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Anisakis simplex: current knowledge

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Summary

Anisakiasis, firstly described in 1960s in the Netherlands, is a fish-borne parasitic disease caused by the consumption of raw or undercooked fish or cephalopods contaminated by third stage (L3) larvae of the Anisakidae family, in particular Anisakis simplex *(As),* A. pegreffii *and* Pseudoterranova decipiens. *Every year, approximately 20,000 cases of anisakiasis were reported worldwide, over 90% are from Japan and most others in Spain, the Netherlands and Germany, depending on the habits of fish consuming. Live As larvae can elicit i) a parasitic infection of the digestive tract or, occasionally, other organs, causing erosive and/or haemorrhagic lesions, ascites, perforations until granulomas and masses, if larva is not removed; and ii) allergic reactions, as anaphylaxis, acute/chronic urticaria and angioedema. Like other parasite infestations, As larva induces an immune adaptive response characterised by T-lymphocyte proliferation with polyclonal and monoclonal (responsible for As allergic symptoms) IgE production, eosinophilia and mastocytosis. Several As allergens, many of which thermostable, were described. In particular the major allergen Ani s 1 and Ani s 7 could characterized a past or a recent infection. There is a general agreement that an active infection is required to initiate allergic sensitivity to* Anisakis. *Until now, the only effective treatment for anisakiasis is the endoscopic removal of live larvae and the best protection against anisakiasis is to educate consumers about the dangers of eating raw fish and to recommend avoiding the consumption of raw or inadequately thermally treated marine fish or cephalopods.*

Introduction

Zoonoses represent approximately 75% of emerging diseases (1). The growing attention on foodborne zoonoses is the result of two main factors. The first is the increased prevalence of these diseases associated with a change in culinary habits, an increasing rate of international travels, commercial trades, and cultural and demographic changes. The second factor is related to improved diagnostic capabilities using advanced techniques and a higher number of instrumental investigations.

Nonetheless, parasitic zoonoses remain under-investigated because their actual and potential economic and health impact is unknown.

Epidemiology

Anisakiasis is a fish-borne parasitic disease caused by the consumption of raw or undercooked fish or cephalopods contaminated by third stage (L3) larvae of the Anisakidae family, in particular *Anisakis simplex* (*As*), *A. pegreffii* and

Pseudoterranova decipiens. The latter parasite is particularly widespread in Canada and the United States. Nematodes, members of the *Anisakis* spp., have a complex life cycle that passes through a number of hosts. Adult stages of the *Anisakis* spp. reside in the stomach of marine mammals, where they are embedded in the mucosa. Unembryonated eggs produced by adult females are expelled through the faeces of marine mammals and be2 come embryonated in seawater, where first-stage (L1) larvae are formed in the eggs. The larvae moult to become free-swimming in the second stage (L2) and are ingested by krill crustaceans, usually Euphausids, in which they mature into the L3 stage. This stage is infective to fish and squid, maintaining the L3 larvae form. Through predation, the larvae are transferred between fishes. Upon the host's death, *As* larvae migrate from the intestine to the tissues in the coelomic cavity and the muscle tissues, growing up to 3 cm in length. *Pseudoterranova* spp. larvae are also able to migrate to the fish flesh during their host life. When marine mammals ingest fish or squid containing L3 larvae, the larvae moult twice and develop into adult worms, completing the nematode lifecycle. Humans become infected by eating raw or undercooked parasitised marine fish and cephalopods, thus, representing an accidental host in which the worms cannot survive or reproduce and die in approximately 3 weeks (2).

Anisakiasis was first described as "worm-herring disease" in 1960s in the Netherlands by Van Thiel, who associated different cases of patients suffering from acute abdominal pain with the consumption of lightly salted herrings (3). Until now, several new cases have been reported; of the approximately 20,000 cases of anisakiasis reported worldwide, over 90% are from Japan (approximately 2,000 cases yearly) and most others occur in Spain, the Netherlands, and Germany (4), depending on the habits of consuming raw or undercooked fish.

The presence of live *As* larvae can elicit two different diseases: i) the parasitic infection (anisakiasis) of the digestive tract or, occasionally, other organs; and ii) allergic reactions with or without digestive symptoms.

