

An Extended Study of Seroprevalence of Anti-*Anisakis simplex* IgE Antibodies in Norwegian Blood Donors

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Abstract

During the last decade, cases of the fish parasite *Anisakis simplex* infection and allergy in human have increased in countries with high fish consumption. Our aim was to perform an extended seroprevalence study of anti-IgE antibodies against this parasite in Norway, one of the high fish-consuming countries. At the Department of Immunology and Transfusion Medicine and the Laboratory of Clinical Biochemistry, Haukeland University Hospital, Bergen, Norway, two main groups of anonymized serum samples were collected; the first ($n = 993$) from recently recruited blood donors (designated 'BDO') and the second ($n = 414$) from patient with total IgE levels ≥ 1000 kU/l (designated 'IGE+'). The sera were analysed by the ImmunoCAP® method for total IgE and IgE antibodies against *A. simplex*, house dust mite (HDM), shrimp, cod, crab, brine shrimp and shrimp tropomyosin. The *A. simplex* positive sera were further tested by an enzyme-linked immunosorbent assay (ELISA) method, which uses 2 recombinant (r) major allergens, rAni s 1 and rAni s 7 as target antigens. SDS-PAGE and Western immunoblotting analyses were also performed. Whereas the prevalences by ImmunoCAP® were 0.4% and 16.2% in the BDO and IGE+ groups, respectively, analyses with recombinant allergens showed only 0.0% and 0.2%. Cross-reactivity and immunoblotting analyses suggested that most of the ImmunoCAP® positive sera were probably false-positive due to cross-sensitization to shrimp and HDM. However, positivity due to other *A. simplex* antigens should also be considered. Compared with other high fish-consuming countries, we observed a very low seroprevalence of anti-*Anisakis* IgE antibodies in a Norwegian population.

Introduction

The nematode *Anisakis simplex* often parasitizes many commercially important fish species, and it causes anisakiasis in humans when accidentally ingested in raw or undercooked infested fish or seafood. This acute gastrointestinal parasitic disease is manifested by clinical symptoms ranging from nausea, vomiting, epigastric pain and diarrhoea to intestinal obstruction [1]. Moreover, ingestion of *A. simplex* may lead to IgE-mediated allergic reactions such as urticaria, angioedema, asthma and sometimes even anaphylaxis in highly sensitized persons [2]. It has been reported that allergic sensitivity to *A. simplex* is initiated by an active infection with live parasite [3]. On the other hand, the intake of even dead parasite or residues in contaminated fishery products has been claimed to evoke allergic symptoms in sensitized individuals by means of its several heat- and pepsin-resistant allergens, which remain active even after cooking or digestion [4–6]. In addition,

inhalation of or contact with *A. simplex* allergens has been reported to cause occupational allergies in fishery and aquaculture workers, fishmongers and cooks, etc. [7, 8].

Anisakis simplex infection and allergy in human have been frequently reported in countries such as Japan, Spain, Italy and Korea where raw or only lightly processed fish are traditionally consumed [9]. Norway is among the countries with high *per capita* fish consumption (65 g/day/person) [10]; however, Norwegian fish consumption is mainly composed of processed and frozen fish products [10]. In recent years, several exotic fish delicacies containing raw or semiraw fish such as sushi, sashimi have gained more interest among the Norwegians. Reports of human anisakiasis are rare in Norway except for a few cases, which were related to the intake of raw or only lightly processed fresh marine fish [11, 12].

Seroprevalence of *A. simplex* sensitization in blood donor populations is reported to be highly variable among the countries or even regions within the same country, for

instance in Spain, ranging from 0.43% [13], 11.7% [14] to 22.1% [15]. These differences in prevalence may partly arise from the different degrees of specificity and sensitivity of the IgE determination methods employed in different studies. Serological methods using *A. simplex* larvae crude extract (CE) as target antigens may either be less specific due to the presence of cross-reacting allergens or less sensitive due to lack of some major excretory secretory (ES) allergens [16]. Ani s 1 and Ani s 7 are the two major ES allergens of *A. simplex* and have no homology with any allergens that humans are known to be sensitized towards [17, 18]. An enzyme-linked immunosorbent assay (ELISA) method using rAni s 1 and rAni s 7 as targets of IgE antibodies has recently been developed and reported to be currently the most specific and sensitive serodiagnostic test for human anisakiasis [16].

