ORIGINAL ARTICLE

Sensitization to *Anisakis simplex* Species in the Population of Northern Morocco

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Abstract

Objective: To investigate sensitization to third-stage *Anisakis simplex* larvae in a randomly selected population in northern Morocco.
Methods: We studied sera obtained from clinical analysis laboratories in Tangier and Tetuouan and from fishermen at Tangier port. The age of the study population ranged from 6 to 83 years. ImmunoCAP and immunoblotting techniques were used to determine total and specific immunoglobulin (Ig) E values and the χ^2 and Fisher exact tests were applied to analyze relationships between study variables.
Results: A seroprevalence of 5.1% was found, with a higher percentage of positive sera in the 31-to-43-year age group. Sensitization was not significantly associated with the origin, sex, occupation, or age of the individuals studied. In sera positive by InmunoCAP, immunoblotting studies detected numerous bands of between 7 kDa and >209 kDa, with a predominance of bands in the ~20-kDa to 24-kDa range.
Conclusions: Although no cases of human anisakiasis have been reported in Morocco to date, part of a randomly selected population in Northern Morocco shows sensitization to *A simplex* proteins.

Key words: *Anisakis*. Morocco. Sensitization. IgE. Allergens.

Resumen

Objetivos: La finalidad de este estudio ha sido la de investigar la sensibilización frente a la larva L3 de *Anisakis*, de una población elegida al azar del norte de Marruecos.
Métodos: Hemos analizado sueros de pacientes atendidos en laboratorios de análisis clínicos de Tánger y Tetuán, incluyendo una subpoblación de pescadores del puerto de Tánger. La edad de la población ha estado comprendida entre 6-83 años. Se han utilizado las técnicas de ImmunoCAP e Immunoblotting para determinar la IgE total e IgE específica y se ha aplicado el estadístico de χ^2 o el test de Fisher, para conocer el grado de significación entre las distintas variables.
Resultados: La seroprevalencia obtenida ha sido del 5.1%, detectándose el mayor nº de sueros positivos en el intervalo de edad de 31-43 años. La sensibilización no está asociada con la procedencia, sexo, ocupación y edad de los individuos estudiados. Entre los sueros positivos por InmunoCAP, se detectaron por inmunoblotting, numerosas bandas entre 7 kDa y >209 kDa, predominando las de ~20 kDa – 24 kDa.
Conclusiones: Aunque la anisakiasis humana por ahora no ha sido denunciada en Marruecos, nosotros aportamos datos acerca de la sensibilización con proteínas de *Anisakis*, de una población elegida al azar en el norte de Marruecos.

Palabras clave: *Anisakis*. Marruecos. Sensibilización. IgE. Alérgenos.

Introduction

Species of the *Anisakis simplex* complex are nematodes whose third-stage larvae (L3) are parasites of numerous fish and cephalopods that form part of the human diet. Inadvertent ingestion of the parasite by humans produces more or less severe gastrointestinal symptoms and allergic reactions that range from urticaria to anaphylactic shock [1]. Cutaneous-mucosal exposure in individuals working with fish can produce occupational allergies, including contact dermatitis, rhinoconjunctivitis, and occupational asthma [2,3]. Certain populations may have developed sensitization to *A simplex*, considering that specific immunglobulin (Ig) E has been detected in individuals with no apparent symptoms. Possible causes of this sensitization include: *1)* active infection after consumption of raw parasitized fish; *2)* consumption of raw, cooked, or canned fish with a large number of thermostable allergens; *3)* frequent contact with parasite allergens among people working with fish or derivatives; and *4)* cross-reactions with other nematodes that share antigens with *A simplex* or arthropods (crustaceans, insects, or mites) [4,5,6].

The few studies on the presence of these nematodes in

fish from Morocco report a high prevalence of species of the *A simplex* complex off the Atlantic and Mediterranean coasts [7,8]. It is widely acknowledged that consumption of raw mackerel can lead to anisakiasis, and indeed consumption of marinated mackerel is one of the main causes of this infection in Japan [9]. Our group has reported that an average of 62.4% of all mackerel caught in Moroccan waters is infected by parasites [10], and mackerel is frequently consumed in marinated form by part of the Moroccan population. The consumption of fresh fish has increased in Morocco since the 1960s, especially in Mediterranean and North Atlantic areas [11]. However, no studies to date have analyzed sensitization to species of the *A simplex* complex in the Moroccan population. The objective of the present study was to conduct a preliminary study on sensitization to this species in a randomly selected group of individuals from the northern Moroccan provinces of Tetouan and Tangiers.

