studies analyzed the presence of *Hysterothylacium* larvae in fish caught in Mediterranean Sea, and data of prevalence are very low in species of Aegean Sea (Chaligiannis et al., 2012) while higher in fish of the Tyrrhenian Sea (Rello et al., 2009; Angelucci et al., 2011) compared with our results. *Hysterothylacium* prevalence and MI data show seasonal pattern where peak value have been observed in coldest season according to other studies (Adroher et al., 1996; Kalay et al., 2009). The prevalence of *Anisakis* larvae found in horse mackerel (9.3%) is much lower than that observed by other authors in the same host in Ligurian Sea (80–100%, Manfredi et al., 2000) and in other Mediterranean areas (Angelucci et al., 2011; Chaligiannis et al., 2012). While we did not find *Anisakis* larvae in other fish species commonly parasitized (Pozio, 2004). *Anisakis* prevalence and MI in *T. trachurus* was higher during the warmer season in agreement with what was reported in other fish hosts (Gutiérrez-Galindo et al., 2010; Mladineo et al., 2012). In agreement to previously published works carried out in Mediterranean Sea (D'Amelio et al., 1999; Abollo et al., 2003; Pontes et al., 2005), *A. pegreffii* is confirmed as the most prevalent species detected in Ligurian Sea; Mattiucci et al. (2004) reported an elevated abundance of *A. pegreffii* in hake from the Ligurian Sea. These findings may be explained by the inclusion of this Sea in a Protected Marine Area, the Pelagos Sanctuary for Mediterranean Marine Mammals, which has the largest population of cetaceans in the Western Mediterranean (Notarbartolo di Sciara et al., 2008), including definitive hosts of *A. pegreffii* (Mattiucci and Nascetti, 2008). Regarding cephalopods, no *Anisakis* and only one *Hysterothylacium* larva were found in *I. coindetii*, suggesting that infection levels are very low in this class of organisms in this area. Only few authors studied the presence of *Anisakis* larvae in cephalopods from Mediterranean. Gestal et al. (1999) found *A. simplex* in the 4.8% of the *I. coindetii* specimens examined from Tyrrhenian Sea, other authors instead found greater positivity in the Tyrrhenian Sea (50%) (Angelucci et al., 2011) and in Adriatic Sea (30.5%) (Petrić et al., 2011). In conclusion, the

finding of a wide distribution of *Hysterothylacium* larvae in fish and squid species of commercial interest in Ligurian Sea seems to indicate the need to pay greater attention at identifying parasite at least at genus level. However, although *Hysterothylacium* is not currently considered a human pathogen, its role in the cause of allergic syndromes in humans is still to be clarified (Fernández-Caldas et al., 1998; Valero et al., 2003), as well as already demonstrated for *Anisakis* (Daschner et al., 2012). The species *A. pegreffii* is the most frequently isolated in many fish species from Italian waters and appears to be responsible for many cases of human anisakiasis in central and southern Italy (D'Amelio et al., 1999; Paggi et al., 2001; Fumarola et al., 2009; Mattiucci et al., 2011). Since the morphological study is not useful for the identification at species level, the application of a biomolecular method such as PCR-RFLP allows to reach a correct identification of the nematode larvae.

5. Conclusions

The data obtained provide new information on the distribution of the *Anisakidae* larvae in fish from Ligurian Sea, and the potential hazard for human health as a consequence of consumption of raw or undercooked fishes. Nevertheless further studies are required to determine as the abundance in given areas changes over time. Investigate on other relevant commercial species of which little is known about should also be necessary.

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