IgE sensitization to the fish parasite *Anisakis simplex* in Norway

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CONTENTS

SCIENTIFIC ENVIRONMENT

This work was performed at the Department of Clinical Science, University of Bergen and the Allergy Research Group of the Laboratory of Clinical Biochemistry (LKB), Haukeland University Hospital, Bergen, and the National Institute of Nutrition and Seafood Research (NIFES), Bergen and the Norwegian Veterinary Institute, Oslo.

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Aung Htun Lin

SUMMARY

Anisakis simplex is a fish parasite which infects many commercially important fish species worldwide. When ingested in contaminated raw or poorly prepared seafood, this parasite may lead to infection and/or allergic reaction in humans. During the last decade, cases of this food-borne infection and allergy have increased in countries with high fish consumption, causing an emerging public health problem. Norway is one of the high fish-consuming countries, but the situation of this parasite-related problem was still unknown. Therefore, we conducted the first pilot study in Norway to investigate the prevalence of IgE sensitization to *A. simplex*.

In this epidemiologic survey, the anonymized serum samples from newly recruited blood donors (designated as "BDO") and from patients with high serum IgE antibodies level ≥ 1000 kU\l (designated as "IGE+") were collected at the Haukeland University Hospital, Bergen. The sera were analyzed by the ImmunoCAP® method for total IgE and IgE antibodies against *A. simplex* and other possible cross allergens such as house dust mite (HDM), shrimp and cod. The results showed 2.0% and 37% of IgE sensitization to *A. simplex* in BDO and IgE+ groups, respectively. But these prevalences might be overestimated owing to cross-sensitization. Later on, we performed an extended study using larger population sizes and a recently developed more specific analysis method. This test is an enzyme-linked immunosorbent assay (ELISA) which utilizes 2 recombinant (r) major and specific allergens of *A. simplex*, namely rAni s 1 and rAni s 7 as target antigens. SDS-PAGE and Western immunoblotting analyses were also performed. As expected, the results of the extended study showed even lower prevalences of sensitization than that obtained in the previous study. Whereas the prevalences by the ImmunoCAP® method were 0.4% and 16.2%, analyses with the more specific test showed very low incidences of 0.0% and 0.2% in the BDO and IGE+ groups, respectively. Cross-reactivity analyses also suggested that most of the ImmunoCAP® positive sera were probably false-positive due to cross-sensitization to HDM and shrimp. The 0.0% prevalence in the BDO group may indicate an absence of sensitization against the fish parasite *A. simplex* in the healthy population. On the other hand, the frequency of 0.2% in the IGE+ group may either represent a certain degree of true sensitization in the "allergic" population or maybe still due to cross-sensitization. The low prevalence of *A. simplex* sensitization in Norway compared to other high-fish consuming countries might be partly explained by the relatively low consumption of raw or uncooked fish among the Norwegians and the low genetic susceptibility of Norwegian population to *A. simplex* allergens.

With respect to food safety, certain regulations for storing and preparing the fish have been implemented to kill this commonly occurring nematode in fish. However, among *Anisakis* allergens, several heat- and pepsin-resistant proteins have been shown to remain active even after cooking or digestion. The ingestion or inhalation of or contact with *A. simplex* allergens has been claimed to evoke allergic symptoms in sensitized individuals.

While directly contaminated fish were accounted for most allergic cases, indirect contamination of food via carry-over from contaminated feed has also been reported to be involved. For this reason, we conducted a zebrafish (*Danio rerio*) feeding trial to investigate the transmissibility of *A. simplex* allergens from feed to fish. Zebrafish was chosen as the study object owing to their rapid maturation and favorable small size affording less space and feed than edible fish such as salmon or trout. The results of feed analyses by both ELISA and immunostaining indicated that certain *A. simplex* allergens have been retained throughout feed processing, confirming the presence of heat and degradation-resistant proteins of this parasite as reported in other studies. The trace amounts of *A. simplex* peptides in the fish flesh were detected by both immunostaining and LCMSMS, thereby providing a positive proof for the transmissibility of *A. simplex* allergens from feed to fish. Nevertheless, considering the relatively higher effect dose of *A. simplex* allergens compared to other food allergens, the low amounts of *Anisakis* proteins transferred via contaminated feed to fish probably do not represent a major food safety problem for consumers with *A. simplex* allergy.

In conclusion, our seroprevalence studies indicated a very low sensitization against the fish parasite *A. simplex* in the studied Norwegian populations as compared with other high fish-consuming countries. Our zebrafish feeding trial suggested the transmissibility of *A. simplex* allergens from feed to fish, but only the low amounts were detected. Given the results from our surveys, the fish parasite *A. simplex* is apparently not an immediate food safety and public health problem in Norway.

LIST OF PUBLICATIONS

1. 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 Apr;75:431-5.
2. Lin AH, Nepstad I, Florvaag E, Egaas E, Van Do T. An extended study of seroprevalence of anti-*Anisakis simplex* IgE antibodies in Norwegian blood donors. Scand J Immunol. 2014 Jan;79(1):61-7.
3. Christiane Kruse Fæste, Arne Levsen, Aung Htun Lin, Natalia Larsen, Christin Plassen, Anders Moen, Thien Van Do, Eliann Egaas. Fish feed as source of potentially allergenic peptides from the fish parasite *Anisakis simplex* (sl). (Submitted)

ABBREVIATIONS

Ab	Antibody
Ag	Antigen
CCD	Cross-reactive carbohydrate
CE	Crude extract
ELISA	Enzyme-linked immunosorbent assay
ES	Excretory secretory
GAA	Gastro-allergic anisakiasis
HDM	House dust mite
Ig	Immunoglobin
kDa	Kilo daltons
L3	Third-stage larvae
LCMSMS	Liquid chromatography mass spectrometry
MW	Molecular weight
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
rAni s	Recombinant *Anisakis Simplex* allergen
SDS-PAGE	Sodium dodecyl sulpahte-polyacrylamide gel electrophoresis
SPT	Skin prick test

1. INTRODUCTION

1. 1. Allergy and sensitization

The European Academy of Allergology and Clinical Immunology (EAACI) has introduced ‘‘A Revised Nomenclature for Allergy” in 2001, in order to standardize the terminology of allergy based on the mechanism initiating the reaction [1]. The report has gained substantial international recognition including the World Allergy Organization (WAO) [2].