Methods

Study Population

We analyzed 333 serum samples: 283 obtained from public clinical analysis laboratories in Tetouan and Tangier (corresponding to 132 females and 104 males and 24 females and 23 males, respectively) and 50 obtained from the general practitioner at the port of Tangier (corresponding to men working in the fishing industry [risk population]). The individuals were randomly selected, with exclusion of patients with severe or chronic diseases. The age of those enrolled in the study ranged from 6 to 83 years, with a mean age of 45 years for the 236 individuals from Tetouan and of 45.9 years for the 97 individuals from Tangier.

Determination of Sensitization to A simplex *(sensu lato)*

Sensitization to *A simplex* sensu lato (s.l.) was determined using 2 serum IgE detection methods: ImmunoCAP and immunoblotting.

Determination of Total and Specific IgE to A simplex *s.l. by ImmunoCAP*

The ImmunoCAP commercial kit (Phadia AB) was used according to the manufacturer's instructions. Total IgE levels of less than 25 kU/L were considered to be low while levels of over 100 kU/L were considered to be high. A specific IgE value of over 0.35 kU/L was considered positive.

Detection of Specific IgE by Immunoblotting
Larvae Identification

L3 larvae of *A simplex* were obtained from blue whiting (*Micromesistius poutassou*) caught in the Northeast Atlantic ocean and were prepared for immunological analysis following morphological identification.

Preparation of Excretory-Secretory Antigens

Live type 1 L3 *A simplex* larvae were successively washed in phosphate-buffered saline (PBS) and then immersed in an antibiotic solution under sterile conditions for 30 minutes [12]. Next, a total of 277 larvae/1.4 mL of sterile PBS were placed in a culture flask and maintained for 7 days at 37ºC and 5% carbon dioxide. The supernatant was then centrifuged at 4000 rpm for 30 minutes at 4ºC and stored at –70ºC until use. Protein content was estimated using the Lowry method [13].

Immunoblotting

Protein electrophoresis [14] was conducted in Precise Protein Gels (Pierce Biotechnology Inc.) in gradient (4%-20%) following the manufacturer's instructions. Excretory-secretory (ES) antigens were mixed at a proportion of 1:1 with dissociation buffer heated at 90ºC for 4 minutes; 40 μg (total protein) was added to each sample well. In the first well, 5 μL of prestained standards (Bio-Rad) was deposited. The separated proteins were transferred onto a nitrocellulose membrane (0.22-μm pore) for 90 minutes at 40 V and 4ºC using a Mini Trans-Blot Cell (Bio-Rad). After blocking with 5% w/v fat-free milk powder, the membranes were placed in a Mini Protean II Multiscreen apparatus (Bio-Rad) and incubated overnight at 4ºC with 600 μL of patient serum (1:5 dilution). After 3 washes, the membranes were incubated with alkaline phosphatase-labeled monoclonal anti-human IgE (Sigma-Aldrich) at 1:6000 dilution. Finally, detection was carried out with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma-Aldrich) for 30 minutes. The following controls were included in each assay: negative control (human serum with specific IgE class 0 [<0.35 kU/L] and very low total IgE levels [<25 kU/L]) and positive control (human serum with specific IgE class 6 [>100 kU/L]).

Risk Factors and Statistical Analysis

Univariate and multivariate logistic regression analyses were used to investigate associations between patient characteristics (place of residence, sex, occupation, and age) as independent variables and sensitization to *A simplex* as the dependent variable. Differences in prevalence between the different categories were examined by means of the χ^2 or Fisher exact test. A *P* value of .05 or less was considered significant. The SPSS (version 15.0) software package was used for all data analyses.

Results

Table 1 shows the characteristics of the study population.

Determination of Total and Specific IgE by ImmunoCap

Positive results were obtained in 17 of the 333 sera tested by ImmunoCAP (5.1% seroprevalence). The positive results corresponded to 13 individuals from Tetouan (8 males and 5 females) and 4 from Tangier (2 males, 1 of whom was a fisherman, and 2 females). Sera from the fishermen showed a positivity of 2%. The age of patients with positive sera ranged from 22 to 70 years (mean age, 38.4 years). The highest percentage of positive sera was detected in those aged between 31 and 43 years.

