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Review Article

Effects of *Anisakis* Allergenic Proteins on Fish-Borne Food Safety Risks

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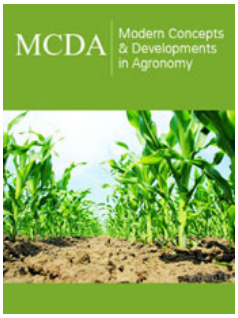
ISSN: 2637-7659



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Submission: April 30, 2021

Published: May 17, 2021

Volume 8 - Issue 4

How to cite this article: Wenqi Du, Dongli Dong, Yi Tan, William W Riley, Guang Yang, Xiyang Wu, Shuze Tang. Effects of *Anisakis* Allergenic Proteins on Fish-Borne Food Safety Risks. Mod Concep Dev Agrono. 8(4). MCDA. 000693. 2021. DOI: 10.31031/MCDA.2021.08.000693

Abstract

Anisakis (mainly *Anisakis* spp.) is a global public health and socio-economic concern. The infective third stage larva (L3) of these nematodes, which may be present in the muscle of fish and squid, is the etiological agent of an underestimated, emerging fish-borne zoonotic disease called anisakiasis (or anisakidosis). With the globalization of the seafood industry and the diversification of eating habits, anisakiasis is distributed worldwide, which seriously threatens human health. However, the risk of humans acquiring anisakiasis in developed countries with the habit of raw fish consumption appears to be underestimated.

Current knowledge of *Anisakis* as a food-borne parasite with special focus on *Anisakis* larvae infection in marine fish, life cycle, geographical distribution, epidemiology, allergenic protein residue of *A. simplex*, are critically reviewed. Research on residues from allergenic proteins of *Anisakis* remained in processed fish products and their health risks to susceptible consumers are discussed for prevention and control of this parasite.

Anisakis is ubiquitous in the world's oceans, but there are differences in the number and species of this nematode with geographical location. Conventional thermal processing like freezing, heating, smoke curing and non-thermal processing such as salting and pressure have shown potential effects on elimination or reduction of food allergenic proteins, however, similar effects on residual *Anisakis* allergenicity are unknown. Potential harm or risk of these parasitic allergenic proteins to consumers is scarce in the published literature. Further research on assessment and technical control of *Anisakis* allergenic proteins in fish-borne food is proposed for fish food safety in aquatic industry.

Keywords: *Anisakis*; Allergens; Anisakiasis; Prevalence of sensitization

Abbreviations: WHO: The World Health Organization; EFSA: The European Food Safety Authority; ES: Excretory-Secretory; IgE: Immunoglobulin E; FDA: The United States Food and Drug Administration; IUIS: International Union of Immunological Societies; GAA: gastro-allergic anisakiasis; ELISA: Enzyme-Linked Immuno Sorbent Assay

Introduction

The World Health Organization (WHO) estimates that more than 56 million cases of parasite infections associated with the consumption of fish products occur annually. There is a group of *Anisakids* among the parasites implicated, and it is responsible for an important foodborne zoonotic parasitic disease that seriously threatens human health [1]. *Anisakids* (mainly *Anisakis* spp.) are of public health and socio-economic concern globally. From the current 9 recognized species, *A. simplex sensu stricto (s.s.)* and *A. pegreffii* are the most frequently reported and hygienically relevant, as they are responsible for anisakiasis [2].

Marine mammals, such as whales and dolphins, are the final hosts of *Anisakis* species, and the adults reside within the stomach of the hosts, laying eggs that are excreted in the feces [3]. Subsequently, second-stage larvae hatch and are eaten by small crustaceans (krill)

before developing into third-stage larvae. These mature larvae are consumed by marine fish, eel and squid, and they then migrate into the viscera and peritoneal cavity. When these intermediate hosts are consumed by marine mammals, the larvae grow into adult worms, thus completing the lifecycle. Humans are incidental hosts, and the larvae cannot grow or replicate inside a human host (Figure 1).