- The term ***hypersensitivity*** should be used to describe *objectively reproducible symptoms or signs initiated by exposure to a defined stimulus at a dose tolerated by normal persons*. ***Sensitivity*** is an acceptable alternative in special circumstances.
- ***Allergy*** is *a hypersensitivity reaction initiated by specific immunologic mechanisms*. The term ***nonallergic hypersensitivity*** should be used in cases where other mechanisms are involved.
- An ***antibody*** (Ab), also known as immunoglobulin (Ig), is a protective protein produced by the immune system upon exposure to any foreign substance called ***antigen*** (Ag).
- ***Allergens*** are referred to antigens which consequently lead to allergic manifestations, for examples, pollens, dust mite, molds, danders and certain foods.
- The term ***sensitization*** is used when a previously encountered foreign substance triggers an immune reaction resulting in a positive allergy test, but possibly without apparent allergic symptoms. For example, a person might show a positive specific antibody to a food but not experience allergic reactions when that food is eaten. People with nasal or multiple allergies might also show false positive food allergy test without having apparent symptoms after eating those foods.

1. 2. Immune mechanism of IgE sensitization and allergy [Fig. 1]

Allergy can be mediated by antibody or cell. In IgE-mediated (type I) allergy, the responsible antibody belongs to immunoglobulin type E, as seen in cases with allergic symptoms from mucosal membranes in the airways and gastrointestinal tract. In none IgE-mediated allergy, allergen-specific lymphocytes or antibodies of the IgG isotype account for the allergic reactions.

In the initial or sensitization phase, type I hypersensitivity reaction occurs; an Antigen-Presenting Cell (APC) presents the allergen entering for the first time to the immune T helper cell called a TH_2 lymphocyte. These interleukin-4 (IL-4) secreting TH_2 cells interact with B lymphocytes which begin to produce a large amount of IgE antibodies. Mast cells and basophils get sensitized when these circulating IgE antibodies bind to specific receptors on their surfaces. Upon later exposure, the same allergen can trigger an acute inflammatory response by directly binding to the IgE molecules already coated on the surface of the mast cells or basophils. Cross-linking of the IgE and Fc receptors activates the sensitized immune

cells which degranulate and release histamine and other inflammatory mediators such as cytokines, interleukins, leukotrienes, and prostaglandins into the surrounding tissue [3]. This event leads to vasodilation, mucous secretion, nerve stimulation, and smooth muscle contraction, resulting in such typical allergic symptoms as rhinorrhea, itchiness, dyspnea, and even anaphylaxis in most severe cases. Sometimes after the acute phase, a late or chronic response may occur when other immune cells such as neutrophils, lymphocytes, eosinophils and macrophages get involved by migrating to the initial site.

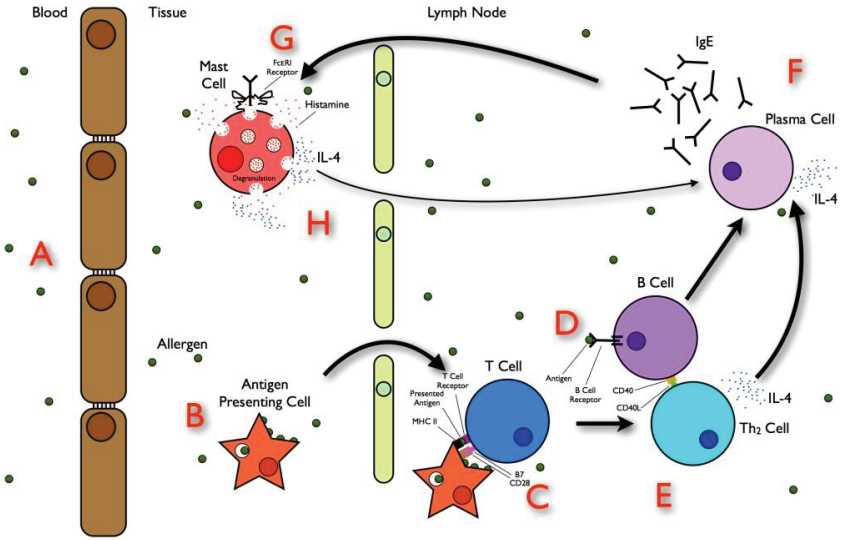


Figure 1. Immune mechanism involved in sensitization and allergy development [3].

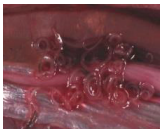
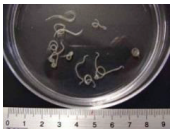
1. 3. Food allergy

Any food protein can trigger an adverse immune reaction and cause food allergy. Even though allergic cases to many different kinds of foods have been reported, most of these reactions have been attributed to only a small group of foods such as eggs, milk, peanuts, soy, fish, shellfish, tree nuts, and wheat [4]. Seafood may also contain potent allergens and cause adverse reactions in sensitized subjects. Fish and shellfish allergies have gained more importance owing to increased popularity of seafood dishes particularly in certain parts of the world [5]. Many commercially important fish species are infected with the nematode *Anisakis simplex* [6]. This parasite, when ingested in contaminated seafood, may lead to an allergic reaction in individuals who produce IgE antibodies against its antigenic proteins [6]. Therefore, in certain allergic cases following seafood consumption, the allergens of the fish parasite *Anisakis simplex* should also be tested in addition to fish allergens since some patients develop allergic reactions to this fish parasite rather than the fish itself [6].

