



Anisakis simplex s.l. parasitization in mackerel (*Scomber japonicus*) caught in the North of Morocco – Prevalence and analysis of risk factors

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ABSTRACT

Consumption of mackerel (*Scomber japonicus*) is very widespread in Morocco, where its production is an important economic pillar. We investigated the prevalence of infection by *Anisakis* spp. in mackerel caught in Moroccan waters, analyzing infection risk factors. The prevalence was generally higher in fish from the Atlantic (67.9%) than from the Mediterranean (57.0%), but they did not differ in the mean abundance, intensity, or prevalence of muscle parasitization. *A. pegreffii* was the predominant species (82.6% of larvae) followed by the hybrid *A. simplex* s.s./*A. pegreffii* (16.3%). Only one L3 of *A. simplex* s.s. was found in a specimen of *S. japonicus* from the Atlantic. Mackerel infection was associated with total fish weight, gonad weight, catch area, and catch season. However, muscle infection was associated solely with total weight and parasite loads. The consumption of lower-weight mackerel may be a good prophylactic measure against human anisakiasis.

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1. Introduction

Fish and fish products are crucial components of the human diet in many countries. Morocco has two coastlines, totaling 3500 km, and the main protein supply for the population derives from fish (Kamili, 2006). Parasites are widely present in fish and impossible to control, except in fish farms, and some are pathogenic for humans. Species arousing health concerns include *Anisakis* spp., since the ingestion of their larvae can injure different parts of the gastrointestinal tract and other tissues and organs, while allergic symptoms range from urticaria/angioedema to recurrent anaphylaxis (Audicana et al., 2002).

Anisakiasis is considered a worldwide human health problem, especially in populations accustomed to eating raw or under-cooked fish (Audicana and Kennedy, 2008; Umehara et al., 2007). Most reports on cases in humans have been associated with the *Anisakis simplex* complex, although it would be more accurate to say *Anisakis* morphotype type I, given the lack of morphological characters to distinguish between the species included in this group (*A. simplex*, *A. pegreffii*, *A. simplex* C, *A. typica*, *A. ziphydarum*, and *A. nascettii*) and the low numbers of larvae from human cases which have been identified specifically by molecular methods (Mattiucci and Nascetti, 2008; Mattiucci et al., 2009). Umehara et al. (2007) studied 100 larvae isolated from 85 patients in Japan and found *A. simplex* s.s. to be the predominant etiological agent of anisakiasis, with only one patient infected by *A. pegreffii*.

Among the few studies on the presence of these nematodes in fish in Morocco, a high prevalence of *Anisakis* spp. was reported in fish from both Atlantic and Mediterranean waters (Mattiucci et al., 2001; Cissé and Belghyti, 2005a; Farjallah et al., 2008a). Nevertheless, there have been no official reports of human infection. One of the most frequently consumed fish in northern Morocco is mackerel (*Scomber japonicus*). The annual mean catch of Scombridae (mackerel and tuna) in Morocco is 20,000 ton, second only to sardine production (Cissé and Belghyti, 2005b). With this background, we conducted an epidemiological study to determine the risk factors and prevalence of infection by *Anisakis* spp. in mackerel obtained directly from Moroccan fishing boats landing at two ports in Northern Morocco. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis was used to identify the parasite species.

2. Material and methods

2.1. Host

We studied 266 mackerels caught off the Moroccan coast in 2007 and 2008 during the different seasons of the year: 135 specimens (50.8%) from the maritime area of Tetouan (Mediterranean) and directly obtained from fishing craft at M'diq port, and 131 fish (49.2%) from Atlantic waters of Tangier and directly obtained from fishing craft at Tangiers port. The fish were identified following Collette et al. (2001) and were measured and weighed. After dissection, the sex was determined, and the viscera and epaxial and hypaxial muscles were removed, weighed and separately subjected to digestion in pepsin/HCl solution, according to the method of McGladdery (1986) at pH 2–2.3 and 36 °C for 3 h.

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2.2. Parasite

Isolated *Anisakis* spp. larvae were washed in 0.9% NaCl solution and rinsed in 70% alcohol and glacial acetic acid for microscopic observation and morphological study following the criteria of Hartwich (1974); Kjøie (1993); Petter and Maillard (1988) and Yoshinaga et al. (1987), mainly: a) presence of boring tooth at anterior end; and b) oesophageal ventricle in first third of the parasite and posterior end of the larva, which differ between morphotypes I and II. Larvae were then individually preserved in labeled Eppendorf tubes at -20°C .