Table 1. Characteristics of the Study Population

Variables	No. (%) of Patients	No. (%) of Positive Patients	*P* Value
Place of residence			.27
Tangier	97 (29.1)	4 (4.1)	
Tetouan	236 (70.9)	13 (5.5)	
Sex			.47
Male	177 (53.2)	10 (5.6)	
Female	156 (46.8)	7 (4.5)	
Occupation			.38
Related to fishery	50 (15.0)	1 (2.0)	
Not related to fishery	283 (85.0)	16 (5.7)	
Age, y			.32
<18	19 (5.7)	0 (0.0)	
18-30	80 (24.0)	3 (3.8)	
31-43	73 (21.9)	8 (11.0)	
44-56	58 (17.4)	4 (6.9)	
57-69	42 (12.6)	1 (2.4)	
>69	61 (18.3)	1 (1.6)	
Total	333	17 (5.1)	

In the 17 patients with positive sera, specific IgE levels were high (>3.5-17.5 kU/L) in 23.5% of cases, moderate (>0.7-3.5 kU/L) in 41.2% of cases, and low (>0.35-0.7 kU/L) in 35.3% of cases (Table 2).

All the positive sera showed markedly elevated total IgE (>5000 kU/L in some cases). In the negative sera, total IgE values were less than 100 kU/L in 62.4% of cases and more than 100 kU/L in 37.6%.

Table 2. Classes of Specific Immunoglobulin (Ig) E in the Study Population Determined by ImmunoCap

Class of Specific IgE	No. of Individuals	%
0 (<0.35 kU/L)	316	94.9
1 (>0.35-0.7 kU/L)	6	1.8
2 (>0.7-3.5 kU/L)	7	2.1
3 (>3.5-17.5 kU/L)	4	1.2
Total	333	100

Immunoblotting

In general terms, a correlation was found between band intensities and ImmunoCAP-determined specific IgE values. In the immunoblotting assay, 3 of the 17 positive sera were negative, 5 showed around 20 bands, ranging from 7 to >209 kDa, and the remaining 9 showed 2 groups of bands: a low-molecular-weight group of bands (7-22 kDa) and a high-molecular-weight group (80->209 kDa). Most of the blots showed strong bands between ~20 kDa and 24 kDa and 2 showed a very strong band of ~40 kDa (Figure).

Statistical Analysis

No significant associations were found between sensitization to *A simplex* and sex (10/169 males, 7/164 females, *P*=.47), age (*P*=.32), or occupation (*P*=.38).