1. 4. *Anisakis* infection and allergy

Anisakis infection, also known as anisakiasis, is a fish-borne zoonotic disease caused by the consumption of raw or poorly prepared seafood which was infested with the parasite *Anisakidae* family, in particular *Anisakis simplex*, *Anisakis pegreffii* and *Pseudoterranova decipiens*. Third-stage larvae (L3) of the parasites are found on the visceral organs, mesenteries and peritoneum of the fish and they appear as flat or tight coils which are approximately 2 cm long [Fig. 2]. Occasionally, some larvae may migrate from the abdominal cavity and burrow deep into the fillet [7]. At this stage, the worms are very difficult to be detected by plain visual inspection [8].

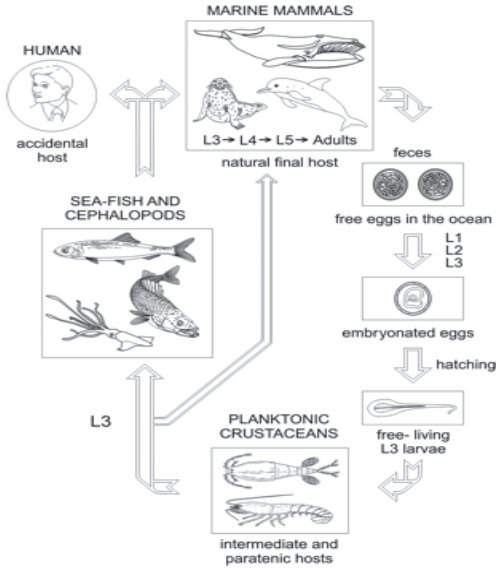
Figure 2. Anisakid nematode larvae;
(a) In the viscera of fresh unfrozen fish
(b) In the petridish showing their lengths

(a)  (b) 

(a) http://bioweb.uwlax.edu/bio203/s2009/papendie_andr/images%202/Anisakids.jpg
(b) http://www.lavinium.com/laviniumblog/*Anisakis_Simplex*.jpg

Anisakis species have a complex life cycle involving a number of hosts [Fig. 3]; their adult stages live embedded in the mucosa of the stomach of marine mammals (whale, seal or dolphin). Adult females produce unembryonated eggs which are expelled through the faeces of the hosts. Embryonations take place in seawater and first-stage larvae (L1) are formed in the eggs. Later on they hatch into the second stage (L2) and these free-swimming larvae are ingested by krill and crustaceans. In these intermediate hosts, they mature into the third stage (L3) which is further transferred to other hosts such as fish and cephalopods, still in the L3 larvae form. When the infected fish or cephalopods are subsequently eaten by marine mammals, the larvae develop into adult worms in the final hosts, thus completing their complex lifecycle.

Figure 3. Anisakis life cycle (http://cmr.asm.org/content/21/2/360.figures-only)



Humans are accidental hosts and become infected upon consumption of raw or under-processed marine fish and cephalopods contaminated with the third stage larvae. The parasites cannot survive in human hosts and usually become regurgitated or expelled or dead in a few days or weeks. Usually within a few hours after ingestion, the burrowing of the worm into the intestinal wall and subsequent death results in an acute and transient infection manifested by such symptoms as abdominal pain, nausea, vomiting, and/or diarrhea [6] . The condition may sometimes mimic several other gastrointestinal disorders such as gastric ulcer and acute appendicitis [9]. At the same time, the parasite can trigger an immune response and provoke clinical manifestations of allergy in some sensitized patients, and this borderline disease with simultaneous clinical manifestations of infection and allergy is termed "gastroallergic anisakiasis" [10]. If the larva passes further into the bowel or large intestine, it may lead to eosinophilic granulomatous response after 1-2 weeks and cause more chronic symptoms [6].

Regarding the *Anisakis* allergy, there has been an interesting debate on whether live or dead larva is necessary to induce the reaction [11, 12]. Several heat- and pepsin-resistant *Anisakis* allergens have been shown to remain active even after cooking or digestion [13, 14]. Therefore, the intake of dead parasite or even residues in contaminated fishery products might cause allergic symptoms. Additionally, inhalation of or contact with *A. simplex* allergens has been reported to cause occupational allergies in fishery and aquaculture workers, fishmongers and cooks etc. [15, 16]. On the other hand, it has been reported that allergic sensitivity to *A. simplex* is initiated by an active infection with live parasite [17]. According to some studies on oral challenges with thermally treated *Anisakis* larvae, the patients did not show any allergic or gastric symptoms during or after the challenge [18, 19]. It is generally accepted that an active infection associated with viable larva is required to induce allergic symptoms in the majority of cases [20].

1. 5. *Anisakis* allergens

Based on the morphological source of the parasite, *Anisakis* allergenic proteins can be defined in three groups [20]; (1) somatic allergens obtained from the larva whole body, (2) Excretory/Secrotory (ES) allergens secreted during larva penetration, and (3) cuticular allergens secreted to protect its body from digestive juices. However, patients could be exposed to different *Anisakis* allergens depending on the larva's status; patients are possibly exposed to all parasite antigens in cases of active penetration of the larva and its subsequent death, or patients are exposed only to ES allergens in cases where there is an expulsion of the parasite intact through the gastrointestinal tract, or patients are mainly exposed to somatic and cuticular allergens and minimally to ES allergens if the larva contained in food is already dead [20].