2.3. Quantitative epidemiology

We calculated the prevalence, mean abundance and intensity of infection as defined by Bush et al. (1997). Differences among prevalence values were evaluated by using the chi-square test or Fisher's test.

2.4. Genetic analysis of parasites for species identification

PCR-RFLP was used to identify the species, extracting genomic DNA by using the RealPure kit (Ref RBMEG01). PCR amplification of rDNA fragment ITS1-5.8S-ITS2 used primers A (forward): 5' GTC GAA TTC GTA GGT GAA CCTBGGC GAA GGA TCA 3' and B (reverse): 5' GCC GGA TCC GAA TCC TGG TTA GTT TCT TTT CCT 3', reported by D'Amelio et al. (2000), and NC5 (Forward), 5' GTA GGT GAA CCT GCG GAA GGA TCA TT 3' and NC2 (Reverse), 5' TTA GTT TCT TTT CCT CCG CT 3' reported by Zhu et al. (1998), followed by digestion with 10 U/ μl restriction enzymes Hinf I (Bioron international), Taq I (Bioron international), and Cfo (Roche). The digestion product was subjected to 3% agarose gel electrophoresis for species identification according to the band pattern (D'Amelio et al., 2000; Kijewska et al., 2000; Martin-Sánchez et al., 2005; Pontes et al., 2005; Umehara et al., 2006).

2.5. Risk factor analysis

The following variables were considered as potential risk factors for infection with *A. simplex* s.l.: sex, weight, length, gonad weight, and liver weight of the fish and the catch area and season. Univariate and multivariate logistic regression analyses were performed, considering total parasitization and muscle parasitization as dependent variables and the above factors (and parasitic load) as independent variables. The multivariate model was constructed by using a stepwise approach, selecting variables according to the statistical significance of their association with parasitization. The goodness of fit of the model was evaluated by using the coefficient of determination R^2 and by graphical representation of the residuals. The possible colinearity of predictor variables was tested by calculating the Pearson correlation coefficient for pairs of variables. We also controlled for possible confounders and for interactions among predictors. SPSS 15.0 (SPSS, Chicago, IL) was used for the data analyses.

3. Results

3.1. Host

The length of the 266 mackerels ranged from 14.5 to 36.0 cm (mean of 24.7 cm) and the weight from 25.1 to 392.7 g (mean value 147.8 g); 155 were male (58.3%) and 111 female (41.7%). Among the seasons, 72 (27.1%) were caught in the autumn, 64 (24.1%) in the summer, and 65 (24.4%) each in spring and winter.

3.2. Quantitative epidemiology

Out of the 266 specimens, 166 were parasitized (62.4% prevalence), detecting 477 L3 larvae of *Anisakis* type I (none of type II). The number of larvae in each fish ranged from 0 to 13, with a mean abundance of 1.8 and intensity of 2.9. Out of the 477 parasites recovered, 451 were detected in the body cavity (prevalence of 61.7%, mean abundance of 1.7, and intensity of 2.8) and 26 in the muscle of 26 fish (9.8% prevalence and mean abundance of 0.1).

The overall prevalence of infection was 67.9% in fish from the Moroccan Atlantic coast, with mean abundance of 2.0 and intensity of 2.9; the prevalence was lower ($p=0.067$) in fish from the Mediterranean coast (57.0%), but the mean abundance (1.6) and intensity (2.8) were similar. No difference in muscle infection prevalence was found between fish from the two coasts ($p=0.456$).

3.3. Identification by PCR-RFLP

PCR was used to amplify the ribosomal fragment ITS1-5.8S-ITS2 in 92 larvae randomly selected from among the 477 larvae morphologically classified as L3 *Anisakis* type I extracted in this study. Table 1 shows the results in accordance with restriction patterns for the three enzymes used.

3.4. Risk factor analysis

Parasitization by *A. simplex* s.l. of the whole fish was associated with the total weight, length, liver weight, gonad weight, catch season, and catch area in the univariate analyses ($p<0.05$), but not with the sex of the fish ($p=0.23$). Length and liver weight were each strongly associated with total weight (Pearson's correlation of 0.73 and 0.86, respectively), and neither entered the multivariate model. Gonad weight was only weakly correlated with total weight (Pearson's correlation of 0.28), and both of these variables remained in the model (Table 2).