In a previous pilot study using the ImmunoCAP® method, we obtained 2.0% and 6.6% prevalence of IgE sensitization to *A. simplex* in blood donors (BDO) and Phadiatop® positive serum groups, respectively. However, a considerable degree of cross-sensitization to shrimp and HDM was suspected [19]. Therefore, we have conducted an extended study using a more specific and sensitive serodiagnostic test and a larger sample size of BDO in order to obtain a more accurate prevalence of IgE sensitization to the fish parasite *A. simplex* in our study region, Bergen, Norway.

Materials and methods

Serum samples. At the Department of Immunology and Transfusion Medicine and Laboratory of Clinical Biochemistry (LKB), Haukeland University Hospital, Bergen, Norway, two main groups of sera were collected from volumes rendered superfluous and normally discarded after the requested analyses had been performed. The first group ($n = 993$) consisted of sera from newly recruited blood donors (designated 'BDO'), and the second group ($n = 414$) contained patient sera with total IgE levels ≥ 1000 kU/l (designated 'IGE+'). All sera were collected consecutively and anonymized; thus, no information was available on patient history. As positive control for the presence of IgE antibodies against *A. simplex*, another group of patient sera from Spain ($n = 10$) was included and they were clinically clearly characterized as gastro-allergic anisakiasis (designated 'GAA'). The Spanish patients had positive skin prick test (SPT; $\geq 3 \times 3$ mm mean diameter), performed with *A. simplex* antigen (Lab IPI, Madrid, Spain). Histamine (1%) and isotonic saline solution (0.9% NaCl) were used as positive and negative controls, respectively. Total IgE and IgE antibodies against *A. simplex* were measured using the ImmunoCAP® method. These samples were kindly provided by Dr. Alvaro Daschner (Servicio de Alergia Instituto de Investigación, Sanitaria-Hospital Universitario de La Princesa, Madrid,

Spain). The immunoCAP® negative sera for *A. simplex* IgE antibodies among the BDO group ($n = 20$) were used as negative control (designated 'NEG'). Additionally, we also used rabbit polyclonal IgG antibodies against *A. simplex* CE (designated 'RAB'), developed by Charles River Laboratories, Research Models and Services, Stolzensee, Germany. The study was approved by the Regional Committee for Medical Research Ethics in Western Norway (REK Vest).

Anisakis crude extract. A protein extract was prepared from *A. simplex* 3rd stage larvae that were collected manually from the viscera, flesh and body cavities of freshly caught blue whittings (*Micromesistius poutassou*). Larvae were rinsed in physiological saline, frozen in liquid nitrogen and smashed by mortar. After addition with phosphate-buffered saline (PBS), *A. simplex* larvae were homogenized by magnetic stirring at 4 °C overnight. After centrifugation for 25 min at 4 °C and 18,000 g, the supernatant was dialysed against distilled water in a dialysis tube with 6–8 kDa pore size (Spectrapor; Spectrum Medical industries, Los Angeles, CA, USA) and then freeze-dried (Heto, Allerød, Denmark) overnight. The protein was dissolved in PBS (pH 7.4), and total protein content was determined by Lowry protein assay (BioRad Laboratories, Hercules, CA, USA). Aliquots were stored at -20 °C until use. HDM and shrimp extracts have previously been prepared by our Allergy Research Group, using the same extraction method described above.

Immunoassays. IgE and IgE antibodies against *A. simplex* (p4, Anisakis spp.), house dust mite (HDM) (d1, *Dermatophagoides pteronyssinus*), cod (f3, *Gadus morhua*), shrimp (f24, *Pandalus borealis*), crab (f23, *Cancer pagurus*), brine shrimp (o202, *Artemia salina*) and shrimp tropomyosin (f351, rPen a 1 *Penaeus aztecus* tropomyosin,) were determined by the ImmunoCAP® system (Thermo Fisher Scientific Inc., Uppsala, Sweden) following the instructions of the manufacturer. The threshold level for *A. simplex* sensitization was defined by an antibody level of ≥ 0.35 kU_A/l. Further, the ImmunoCAP® positive sera were analysed for IgE antibodies against rAni s 1 and rAni s 7 allergens by Trisakis 170 ELISA (Laboratorio de Parasitología, Facultad de Farmacia, Universidad de Santiago de Compostela, Santiago de Compostela, Spain). Briefly, the Trisakis 170 ELISA is a solid-phase double-antibody indirect immunoassay for the qualitative determination of IgE class antibodies against *A. simplex* in human sera. The test uses 96-well microtitration plates coated with the recombinant allergens rAni s 1 or rAni s 7 to bind anti-*Anisakis* IgE antibodies in human sera. The wells of columns 1, 4, 7 and 10 contain the rAni s 1 allergen; the wells of columns 2, 5, 8 and 11 contain the rAni s 7 allergen, whereas the wells of columns 3, 6, 9 and 12 contain no *Anisakis* allergens and serve as negative control for each sample. The ELISA consists of three main steps: (1) incubation with undiluted human serum, (2)

