

Occurrence of nematodes of the genus *Anisakis* in Mediterranean and Atlantic fish marketed in Sardinia

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Abstract

Anisakiasis is a gastrointestinal fish-borne zoonosis caused by the ingestion of third stage larvae of the genus *Anisakis*. Between January and December 2013, 1112 specimens of four commercial fish species (*Engraulis encrasicolus*, *Merluccius merluccius*, *Scomber colias* and *Trachurus mediterraneus*) marketed in Sardinia (Italy) were examined for *Anisakis* sp. The overall prevalence of *Anisakis* spp larvae was 39.9%, all morphologically identified as Type I. *Scomber colias* showed the highest prevalence (100%), followed by *M. merluccius* (Atlantic 91.0%, Mediterranean 71.2%), *T. mediterraneus* (32.7%) and *E. encrasicolus* (25.9%). All the larvae found in Mediterranean hosts were genetically identified as *Anisakis pegreffii*, whereas 90.0% of the larvae found in the Atlantic *M. merluccius* belonged to *Anisakis simplex sensu stricto* and 10.0% to *A. pegreffii*. The mean abundance of *Anisakis* sp. larvae was positively correlated with fish size in *E. encrasicolus*, Atlantic *M. merluccius* and local *M. merluccius*. The prevalence of infection was greater in the body cavity (37.9%) than in the edible muscle (9.4%). However, 1.8% of the examined fish were infected exclusively in the muscle. Therefore, the risk associated to the consumption of raw or undercooked fishery products poses the need of measures such as visual inspection and preventive treatments to guarantee consumers' health.

Introduction

Anisakiasis is a fish-borne zoonosis caused by the ingestion of third stage larvae (L_3) of the genus *Anisakis* (Nematoda: Anisakidae), commonly present in the body cavity and muscle of many fish species and cephalopods (Chai *et al.*, 2005). The infection is often associated with gastrointestinal

symptoms such as abdominal pain, diarrhea, nausea and vomiting. There have also been reported extra-gastrointestinal or ectopic forms, where the parasite localizes in different organs following the initial gastrointestinal penetration (*larva migrans visceralis*) (Griglio *et al.*, 2012). The exposure to the antigens of this parasite can cause hypersensitivity reactions characterized by urticaria and angioedema referred to as Syndrome Urticaria Angioedema. The onset of symptoms begins after the ingestion of infected fish, with formation of transient and pruritic wheals, dermic and subcutaneous edema. In the most severe cases, the reaction can lead to a potentially deadly anaphylactic shock (AAITO-IFIACI, 2011). Anisakiasis occurs after the ingestion of alive L_3 with raw or undercooked fishery products (*i.e.* marinated, smoked, dried or salted). The risk of anisakiasis is associated to the increased exposure to the parasite, which in turn depends on the high prevalence of parasitism in fish (Murrell and Fried, 2007) and the growing demand of gastronomic preparations based on raw or lightly cooked fish at global level (Broglia and Kapel, 2011). Parasitic infections account approximately for 56 million cases worldwide with an estimated population of 400 million individuals at risk (WHO, 2013). Products such as salted or pickled herring (Holland and Nordic countries), gravlax (Nordic countries), boquerones (Spain), lomi-lomi salmon (Hawaii) and ceviche (South America), have been implicated in human anisakiasis all over the world (Baird *et al.*, 2014). In the European Union, during the period 2009-2013, the Rapid Alert System for Food and Feed reported 333 notifications for the presence of parasites 78.5% of which were accountable to *Anisakis* spp. (RASFF, 2014). Host species most frequently implicated were *Engraulis encrasicolus*, *Merluccius merluccius*, *Scomber scombrus*, *Lophius* spp, *Xiphias gladius* and *Lepidopus caudatus*. Several cases of human anisakiasis have been reported in Italy since 1996, mainly in the southern regions, where the consumption of raw marinated anchovies (*Engraulis encrasicolus*) and pilchards (*Sardina pilchardus*) is common (Griglio *et al.*, 2012; Mattiucci *et al.*, 2011, 2013). Cases of human anisakiasis reported in Italy were associated with *Anisakis pegreffii* (Fumarola *et al.*, 2009; Mattiucci *et al.*, 2013). This is supported by available data that indicates that *A. pegreffii* is mainly distributed in the Mediterranean Sea while *Anisakis simplex sensu stricto* in the northern Atlantic and Pacific Oceans (Mattiucci and Nascetti, 2007; Abollo *et al.*, 2001). According to the Regulation (EC) No.