Parasitization by *A. simplex* s.l. of the muscle was associated with the total weight, fish length, liver weight, and total number of parasites in the fish in the univariate analyses ($p<0.05$). In the multivariate analysis, only total fish weight ($\text{OR}=1.01$; $\text{CI}=1.01\text{--}1.02$; $p<0.01$) and parasite load ($\text{OR}=1.52$; $\text{CI}=1.27\text{--}1.84$; $p<0.01$) remained significantly associated with the presence of L3 larvae in the muscle of these fish.

Table 1

Genotypes of *Anisakis* identified by using genetic markers in *S. japonicus* caught off the Atlantic and Mediterranean coasts of Morocco. N: number of *Anisakis* L3 identified, %: percentage of L3 identified.

Parasites	Mediterranean Sea		Atlantic Ocean		Total	
	N	%	N	%	N	%
<i>A. pegreffii</i>	31	81.6	45	83.3	76	82.6
<i>A. simplex</i> s.s.	0	0	1	1.9	1	1.1
Hybrid <i>A. pegreffii/A. simplex</i> s.s.	7	18.4	8	14.8	15	16.3
Total	38	100	54	100	92	100

Table 2

Factors associated with the presence of *Anisakis* larvae in mackerel (total parasitization) caught in Moroccan waters, final model determined by multivariate analysis. Dependent variable: presence/absence of *A. simplex* s.l. in the whole fish. OR: odds ratio, CI: confidence interval.

Variables (N)	OR (95%CI)	p value
Total weight (261)	1.02 [1.01–1.03]	<0.01
Gonad weight	1.28 [1.02–1.61]	0.04
Catch area		<0.01
Mediterranean ref. (134)	–	–
Atlantic (127)	3.25 [1.71–6.18]	–
Season		0.03
Spring ref. (65)	–	–
Summer (60)	1.66 [0.69–4.00]	0.26
Autumn (71)	2.91 [1.27–6.67]	<0.01
Winter (65)	2.88 [1.13–7.38]	0.03

4. Discussion

This study reveals an elevated prevalence of infection by *A. simplex* s.l. in *S. japonicus* from Northern Moroccan waters, with a higher prevalence in Atlantic (67.9%) than in Mediterranean (57.0%) waters ($p=0.07$). Costa et al. (2003) reported a similar prevalence in mackerel from the Atlantic waters off Madeira (69.5%).

By far the most dominant *Anisakis* species in these mackerels was *A. pegreffii*, which represented 82.6% (76/92) of detected larvae, followed by the hybrid *A. pegreffii*-*A. simplex* genotype (16.3%). The proportion of *A. pegreffii* and hybrids did not differ between mackerel from the Mediterranean and Atlantic ($p=0.21$). *A. simplex* s.s. appeared in one specimen from the Atlantic, probably reflecting a migration from higher latitudes, since its southern limit in this part of the Eastern Atlantic, where it is widespread, is considered to be the Strait of Gibraltar, north of our catch area (Mattiucci and Nascetti, 2008). In contrast, the northern limit for *A. pegreffii* in Atlantic waters is the northern Spanish coast, and it is the dominant species in the Mediterranean Sea (Mattiucci and Nascetti, 2008). F1 hybrid individuals are characterized by having one DNA strand characteristic of *A. simplex* s.s. (T, T in positions 255 and 271 of the ribosomal fragment ITS1–5.8S–ITS2) and another strand of *A. pegreffii* (C, C); only one of these positions showed this hybrid genotype (by PCR-RFLP) in 73.3% of the hybrids, with the other corresponding to *A. pegreffii*, demonstrating the backcrosses of F1 hybrids with this progenitor (unpublished results).

Similar to our findings in Moroccan Mediterranean waters, Farjallah et al. (2008b) detected only *A. pegreffii* in their study of *Anisakis* species in 13 *S. japonicus* specimens from Tunisian Mediterranean waters (Kelibia), while Abollo et al. (2003) reported 83.3% *A. pegreffii* larvae and 16.7% hybrids in mackerel from Spanish Mediterranean waters. However, our results for *A. simplex* s.s. in mackerel from the Moroccan Atlantic differed from findings by Farjallah et al. (2008a) of 15.4% *A. simplex* s.s. larvae and 7.7% hybrids in mackerel from the Atlantic coast off Tangiers.