incubation with fluorescein isothiocyanate (FITC) labelled anti-human IgE monoclonal conjugate (first antibody) and (3) incubation with horseradish peroxidase (HRP) labelled anti-FITC monoclonal conjugate (second antibody). Finally, the chromogen o-phenylenediamine dihydrochloride (OPD) is added to reveal bound IgE antibodies. The intensity of this coloured (yellow) product is proportional to the concentration of anti-*A. simplex* IgE antibodies in serum. Samples are considered positive if the calculated absorbance values are higher than the cut-off values of 0.09 and 0.05 for rAni s 1 and rAni s 7, respectively.

SDS-PAGE and IgE/IgG immunoblotting. *Anisakis simplex*, HDM and shrimp extracts were separated by SDS-PAGE. The samples along with molecular weight standards were resolved in a 12% separating gel at 200 V. Proteins were visualized by Coomassie brilliant blue R-250 staining (Sigma Chemical Co., St. Louis, MO, USA). For immunoblot analyses, proteins were transferred onto nitrocellulose membranes (0.45 mm) using a minitrans-blot cell (BioRad Laboratories) for 1 h at 100 V. Immunodetection was performed with the serum pools of the ImmunoCAP[®] positive BDO ($n = 4$), IGE+ ($n = 20$), GAA ($n = 10$), NEG ($n = 20$) and RAB ($n = 2$) sera. After antibody binding, the colour reaction was developed with SigmaFast BCIP/NBT (5-Bromo-4-chloro-3-indolyl phosphate dipotassium/nitrotetrazolium blue chloride) tablets (Sigma Chemical Co, St Louis, Mo, USA).

Statistical analysis. Mann–Whitney *U*-test was used to determine the difference in prevalence of sensitization between the groups. Values of $P < 0.05$ were considered statistically significant. Medians and interquartile ranges (IQR) were calculated for IgE antibodies and compared to evaluate the levels of sensitization among the groups. All statistical analyses were carried out with Statistica software program, version 9 (StatSoft, Inc., Tulsa, OK, USA).

Results

Prevalences and degrees of IgE sensitization

The prevalences of IgE sensitization to *A. simplex* in the BDO and IGE+ sera are summarized in Table 1. As expected, the prevalence was higher in the IGE+ than the BDO group. According to the ImmunoCAP[®] method, the median of anti-*Anisakis* IgE-antibody levels was 0.45 kU_A/l

Table 1 The prevalences given as number of sera (%) with IgE antibodies against *Anisakis simplex* in the blood donors (BDO) and IGE+ sera (patient sera with total IgE levels ≥ 1000 kU/l). (Cut-off values; ≥ 0.35 kU_A/l in the immunoCAP method and 0.09 and 0.05 ODs for rAni s 1 and rAni s 7, respectively, in the Trisakis ELISA method).

Source	BDO ($n = 993$)	IGE+ ($n = 414$)
ImmunoCAP [®] method	4 (0.4%)	67 (16.2%)
Trisakis ELISA method	0 (0.0%)	1 (0.2%)

