SURVEY ON THE PRESENCE OF ANISAKID NEMATODES IN COMMERCIAL FISH SPECIES OF SOUTHERN ITALY COASTS (SICILY)
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Abstract
In this work a total of 6612 commercial fish samples (anchovies, sardines, mackerel and horse mackerel) were analysed for the detection and species identification of Anisakid nematodes. All the fish samples came from the Regional Monitoring Plan for Anisakidae detection in fish products. The initial visual inspection phase and artificial digestion of the fish samples revealed the presence of 3452 nematodes larva, with a 17% of larva detected after artificial digestion compared to visual method. All the fish species revealed different infestation parameters, with maximum prevalence value in horse mackerel and minimum in sardines. All the nematodes larvae that infested the mackerel were found in the gonads of their fish host, showing a marked tropism. All the larvae examined by optical microscopy belonged to the morphotype I of Anisakis. The molecular identification of the larvae, conducted by RFLP-PCR of the nuclear ITS region (ITS-1, ITS-2 and 5.8 S subunit) showed a major presence of Anisakis pegreffii in the fish samples examined, confirming the prevalence of this species in Mediterranean Sea. The 7% of the larvae examined belonged to the A. simplex s.s. species. Furthermore, the 5% of restriction patterns revealed the presence of Anisakis pegreffii and Anisakis simplex s.s. hybrid forms, with a digestion pattern common to the two analysed species. Two larvae of Anisakis typecs were identified in horse mackerel and mackerel samples. Five nematodes isolated from anchovies and horse mackerel were identified as Hysterothylacium aduncum. A marked seasonal infestation rate was verified in all the fish samples analysed. Mackerels and horse mackerels showed a high infestation value during spring, with a sudden decrease during summer months. Results obtained confirmed a correlation between ecology of fish hosts and their parasites.

Introduction
Nematodes of the family Anisakidae have a widespread distribution among fish species and cephalopods as paratenic hosts (Kats et al., 1995; Mattiucci and Nascetti, 2008). The fishery products are essential elements of the Mediterranean diet and the consumption of raw fish is a common practice of the Italian tradition. The principal category of fish consumed raw by Italian people is the small pelagic fish: these include several fish species, such as sardines, anchovies and mackerels which have a high content of unsaturated fats. Ingestion of raw, partly cooked, salted, marinated or smoked fish, infected with alive larvae of Anisakis, can provoke gastrointestinal and allergic symptoms (Baird et al, 2014, Cavallero et al. 2015). The main source of human infections in Italy seems to be the home-made fishes marinated (sardines and anchovies). The marinating process does not kill larval nematodes (Pozio, 2013). The aim of the present study was to investigate the prevalence of parasitic nematodes of Anisakidae family in pelagic fish samples (anchovies, sardines, mackerel and horse mackerel) caught off the Southern Italy coasts in order to have an exhaustive risk analysis on the consumption of this products.

Materials and methods

Detected larvae are subjected to identification at genus level, through the optical microscopy (Leica DM 2000), according to morphological characters (Berland 1961). Subsequently, the larvae were washed, fragmented with a scalpel and frozen at -20 °C for 24 hours. The extraction of DNA were subjected to special kits based on the use of affinity columns. For the genus Anisakis we proceeded to the amplification of the ITS regions (ITS-1, ITS-2 and 5.8 S subunit) of nuclear rDNA by the primers NCS (5'GTA GGT GAA CTC GGC ACA GGA TCA TT3'), NCS (5'TTA GTT TCT TTT CCT CCG CT3') (Zhua et al, 1998). The DNA samples have been subjected to restriction enzyme digestion with two restriction enzymes, HinfI and HhaI, for the identification of Anisakis spp. according to genetic key of D’Amelio et al. (2000). The digestion was performed over night at 37°C and the digestion products were electrophoresed in 2% agarose gel (Invitrogen) stained with SYBR safe and visualized by UV transilluminator. For the identification of Hysterothylacium species a sequence analysis were conducted. Purification of ITS gene amplification products was carried out with Illustra GFX PCR DNA and Gel Band Purification kit (GE Healthcare) following the manufacturer’s instructions. The purified products were sent to Macrogen company (Amsterdam, Holland) for Sanger sequencing.

Results and Discussion
The results are shown in table 1. The maximum prevalence values were found in horse mackerel Trachurus trachurus (48.5%) and mackerel Scomber scombrus (52%) while very low prevalence rate was found in sardines (2.5%). The horse mackerels revealed the highest infestation rate for each individual sample, in fact only one sample of horse mackerel revealed the presence of 189 Anisakid larvae. About 97% of the larvae examined at morphological analysis belonged to the morphotype I of Anisakis genus detecting the presence of a boring tooth on the cephalic part, a cylindrical ventricle and a mucrone on the caudal part. The RFLP-PCR analysis of ITS region (Fig 1), obtained with HinfI restriction enzyme produced four different patterns: one pattern with three strong bands (650, 250) and a weak 80 bp respectively linked to Anisakis simplex s.s., one pattern with three bands at 370, 300 and 250 bp corresponding to Anisakis pegreffii and a restriction pattern corresponding to a combination of the bands observed in Anisakis simplex s.s. and Anisakis pegreffii, recognised as hybrid form. The digestion with HhaI produced only two patterns with very slightly differences (550-430 bp and 540-420 bp respectively). The molecular analysis confirmed A. pegreffii as the Anisakidae parasite more present in the Mediterranean, with a prevalence of 80% (280 larvae). The 5% of the examined larvae revealed a restriction pattern comparable as Anisakis simplex s.s./Anisakis pegreffii hybrid form. The 7% of the larvae examined belonged to the A. simplex s.s. species. Two larvae were identified as A. typeca and five nematodes as Hysterothylacium aduncum. The results offer interesting insights on the possible correlation between the presence of Anisakidae parasites in fish products and consumer health effects, suggesting further investigations aimed to a better understanding of the subjects under consideration and to the identification of appropriate prevention measures.

References

Table 1: Epizootiological parameter for parasites infestation. Np: the number of parasites; P: prevalence; ml: mean intensity.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Np</th>
<th>P(%)</th>
<th>ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scomber scombrus</td>
<td>720</td>
<td>52</td>
<td>6.2</td>
</tr>
<tr>
<td>Trachurus trachurus</td>
<td>1985</td>
<td>48.5</td>
<td>13.6</td>
</tr>
<tr>
<td>Engraulis encrasicolus</td>
<td>664</td>
<td>19</td>
<td>1.1</td>
</tr>
<tr>
<td>Sardina pilchardus</td>
<td>83</td>
<td>2.5</td>
<td>1.14</td>
</tr>
</tbody>
</table>

Figure 1: Restriction enzyme analysis with HinfI and HhaI
Lanes 1-8: Anisakis typera; lane 2: Anisakis simplex s.s./Anisakis pegreffii hybrid form; lanes 3,4,5,7: Anisakis pegreffii; lane 6: Anisakis simplex s.s.; L: ladder 100 bp.