Clinical features of anisakiasis

The ingestion of a parasitised fish with a nematode of the *Anisakis* genus may elicit symptoms within few hours. Four principal clinical syndromes associated to anisakiasis have been described: gastric, intestinal, ectopic (or extra-gastrointestinal), and allergic. The onset of gastric anisakiasis begins within few hours, generally 1 to 2, when a live *As* larva reaches the human stomach. Here, it adheres to the gastric mucosa by a projection surrounding its mouth and produces proteolytic enzymes, mainly secreted by a dorsal oesophageal gland and other excretory glands around the mouth. These proteases cause erosive and/or haemorrhagic lesions in or near the main lesion, forming a tunnel through the gastric mucosa to the submucosa. This acute phase of the infection elicits severe epigastric pain, vomiting, diarrhoea, and a mild fever. Generally, acute symptoms resolve within a few days, but untreated gastric disease can lead to chronic, ulcer-like symptoms lasting for weeks to months. Intestinal anisakiasis is characterised by intermittent or constant abdominal pain starting 5 to 7 days after the larva ingestion. Infected individuals may develop ascites and/or peritoneal signs. Intestinal infection and inflammatory responses mainly occur in the terminal ileum and less commonly in the colon or jejunum. Rare complications include small bowel obstructions, ileal stenosis, intussusception, intestinal perforation, and pneumoperitoneum.

Although less common, the larva penetration through gastric or gut mucosa can lead to its migration into the peritoneal or pleural cavity, mesentery, liver, pancreas and ovary. Chronic infection may present with mesenteric masses (5).

The clinical manifestations of anisakiasis vary depending on the organ where the person was infected and which *Anisakis* spp.caused the infection. In Japan, a gastric infection occurs primarily, whereas intestinal disease is more common in Europe (6).

In addition to directly visualising the larva(e) embedded in the gastric mucosa, endoscopy may reveal erythema, oedema, severe erosive gastritis, a tumour-like nodule, or ulcerations. Biopsy can show an early inflammatory infiltrate of eosinophils and lymphocytes in the mucosa and submucosa as well as phlegmon formation. Although larvae may be found up to 6 days after the consumption of seafood, if endoscopy is delayed, the worm may degenerate, be eliminated, or pass through the mucosa (resulting in ectopic disease), preventing it from being visualised; the only signs may be thickened gastric folds and inflammation. Chronic infection can result in abscess and/or granuloma formation in response to degenerating larvae. Radiographic findings depend on the site of infection. Thread-like filling defects and mucosal oedema can be observed on barium studies with a gastric infection. Intestinal infection can cause non-specific, irregular bowel-wall thickening with a disappearance of Kerckring folds, mucosal oedema, and luminal narrowing, detectable by

scanning or CT (7,8). CT findings also include lymphadenopathy, focal masses, and/or ascites. Ascitic fluid obtained by paracentesis may demonstrate an eosinophilic predominance. A gastric infection is frequently accompanied by leucocytosis; eosinophilia is more commonly observed in gastric compared to intestinal infections, particularly if the worm remains in place (6). Continual exposure to the offending nematode or massive infestation causes chronic intestinal mucosa and submucosa inflammation or multiple or wide granulomas, simulating a subocclusive neoplastic lesion (5,9,10).

In summary, after 4 hours to 6 days, *As* larva penetrates mucosa and submucosa. The excretory/secretory proteases as well as the *As* surface and somatic components induce an immune response, mast cell (IgE-independent) degranulation, immunosuppression, anticoagulant activity, eosinophil chemotaxis and mutagenic effects. These features induce erosive lesions and eosinophilic phlegmon without any damage to the *As* body surface.

After 7-14 days, granulomas, ulcerative lesions and the induction of a hypersensitivity response occur.

After 14 days, *As* larvae die, but a persistent inflammation or granulomas remain. Two situations could occur: the loss of parasite with ulcerative lesions or the endoving of a dead larva into a granuloma.

As with other parasite infestations, *As* larva induces an adaptive response characterised by T-lymphocyte proliferation with polyclonal and monoclonal (responsible for *As* allergic symptoms) IgE production, eosinophilia and mastocytosis.

