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Anisakis sp. and *Hysterothylacium* sp. larvae in anchovies (*Engraulis encrasicolus*) and chub mackerel (*Scomber colias*) in the Mediterranean Sea: Molecular identification and risk factors

Alessia Libera Gazzonis [a, *, 1], Serena Cavallero [b, 1], Sergio Aurelio Zanzani [a], Emanuela Olivieri [a, c], Renato Malandra [d], Valerio Ranghieri [e], Stefano D'Amelio [b], Maria Teresa Manfredi [a]

[a] Department of Veterinary Medicine, Università degli Studi di Milano, via Celoria 10, 20133 Milan, Italy
[b] Department of Public Health and Infectious Diseases, Sapienza University of Rome, P.le Aldo Moro 5, 00185 Roma, Italy
[c] Department of Veterinary Medicine, University of Perugia, Via S.Costanzo 4, 06126 Perugia, Italy
[d] Veterinary Department, ATS Milano Metropolitana, Italy
[e] Veterinary Practitioner, Milan, Italy

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ABSTRACT

Larval ascaridoids in fish destined to human consumption represent an important public health issue, besides to be an economical problem. Indeed, marine ascaridoids are the etiological agents of the fish-borne zoonosis anisakidosis. Due to an increase of new cases reported worldwide, a continuous monitoring of infection in fish is mandatory. The study was aimed to evaluate the risk of infection by larval ascaridoids in fishes from Mediterranean Sea. Two species of fishes among those representing a major potential threat for human health were selected. Epidemiological and molecular study was carried out. At Milan Fish Market, Italy, 179 anchovies (*Engraulis encrasicolus*) and 84 chub mackerels (*Scomber colias*) caught in different fishing areas in the Mediterranean Sea were sampled and inspected for the presence of larvae. For each fish, larvae were counted and morphologically identified. Predictors of infections were investigated through general linear models. A subsample of 100 larvae was molecular characterized with PCR–RFLP targeting the nuclear ribosomal internal transcribed spacer (ITS) region. Moreover, 26 *Hysterothylacium* spp. larvae were analyzed by sequencing of both nuclear ITS and mitochondrial ribosomal *rrn*S regions.

Overall, 1080 anisakids larvae were collected from 103 anchovies (P = 57.5%) and 53 chub mackerels (P = 63.09%). Larvae were morphologically identified as *Anisakis* Type I larvae (P = 6.14% in anchovies and P = 55.95% in chub mackerels) and as *Hysterothylacium* spp. (P = 54.18% in anchovies and P = 13.09% in chub mackerels). Fishing area and fish weight resulted predictors of both *Anisakis* Type I land *Hysterothylacium* spp. infections in anchovies; in chub mackerels, only fishing areas resulted to be associated to both infections. Molecular analysis on ITS region identified *Anisakis pegreffii*, heterozygote genotype between *A. pegreffii* and *A. simplex* sensu stricto, and *Hysterothylacium aduncum*. Sequences analysis on *Hysterothylacium* specimens revealed a great homogeneity in *rrn*S marker, with eight variable nucleotides and an average evolutionary divergence over all sequence of 0.3%.

1. Introduction

The presence of larval parasitic nematodes in fish or fish products intended for human consumption causes economic and medical problems: alongside with the loss of marketability of fish, larval nematodes belonging to Anisakidae family may cause a fish-borne zoonosis known as anisakidosis, while nematodes of Raphidascarididae family are commonly considered not zoonotic or of negligible concern (Klimpel & Palm, 2011).

These nematodes comprise a parasitic group widely distributed at geographical level, with a complex life cycle depending on aquatic ecosystem and various intermediate, paratenic and

* Corresponding author.
E-mail address: alessia.gazzonis@unimi.it (A.L. Gazzonis).
[1] These two authors have contributed equally to the work.

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definitive hosts at different levels in the food-web (Anderson, 1992; Koie, 2001).

Humans may become accidental hosts acquiring the infection by consuming raw or lightly cooked fish and cephalopods, paratenic hosts for anisakids, infected with third-stage larvae. Considering the zoonotic potential, the relevant genera of the family Anisakidae are *Anisakis* and *Pseudoterranova*, in particular the *Anisakis simplex* and *Pseudoterranova decipiens* complexes of species, although larvae of *Contracaecum* have been rarely associated with the disease in humans (Hochberg & Hamer, 2010; Shamsi & Butcher, 2011). Larvae of *Hysterothylacium* spp. (Raphidascarididae) are not considered pathogenic for human, although members of this genus may be involved in allergic reactions due the ingestion of infected fish (Valero, Terrados, Diaz, Reguera, & Lozano, 2003).

In Mediterranean countries, *Anisakis pegreffii* is the main etiological agent of anisakiasis, due to the widespread presence of this species in paratenic and definitive hosts of Mediterranean waters (Mattiucci & D'Amelio, 2014). Among traditional fish dishes considered to be of high risk for human disease, Spanish boquerones and Italian marinated anchovies are mentioned. In recent years, new cases of anisakiasis have been increasingly reported worldwide and it is now considered an emerging disease (Carrera, Gallardo, Pascual, Gonzalez, & Medina, 2016; Mladineo, Popovic, Drmic-Hofman, & Poljak, 2016).