(IQR 0.37–0.52) in the BDO group, indicating a very low sensitization level and that of the IGE+ group was 1.25 kU_A/l (IQR 0.6–3.22), suggesting a low sensitization level as well. There was a significant difference of the median levels of IgE antibodies to *A. simplex* between the two groups (Mann–Whitney *U*-test, $P < 0.05$). Moreover, the GAA group had a relatively much higher median IgE-antibody level of 37.5 kU_A/l (IQR 23.78–88.75; Mann–Whitney *U*-test, $P < 0.001$). Classes and medians of anti-*Anisakis* IgE antibodies in the BDO, IGE+ and GAA serum groups are shown in Table 2. All BDO sera ($n = 993$) were further tested for rAni s 1 and rAni s 7 allergens, using Trisakis ELISA. All of them showed negative, indicating 0.0% prevalence of sensitization against *A. simplex* in the blood donor population as determined by this method. Among the IGE+ group ($n = 414$), 67 ImmunoCAP[®] positive sera were further tested for rAni s 1 and rAni s 7 allergens and the results showed only one positive sample, giving a 0.2% prevalence of sensitization. Interestingly, this particular sample was highly positive by both methods: by the ImmunoCAP[®] method, it showed a high IgE-antibody level of 38.7 kU_A/l and by the Trisakis ELISA method, very high OD values of 2.97 and 2.90 to rAni s 1 and rAni s 7, respectively. In the negative control group of the ImmunoCAP[®] negative sera of the IGE+ group ($n = 49$), all sera were tested negative. In the positive control GAA group ($n = 10$), all samples exhibited highly positive signals; with IgE-antibody levels (ODs) of 1.22; 2.02 and 1.03; 2.04 (median; IQR) against rAni s 1 and rAni s 7 allergens, respectively. Among them, 8 sera were positive to both rAni s 1 and rAni s 7 and 2 sera were positive to only Ani s 7, but not to Ani s 1.

Cross-reactivity analyses

All the ImmunoCAP[®] positive sera from the BDO group ($n = 4$) showed low levels of anti-*Anisakis* IgE antibodies, and at the same time, they were positive (IgE antibodies ≥ 0.35 kU_A/l) to one or more of the cross-reactive allergens. In fact, most of those samples (three of four) had higher IgE levels to the cross-allergens than *A. simplex* (Table 3).

SDS-PAGE and Western blottings

Three different allergen extracts, *A. simplex* CE, HDM and shrimp, were blotted with five serum pools, BDO, IGE+, GAA, NEG and RAB, to investigate possible cross-reactions. In the Western blotting (Fig. 1), a correlation seemed to exist between band intensities and IgE-antibody levels determined by the ImmunoCAP[®] method. The BDO serum pool showed weaker protein bands to *A. simplex* CE than the IGE+ and GAA serum pools, as expected. Both BDO and the IGE+ pools exhibited around five bands ranging between 40 and 100 kDa to *A. simplex* CE, but they showed certain similar but slightly stronger

Table 2 Number of sera (medians and IQRs of IgE-antibody levels to *Anisakis simplex*) in the BDO (blood donors), IGE+ (patient sera with total IgE levels ≥ 1000 kU/l) and gastro-allergic anisakiasis (GAA) serum groups, according to the classes of IgE levels measured by the ImmunoCAP[®] method.

Class of IgE-antibody level (kU _A /l)	BDO (<i>n</i> = 993) (median; IQR)	IGE+ (<i>n</i> = 414) (median; IQR)	GAA (<i>n</i> = 10) (median; IQR)
0 (<0.35)	989	347	0
1 (0.35–<0.7)	4 (0.45; 0.14)	21 (0.43; 0.12)	0
2 (0.7–<3.5)	0	31 (3.94; 1.06)	0
3 (3.5–<17.7)	0	11 (6.96; 4.72)	2 (12.4; 3.9)
4 (17.7–<50)	0	3 (38.7; 12.05)	4 (30.9; 5.95)
5 (50–<100)	0	1 (63.50)	1 (55.00)
6 (≥ 100)	0	0	3 (366.0; 437.5)

Table 3 Total IgE (kU/l) and IgE-antibody levels (kU_A/l) against *Anisakis simplex* and six different cross-allergens in the four ImmunoCAP[®] positive sera of the BDO (blood donor) group.

Sample no.	1	2	3	4
Total IgE	308	426	3954	576
<i>Anisakis simplex</i>	0.53	0.51	0.38	0.36
HDM (<i>Dermatophagoides pteronyssinus</i>)	0.41	1.33	18.6	1.28
Shrimp	<0.35	3.54	0.76	0.74
Crab	<0.35	1.07	<0.35	2.23
Cod	<0.35	<0.35	<0.35	<0.35
Brine Shrimp (<i>Artemia</i>)	<0.35	<0.35	<0.35	0.53
Shrimp tropomyosin	<0.35	0.4	<0.35	<0.35