To date, many *A. simplex* allergens have been described and most of them have been well characterized by molecular methods (Table 1) [12]. Ani s 1 and Ani s 7 are the important

major allergens of *A. simplex* which are recognized by >50% of patients [21, 22]. Ani s 1 is an ES allergen with a molecular mass of 24 kDa, and shared certain similarities in amino acid sequence with Kunitz-type serine protease inhibitors, but without appropriate inhibitory activity [23]. It is highly specific for *Anisakis* allergic patients and was recognized by serum IgE antibodies in 67-85% of patients with gastroallergic anisakiasis, but not by asymptomatic individuals [23]. Ani s 7 is also an ES product of approximately 139 kDa, and is a glycoprotein which lacks any significant homology with other known allergens. This highly specific molecule was recognized by serum IgE antibodies in up to 100 % patients with *Anisakis* allergy [22, 24]. The two pan-allergens associated with *Anisakis* are Ani s 2 (100 kDa) and Ani s 3 (41 kDa), and they are the muscle protein paramyosin and tropomyosin, respectively [25, 26]. Due to a high degree of homology with other muscle proteins, these allergens are considered to be primarily responsible for cross-reactivity between *Anisakis* and other invertebrates, such as crustaceans, house dust mite, cockroaches and insects [27, 28].

Table 1. Characterized allergens of *Anisakis simplex* [12].

	Allergen	MW (kDa)	Compartment	Function	Positivity	Major allergen	Pan-allergen	Refs.
1	Ani s 1	24	ESP	Kunitz-type trypsin inhibitor	85%	Yes		[21]
2	Ani s 2	97	Somatic	Paramyosin	88%	Yes	Yes	[25]
3	Ani s 3	41	Somatic	Tropomyosin	4%?		Yes	[26]
4	Ani s 4	9	ESP	Cystatin	27%			[29]
5	Ani s 5	15	ESP	SXP/RAL protein	25–49%			[30]
6	Ani s 6	7	ESP	Serpin	18%			[30]
7	Ani s 7	139	ESP	Glycoprotein	83–100%	Yes		[22]
8	Ani s 8	15	ESP	SXP/RAL protein	25%			[31]
9	Ani s 9	14	ESP	SXP/RAL protein	13%			[32]
10	Ani s 10	22	Somatic?	?	39%			[33]
11	Ani s 11	55	Somatic?	?	47%			[34]
12	Ani s 11-li	?	Somatic?	?	?			[34]
13	Ani s 12	?	?	?	57%	Yes		[34]

MW, molecular weight; ESP, protein from excretory–secretory products; Major allergen, *A. simplex* protein recognized by IgE antibodies in >50% of patients; Pan-allergen, highly conserved protein, which can explain cross-reactivity with other food sources;? = unknown.

Recently, high-resolution protein purification methods and immunoscreening of protein-expressing cDNA libraries or phage display systems constructed from *A. simplex* larvae have detected several new allergens; namely, Ani s 24 kDa, Ani s CCOS3, Ani s Cytochrome B, Ani s FBPP (Fructose 1,6-bisphosphatase), Ani s NADHDS4L, Ani s NARaS, Ani s PEPB, Ani s Troponin, but they have yet to be confirmed experimentally [35]. A new study combining IgE-based immunoscreening with mass spectrometry-based proteomics, has also characterized a number of potential novel allergens of this fish parasite including myosin,

heat shock protein 70, α-amylase, disulphide isomerase, myophilin, enolase, arginine kinase, haemoglobin and fructose-1,6-biphosphate aldolase 1 [36].

1. 6. Prevalence

The consumption of seafood products has increased considerably over the last decades due to the development of new modes of aquaculture, packaging, transport, population growth and globalization. According to the World Health Organization (WHO) statement, the average apparent consumption per capita has nearly doubled (from ~ 9 kg to 16 kg) in the period of 40 years (from 1960-1997) [37]. Majority of the fish consumed (~2/3) is supplied by marine captures and the rest is attained from aquaculture. All wild-caught marine and freshwater fishes carry the risk of *Anisakis* nematode infections [38]. Therefore, consumers are considered to be more at risk of anisakiasis from eating wild fish than farmed fish.

The first case of *Anisakis* infection in human was reported in the Netherlands by Van Thiel et al., in a patient who suffered from acute abdominal pain after eating raw herring [39]. Since then, more than 20,000 cases have been reported worldwide, most frequently in the countries where fish is consumed raw, lightly pickled or salted. The country with the highest prevalence (~90%) of *Anisakis* infections is Japan, with 2000-3000 cases being reported annually [20]. Traditional raw fish dishes such as sushi and sashimi when contaminated with *Anisakis* are a significant source of human infections in Japan. Other regional cuisines, such as salted or pickled herring (the Netherlands and Scandinavia), gravlax, cod livers (Scandinavia), boquerones (Spain), marinated and/or raw fish (Italy), lomi-lomi (Hawaii) and ceviche (South America), have also been associated with anisakiasis in humans [40]. The globalization of provincial cuisine, development of better diagnostic tools and greater awareness has led to more frequent reporting of anisakiasis in those countries as well as in other countries like Korea, Australia, China, Croatia and the United States of America [41-45]. In Norway, only few cases of human anisakiasis have been recorded in association with the intake of raw or only lightly processed fresh marine fish [46, 47].

1. 7. Diagnosis and treatment

The diagnosis of anisakiasis and/or *Anisakis*-related allergy can be made upon a compatible history with typical symptoms following seafood intake, a positive skin prick test (SPT) and/or positive specific IgE test against *Anisakis* and a negative history and tests for allergy to fish and/or other possible cross-reactive allergens (dust mites, crustaceans, insects) [20]. The definite diagnosis can be made by gastroscopic finding of the *Anisakis* nematode or by histopathological examination of tissue removed at biopsy or during surgery. The treatment can also be done at the same time with diagnostic endoscopy by removing the larva itself or the affected tissue in case of eosinophilic granuloma formation [20]. Other alternative therapies with various compounds such as albendazole and

monoterpenic derivatives from several essential oils have been reported in several in-vitro and in-vivo studies showing their different degrees of therapeutic efficacies [48-50].