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853/2004 (European Commission, 2004), food business operators must submit their product to visual examination for the detection of visible parasites in order not to place fishery products that are obviously contaminated with parasites on the market for human consumption. Successively to the Reg. 853/2004 European Union and Member States have implemented a series of disposition to regulate the control of parasites in fishery products. D'Amico *et al.* (2014), conducted a comprehensive review of the legislation aimed to manage the risk of *Anisakis*. The food safety implication for human anisakiasis concerns the presence of larvae in the edible part of fish (muscle) rather than in the non-edible (viscera). Therefore, when assessing the risk of exposure to the parasite, it is important to consider whether parasites are detected or not in the edible part. However, as stated by the European Food Safety Authority report (EFSA, 2010), conditions necessary for *intra-vitam* and *post-mortem* migration of larvae from the viscera into the muscle is still controversial. Few studies have been conducted to assess the level of infection and the species of *Anisakis* larvae in fish species commercialized in Sardinian market

(Meloni *et al.*, 2011; Tedde *et al.*, 2011; Piras *et al.*, 2014). The objective of the present study was to gain epidemiological information for the risk of anisakiasis associated with the consumption of fish marketed in Sardinia (Italy). With this aim, the levels of infection of *Anisakis* larvae in four commercial fish species at retail level were evaluated.

Materials and Methods

Fish sampling and *Anisakis* larvae detection and identification

During the period between January and December 2013, a total of 1112 fish specimens were acquired from Sardinian fish markets and examined for the presence of *Anisakis* sp. larvae. The fish belonged to four commercial species, *Engraulis encrasicolus* (n.750), *Merluccius merluccius* (n. 218), *Scomber colias* (n. 40) and *Trachurus mediterraneus* (n.104). The first three species and 118 specimens of *M. merluccius* were from the Gulf of Asinara (North Sardinia, western Mediterranean Sea), while the remaining 100 *M. merluccius* were imported from the northeastern Atlantic Ocean. After collection, samples were transported to the laboratory under refrigeration ($4\pm 2^{\circ}\text{C}$) and either analysed within 24 h or frozen at -20°C for subsequent examination. Weight and length were determined on each sample. After dissection, the body cavity of fish was visually inspected for free larvae, then rinsed with saline solution and the rinse examined under stereomicroscope; the internal organs were separated and observed under a stereomicroscope. The presence of *Anisakis* sp. larvae in the muscle was investigated by artificial digestion (Food and Drug Administration, 1984). *Anisakis* sp. larvae were morphologically identified as Type I or II (*sensu* Berland, 1961), then stored in 70% ethanol for molecular analysis. DNA was extracted from larvae using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). ITS region (ITS-1, 5.8S e ITS-2) of rDNA was amplified using the primers described by D'Amelio *et al.* (2000) and Pontes *et al.* (2005). PCR was carried out according to Sanna *et al.* (2012); the annealing temperature was set at 52°C . PCR products were digested with the restriction enzymes *Hinf*I and *Taq*I using the genetic markers defined by D'Amelio *et al.* (2000) and Pontes *et al.* (2005). Digestion reactions had a final volume of 20 μL , containing 10 U of enzyme, and 2 μL of buffer $10\times$ and were incubated at 37°C for 3-4 h.