As immune stimulation

Different authors aimed to investigate cytokine expression induced by the *Anisakis* parasitism. Cuéllar et al. (11) found low levels of IL-6, IL-10, IL-17, TNF and IFNγ cytokines, mainly indicating a Th1 response, in sera from *As*-sensitised patients after *As* crude extract and mitogen stimulation during a cytometric bead array (CBA) analysis. Higher values of IL-2 (important for growth and survival of both Th1 and Th2 lymphocytes) were detected after exposure to the crude extract, emphasising the relevant immune stimulation of this extract.

Therefore, *As* is able to induce both a Th1- and Th2-type immune response, with different levels in sensitised patients. Gonzalez-Munoz et al. (12) found an increased *in vitro* production of IL-2, IL-4, IL-5, IFNγ cytokines in *As*-sensitised patients with respect to control patients after both crude and thermally treated extract exposure. Moreover, IL-10 levels were higher after crude extract incubation, and there was a correlation between the symptoms and cytokine patterns. Patients with urticaria/angioedema and/or anaphylaxis had higher levels of Th2 cytokines (IL-4, IL-5) and IgE, while patients with predominantly gastrointestinal symptoms had higher levels of IFNγ, which inhibited the IL-4-dependent responses.

In a more recent paper (13), cytokine responses in patients with gastro-allergic anisakiasis (GAA) or chronic urticaria with (CU+) and without (CU-) *Anisakis* sensitisation were investigated. IL-10 was low in CU+ and CU- patients and higher, but not statistically significant, in GAA patients. Higher levels of TGFβ, a marker of Th1 response, and IL-17 were found in GAA patients compared to CU+ and CU- patients. The authors correlated TGFβ levels with a previous exposure to *As* and IL-17 values with positive urticaria outcomes rather than to parasite exposure. Because the expression of the two cytokines was similar, we hypothesised that both molecules correlate with a previous *Anisakis* exposure.

In 2010, Daschner et al. (14) investigated the immunological pattern expressed in GAA, prolonged acute urticaria (PROL, 3 days-6 weeks), and CU *As*-sensitised patients by *As*-specific IgE, IgG and IgG4 detection. There were no differences found in the antibody levels between PROL and CU patients, but GAA patients showed significantly higher levels for all the tested immunoglobulins. The authors concluded that there was similar immunological stimulation both in PROL and CU.

Focusing on *As* sensitivity and CU, in a previous study, Daschner et al. (15) analysed the effect of a two-month fish-free diet in an *As*-endemic Spanish region. Among the 65 CU *As*-positive and 11 CU *As*-negative patients, there was a statistically significant symptom improvement ($p<0.001$) in the first group. Moreover, a clear improvement occurred due to diet in patients presenting with positive specific IgG4 levels to *As*. Because high IgG4 results from continual allergen exposure, CU *As*-positive patients are likely to improve with a fish-free diet when presenting this immunological pattern.

IgE-mediated allergy to *As*: clinical diagnosis and symptoms

The correct diagnosis of IgE-mediated *As* allergy is based on the following criteria: i) a compatible history, such as typical allergic symptoms following fishery product con-

sumption; ii) a positive skin prick test (SPT) and/or positive serum-specific IgE levels to *As*; and iii) a negative history of allergic symptoms and *in vivo* and *in vitro* tests to fish and/or other possible cross-reactive allergens (crustaceans, dust mites, insects).

Allergic reactions to *As* may elicit different clinical symptoms. The most severe is anaphylaxis, as described in many papers and mainly occurring in Mediterranean (16,17) and Asian countries (18,19). In 12 studies describing allergic and anaphylactic reactions in a total of 448 *As*-allergic patients, 130 (29%) experienced anaphylaxis. Interestingly, Audicana and Kennedy (20) retrospectively analysed the causes of anaphylaxis in two different hospitals in Spain and concluded that if *As* was considered as a causative agent, idiopathic anaphylaxis dropped from 14 to 4%. Therefore, as a food allergy, *As* accounted for 10% of the total recorded anaphylactic reactions.

Another very common allergic reaction to *As* is acute urticaria, as demonstrated by Choi et al. (18) in 10 *As*-allergic patients. All patients had acute urticaria (100%) followed by abdominal pain (30%) and anaphylaxis (30%). In the Basque region (Spain), *As* is considered the main cause of urticaria and angioedema in adults with fish consumption and is responsible for 8% of acute urticarias (21). In a high-risk population of fishmongers, Purello D'Ambrosio et al. (22) observed 72% of subjects presenting with urticaria and angioedema.