1. 8. Prevention

The most important prevention method is to raise the awareness of consumers and producers about the existence of anisakid worms in fish and to recommend avoiding the consumption of raw or poorly cooked, marinated, or salted marine fish or squid. Anisakid larvae can be easily killed by adequate cooking at temperatures > 60°C or freezing, but most probably not by salting and marinating. The US Food and Drug Administration (FDA) recommends that all shellfish and fish intended for raw consumption should be blast frozen to -35°C or below for 15 hours or be regularly frozen to -20°C or below for seven days [51]. Other countries in EU, UK, Australia and Canada also have similar national food safety guidelines [38]. Yet, The current preventive regulations may not be enough for some patients who are particularly sensitized to heat-stable *Anisakis* allergens and these patients might need to avoid marine fish altogether [11, 52]. However, several studies have indicated that proper-thermally treated fish consumption is tolerated by the majority of patients [18, 19]. Concerning the detection of parasites in food production, visual inspection of unprocessed fish gut cavity was implemented but was shown to be unreliable as a Hazard Analysis Critical Control Point (HACCP) [53]. Other methods such as UV fluorescent imaging [54], real-time PCR [55, 56], immunoblotting [57] and enzyme-linked immunosorbent assay (ELISA)[58, 59] have been proven effective in the detection of anisakids in fresh and processed fish products.

10. AIMS OF THE STUDY

The main aims of the studies were:

1. To study the prevalence of IgE sensitization to the fish parasite *A. simplex* in the Norwegian Population
2. To investigate the transmissibility of *A. simplex* allergens from feed to fish

MATERIALS AND METHODS

3. 1. Patient 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. One group consisted of sera from newly recruited blood donors (designated "BDO"), and the other of patient sera from the routine Allergy laboratory (designated "ALL"). All sera were collected consecutively and anonymized; thus, no information was available on patient history. The latter group was further divided into three series; the first one sampled unsorted without any additional information on analytical results (designated "USO"), the second similarly sampled apart from sorting out sera with Phadiatop® (Thermo Fisher Scientific Inc, Uppsala, Sweden) positive results ≥0.35 kU_A/l (designated "PHA+"), and the third sampled like the former but including only sera with total IgE levels ≥1000 kU/ l (designated "IGE+"). For comparison, PHA+ patient sera were similarly collected at the Clinical Allergy Research Unit of the Karolinska University Hospital, Stockholm, Sweden. As positive control for the presence of IgE antibodies against *A. simplex*, another group of patient sera from Spain was included and they were clinically clearly characterized as gastro-allergic anisakiasis (designated "GAA"). The immunoCAP® (Thermo Fisher Scientific Inc, Uppsala, Sweden) negative sera for *A. simplex* IgE antibodies among the BDO group were used as negative control (designated "NEG"). The collection of sera was approved by the respective committees for medical research ethics. (See publications 1 and 2)

3. 2. Rabbit serum polyclonal antibodies

Additionally, we also used rabbit polyclonal IgG antibodies against *A. simplex* CE (designated "RAB"), developed by Charles River Laboratories, Research Models and Services, Stolzenseeweg, Germany. (See publication 2)

3. 3. *Anisakis* and other allergen crude extracts

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 whitings (*Micromesistlus poutassou*). Larvae were rinsed in physiological saline, frozen in liquid nitrogen and smashed by mortar. After being added 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 18,000g, the supernatant was dialyzed 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 similar extraction method described above. (See publication 2)

3. 4. Zebrafish, feed and design of the experiment

The zebrafish used in the feeding trial were young adults of a F4 generation of the "Tupfel long-fin" wild-type strain line (ZFIN ID: ZDB-GENO-990623-2). For this experiment, three types of feed were prepared; the first group (F1) containing basic commercial zebrafish feed (Aqua Schwarz GmbH, Göttingen, Germany), gelatin and freeze-dried *A. simplex* larvae, the second group (F2) containing basic zebrafish feed, gelatin and fish meal (Nofima AS, Bergen, Norway), and the third group (F3) consisting of basic zebrafish feed and gelatin. Feeds were analyzed for *Anisakis* content using ELISA, PCR, LCMSMS and Immunostaining. Based on the respective type of feed given, the zebrafish were accordingly divided into the three different trial groups as Z1, Z2 and Z3.

The trial setup consisted of totally six plastic tanks (i.e. two tanks for each of three groups) with continuous slow water exchange. At trial start, each tank was stocked with 15 randomly chosen zebrafish which had been exclusively fed with commercial zebrafish meal before the experiment. The fish were fed twice a day (10 mg per fish) throughout the 2-weeks trial period. At each sampling, five zebrafish were randomly removed from each tank and then transferred to the laboratory for immediate sample extraction. After removing the heads, gills and the visceral organs, the remaining carcasses were thoroughly washed and then stored in small sealed plastic bags in -20°C freezer. The samples were then analyzed for the presence of *A. simplex* protein traces by ELISA, LCMSMS, and Immunostaining. (See publication 3)

3. 5. ImmunoCAP® and Enzyme-Linked Immunosorbent Assay (ELISA)