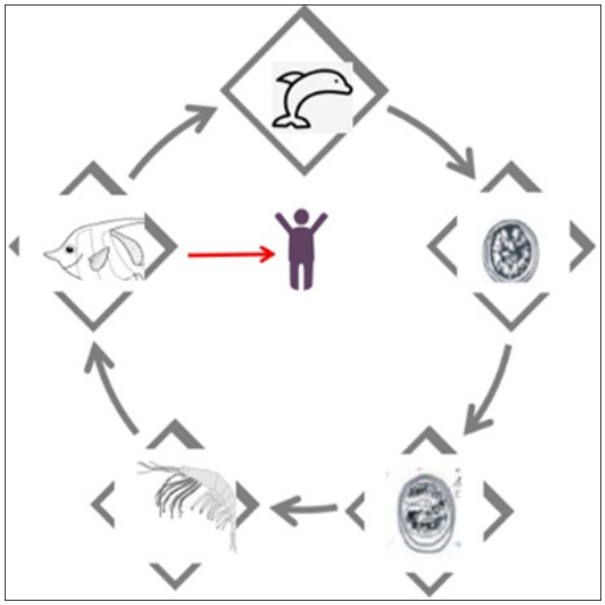

Figure 1: Life cycle of *Anisakis simplex*, including accidental human host.

Humans may be infected and develop disease after eating raw or under cooked parasitized fishery products, such as traditionally marinated anchovies in Spain (boquerones en vinagre) and Italy (alici marinate), Japanese sushi and sashimi (eaten worldwide) and South American ceviche.

Geographical and host distributions

Since 1960, when anisakiasis was first described, thousands of cases have been reported in Japan and hundreds in Europe, as well as in other parts of the world [4]. The European Food Safety Authority (EFSA) summarized that prior to 2010, approximately twenty thousand human cases have been reported worldwide, with most occurring in Japan and Spain [5]. However, this number is thought to be an underestimation, since in Spain alone, ~ 8,000 cases per year have been estimated [6]. The large majority of the world's cases are reported in Japan [5], where more than 20,000 people have been infected with this disease, and it is increasing at a rate of > 2,000 cases per year [7], due primarily to Japan's traditional raw culinary eating habits in association with one of the highest seafood consumption levels in the world. Other countries with high incidences of anisakiasis include the Netherlands and Germany [8]. China reported its first case of anisakiasis in 2013 [9]. Between 2010 and 2014, ~80 human anisakiasis reactions (the human allergy caused by *Anisakis* sp. infestation) have been recorded by the French, with 4-14 cases each year [10]. In Poland, the first instance of this disease was described in 2020 [11].

Anisakis is widely distributed, with infections of the terminal host or the intermediate host having been reported in all the world's major waters, but mainly distributed along the North Pacific and North Atlantic coasts and their islands. At present, more than 30 countries or regions have identified >200 species of fish and 25 species of cephalopods that can be infected with *Anisakis* [12]. Distribution of Anisakid larvae species differs between locations [13]. The presence of the final host plays a significant role, as each Anisakid species has a preference for certain marine mammal hosts which are linked to different geographic regions [14]. *A. simplex* is more abundant in the Atlantic Ocean and *A. pegreffii* predominates in the Mediterranean Sea [15]. *Pseudoterranova* spp. have only been reported in low numbers in the North East Atlantic and South Pacific [16]. Hybrids of *A. simplex* and *A. pegreffii* have been found in the North West Atlantic along with *A. simplex* and *A. pegreffii*. Species of fish that occur in multiple oceans have different larvae distribution profiles between oceans. For example, European hake (*Merluccius merluccius*) and blue whiting (*Micromesistius poutassou*) are predominantly infected with *A. simplex* in the Atlantic Ocean, while in the Mediterranean Sea, *A. pegreffii* (and to some level *A. physeteris*) infections were found in these two species [14].