Statistical analysis

Epidemiological data on the infection levels of *Anisakis* larvae included the following quantitative descriptors: prevalence (P%), mean intensity (mI) and mean abundance (mA) as described by Bush *et al.* (1997). The P% was defined as the number of hosts infected with 1 or more individuals of a particular parasite species divided by the number of hosts examined. The prevalence, with 95% CI, was determined as total infection (larvae recovered from viscera and muscles) and independently for the viscera and the muscles. Computation was performed with the Sterne's method. The mI (and the 95% CI) was determined as the average number of individuals of a particular parasite species among the infected members of a particular host species. The mA (and 95% CI) was defined as the total number of individuals of a particular parasite species in a sample of a particular host species divided by the total number of host of that species examined (infected and uninfected hosts). Differences in the P% and mI values of larvae infection between the four fish species were assessed respectively with the Fisher's exact test and bootstrap t-test. The same statistical test was also used to compare for each fish the P% and mI between the body cavity and the muscles. The association between total mA of *Anisakis* spp with fish size was evaluated for each fish species with the Spearman's coefficient (Reiczigel *et al.*, 2013). The significance level was set at $P < 0.05$. All statistical analyses were performed using the Software Quantitative Parasitology QPweb implemented for the web (Reiczigel and Rozsa, 2005).

Results

Levels of infection

Out of 1112 fish examined, 443 (39.9%) were infected with 5,866 *Anisakis* sp larvae, all morphologically identified as Type I (*sensu* Berland, 1961). *Scomber colias* showed the highest prevalence (100%), followed by *M. merluccius* (Atlantic 91.0%, Mediterranean 71.2%), *T. mediterraneus* (32.7%) and *E. encrasicolus* (25.9%). The total number of each fish species examined, the mean and range values of length and weight, total number (N_{tot}) and levels of infection of larvae recovered are reported in Table 1. Pairwise differences in the overall P% and mI between host species are reported in Table 2. Prevalence and mean intensity of *Anisakis* spp. larvae according to location (body cavity or muscle) are reported in Table 3. No larvae were detected in the muscle of *S. colias*. A significant difference in the P% of infection between body cavity and muscle was observed in all the species with the exception of Atlantic *M. merluccius*, where the prevalence was comparable (Table 3). As well as the P%, significant differences were always observed for the mI, apart from the Atlantic *M. merluccius*. Despite the overall prevalence in the body cavity was greater than that in the muscle, respectively 37.9 and 9.4%, in 1.8% of the hosts the presence of larvae was only observed in the muscle. The correlation between abundance of *Anisakis* spp. with fish size was significantly positive for *E. encrasicolus*, Atlantic *M. merluccius* and local *M. merluccius*, while it was negative correlation for *T. mediterraneus* (Figure 1).

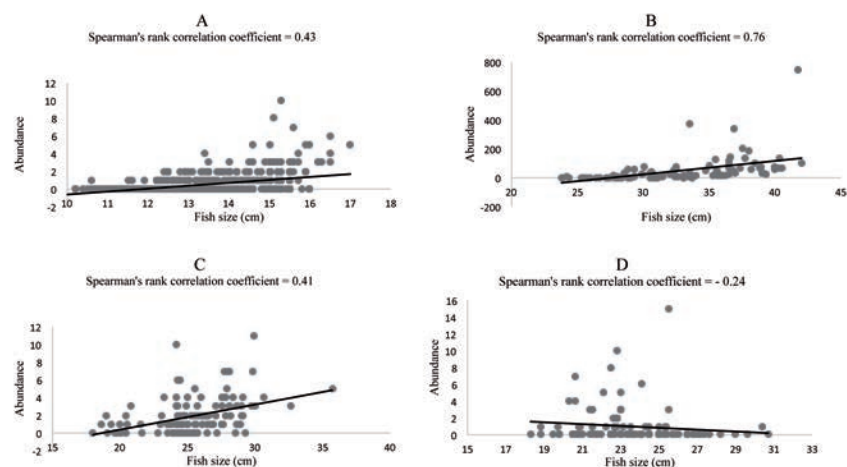


Figure 1. Relationship between *Anisakis* spp. abundance and fish size of *Engraulis encrasicolus* (A), Atlantic *Merluccius merluccius* (B), Mediterranean *Merluccius merluccius* (C), and *Trachurus mediterraneus* (D).