Only 5.5% (26/477) of the total number of larvae isolated were found in muscle. Suzuki et al. (2010) reported that *A. pegreffii* has a lesser capacity to penetrate fish muscle in comparison to *A. simplex* s.s., which may help to explain this low prevalence.

With regard to the analysis of risk factors, the likelihood that mackerel caught in Moroccan waters were parasitized by *A. simplex* s.l. depended on the weight of the fish and its gonads and the catch area and season, but not on the sex of the fish ($p=0.37$). Cremona and Sardella (1997) and Silva and Eiras (2003) also found no significant relationship between parasitism by *Anisakis* spp. and the sex of mackerels caught in Argentinean waters. According to our multivariate model, the risk of parasitization increases by 2% per gram fish weight. The risk also increases gradually with fish length, because the high correlation between fish length and weight means that a model can be constructed with either variable (but not both together). We depict here the model with fish weight, which is more readily evident to the consumer at the time of purchase. The association between length and *Anisakis*

parasitization was previously reported. A significant correlation between these variables was found by Cremona and Sardella (1997) in *S. japonicus* from Argentinean waters and by Gutiérrez-Galindo et al. (2010) in a closely related species (*S. scombrus*) from Spanish Mediterranean waters (Tarragona). An increase in *Anisakis* prevalence with greater length has been reported in various fish species (McGladdery and Burt, 1985; Takao, 1990; Valero et al., 2006). This relationship can be explained by the accumulation of parasites over the life of the fish, as proposed by Bahar et al. (2007) and Busmann and Enrich (1979), but differences in prey selection at different sizes may also play a role.

In our multivariate model, the risk of parasitization increased by 28.0% per gram gonad weight. According to Durif et al. (2000), there is an increase in the gonadosomatic index (determined by gonad weight and total weight) when the fish prepares for reproduction. The main proportion of its energy is devoted to this process, to the detriment of other physiological functions, including immune defense, increasing vulnerability to parasitic infections. Liver weight did not enter our multivariate model due to its strong correlation with total weight, which was again selected for the model as the more relevant variable for consumers.

For the same total weight, gonad weight, and catch season, the risk of parasitization by *A. simplex* s.l. was more than threefold greater in Atlantic than in Mediterranean waters, consistent with findings on the prevalence of *Anisakis* in other species (Adroher et al., 1996; Valero et al., 2000, 2006). The parasitization risk also varied with the catch season ($p=0.03$), being threefold higher in winter and autumn than in spring or summer for the same total weight, gonad weight, and catch area. Akmirza (2003) reported that the prevalence of infection by *A. simplex* s.l. was higher in mackerel from two areas of Turkey during the spring (77.3% and 12.5%) than during the summer (9.1% and 4.1%). In contrast, a study in the Spanish Mediterranean by Gutiérrez-Galindo et al. (2010), found a higher prevalence in mackerel during the summer (19.6% and 14.3%) than during the winter (7.0% and 8.1%), although they observed no correlation between catch month and infection in *S. scombrus*. Seasonal variations may be explained by differences in the prey consumed by the fish at distinct times of the year. Thus, Castro and Santana del Pino (1995) found that the stomach contents of mackerel caught in the Canary Islands were influenced by the season and habitat. Seasonal fluctuations in the quality and quantity of plankton may also play a role (Cognetti, 2001).

The odds of the presence of *A. simplex* s.l. in the muscle of these mackerel rose by 1% per gram of total fish weight and by 52% for every *Anisakis* present in the whole fish. One explanation may be that the increase in weight would be accompanied by an increase in muscle fat, to which more larvae would migrate, since Smith (1983) found that *Anisakis* larvae migrate towards the muscle in fatty species.

The relatively low prevalence of *Anisakis* in the muscle is of major health interest because fish muscle is preferentially consumed, implying that there are less probabilities of human infection. With regard to the choice of fish, although the prevalence of *Anisakis* was higher in mackerel caught in Atlantic than Mediterranean Moroccan waters, the risk of finding the parasite in muscle did not differ between them, which may be due to their similar parasitization intensity and diversity of parasite species. According to our study, a useful prophylactic method against anisakiasis would be to select fish of lower weight.

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