The fish with the highest infection rates are cod (*Gadus morhua*) - 88%, herring (*Clupea harengus*) - 88%, rockfish (*Sebastes spp.*) - 86% [17]. While some fish species are more abundant in one specific ocean or sea, such as cod in the North Atlantic, or mackerel (*Scomber scombrus*) in the North Sea, most fish have a more widespread distribution, where differences in the prevalence of Anisakidae between the bodies of water are readily evident. For example, the prevalence of larvae in blue whiting and anchovy (*Engraulis encrasicolus*) are 23% and 1%, respectively, in the Mediterranean Sea compared to 92% and 78%, respectively, in the North East Atlantic Ocean. This could be related to the presence and infective status of the terminal host, the presence of the intermediate hosts, and the interactions among all hosts, which differ in each body of water [18]. The general occurrence of Anisakidae is thereby considered higher in the Atlantic Ocean compared to the Mediterranean Sea due to the higher abundance of cetaceans (final hosts), among other factors [19-21].

In the waters near East Asia, *Anisakis* also exhibits regional differences. *A. simplex sensu stricto* is mainly distributed off the Pacific coast of north western Japan, and *A. pegreffii* is mainly found in the area from the Sea of Japan to the coast of China [22]. Quiazon et al. [23] conducted a comprehensive survey of various marine fish in six different locations near Japan and found that *A. simplex sensu stricto* and *A. pegreffii* predominate around the coastline of Japan, although there are a small number of other species of *Anisakis* [24]. Among them, *A. simplex sensu stricto* was found distributed in the North and on the Pacific coast, and *A. pegreffii* was discovered in the South on the coast of the Sea of Japan. In the waters near South Korea, the infection rate of *A. pegreffii* in sea eels is 86.8%, while the infection rate of typical *Anisakis* is 7.8%, and 5.4% are unspecified, indicating that *A. pegreffii* has a selective advantage in the waters near South Korea (Table 1) [25-30].

Table 1: Investigation of *Anisakis* larvae infection in marine fish around the world.

Origin	Year	Anisakis	Fish species	Infection Rate (%)	Ref.
Ligurian Sea （Mediterranean Sea）	2013	*A. pegreffii*	*Mullus barbatus* *Serranus scriba* *T. trachurus*	21.5	Serracca et al. [26]
Alborán Sea (Mediterranean Gibraltar area)	2014	*A. pegreffii* *A. simplex sensu stricto*	*T. trachurus*	61.5	Piras et al. [27]
Iberian waters （Atlantic Ocean）	2015	*A. simplex sensu stricto* *A. pegreffii*	*Sardina pilchardus*	10	Molina -Fernández et al. [28]
Papua New Guinea （Western Pacific）	2013	*Anisakis* Type I larvae （*A. typica*）	*Selar crumenophthalmus* *Gerres oblongus* *Thunnus albacares*	7.6	Koinari et al. [29]
Pacific stock (the Pacific coast of Japan)	2010	*A. pegreffii* *A. simplex sensu stricto*	*Scomber japonicus*	74.3	Suzuki et al. [30]
Republic of Korea	2015	*A. pegreffii* *A. typica*	*Astrocongermyriaster*	100	Cho et al. [25]

Note: Infection rate (%)=Number of infected fish / total number of checked fish x 100.

The infection rate of *Anisakis* in fish on the Pacific coast of China is also very high. Zheng [31] studied a variety of marine fish near Xiamen, Fujian Province, some of which had an infection rate as high as 100%, including *A. pegreffii* and *A. simplex*. Li et al. [32] investigated 40 fish species (311 fish in total) in the Yellow Sea and found a total of 1709 *A. pegreffii* larvae, which accounted for 98.7% of the total number of *Anisakis* larvae enumerated, indicating that *A. pegreffii* is most abundant in the Yellow Sea. Li [33] investigated hairtail (*Trichiurus lepturus*), yellow croaker (*Larimichthys polyactus*), and mackerel in the East China Sea and found that the East China Sea is dominated by *A. pegreffii*. Shi et al. [34] investigated 35 species of marine fish (327 fish in total) in the South China Sea. Most of these fish were infected with *A. typical*, while a few were infected with *A. pegreffii* and *A. physeteris*, and others were unspecified. The infection situation is different from other sea areas around China.