Molecular identification

After molecular analysis, all the larvae found in the Mediterranean hosts were identified as *A. pegreffii*, while 10.0% of the larvae found in the Atlantic *M. merluccius* belonged to *A. pegreffii* and 90.0% to *A. simplex s.s.*

Discussion

In the present study, differences were observed in the prevalence and in the mean intensity of infection between host species. All examined specimens of *S. colias* were infected, whereas *E. encrasicolus* was the least infected host species. Despite the lowest prevalence in anchovies, these fish are traditionally consumed marinated, which poses a serious risk for human anisakiasis. Under the food safety perspective, the concern is the presence of larvae in the edible part of fish (muscle) rather than in the non-edible part (viscera). According to the

Regulation (EC) No. 853/2004, food business operators must not place on the market for human consumption fishery products that are obviously contaminated with parasites. With this regard, the European Commission (SANCO, 2013) in its working document specify that the food business operator can place on the market fish with visible parasites only in non-edible parts. However, the experience demonstrated that the visual inspection is not reliable since only 50% of the parasites can be detected comparing with candling or enzymatic digestion (Llarena-Reino *et al.*, 2012). The results of the present study showed the presence of infected host of Mediterranean and Atlantic origin, confirming EFSA conclusion pointed out in the 2010 report, that no maritime area can be considered free from Anisakids and caught fish should be regarded as presumptively infected. In all fish of Mediterranean origin, no association in the level of infection was observed between the body cavity and the muscle. On the contrary, in *M. merluccius*, the only analyzed

fish with specimen of both origin, the prevalence and mean intensity of infection in the viscera and in the edible part were comparable. Furthermore, this was the only host in which the presence of *A. simplex s.s.* was observed while the other hosts were exclusively infected with *A. pegreffii*. This is a further confirmation that *A. simplex s.s.* is the prevalent species in the northeastern Atlantic Ocean while *A. pegreffii* is prevalent in the Mediterranean Sea. Differences in the ability of larvae migration from the body cavity into the muscle may exist between the two *Anisakis* species, justifying the high prevalence of infection observed in the muscle of the Atlantic *M. merluccius*. This could also be due to the longer time elapsed between catching and commercialization as compared to local species. The absence of association existing in most of the cases between the infection in the body cavity and the muscle is confirmed by the observation in 1.8% of the examined fish specimen of larvae exclusively in the muscle. It has to be noticed that the conditions

Table 1. Level of infection of *Anisakis* spp. larvae in four fish species from the Mediterranean Sea and the Atlantic Ocean.

Host	N	Mean length ^o (cm)	Mean weight ^o (g)	Prevalence [#] (%)	N _{Tot}	mI [#]	mA
<i>Engraulis encrasicolus</i>	750	13.2 (9.7-17)	16.8 (5-36.6)	25.9 (22.8-29.1)	326	1.7 (1.5-1.9)	0.43
<i>Merluccius merluccius</i> Med	118	25.3 (18-35.7)	115.4 (40.7-299.3)	71.2 (62.3-78.9)	222	2.6 (2.2-3.1)	1.88
<i>Merluccius merluccius</i> Atl	100	32.7 (23.8-42)	266.6 (90.8-606.6)	91.0 (83.6-95.4)	4827	53.0 (38.8-85.8)	48.27
<i>Scomber colias</i>	40	26.5 (21-31)	178.3 (110.2-275.7)	100.0 (91.6-100.0)	369	9.2 (7.7-11.0)	9.23
<i>Trachurus mediterraneus</i>	104	23.7 (18.3-30.7)	107.6 (17.2-251.5)	32.7 (24.0-42.3)	102	3.0 (2.2-4.4)	0.98
Total	1112	17.7 (9.7-42)	54.46 (5-606.6)	39.9 (37.0-42.8)	5846	13.2 (9.9-20.1)	5.26

N_{Tot}, number of larvae of *Anisakis* spp collected; mI, mean intensity of infection; mA, mean abundance. ^oValues within parenthesis represent range; [#]values within parenthesis represent 95% confidence interval.