HDM, house dust mite

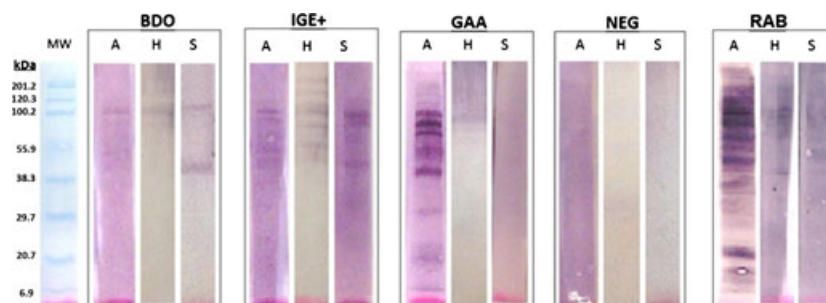
bands to HDM and shrimp extracts. The GAA pool revealed more distinct and a larger numbers of bands to *A. simplex* CE, including several additional low molecular-weight bands (~7–30 kDa) and high molecular-weight bands (~40–130 kDa); however, much weaker or no obvious bands were seen to HDM or shrimp extracts, respectively. The NEG pool demonstrated no apparent bands to any of the allergen extracts. In the immunoblot with RAB pool, *A. simplex* CE revealed many distinct bands, indicating a high antigenicity of the extract. Moreover, the findings of several similar but weaker protein bands obtained with HDM and shrimp extracts

suggested the presence of several cross-allergens between the extracts used.

Discussion

This study corroborates and extends our earlier pilot study on the seroprevalence of anti-*A. simplex* IgE antibodies in Norway [19]. In this extended study, we used a larger population of BDO (*n* = 993) than the previous one (*n* = 100) in the pilot study. In addition to the ImmunoCAP[®] method, we also utilized a highly specific and sensitive ELISA method using rAni s 1 and rAni s 7 allergens [16] to obtain a more accurate estimate of IgE sensitization to the fish parasite *A. simplex*. Whereas the prevalences by ImmunoCAP[®] were 0.4% and 16.2% in the BDO and IGE+ groups, respectively, analyses with recombinant allergens showed only 0.0% and 0.2%. Cross-reactivity and immunoblotting analyses suggested that most of the ImmunoCAP[®] positive sera might be false-positive due to cross-sensitization to shrimp and/or HDM or due to the unspecific binding of very high total IgE levels. However, other possibilities such as positivity due to other minor *A. simplex* antigens should also be considered.

In the pilot study, we reported a seroprevalence of 2.0% and 37.0% in the BDO and the IGE+ sera, respectively, using the ImmunoCAP[®] method [19]. In this extended study, a prevalence of 0.4% and 16.2% was obtained in the

**Figure 1** Western blot analysis of three allergen extracts with five different pools of sera. MW, molecular mass standard (kDa); BDO, pool of the ImmunoCAP[®] positive blood donor sera (*n* = 4); IGE+, pool of the ImmunoCAP[®] positive patient sera with total IgE levels ≥ 1000 kU/l (*n* = 20); GAA, pool of the Spanish gastro-allergic anisakiasis sera (*n* = 10); NEG, pool of the ImmunoCAP[®] negative sera (*n* = 20); RAB, pool of rabbit sera with anti-*Anisakis* polyclonal IgG antibodies (*n* = 2); Lane A, *Anisakis simplex*; H, House Dust Mite (*Dermatophagoides pteronyssinus*); S, Shrimp.

BDO and IGE+ sera, respectively, with the same method. The fact that the method used CEs as target allergens and thus might contain several cross-reactive allergens [20, 21] could lead to an overestimation of the seroprevalence. The results of our cross-reaction analyses both in the current and previous studies also suggested the possibility of such false positives. It is further confirmed by the results of rAni s 1 and rAni s 7 ELISA, which showed a much lower prevalence of 0.0% and 0.2% in the BDO and IGE+ sera, respectively. The 0.0% prevalence in the BDO group may indicate an absence of sensitization against the fish parasite *A. simplex* in the healthy population. It is also lower than the prevalence of IgE sensitization to cod fish, previously shown to be <0.2% among Norwegian BDO [22]. On the other hand, the frequency of 0.2% in the IGE+ group may represent some degree of true sensitization in the 'allergic' population or maybe due to cross-sensitization.