In addition, at China's Zhoushan, Taizhou and Guangzhou import and export ports, different types of marine fish have been found to have *Anisakis* infections, mainly *A. simplex (s.s.)* and *A. pegreffii*, both of which are the most important pathogens that cause human anisakiasis, indicating that there is a risk to consumers of contracting *Anisakis* disease from imported marine fish [35-37]. In the domestic market, there is still a potential risk of *Anisakis* infection from fresh sea fish (Table 2). Data show that hairtail, mackerel and small yellow croaker have the highest infection rates [38-41]. Disease of *Anisakis* is a second type of parasitic disease that is prohibited from entering China. Statistics show that the main detection parts of *Anisakis* are distributed in the body cavity, stomach, intestine, liver and pancreas, and gonads of marine fish, while there are few or even no parasites in the muscle tissue.

Table 2: Investigation of *Anisakis* larvae infection in marine fish in China.

Origin	Year	Fish Species	Infection Species	Check Sum	Infection Rate (%)	High Intensity Infection	Ref.
Zhoushan (Import)	2015	19	14	660	56.97	*Gadus* (New Zealand)	Li et al. [32]
Zhoushan (Export)	2015	19	14	378	57.94	*Pneumatophorus japonicus, Lophius litulon, Gadus, Nemipterus virgatus*	Li et al. [32]
Guangzhou (Import)	2012-2015	9	9	63	93.7	*Ephippus orbis*	Huang et al. [35]
Yantai (Domestic market)	2016-2017	20	6	708	15.82	*Larimichthys polyactis* *Trichiurus lepturus*	Gong et al. [38]

Dongtai (Domestic market)	2018	5	4	149	52.35	*Trichiurus lepturus* *Scomberomorus niphonius* *Pneumatophorus japonicus* *Larimichthys polyactis*	Zhang et al. [41]
Fujian Province (Domestic market)	2016-2018	32	19	810	34.2	*Trichiurus lepturus* *Scomberomorus niphonius*	Lin et al. [39]
Jiangsu Province (Domestic market)	2018	Unknown	Unknown	494	64	*Trichiurus haumela* *Pneumatophorus japonicas*	Mao et al. [40]

Note: Infection rate (%)=Number of infected fish (not shown) / sum of checked fishx100.

Routes of exposure and epidemiology

Adult *Anisakis* is parasitic in the digestive tract of marine mammals, while the larvae are widely parasitized in different species of marine fish [44]. The infestation in humans results from accidental ingestion of the larvae. If a person eats raw or inadequately cooked marine fish containing *Anisakis* larvae, such as sushi, sashimi, etc, it can cause anisakiasis symptomized clinically as four main clinical types: gastric, intestinal, extra-gastrointestinal and allergic. Within 8-12 hours, the *Anisakis* larvae bore into the digestive tract or migrate to other tissues, causing acute abdominal pain, indigestion, nausea, vomiting and diarrhoea due to pathologic index of edema, hyperaemia, and bleeding in the surrounding mucosa [45-47]. This acute gastrointestinal form of *Anisakis* infection is usually transient, with the worm dying within a few weeks. Diagnosis is generally obtained through anamnestic data, endoscopy, radiography, serum specific anti-*Anisakis* IgE determination, or surgery if the worm has become embedded.

The presence of the parasite in the extra-gastrointestinal omental fat is unexpected and unusual. The invasive capacity of *Anisakis* is nonetheless known, and cases of larvae penetrating the abdominal cavity through the abdominal wall, and its association within inguinal hernias, have been described [49]. Hajjar et al. [42] presented the case of a female Canadian patient with an *Anisakis* larvae in an incarcerated ventral hernia, while Kawashima [43] reported a case of gastrointestinal hemorrhage due to ulcer formation caused by the invasion of the bowel wall by an *Anisakis* larva.