Along with occupational exposure, other sensitisation routes, such as inhalation or skin contact, can be involved, and allergic conjunctivitis, dermatitis and asthma have been described (22-25). Nevertheless, among fishery and aquaculture workers, fishmongers, and seafood handlers, *As* allergy is quite rare, considering that over 38 million people work in this field. *As* is a stronger sensitiser than fish, since *As* allergy prevalence exceeds fish allergy (8% vs. 6%) (25). The incidence of *As* sensitisation in fish workers is higher, up to 64% (28), than in the general population, in which *As* allergy remains rare. In the Italian population, of the 10570 screened individuals, 4.5% had *As*-positive skin prick tests, but only 0.6% experienced *As* allergy symptoms (26).

Anisakis allergens

Considering the *As* body morphology, three groups of possible allergenic proteins can be defined: i) proteases and protease inhibitors secreted during larva penetration, namely the excretory/secretory (ES) allergens; ii) somatic allergens obtained from the *As* whole body; and iii) cuticular allergens, secreted to protect the *As* body from digestive juices.

Depending on the *As* larva's fate, individuals could be exposed to different *As* allergens. During active penetration of the larva and its subsequent death, patients are exposed to all *As* allergens, while if *As* larva is eliminated intact through the gastrointestinal tract, patients are exposed only to ES allergens. Finally, if the *As* larva that is ingested is already dead, patients are mainly exposed to somatic and cuticular allergens and minimally to ES allergens.

To date, several *As* allergens have been described (from Ani s 1 to Ani s 12), but only the first 9 allergens have been identified and characterised. Seven (Ani s 1, Ani s 4, Ani s 5, Ani s 6, Ani s 7, Ani s 8, Ani s 9) are ES allergens, while two are somatic allergens (Ani s 2, Ani s 3). Ani s 1, Ani s 2, Ani s 3 and Ani s 7 are major allergens, and Ani s 4, Ani s 5, Ani s 6, Ani s 8, Ani s 9 and Ani s 10 are minor allergens. Ani s 1 is a major ES allergen with a molecular mass of 24 kDa, lacking any significant homology with other known allergens. It is highly specific for *As*-allergic patients; 85% of patients develop IgE to this protein. Interestingly, sera from patients with positive SPT or with serum-specific IgE levels but no clinical allergy to *As* did not bind purified Ani s 1 in SDS-PAGE immunoblots (27).

Ani s 7 is a major ES allergen as well, with a molecular mass of approximately 139 kDa and no significant homology with other known allergens, but it is characterised by a repeated cysteine residue-rich motif (28). Rats inoculated intraperitoneally with either live or dead L3 larvae produced IgE, IgA and IgM only against live larvae, even when re-infected with the same type of larvae. Authors suggested that the Ani s 7 allergenicity does not last in dead larvae and that immunisation occurs during the acute phase of infection when the ES allergens are released. In the same study, the authors evaluated the species specificity of Ani s 7 in rats infected with either *Anisakis* spp. or *Pseudoterranova* spp. larvae. While the crude extracts of the two nematodes elicited a positive response in both groups of infected rats, Ani s 7 was specific for *As* infection.

In a further study (29), IgE levels to rAni s 7 decreased more rapidly than those to rAni s 1; the authors suggested that rAni s 1 might be a marker of a previous infection, while high rAni s 7 IgE levels may indicate a recent infection. Ani s 2 and Ani s 3, paramyosins and tropomyosins, respectively, present a high degree of homology with other myosins. In particular, Ani s 2 is highly cross-reactive

with *Blomia tropicalis* and *Dermatophagoides* spp., while Ani s 3 presents a wide range of cross-reactivity with tropomyosins of major and minor house dust mites, crustaceans, molluscs, cockroaches and insects (30). Asturias (31) investigated patients' response to Ani s 3 in those with a clear history of an allergic reaction following the ingestion of *As*-contaminated food, in patients with possible *As* allergy and in patients hypersensitive to mites. No sera from *As*-allergic patients reacted to Ani s 3, while 13% of the patients with a possible *As* allergy reacted to Ani s 3. Moreover, pre-incubation of sera with other tropomyosins completely inhibited the Ani s 3 binding in *As* immunoblotting. Thus, Ani s 3 cannot be regarded as a relevant allergen in *As* sensitisation.