Anisakidosis is considered an emerging disease in Europe, with an increasing of notified cases also in countries where the disease was sporadically reported, due to the consumption of traditional dishes and to the increasing consumption of exotic food products with raw fish (i.e. sushi, sashimi, etc.). In Italy, particularly, few cases have been reported mainly from the southern regions and associated to the consumption of raw fish (Fumarola et al., 2009; Maggi et al., 2000; Mattiucci et al., 2011; Pampiglione et al., 2002).

Therefore, a continuous monitoring of anisakid infections in fish destined to human consumption appears needed, particularly regarding certain species.

Panel of experts from the European Food Safety Authority (EFSA) released a scientific opinion on zoosanitary parasite control of fishery products for human consumption. They indicated protection and prevention as priorities and recommended a continuous research in parasites of public health importance in fishery products, regarding prevalence, intensity, anatomical location, as well as geographical and seasonal distribution (EFSA, 2010; Pico-Duran, Pulleiro-Potel, Abollo, Pascual, & Munoz, 2016).

Following the EFSA guidelines (2010), the study was aimed to evaluate the risk of infection by larval ascaridoids in fishes from Mediterranean Sea. For the present survey, fish originating from different areas of Mediterranean Sea were collected at Milan Fish Market, thus depicting an example of fish consumed in Northern Italy. Among fishes representing a major potential threat for human health, two species were selected: anchovies and chub mackerels. In particular, anchovies are often consumed raw in Italian regions. Further, these are among the most commonly consumed fish in Italy, representing 23% of the national fishery production (data of Ministry of Agricultural Food and Forestry Policies); in some Italian regions anchovies are often prepared cured or marinated and their consumption is assumed as the major cause of anisakiasis in Italy (Mattiucci & D'Amelio, 2014). Chub mackerel is a pelagic-neritic fish and it is recognized as one of the species more at risk of infection by anisakids, being at the top of the trophic chain in Mediterranean Sea (Piras et al., 2014). In the present study, epidemiological study and molecular identification were carried out in order to analyze the risk factors that may influence the infection in fish and infer the human risk for anisakiasis posed by the consumption of the surveyed fishes.

2. Material and methods

2.1. Fish sampling and visual inspection

A total of 179 anchovies (*Engraulis encrasicolus*) and 84 chub mackerels (*Scomber colias*) were sampled at Milan Fish Market, between April and December 2014. Fish originated from Adriatic and Tyrrhenian Seas: specifically, anchovies were caught in Tyrrhenian Sea (FAO zone 37.1.3) and Adriatic Sea (FAO zone 37.2.1, FAO zone 37.2.2), whereas mackerels came from the Adriatic Sea (FAO zone 37.2.2).

Each fish was measured, weighted and submitted to inspective analysis for the presence of nematodes larvae. Third stage larvae of nematode ascaridoids were isolated from the visceral surface and body cavity of the fresh fish; larvae encysted in fillets were carefully removed. The visceral organs were separated and then carefully observed with a stereomicroscope. Collected larvae were washed with saline solution and stored in 70% ethanol until further examination. For each specimen and irrespectively of the localization in the fish body, larvae were counted and identified according to their morphological features by a light microscope at 100 or 400 × magnification (Hurst, 1984; Petter, 1969).

2.2. Statistical analysis

Epidemiological parameters including prevalence, intensity, abundance and the parameter k of the negative binomial distribution were calculated for *Anisakis* spp. and *Hysterothylacium* spp. larvae recorded in both anchovies and chub mackerels (Bush, Lafferty, Lotz, & Shostak, 1997; Wilson et al., 2001). Pearson's chi-square was used to test for the difference between prevalence values for both larval genera. General linear models (GLMs) with binomial negative distribution and logarithmic link were performed separately for anchovies and chub mackerels to investigate on predictors of *Anisakis* and *Hysterothylacium* infections, using the number of larvae as independent variable. For chub mackerels, a second GLM was run to verify the influence of considered variables on the number of *Anisakis* larvae found encysted in the fillet.

The following explanatory variables were inserted in each full model: fish body length (continuous variable, measured in centimetres), fish weight (variable, measured in grams), and fishing area; in addition, the interactions between length/weight and fishing area were considered. Final models were developed by backward elimination. Statistical analysis was performed with SPSS 22.0 software (IBM, Chicago, IL).

2.3. Molecular identification of species

2.3.1. PCR-RFLP

A selected subsample of 100 third stage larvae of ascaridoids (80 from anchovies and 20 from chub mackerels), randomly selected, 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 (Abollo, Paggi, Pascual, & D'Amelio, 2003; D'Amelio et al., 2000; De Liberato et al., 2013; Pontes, D'Amelio, Costa, & Paggi, 2005). Genomic DNA was isolated from entire larvae using the Wizard Genomic DNA purification kit (Promega, Madison, WI), according to the manufacturer's protocol.

The entire ITS region (ITS-1, 5.8S, ITS-2), of around 1000 base pairs, was amplified using 20 ng 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 NC5 primer forward (5-GTAGGTGAACCTGCGGAAGGATCAT-3) and NC2 reverse primer (5-