The serum levels of IgE and IgE antibodies against *A. simplex* (*Anisakis* spp.), house dust mite (HDM) (*Dermatophagoides pteronyssinus*), cod (*Gadus morhua*), shrimp (*Pandalus borealis*), crab (*Cancer pagurus*), brine shrimp (*Artemia salina*) and shrimp tropomyosin (*Penaeus aztecus* tropomyosin) were analyzed using the ImmunoCAP® system following the instructions of the manufacturer. Briefly, this system consists of ImmunoCAP® flexible hydrophilic CNBr-activated cellulose derivative to which the target allergen preparation is attached. Specific IgE antibodies are first detected by incubation with serum samples and then revealed using an anti-IgE MAb labeled with β-galactosidase, which generates fluorescence. The threshold level for *A. simplex* sensitization was ≥0.35 kU_A/l. (See publications 1 and 2)

Patient sera which were *A. simplex* positive by the ImmunoCAP®, were further analyzed for IgE antibodies against two major and specific *A. simplex* antigens, rAni s 1 and rAni s 7, by utilizing the commercial Trisakis 170 ELISA kits (Laboratory of Parasitology, Faculty of

Pharmacy, University of Santiago de Compostela, Santiago de Compostela, Spain) [60]. 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. (See publication 2)

For the detection of *A. simplex* protein in zebrafish and different types of feed, samples were analyzed using a previously developed polyclonal sandwich ELISA which specifically detects *A. simplex* proteins [59]. Fish and feed samples (2 g) were homogenized and extracted under shaking with PBS pH 7.4 (Oxoid, Basingstoke, UK) at room temperature for 1 hour. Extracts were diluted at least 1:20 in PBS before analysis. The standard curve of the ELISA was constructed with 12 concentrations (from 0 to 1000 mg/l) of PBS-extracted total *A. simplex* protein. (See publication 3)

3. 6. SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) and IgE/IgG–immunoblotting

In the seroprevalence study, 3 allergen extracts; *A. simplex*, HDM and shrimp, were separately blotted with 5 serum pools; the ImmunoCAP® positive BDO, IGE+, GAA, NEG and RAB sera. First, the extracts along with molecular weight (MW) standards were separated by SDS-PAGE by resolving in a 12% separating gel at 200 V for 45-55 min. Proteins were visualized by Coomassie brilliant blue R-250 staining. For immunoblot analyses, proteins were transferred onto nitrocellulose membranes using a minitrans-blot cell (BioRad Laboratories, Hercules, CA, USA) at 100 V for 1 hour. Immunodetection was then performed with the serum pools. (See publication 2)

For the detection of *A. simplex* protein in zebrafish and feed, *A. simplex* standard protein and zebrafish or feed samples were analyzed by gradient gel electrophoresis and subsequent immunostaining with either polyclonal anti-*A. simplex* IgG antibodies (IgG_1) (self-produced in rabbit against semi-purified *A. simplex* extract) or serum of a Spanish patient with *A. simplex* allergy (IgE_1). In the second experiment, a serum pool (IgE_2) from ten Spanish gastro-allergic anisakiasis patients or rabbit polyclonal IgG antibodies against *A. simplex* (IgG_2) (Charles River Laboratories, Sulzfeld, Germany) were used. (See publication 3)

3. 7. Real-time polymerase chain reaction (rtPCR) assay

The rtPCR analyses were performed for the detection of *A. simplex* DNA-content in the different feed types. First, DNA was isolated from *A. simplex* larvae for the preparation of standard DNA and from feed samples using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). The extracted DNA was then purified using the Wizard DNA Clean-Up System and eluted with sterile water. The final DNA concentration was measured by absorbance at 260 nm. Positive controls of 5 and 10 ng/µl *A. simplex* DNA, negative extraction controls and water controls were included in all assays. In total 100 ng purified DNA were analyzed per feed sample in duplicates in multiple assays. The rtPCR assays were performed as described in Lopez and Pardo's study [61]. (See publication 3)

3. 8. Liquid chromatography tandem mass spectrometry (LCMSMS)

For the detection of *A. simplex* protein, the fish and feed samples were prepared and analyzed by LCMSMS as described earlier [62]. Protein extracts (50 µl/sample, 1 mg/ml) were digested with trypsin overnight at 37°C on ultrafiltration filters and peptides were eluted, dried and re-dissolved in 20 µl 0.1 % formic acid. Peptides (3 µl/sample) were injected with 10 µl/min onto Zorbax 300 SB-C18 pre-column, separated with 0.2 µl/min on a GlycproSIL C18–80Å column using a gradient from 5 to 55% acetonitrile in water/0.1 % formic acid in 68 min, and analyzed on a nano-electrospray LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Mass spectra were acquired in the positive ion mode in the mass range of m/z 200–2000, followed by MS/MS using collision-induced dissociation of the most intense parent ions with 10 ppm accuracy and 3 m/z isolation width. Data analysis was performed by Xcalibur V2.0. Previously identified marker peptides [62] of *A. simplex* haemoglobin were extracted with 10 ppm accuracy and spectra were manually verified. Standard *A. simplex* protein in buffer was used for semi-quantitatively extern calibration. Zebrafish and feed samples with and without *A. simplex* contamination were analyzed and compared. (See publication 3)

3. 9. Statistical analysis

The difference in prevalences of sensitization between the groups and genders was determined using Mann–Whitney U test. Spearman's rank test was used to evaluate correlation between total IgE, IgE antibodies to *A. simplex* and CCDs in addition to the association between IgE antibodies to *A. simplex* and age. Means and standard deviations, medians and interquartile ranges (IQR) for IgE antibodies were calculated to assess the levels of sensitization among the groups. *P* values of < 0.05 were considered statistically significant and all statistical analyses were carried out with Statistica software program version 9 (StatSoft, Inc., Tulsa, OK, USA).