Dead or live *As* larva: which is necessary to induce *As* allergy?

Currently, to avoid the parasitism risk, the European Community recommends cooking fish at 60°C for at least 10 minutes or freezing at -20°C for at least 24 h; the USA Food and Drug Administration (FDA) agency demands the same cooking conditions but freezing at -20°C for at least 7 days. Discrepancies could be explained considering the higher resistance of *Pseudoterranova* spp., typically diffused in Canada and the northern USA.

Many *As* allergens have been found to resist heating and freezing. Therefore, some authors demonstrated the *in vitro* IgE reactivity of *As*-allergic patients to thermally treated *As* extracts (32,33). *As* allergens may also be present in the fish flesh near the larvae (20); therefore, parasite allergens can be present in edible fish muscle and might cause allergic symptoms irrespective of larvae ingestion.

Recently, *As* allergens and in particular Ani s 4 were quantified in fresh or different thermally treated fish muscle using IgG dot blot analysis. Ani s 4 appeared to suggest the presence of *As* larvae in fish flesh (33).

Tejada et al. (34) submitted hake steaks artificially parasitised with *As* larvae to different treatments (chilling, freezing, heating at 86.3°C and microwave cooking). By scanning electron microscopy, no apparent changes in the frozen larvae or disruptions in the cuticle were found.

Few studies are available on oral challenges with thermally treated *As* larvae. In one study, 11 *As*-allergic patients with positive tests and allergic symptoms after heating marinated fish were submitted to double-blinded, placebo-controlled oral challenges with lyophilised *As* larvae (35). The ultimate dose corresponded to 100 larvae, and five patients were also challenged with an aqueous extract corresponding to 105 or 210 larvae. None of the patients experienced a positive reaction during or after the challenge. In another study (36), 5 *As*-allergic patients with one or more nematodes detected by gastroscopy in the stomach were submitted to two different single-blinded challenges versus placebo. The first challenge was performed with 11 *As* larvae frozen at -20°C for 48 h, while the second was performed with the offending seafood after freezing at -20°C for 48 h. All patients tolerated both challenges without any allergic or gastric symptoms. Patients were advised to consume deep-frozen fish at least once a week. After 6 months, no reaction had been reported, even if SPT and serum-specific IgE to *As* remained positive. Alonso-Gomez et al. (37) challenged 22 *As*-allergic patients with up to 20 frozen larvae without reporting any allergic reactions. The patients were followed-up for more than two years, in which patients consumed deep-frozen fish without problems.

There is a general agreement that in the majority of cases, an active infection is required to initiate allergic sensitivity to *Anisakis*, even if a prior sensitisation via exposure to thermally resistant *As* allergens, including dead larvae, could not be excluded.

Therapy and prevention

Until now, the only effective treatment for anisakiasis is the endoscopic removal of live larvae, as they naturally die after approximately three weeks in the human body. When eosinophilic granulomas occur, surgical removal is necessary to avoid a subocclusive emergency. Arias-Diaz et al. (38) studied the *in vitro* activity of different concentrations of albendazole against *As* larvae under different pH levels. Albendazole dose-dependently reduced the survival of the larvae, but acidic pH media significantly reduced its efficacy.

The lower prevalence of anisakiasis in certain Asiatic populations who season raw fish with aromatic plants, such as perilla or ginger, prompted several authors to investigate the biocidal effects of natural products. Navarro et al. (39) studied different monoterpenic derivatives from several essential oils, concluding that α–pinene significantly reduced the lesion-treated rats. Hierro et al. (40) demonstrated that citral damaged *As* L3 larvae.

However, the best protection against anisakiasis is to educate consumers about the dangers of eating raw fish and to recommend avoiding the consumption of raw or inade-

quately thermally treated (see above), marinated, or salted marine fish or squid. Unfortunately, the current regulations do not protect the consumers against allergic hazards from ingesting killed parasites, but research has indicated, in the majority of cases, the safety of thermally treated fish consumption.

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