In addition to gastrointestinal symptoms, anisakiasis may sometimes cause allergic symptoms, such as urticaria, angioedema and even anaphylaxis [50-53]. Kasuya et al. [48] reported that sensitization to *A. simplex*, but not to a fish allergy, was the cause of some urticaria episodes present in Japanese patients who consumed cooked mackerel. The larvae penetrate the gastrointestinal mucosa-leading to an inflammatory response with a generalised immunoglobulin E (IgE)-mediated allergic reaction, resulting in an ulcer, an eosinophilic granuloma, or even gastrointestinal perforation [54]. Following the description in Spain of a case of recurrent anaphylaxis caused by *A. simplex* parasitizing fish, it became clear that both skin tests and specific IgE determination were useful tools in the diagnosis of this type of sensitization [55]. *A. simplex* is, so far, the only described parasite associated with fishery products that can cause allergic responses [5], gastro-allergic reactions by *A. pegreffii* have been reported in Italy [56].

Allergenic sources

Patients may be exposed primarily to somatic antigens from dead larvae in food, excretory-secretory (ES) antigens when there is expulsion or surgical removal of the intact larvae, or both [57]. In the case of anisakiasis, excretory/secretory products contain proteins with higher allergenic potential than somatic components [60].

In human anisakidosis, the patient may be exposed to *A. simplex* antigens from any of three sources:

i. Proteases and protease inhibitors secreted when the larvae invade, that is, excreted and secreted allergens, which lead to exposure to the complete profile of the parasite's antigens.

ii. Allergens from the worm itself, which are cuticular and somatic antigens from dead larvae contained in food.

iii. Epidermal allergens which are used to protect the digestive juice of the host.

To date, there are 19 allergens that have been identified and described in *Anisakis* [61]. However, a recent proteomic study combining 2D gel analysis and western blotting described 28 immunoreactive proteins present in the species complex (*A. simplex*, *A. pegreffii*, and their hybrids), including intraspecies variations which could be assessed as potential allergens. The result also showed that *A. simplex (s.s.)* (34 different protein spots) was more allergenic than *A. pegreffii* (11 different protein spots) and that both species were more allergenic than their hybrids (6 protein spots) [62].

Allergens of *Anisakis simplex*

To date, 14 allergens of *A. simplex* (s.s.) have been confirmed and described in detail, many of which have been well characterised by molecular methods [63] (Table 3). Officially designated Ani s 1 to Ani s 14, allergenic activity, according to the criteria of the WHO/IUIS Committee, has been verified in these allergens. Eight allergens, Ani s 1, Ani s 4, Ani s 5, Ani s 6, Ani s 7, Ani s 8, Ani s 9 and

Ani s 13 are parasite excretion/secretion molecules, while Ani s 2, Ani s 3 and Ani s 10 are somatic antigens. Ani s 1, Ani s 2, Ani s 3 and Ani s 7 are the main allergens, and Ani s 4, Ani s 5, Ani s 6, Ani s 8, Ani s 9 and Ani s 10 are minor allergens.

Table 3: *Anisakis simplex* allergens approved by the WHO/IUIS Allergen Nomenclature Sub-Committee.

Allergen	Molecular Weight (kDa)	Nematode Antigen	Protein	Reactivity in Infected Patients (%)
Ani s 1	24	ES	Kunitz-type trypsin inhibitor	85
Ani s 2	100	Somatic	Paramyosin	88
Ani s 3	41	Somatic	Tropomyosin	13
Ani s 4	9	ES	Cystatin	27
Ani s 5	15	ES	SXP/RAL protein	25
Ani s 6	7	ES	Serine protease inhibitor	18
Ani s 7	139	ES	Ua3 recognized allergen	94
Ani s 8	16	ES	SXP/RAL protein	25
Ani s 9	15	ES	SXP/RAL protein	14
Ani s 10	23	Somatic	Not given	39
Ani s 11	30	Unknown	Not given	50
Ani s 12	33	Unknown	Not given	57
Ani s 13	37	ES	Haemoglobin	64
Ani s 14	23.5	Unknown	Not given	54

Note: ES, excretory/secretory. Reactivity, percentages of infected patients positive to individual allergenic protein.