4. SUMMARY OF RESULTS FROM THE PUBLICATIONS

4. 1. Publication 1

IgE sensitization to the fish parasite *Anisakis simplex* in a Norwegian population: a pilot study

Prevalences and levels of IgE sensitization: The prevalences were similar in the BDO and USO groups (2.0% and 2.2%, respectively), but lower than that of the PHA+ group (6.6%). The prevalence of IgE antibodies to *A. simplex* in the Swedish PHA+ sera (13.5%) was significantly higher than that of Norway (6.6%). Among the Norwegian IGE+ sera, 37.0% were sensitized to *A. simplex*. The mean levels of IgE antibodies to *A. simplex* in all serum groups were found to lie within the range of 0.7–3.5 kU_A/l, indicating a low level of sensitization.

Cross-reactivity and unspecific binding: In eight of the nine samples with the highest IgE antibody levels to *A. simplex*, the levels of IgE antibodies to HDM and/or shrimp were higher than that of *A. simplex*. Levels of IgE antibodies to cod were low in all samples. A weak positive correlation between *A. simplex*-specific IgE and total IgE was observed (Spearman's rank test, r = 0.2843, *P* = 0.00001). Analyses for cross-reactive carbohydrate determinants revealed no significant association between the antibody levels to *A. simplex* and CCDs (Spearman's rank test, r = 0.0145, *P* = 0.9212).

Age and gender: The mean age of all the *A. simplex* positive serum donors was 30 years, (range: 1–88 years), but no significant association between patient age and sensitization was observed (Spearman's rank test, r = 0.0612, P = 0.6667). Our results also showed no difference in the prevalence of sensitization between the genders (Mann–Whitney U test, P > 0.54).

4. 2. Publication 2

An extended study of seroprevalence of anti-*Anisakis simplex* IgE antibodies in Norwegian blood donors

Prevalences and levels of IgE sensitization: The ImmunoCAP® results showed that 0.4% prevalence in the BDO group was significantly lower than 16.2% of the IGE+ group, as expected. The medians of anti-*Anisakis* IgE-antibody levels were 0.45 kU_A/l (IQR 0.37–0.52) and 1.25 kU_A/l (IQR 0.6–3.22) in the BDO and IGE+ groups, respectively, indicating a low sensitization levels in both groups. However, there was a significant difference of the medians between the two groups (Mann–Whitney U-test, P < 0.05). The GAA group expectedly 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). All the BDO sera were further tested for rAni s 1 and rAni s 7 allergens, using Trisakis ELISA and the results were all negative (i.e. 0.0%

prevalence). The Trisakis ELISA analyses of the immunoCAP® positive sera of the IGE+ group showed only one highly positive sample, giving a 0.2% prevalence of sensitization.

Cross-reactivity analyses: Three of four ImmunoCAP® positive sera from the BDO group showed higher IgE levels to the cross-allergens HDM and/or shrimp than to *A. simplex*. They all had low levels of anti-*Anisakis* IgE antibodies.

SDS-PAGE and Western blottings: A correlation was observed between band intensities and IgE-antibody levels determined by the ImmunoCAP® analyses. 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 several similar but slightly stronger bands to HDM and shrimp extracts. Distinct and more bands to *A. simplex* CE were revealed in the GAA pool, but no obvious bands were seen to HDM or shrimp extracts. The NEG pool demonstrated no apparent bands to any of the allergen extracts whereas the RAB pool revealed many distinct bands to *A. simplex* CE and several similar but weaker protein bands to HDM and shrimp extracts.

4. 3. Publication 3

Fish feed as source of potentially allergenic peptides from the fish parasite *Anisakis simplex* (sl)

Content of A. simplex proteins in different feed types: The different analysis methods; rtPCR, ELISA, immunostaining and LCMSMS were consistent and mutually corroborative detecting a high level of *A. simplex* substances in the feed type F1, but none in F2 and F3. The parasite DNA contents were determined to be 63 pg/100ng in F1, but < 0.4 pg/100ng in both F2 and F3 by the rtPCR analyses. The ELISA results showed the *A. simplex* protein content of > 10000 mg/kg in F1, but < 5 mg/kg in F2 and F3. The immunoblotting of the three feed samples with four antibody preparations showed coherent results although different *Anisakis* protein bands were detected by the individual fractions. By the LCMSMS feed analysis, the two haemoglobin marker peptides HSWTTIGEEFGHEADK (m/z=615.27) and LFAEYLDQK (m/z=563.79) were detected with relative strong intensities ($2.3*10^6$ and $6.7*10^5$, respectively) in F1, but not in F2 and F3 ($< 10^2$).

Detection of A. simplex proteins in exposed zebrafish: Both the immunostaining and LCMSMS for contents of *A. simplex* proteins in the three different zebrafish trial groups (Z1-Z3) exhibited correlating results of positive read-outs for Z1 and negative for Z2 and Z3. However, The ELISA could not differentiate between the samples because the method, calibrated with a limit of quantitation at 2 mg/kg, was apparently not sensitive enough. In the immunostaining experiments with zebrafish samples, all the four antibody preparations (IgG_1, IgE_1, IgG_2, IgE_2) detected weak binding signals for Z1, but not in Z2 and Z3. The

LCMSMS analysis detected the apparently more sensitive *anisakid* haemoglobin marker LFAEYLDQK (m/z=563.79) with an intensity of $5*10^3$, but not the second marker in Z1 whereas no marker peptides were found in Z2 and Z3.

5. GENERAL DISCUSSION

5. 1. Seroprevalence of IgE sensitization to the fish parasite *Anisakis simplex*

During the last decade, cases of the fish parasite *Anisakis simplex* infection and allergy in human have increased in countries with high fish consumption. Norway is one of the high fish-consuming countries, but the situation of this parasite-related problem was still unknown. Therefore, we conducted the first pilot study in Norway to investigate the prevalence of IgE sensitization to *A. simplex*. The results showed that the prevalences of IgE sensitization to *A. simplex* was 2.0%, 2.2% and 6.6% in BDO, USO and PHA+ serum groups, respectively, indicating a lower incidence in the Norwegian population than in other high fish-consuming countries. But they might still be overestimated due to the possible false-positivity caused by the cross allergens. The employed ImmunoCAP® system, 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 [60]. Our cross-reactivity analyses also suggested a considerable degree of cross-sensitization to shrimp and HDM.