Comparing the prevalences of *A. simplex* sensitization in healthy subjects in different countries determined by the ImmunoCAP method, 0.4% prevalence in Norway as indicated by our results is lower than 10% in Japan [23] and 22.1% in Spain [15]. Using the same method, 5.1% prevalence was reported in a randomly selected population in northern Morocco [24] and with a similar method using *A. simplex* CE, a prevalence of 5.0% was estimated in the health-examined residents in southern Korea [25]. One possible explanation for the relatively low prevalence we obtained could be the relatively low consumption of raw or uncooked fish among the Norwegian. In fact, *A. simplex* sensitization has been reported to be closely associated with high frequency of raw or uncooked fish consumption in several studies [14, 26–28], and an initial infection with live larvae has been claimed to be required for subsequent sensitization [3]. In this aspect, Norwegian fish diet, being largely composed of processed, canned and frozen products or farmed fish [10, 29], thus carrying less risk of infection with *A. simplex* live parasite could explain this low prevalence of sensitization. On the other hand, some studies have reported several thermo- and pepsin-resistant allergens of *A. simplex* [4, 5] and even the ingestion of well-cooked fish contaminated with *A. simplex* larvae has been claimed to induce sensitization or allergic symptoms [6]. However, such claim seems less likely at least in our country, due to the very low prevalence we obtained in spite of the comparatively high Norwegian fish intake [10] and the high frequency of *A. simplex* parasitization of Norwegian wild catch marine fish [30, 31]. Another possible reason for the low seroprevalence may be explained by the low genetic susceptibility of Norwegian population to *A. simplex* allergens. A significant association has been shown between sensitization to *A. simplex* and the DRB1*1502-DQB1*0601 haplotype, which is absent or very rare in certain populations including Norwegians [32].

In the BDO group, all 4 ImmunoCAP® positive sera showed very low IgE-antibody levels (median 0.45 kU_A/l, IQR 0.37–0.52), and at the same time, they were positive to one or more of the cross-reactive allergens. In fact, 3 of 4 sera had higher IgE-antibody levels to other allergens than *A. simplex* (Table 3), thus suggesting primary sensitization caused by other allergen exposure than *A. simplex*. It was therefore not surprising that all of them showed negative when further tested by rAni s 1 and rAni s 7 ELISA. In the IGE+ group, only 1 of 67 ImmunoCAP® positive sera showed positive when further tested by rAni s 1 and rAni s 7 ELISA. Interestingly, this single positive sample was highly positive both by the ImmunoCAP® and Trisakis ELISA methods, with the OD values, which were even higher than those of most Spanish GAA sera. Hence, this particular sample might represent a highly sensitized subject in the IGE+ group.

Several studies have shown cross-sensitization of *A. simplex* with HDM and crustaceans [20, 21]. Our Western blotting results (Fig. 1) also suggested the presence of cross-reactivity to HDM and shrimp in the ImmunoCAP® positive sera. Both the BDO and IGE+ pools exhibited around five bands ranging between 40 and 100 kDa in blotting with *A. simplex* CE. But they showed certain similar but slightly stronger bands to HDM and shrimp extracts, suggesting possible cross-reactions with these antigens. Certain blots exhibited bands of ~40 kDa, which may correspond to Ani s 3 (tropomyosin, 41 kDa). This protein is a somatic allergen of *A. simplex* and is responsible for cross-sensitization to dust mites, cockroach and crustaceans in certain sensitized subjects [20, 21]. Furthermore, most blots of the BDO and IGE+ serum pools revealed the bands of ~100 kDa, possibly corresponding to Ani s 2 (paramyosin, 97 kDa), a major somatic *A. simplex* antigen and a pan-allergen responsible for similar cross-sensitization [20]. Blotting of the *A. simplex* extract with the GAA pool revealed more distinct and larger numbers of bands, whereas weaker or no bands were identified in the HDM or shrimp extracts, suggesting a primary and high sensitization to a wider variety of the *A. simplex* antigens. It is especially interesting to observe two additional bands; one band around 20–24 kDa that may correspond to the Ani s 1 (24 kDa), a major thermostable allergen produced by the excretory gland of *A. simplex* [17], and another slight band around 130 kDa corresponding to the major ES allergen, Ani s 7 (139 kDa) [18]. The appearance of those bands for Ani s 1 and Ani s 7 is in conformity with the positive signals shown by the rAni s 1 and rAni s 7 ELISA in this group. According to the cross-reactivity and immunoblotting results, most of the ImmunoCAP® positive sera were probably false-positive due to cross-sensitization to shrimp and/or HDM. However, several *A. simplex* allergens (from Ani s 1 to Ani s 12) have been described (www.allergome.org) and depending on whether the ingested larva is alive or dead, individuals could be exposed to different types of

allergens such as ES, somatic or cuticular [33]. Therefore, an option still remains that the immunoCAP[®] positive but Trisakis negative results can be due to sensitization to other minor allergens of the parasite.