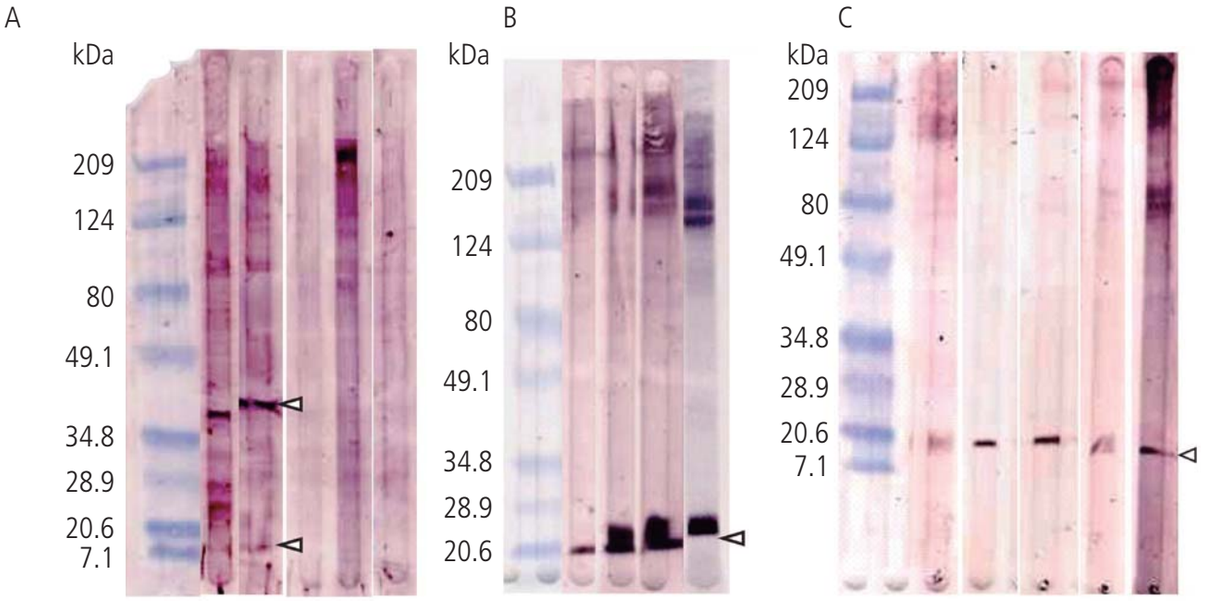

Figure. Different patterns of immunoglobulin E immunoblotting against type 1 *Anisakis simplex* excretory-secretory antigens. Each lane was probed with the serum of each patient. A) Blots showing bands of between 7 and >209 kDa; in 2 blots a strong band of ~40 kDa was observed (the arrowheads show the ~40- and ~9-kDa bands). B and C) Blots with 2 groups of high- and low-molecular-weight bands. Blots showing strong bands of between ~20 and 24 kDa (arrowheads). The molecular weight markers are indicated on the left.

Discussion

Human anisakiasis is characterized by the production of IgE antibodies against somatic and ES parasite antigens, which can be detected by skin prick testing, enzyme-linked immunosorbent assay, or immunoblotting, among other techniques [15,16]. We used ImmunoCAP and immunoblotting to determine subclinical sensitization to type 1 *A simplex* in a randomly selected population from northern Morocco aged over 6 years and with no diagnosis of anisakiasis or allergy to species of the *A simplex* complex. A prevalence of 5.1% was detected by InmunoCAP (Table 1); this is lower than the values of 0.43% to 22.1% reported for most healthy populations studied in neighboring Spain, which also has Mediterranean and Atlantic coasts [6,17,18]. This wide variation in results may be due to differences in the sensitivity of the techniques used to detect specific IgE and to differences in risk factors, such as the fish species consumed, their origin, and the form of preparation, among others. Spain is a country with a high consumption of fish (85 g/inhabitant/d); fish in Spain is generally eaten cooked, but raw anchovies in vinegar are widely consumed [19]. In Morocco, there is also a high consumption of fresh fish, especially in the Mediterranean and North Atlantic areas, where it is caught exclusively for human consumption. The estimated annual consumption per capita is 8.6 kg and this is on the increase due to changes in the population's eating habits [11]. The fish is usually eaten cooked, but raw mackerel seasoned with lemon and spices is also a popular dish.

The comparatively low sensitization of the Moroccan population may be related to cooking habits, the specific parasite species studied, and the low prevalence of this species in fish muscle. Distinct authors have detected a predominance of *Anisakis pegreffii* over *A simplex* sensu strictu (s.s.) [8,10,20]. Hence, the low sensitization in the northern Moroccan population studied may be in part attributable to the lower pathogenic potential of *A pegreffii*. Suzuki et al [9] observed that this species of *Anisakis* has a lower power of penetration into fish muscle than *A simplex* s.s. This observation is highly relevant, as humans preferentially consume the muscle of fish. Furthermore, we think that the larvae of *A pegreffii* has a lower capacity of fixation and penetration in the gastrointestinal tract, meaning that even when humans are infected, there may be a lesser likelihood of sensitization to the ES antigens released. Another important factor to take into account is the susceptibility of humans to allergic reactions to this parasite, which some have suggested could be genetically determined. An association has been shown between the presence of certain human leukocyte antigen class II alleles and sensitization to species of the *A simplex* complex [1]. Sanchez-Velasco et al [21], in turn, suggested that the haplotype DRB1*1502-DQB1*0601 might be a susceptibility factor for hypersensitivity to *A simplex* allergens. In Morocco, the frequency of alleles DRB1*1502 and DQB1*0601 is low, with values of 1.5% and 1%, respectively [22]. This may also explain the absence of clinical cases, as has been suggested for Norway [21].