Ani s 1 is considered the main allergen of this species and is known to occur in different isoforms [64]. Moneo et al. [58] purified crude parasite extracts by ethanol precipitation and reversed-phase high-performance liquid chromatography to obtain a protein with a molecular weight of 24kDa (belonging to the nematode troponin family). At the same time, Arrieta et al. [59] also found a 21kDa protein by constructing the cDNA library, Western blot hybridization analysis and conducting other experiments. The 24kDa allergens discovered by Moneo et al. [58] and the 21kDa allergens discovered by Arrieta et al. [59] were all named Ani s 1. More than 50% of anisakiasis patients have antibodies to Ani s 1, indicating that it is a major allergen. Most gastro-allergic anisakiasis patients have anti-Ani s 1 specific immunoglobulin (Ig) E (67-85%), but Ani s 1 antibodies are not found in the serum of asymptomatic individuals. Ani s 1 is heat stable and can act as a food allergen, causing clinical reactions upon secondary exposure, immediately following the ingestion of cooked fish [58].

Ani s 2 and Ani s 3 are the two somatic allergens described so far. They are paramyosins and tropomyosin proteins, respectively, and they are similar to the paramyosins and tropomyosin's of other species [67]. The molecular weight of Ani s 2 is 100kDa. Because of its high sequence homology with flukes and arthropods, it can cause a cross-immune reaction [70]. Asturias [71-72] discovered the allergen Ani s 3, which is considered to be a less important protein allergen in *A. elegans*. The molecular weight of this protein is 41kDa. Interestingly, nematode tropomyosin's may also represent vaccine candidates, as they have been shown to elicit antibody-mediated protective immunity against different larval stages of the *Trichostrongylus colubriformis* (*Trichostrongyloid* nematode) and the *Onchocerca volvulus* (*Filarioid* nematode) [73]. In a similar manner, Sereda et al. [65] found that serum antibodies against haemoglobin of *A. pegreffii* induced an immunogenic response in mice against infection by *Nippostrongylus brasiliensis* (hookworm).

Moneo et al. [66] extracted an allergen from the E/S protein of *Anisakis*. Ani s 4 is located both in the excretory gland and below the cuticle, and it is heat stable (boiling for 30 min) and resistant to pepsin digestion [75]. Protein glycosylation is not a part of the allergic reaction. The molecular weight of the Ani s 4 allergen is 9kDa. In that study, it was found that after heating the extract of the parasite, some patients still had a reaction, with Anis 4 being recognized by 27% of allergic patients. Margarita et al. [68] used various technological and food processing treatments on parasitized fish to kill the *Anisakis* larvae and prevent infection and consumer sensitization. However, dot blot analysis indicated a high loss of Ani s 4 recognition post-canning, but residual antigenicity was still present. In addition, patients who can recognize this allergen are observed to have more frequent allergic attacks than those who are not sensitive to it.

Kobayashi et al. [69] used the serum of patients with anisakiasis to construct a cDNA expression library and do immune-screening, and they obtained two positive clones that encoded allergens (namely, Anis 5 and Anis 6). The molecular weight of Anis 5 is 15kDa, and it is a thermostable ES allergen. Ani s 6 (7kDa), is homologous to serine protease inhibitors from *Boophilus microplus* (cattle tick), *Anopheles stephensi* (mosquito) and *Glossina morsitans* (tsetse fly), including the *Apis mellifera* (honeybee) allergen Api m 6 [63]. Neither of them is major allergens of *A. simplex* [77]. However, in one study, Ani s 5 was recognised by serum antibodies in 49% of patients (41/84).

Ani s 7 is also a major allergen of *Anisakis*, which is an ES product of approximately 139kDa [79]. The native form is glycosylated and recognized by IgE from 100% of infected subjects, which gives it a great diagnostic value [80]. However, there is no experimental support for the allergenicity of this molecule [50].