Table 2. Differences in the prevalence (Fisher's exact test) and mean intensity (bootstrap t-test) of *Anisakis* spp. larvae infection among fish species.

Prevalence (%) / mI	<i>Engraulis encrasicolus</i>	<i>Merluccius merluccius</i> Atl	<i>Merluccius merluccius</i> Med	<i>Scomber colias</i>	<i>Trachurus mediterraneus</i>
<i>Engraulis encrasicolus</i>	-	*	*	*	ns
<i>Merluccius merluccius</i> Atl	*	-	*	ns	*
<i>Merluccius merluccius</i> Med	*	*	-	*	*
<i>Scomber colias</i>	*	*	*	-	*
<i>Trachurus mediterraneus</i>	ns	*	ns	*	-

mI, mean intensity of infection. *P<0.05; ns, not significant.

Table 3. Prevalence and mean intensity of *Anisakis* spp. larvae in four fish species according to the location of infection (body cavity and muscle).

Host	Prevalence ^o (%)		P [#]	mI ^o		P [§]
	Body cavity	Muscle		Body cavity	Muscle	
<i>Engraulis encrasicolus</i>	25.3 (22.3-28.6)	0.9 (0.4-1.9)	**	1.7 (1.5-1.9)	1.1 (1.0-1.4)	*
<i>Merluccius merluccius</i> Atl	81.0 (72.1-87.6)	76.0 (66.6-83.6)	ns	30.8 (24.3-39.8)	30.7 (19.6-64.6)	ns
<i>Merluccius merluccius</i> Med	67.8 (58.9-75.9)	13.6 (8.4-21.1)	**	2.6 (2.2-3.0)	1.1 (1.0-1.2)	**
<i>Scomber colias</i>	100 (96.1-100)	nd	-	9.1 (7.4-10.8)	nd	-
<i>Trachurus mediterraneus</i>	29.8 (21.5-39.4)	5.8 (2.5-12.3)	**	3.1 (2.2-4.5)	1.0 (***)	*
Total	37.9 (35.1-40.9)	9.4 (7.8-11.3)	**	8.2 (6.7-10.4)	22.5 (13.9-44.7)	ns

mI, mean intensity of infection; nd, not determined. ^oValues within parenthesis represent 95% confidence interval (CI); [#]significance level (Fisher's exact test) of differences between prevalence; [§]significance level of differences between mean intensity (bootstrap 2-sample t-test). *P<0.05; **P<0.001; ns, not significant; ***intensity was constant, CI could not be calculated.

necessary for *intra-vitam* and *post-mortem* migration of larvae from the viscera into the muscle is still controversial (EFSA, 2010). Disqualification of fish placed on the market is based on the presence of obviously contaminated fish after the visual inspection. On the other hand, even if food safety represents a primary condition in the decision whether to exclude or not a product from being placed on the market, another important aspect is its suitability for human consumption. For these reasons, in Sardinia and other Italian regions the presence of zoonotic visible parasites during visual inspection of the body cavity disqualify the product, which should be seized and destroyed. The positive correlation existing between fish size and abundance of larvae indicates a reduced risk for small-sized fish in *E. encrasicolus*, Mediterranean and Atlantic *M. merluccius*. However, the presence of *Anisakis* larvae in the muscle cannot be excluded regardless the host species, the origin, the body part or the size. For these reasons, it is necessary the adoption of preventive treatments for the inactivation of the parasites when fishery products are to be consumed raw or undercooked, such as freezing at -20°C for at least 24 h or at -18°C for 96 h in domestic freezers. Preventive treatments could be omitted in *Anisakis* free fish, which is the case only under particular farming conditions.

Conclusions

It is clear that either the food business operator or the official control need a specific qualification for detecting visible parasites in order to guarantee the consumers' health and the local economic activities related with fishing and fishery product preparations.

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