The age of sensitized individuals in our series ranged from 22 to 70 years. The highest proportion of positive results was in the 31-to-43-year age group and only a small percentage of sensitized individuals were aged over 57 years (Table 1). Gastric acid secretion is known to decrease with age, and this may reduce the secretion of proteins secreted by the larvae after gastric infection [23]. It is also possible that continual contact with ES products from species of the *A simplex* complex throughout a lifetime may induce a modified type 2 helper cell response, leading to the development of interleukin-10-producing regulatory T cells. A limitation of our investigation is that no data were available on the cooking habits or the specific occupations of the study population, precluding examination of possible associations between these variables and sensitization.

We highlight the low prevalence of sensitization to *A simplex* (2%) in the fishermen from Tangier port, with no significant differences observed with the rest of the series. Although we had considered them to be a risk group due to their continuous occupational contact with fish, they do not eviscerate or fillet specimens, unlike fishmongers and catering workers, who are known to develop asthmatic and contact dermatitis due to inhalation of and contact with parasite allergens [2,3].

Specific IgE levels were high (>3.5-17.5 kU/L) in only 23.5% of positive sera, but total IgE was high in all samples, reaching levels of over 5000 kU/L in some cases; 37.6% of the negative sera showed total IgE values of over 100 kU/L and none had values of over 5000 kU/L. The slightly elevated levels of total IgE in the negative sera may be due to allergic processes caused by other allergens or to infection with helminths that are not members of the Anisakidae family, such as *Trichuris trichiura*, *Enterobius vermicularis*, *Ascaris lumbricoides*, among others, which are frequently detected in the Moroccan population [24].

Our immunoblotting assays used laboratory-prepared ES antigens of type 1 *A simplex* (concentrations of 4.7 μg/μL), because the sensitivity and specificity of the technique have been demonstrated to be higher with ES than with somatic antigens [25]. A varied band profile was obtained, indicating sensitization to different allergens of the parasite (Figure). No correlation between positive immunoblot results and specific bands were detected, as shown in the Figure. None of the blots showed all bands simultaneously. In addition to *A pegreffii*, the predominant species in mackerel from Moroccan waters, these individuals may have consumed fish infected with larvae of other anisakids associated with common allergens [26]. Moreover, we cannot rule out the possibility of infection of the population with other ascarids such as *Ascaris lumbricoides* or larva migrans due to *Toxocara* species, meaning that some bands may be due to cross-reactions with antigens of these parasites. The band of ~40 kDa detected in 2 blots may correspond to Ani s 3 (tropomyosin, 41 kDa), a somatic allergen of *A simplex*, which is a thermostable protein that may be responsible for allergic reactions in sensitized individuals after the consumption of cooked parasitized fish. This protein shows significant homology with the proteins of various organisms, including dust mites, cockroach, molluscs, and helminths, and is a cause of cross-reactions [27,28]. Most of the blots showed bands of between 20 kDa and 24 kDa, and some of these may correspond to the major thermostable allergen Ani s 1 (21 kDa), produced by the excretory gland of *A simplex* [29], or to the thermostable somatic or cuticular allergen Ani s 10 (22 kDa) [30]. These allergens

are considered in the differential diagnosis of hypersensitivity reactions because they behave as food allergens in raw, cooked, and canned fish [31]. The detection of specific IgE to low-molecular-weight antigens by immunoblotting is of diagnostic interest [32]. Some of the sera in our series recognized proteins of 7 to 9 kDa that may correspond to minor ES allergens, ie, protease inhibitors corresponding to Ani s 4 (9 kDa). This allergen is thermostable and resistant to pepsin digestion and was the first nematode cystatin identified as a producer of human allergies, with a high diagnostic value [33].

Three sera were positive by immunoCAP but negative by immunoblotting, which probably reflects inadequate sensitivity of the immunoblotting technique, given that they were all classified as Class 1 by ImmunoCAP.

In conclusion, the present data indicate that sensitization to species of the *A simplex* complex is present in Morocco, although a low proportion of our study population showed sensitization to different somatic and cuticular proteins of the parasite and to those derived from their metabolism. The sensitization would be due to contact with the parasite, attributable to the high prevalence of parasitization in some fish species commonly eaten in the north of Morocco. No cases of human anisakiasis have been diagnosed in Morocco to date. This may be due to the absence of specific symptoms and signs of the disease, which could be confused with other diseases with similar acute gastric or abdominal symptoms. Moreover, allergic symptoms produced by *A simplex* allergens can be confused with those seen in allergies to fish or other foods.

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