Ani s 8 (15kDa) and Ani s 9 (14kDa), which share protein sequence homology, are recognized by 25% and 13% of IgE from subjects sensitized to *Anisakis,* respectively. Both belong to the SPX/RAL-2 family [81], which also includes Ani s 5, and they are heat-stable and present in excretory/secretory products. Five of thirty-six *Anisakis* allergic patients (13.8%) were positive for Ani s 9 [81]. Cho used an experimental mouse model to evaluate the allergenicity of Ani s 9 allergens from *A. simplex* (whale worm) third stage larvae. The result showed that repeated treatment with the allergen could elicit airway inflammation, including eosinophilia and high Ovalbumin-specific IgE levels [83]. Although Ani s 9 is reportedly more abundant in crude somatic extracts from *Anisakis*, its biological function is unknown.

Anisakis haemoglobin has been described as a major allergen (Ani s 13), and there is an absence of IgE cross-reaction to Ascaris haemoglobin in *Anisakis* patients. In addition, Ani s 13 has shown high rates of recognition (80.9% of the GAA patients) using a specific antigen-capture ELISA [84]. However, further confirmatory studies are needed.

Kobayashi et al. [76] identified Ani s 14 as a new major allergen of *A. simplex* by means of the chemiluminescent immune-screening method. As a result, an IgE-positive clone coding for a 23.5kDa protein composed of 217 amino acid residues was isolated. Recombinant Ani s 14 was verified to be IgE reactive, and hence could be useful as a diagnostic tool for *A. simplex* allergy. Although the amino acid sequence of Ani s 14 is partly homologous to those of Ani s 7 and 12, it is structurally unique and does not belong to any known protein families [55].

So far, as many as 14 types of proteins (Ani s 1-14) have been identified as *A. simplex* allergens, but more unknown allergens may exist. Future studies with this immune-screening method using sera from other patients would further identify more unknown *A. simplex* allergens.

Allergenic protein residue

Control measures against Anisakidae focus on the prevention of their post-mortem migration from the viscera to the muscles and on the removal or inactivation of the larvae present in captured fish. The United States Food and Drug Administration (FDA) has listed guidelines for seafood freezing below -35 °C for more than 15 hours or freezing below -20 °C for more than 7 days. Additionally, food business operators must conduct a visual inspection of all fishery products, and they must remove all visible parasites during industrial processing. However, the *A. simplex (s. s.)* L3 larvae may be freeze tolerant despite the rapid cooling of nematodes to -20 °C, according to the sanitary authorities of the USA and the EU [85].

Podolska et al. [78] used malachite green staining reaction to identify the viability of frozen *A. simplex (s.s.)* larvae. Results showed that 84% *A. simplex (s.s.)* larvae was dead but 16% uncertain due to motionless but also colourless [78,87]. This phenomenon indicated that freezing may not completely kill the *A. simplex (s.s.)* larvae and the allergenic proteins may remain unchanged [89].

Freezing conditions may have a profound effect on the subsequent safety of fish products. Łopieńska-Biernat et al. [82] found that a rapid freezing rate is optimal to ensure the safety of fish products relative to *Anisakis* larvae mortality. However, although high freezing rates may be preferable for the rapid killing of *Anisakis* larvae and in maintaining optimum eating quality, they may cause a greater release of antigens to the surrounding medium [86].

Heating to ≥60 °C at the core of the product for at least 1 min also ensures the destruction of the larvae [90,91]. However, Sánchez-Alonso et al. [92] found that conditions reported in the EU Regulation should be revisited, since reaching 60 °C for 1 min in the thermal centre would not be sufficient to kill all L3, and at least 8 min of heating are needed. Simultaneously, immunoblot studies using sera from *Anisakis*-sensitized patients have shown that some allergenic proteins of *Anisakis* are not inactivated after thermal treatments [75]. Some authors claim that exposure to nonviable *Anisakis* material can result in allergic symptoms in previously sensitized patients, indicating that parasite allergens are resistant to the thermal treatments of the usual cooking procedures [50,60,93]. *Anisakis* larvae can also be a source of allergens in what is considered secured food in the canning industry (following processing with technological treatments). This appears related to the existence of thermostable and pepsin resistant parasite allergens. Tejada et al. [88] analyzed allergen stability during heating to 121 °C in an autoclave to simulate the thermal processing applied to canned fish. Some relevant *A. simplex* allergens retained their capacity to bind immunoglobulin E and activate basophils post-autoclaving. It can be assumed that the salting and heating process during canning kills the nematode larvae and eliminates the zoonotic potential for anisakiasis. However, an allergenic potential remains through the presence of Ani s 1 and Ani s 4 in the canned products [94]. Therefore, heat-stable allergens are important (such as Ani s 4), even if classified as "minor allergens" (due to the frequency of recognition by patient sera), because these allergens relate to allergic reactions to cooked or canned (anisakid infected) fish [90].