Recently, an ELISA method was developed using two major and specific recombinant allergens of *A. simplex*, namely rAni s 1 and rAni s 7, and was shown to provide currently the most specific and sensitive results [60]. Therefore in order to obtain a more accurate estimate of IgE sensitization to *A. simplex* in our region, we performed another extended study by utilizing this better serodiagnostic test and by enlarging the population size of blood donors (from 100 to 993). The results showed the prevalences of 0.4% and 16.2% in the BDO and IGE+ groups, respectively, as determined by the ImmunoCAP® method, but analyses with specific allergens showed much lower prevalences of only 0.0% and 0.2% in the respective groups. Cross-reactivity analyses suggested that most of the Immuno-CAP® positive sera are possibly false-positive due to cross-sensitization to shrimp and/or HDM or due to the unspecific binding of very high total IgE levels. This was as expected, also based on our previous pilot study findings. However, another possibility of being true-positive due to other major/minor *A. simplex* antigens which were not present in the employed tests cannot be ruled out. 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 [63]. On the other hand, the frequency of 0.2% in the IGE+ group, represented by a highly positive result, may indicate a certain degree of true sensitization in the “allergic” population.

Comparing the prevalences among the healthy populations in different countries, that of 0.4% in Norway is still much lower than 10% in Japan [64] and 22.1% in Spain [65]. High incidences, but in different population types were reported elsewhere; 5.1% in a randomly selected population in northern Morocco [66] and 5.0% in the health-examined

residents in southern Korea [67]. This low prevalence of *Anisakis* sensitization in Norway might be partly explained by the relatively low consumption of raw or uncooked fish among the Norwegians in spite of high total fish intake [68], and the low genetic susceptibility of Norwegian population to *A. simplex* allergens [69] .

A. simplex sensitization has been reported to be closely associated with high frequency of raw or uncooked fish consumption in several studies [70-72]. In this context, Norwegian fish diet, being largely composed of processed, canned and frozen products [68], can be considered to carry less risk of infection with *A. simplex* live parasite. Additionally, Norwegians consume a considerable amount of farmed fish in their raw or lightly processed fish dishes and one study has shown that anisakid nematodes seem to be absent from the flesh of farmed Atlantic salmon in Norway [73]. With regard to the genetic predisposition, a significant association has been shown between sensitization to *A. simplex* and the DRB1*1502-DQB1*0601 haplotype which is however absent or very rare in certain populations including Norwegians [69]. Nonetheless, many *A. simplex* allergens 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 [20]. Therefore, an option remains that our prevalence results might still not include certain subjects sensitized to other major and/or minor parasite allergens which are not present in the employed tests.

5. 2. Fish feed as source of potentially allergenic peptides from *Anisakis simplex*

Contamination of *A. simplex* proteins in food can pose health hazard to some consumers who are highly allergic to this fish parasite [38]. While most allergic cases were caused by directly contaminated fish [74], indirect contamination of food via carry-over from contaminated feed has also reported to be involved [75]. The evaluation of a contaminant's potential to be transported through the food chain is an integral part of risk assessment, and foreign proteins in a given product have to be consistently considered.

In the zebrafish feeding trial, the transmissibility of *A. simplex* peptides from feed to fish was investigated. Before being added to the trial feed, *A. simplex* larvae had been deep-frozen, freeze-dried, fine-grinded, and heated to 40°C for several hours. Yet, measurable *A. simplex* peptides or DNA fragments were detectable in the feed by all four specific detection methods used, indicating their stability in conformity with other earlier findings [13, 14, 76].

The trace amounts of *A. simplex* peptides in the fish flesh were detected by both immunostaining and LCMSMS whereas the ELISA method was not sensitive enough. The four different immunoblottings using patient and rabbit sera all revealed weak but distinct binding signals in the zebrafish extracts. These results were further confirmed by the

LCMSMS findings which showed that the most sensitive anisakid haemoglobin marker peptide was detected with a significant intensity. Considering the great specificity of the used high-resolution LCMSMS method, this result could be regarded as a positive proof for the transmissibility of *A. simplex* peptides from feed to fish.

6. CONCLUSIONS

- Seroprevalence of IgE sensitization to the fish parasite *A. simplex* appears to be very low in the Norwegian population in comparison with other high fish-consuming countries. At least, 0.0% prevalence in our blood donor group which may represent the general healthy population, suggests that *A. simplex* is apparently not an immediate public health problem in Norway.
- The detection of immunoreactive *A. simplex* peptides in the flesh of experimentally exposed zebrafish can be regarded as proof-of-principle that allergenic peptides may be transferred via the feed into the final animal food products. Nevertheless, considering the relatively higher effect dose of *A. simplex* allergens compared to other food allergens, the low amounts of *anisakid* proteins transmitted from the contaminated feed to the fish probably do not represent a major food safety problem for consumers with *A. simplex* allergy.

7. FURTHER RESEARCH PERSPECTIVES

- Similar prevalence studies could be carried out in other regions of Norway.
- Different populations such as allergic patients with unconfirmed diagnosis, or workers in fishery industry could be studied.
- Other major allergens of *A. simplex* parasite in addition to rAni s 1 and rAni s 7 could be included and studied in the immunoassays to develop better diagnostic tests.
- The zebrafish trial model could be conducted on commercially relevant fish species such as Atlantic salmon, over an extended exposure period and using feed with a lower *A. simplex* content in order to better reflect authentic feeding conditions in the marine aquaculture industry.

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