In conclusion, this follow-up study showed that the seroprevalence of IgE sensitization to the fish parasite *A. simplex* appears to be very low in a Norwegian population in comparison with other high fish-consuming countries; in fact, 0.0% prevalence given that our BDO group is representative of the general healthy population.

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Authors contributions

Aung Htun Lin, Erik Florvaag and Thien Van Do have contributed with study design, acquisition and interpretation of data. Eliann Egaas has contributed with interpretation of data and revision of the draft. Ina Nepstad has contributed with collection and analysis of serum samples. All authors have participated in drafting and revising the article critically and have given the final approval of the version to be published.

Conflict of interest

There is no conflict of interest for any of the authors.

References

- Hochberg NS, Hamer DH. Anisakidosis: perils of the deep. *Clin Infect Dis* 2010;51:806–12.
- Daschner A, Pascual CY. *Anisakis simplex*: sensitization and clinical allergy. *Curr Opin Allergy Clin Immunol* 2005;5:281–5.
- Cuellar C, Daschner A, Valls A *et al.* Ani s 1 and Ani s 7 recombinant allergens are able to differentiate distinct *Anisakis simplex*-associated allergic clinical disorders. *Arch Dermatol Res* 2012;304:283–8.
- Caballero ML, Moneo I. Several allergens from *Anisakis simplex* are highly resistant to heat and pepsin treatments. *Parasitol Res* 2004;93:248–51.
- Tejada M, Vidacek S, de las Heras C *et al.* *Anisakis simplex* allergens remain active after conventional or microwave heating and pepsin treatments of chilled and frozen L3 larvae. *J Sci Food Agric* 2009;89:1997–2002.
- Falcao H, Lunet N, Neves E, Barros H. Do only live larvae cause *Anisakis simplex* sensitization? *Allergy* 2002;57:44.
- Nieuwenhuizen N, Lopata AL, Jeebhay MF, Herbert DR, Robins TG, Brombacher F. Exposure to the fish parasite *Anisakis* causes allergic airway hyperreactivity and dermatitis. *J Allergy Clin Immunol* 2006;117:1098–105.
- Scala E, Giani M, Pirrotta L *et al.* Occupational generalised urticaria and allergic airborne asthma due to *Anisakis simplex*. *Eur J Dermatol* 2001;11:249–50.
- Audicana MT, Kennedy MW. *Anisakis simplex*: from obscure infectious worm to inducer of immune hypersensitivity. *Clin Microbiol Rev* 2008;21:360–79, table of contents.
- Alexander J, Frøyland L, Hemre GI. *Fish and Seafood Consumption in Norway – Benefits and Risks*. Norway: Norwegian Scientific Committee for Food Safety, 2006.
- Eskenes A, Strand EA, Andersen SN, Rosseland A, Hellum KB, Strand OA. Anisakiasis presenting as an obstructive duodenal tumor. A Scandinavian case. *Scand J Infect Dis* 2001;33:75–6.
- Kroese AJ, Larsen KA, Berland B. [Human anisakiasis]. *Tidsskr Nor Lægeforen* 1980;100:1340–4.
- Valinas B, Lorenzo S, Eiras A, Figueiras A, Sanmartin ML, Ubeira FM. Prevalence of and risk factors for IgE sensitization to *Anisakis simplex* in a Spanish population. *Allergy* 2001;56:667–71.
- Puente P, Anadon AM, Rodero M, Romaris F, Ubeira FM, Cuellar C. *Anisakis simplex*: the high prevalence in Madrid (Spain) and its relation with fish consumption. *Exp Parasitol* 2008;118:271–4.
- Del Rey Moreno A, Valero A, Mayorga C *et al.* Sensitization to *Anisakis simplex* s.l. in a healthy population. *Acta Trop* 2006;97:265–9.
- Anadon AM, Rodriguez E, Garate MT *et al.* Diagnosing human anisakiasis: recombinant Ani s 1 and Ani s 7 allergens versus the UniCAP 100 fluorescence enzyme immunoassay. *Clin Vaccine Immunol* 2010;17:496–502.
- Moneo I, Caballero ML, Gomez F, Ortega E, Alonso MJ. Isolation and characterization of a major allergen from the fish parasite *Anisakis simplex*. *J Allergy Clin Immunol* 2000;106:177–82.
- Rodriguez E, Anadon AM, Garcia-Bodas E *et al.* Novel sequences and epitopes of diagnostic value derived from the *Anisakis simplex* Ani s 7 major allergen. *Allergy* 2008;63:219–25.
- Lin AH, Florvaag E, Van Do T, Johansson SG, Levsen A, Vaali K. IgE sensitization to the fish parasite *Anisakis simplex* in a Norwegian population: a pilot study. *Scand J Immunol* 2012;75:431–5.
- Guarneri F, Guarneri C, Benvenega S. Cross-reactivity of *Anisakis simplex*: possible role of Ani s 2 and Ani s 3. *Int J Dermatol* 2007;46:146–50.
- Johansson E, Aponno M, Lundberg M, van Hage-Hamsten M. Allergenic cross-reactivity between the nematode *Anisakis simplex* and the dust mites *Acarus siro*, *Lepidoglyphus destructor*, *Tyrophagus putrescentiae*, and *Dermatophagoides pteronyssinus*. *Allergy* 2001;56:660–6.
- Johansson SG, Nopp A, Florvaag E *et al.* High prevalence of IgE antibodies among blood donors in Sweden and Norway. *Allergy* 2005;60:1312–5.
- Kasuya S, Koga K. [Significance of detection of specific IgE in *Anisakis*-related diseases]. *Arerugi* 1992;41:106–10.
- Abattouy N, Valero A, Martín-Sánchez J, Peñalver MC, Lozano J. Sensitization to *Anisakis simplex* species in the population of Northern Morocco. *J Invest Allergol Clin Immunol* 2012;22:514–9.
- Kim J, Jo JO, Choi SH *et al.* Seroprevalence of antibodies against *Anisakis simplex* larvae among health-examined residents in three hospitals of southern parts of Korea. *Korean J Parasitol* 2011;49:139–44.