Some researchers have used IgG hybrid analysis to analyse the simple *Anisakis* allergens and found that Ani s 4 can be quantified in frozen, fresh, and heated fish at different temperatures, which can be used as a signal for the presence of *Anisakis* in fish [95]. Leticia showed a different intensity and frequency of response to *Anisakis*-IgE measured by Immuno CAP and Ani s 1 by ELISA, between *A. simplex* allergic patients and asymptomatic sensitized populations. Also, higher frequency of recognition of the r Ani s 1 allergen was

found in patients who have experienced a severe reaction compared to those who had suffered a mild to moderate reaction [96].

Thus, the very high prevalence of *Anisakis* in the imported frozen mackerel still poses a significant risk for consumers because *Anisakis* allergens are heat-stable or resistant to pepsin and deep-freezing methods even in processed food. Two cases of immediate allergies to *Anisakis* after ingestion of processed seafood have been reported. In case 1, a 75-year-old man was diagnosed with IgE-mediated allergy due to *A. simplex* after the ingestion of salted fish guts made of Sagittated calamari, indicating that Ani s 3 was the causative allergen in this case [97]. In case 2, a 62-year-old man ingested dressed salmon and its roe (ikura) and grilled mackerel, ELISA with 11 natural or recombinant *A. simplex* allergens (Ani s 1-6, 8, 9, 11 and 12 and troponin C-like protein) showed that the patient serum strongly reacted to Ani s 1 and Ani s 12 and weakly to Ani s 2 and troponin C-like protein [98].

To date, freezing and heating are the most effective processes available to inactivate anisakid larvae. Many traditional cooking methods that have been used to kill anisakid larvae, such as salting [99], smoke curing, high hydrostatic pressure [100] and marinating [101] have proven to be ineffective if the treatment period is too short or they lower the product quality during the killing process. Air-dried stockfish do not carry viable anisakid nematodes, but the potential presence of allergenic proteins in the product may still pose a health risk for sensitized consumers and, thus, this source warrants further investigation [102].

Conclusion and Future Prospects

With the promulgation of new international standards on food safety, risk assessment methods are now based on scientific principles and directed at solving global public health problems, and the safety standards and monitoring standards as well as methods of canning fish are continuously improving. To date, the detection of *Anisakids* in fresh and processed fish products has been successful using real-time polymerase chain reaction (PCR [103], ultraviolet fluorescent imaging [104], IgG antibody immunoblotting [105] and enzyme-linked immunosorbent assay (ELISA) [106]. Different countries have different regulations and/or non-mandatory guidelines for the control of nematodes, including *A. simplex*, in fish and fish products, particularly for importation. In some countries, such as South American nations, the judgment that "canned fish is dangerous for consumers' health due to the presence of parasites" appears, but the parasites associated with marine fish are inevitable [107]. Therefore, whether the activity of allergenic proteins still exists after being processed by high-temperature and/or high-pressure processes such as canning, bringing with it food safety risks, is a very important question. A recent study identified an increased importance of *Anisakis* during the last few decades, with potential adverse effects on fish and human health, and on the world's fisheries [108].

Acknowledgement

The authors are grateful to the National Key Research and Development Project (2018YFG1602500) for its support. This research was also supported by the Joint Innovation Project of the Hong Kong-Guangdong Province (2016A05053031).

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