- 26 Fernández de Corres L, del Pozo MD, Aizpuru F. Prevalence of *Anisakis simplex* sensitisation in three Spanish areas, in relation to the fish intake rates. *Alergol Immunol Clin* 2001;16:337–46.
- 27 Daschner A, De Frutos C, Valls A, Vega de la Osada F. Different clinical presentation of *Anisakis simplex* associated urticaria is dependent on the frequency of raw fish intake. *Allergol Immunopatbol (Madr)* 2010;38:166–7.
- 28 AAITO-IFIACI Anisakis Consortium. Anisakis hypersensitivity in Italy: prevalence and clinical features: a multicenter study. *Allergy* 2011;66:1563–9.
- 29 Lunestad BT. Absence of nematodes in farmed Atlantic salmon (*Salmo salar* L.) in Norway. *J Food Prot* 2003;66:122–4.
- 30 Levsen A, Lunestad BT. *Anisakis simplex* third stage larvae in Norwegian spring spawning herring (*Clupea harengus* L.), with emphasis on larval distribution in the flesh. *Vet Parasitol* 2010;171:247–53.
- 31 Stromnes E, Andersen K. Distribution of whaleworm (*Anisakis simplex*, Nematoda, Ascaridoidea) L3 larvae in three species of marine fish; saithe (*Pollachius virens* (L.)), cod (*Gadus morhua* L.) and redfish (*Sebastes marinus* (L.)) from Norwegian waters. *Parasitol Res* 1998;84:281–5.
- 32 Sanchez-Velasco P, Mendizabal L, Anton EM, Ocejo-Vinyals G, Jerez J, Leyva-Cobian F. Association of hypersensitivity to the nematode *Anisakis simplex* with HLA class II DRB1*1502-DQB1*0601 haplotype. *Hum Immunol* 2000;61:314–9.
- 33 Pravettoni V, Primavesi L, Piantanida M. *Anisakis simplex*: current knowledge. *Eur Ann Allergy Clin Immunol* 2012;44:150–6.