For: *Research in Veterinary Science*, Special Issue: "Foodborne and Waterborne Zoonotic Parasites of Veterinary and Medical Importance"

Title: Anisakiasis and *Anisakis*: an underdiagnosed emerging disease and its main etiological agents

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Highlights

1.- Anisakiasis is an emergent, cosmopolite, subdiagnosed seafood-borne parasitic zoonosis.

2.- Awareness and training of health personnel is decisive for the diagnosis of anisakiasis.

3.- Anisakiasis prevention and control measures are effective if implemented.

4.- The development of less invasive and more specific diagnostic methods is required.

5.- The relationship of anisakiasis with cancer and other diseases needs to be clarified.

Abstract

Anisakiasis or anisakiosis is a human parasitic infection caused by the third-stage larvae (L3) of nematodes of the genus *Anisakis*, although the term is also used in medical literature for the much less frequent (<3% of cases) infection by L3 of other genera of anisakids, particularly *Pseudoterranova*. These parasites have a marine lifecycle. Humans are infected by the L3 through ingesting of fish and squid, the intermediate/paratenic hosts. The live larvae generally penetrate the wall of the stomach or intestine causing, amongst other symptoms, intense pain or allergic symptoms. These are emerging, cosmopolite illnesses. Diagnosis and treatment is usually by endoscopy and extraction and identification of the larvae. Allergic forms are usually diagnosed by prick-test and/or allergen-specific IgE detection and treated with a suitable anti-allergy treatment. The patient is also warned against further consumption of marine fish or squid, as these may be infected with *Anisakis*. The most common method of prevention is thermal treatment of the entire fish or squid prior to consumption (>60 ºC, >1 min or -20 ºC, >24 h). Useful measures for the control of anisakiasis would be to establish a national register of cases, to initiate educational campaigns for the general public and consciousness-raising and training campaigns for health professionals. These would be complemented by control measures for the relevant sectors of the economy: fish operators, fish farming, fishermen, fishmongers, fish industry and catering facilities. Possible genetic predisposition for allergy to *Anisakis* and the possible relationship between anisakiasis and cancer would also require further investigation.

Keywords: Anisakiasis; allergy; seafood-borne disease; *Anisakis*; diagnosis; control.

Introduction.

Anisakidosis or anisakiasis is an infradiagnosed, emerging, cosmopolite illness. It entails the accidental infection of humans by the third larval stage (L3) of parasitic nematodes of the family Anisakidae (genera *Anisakis*, *Pseudoterranova* and, very rarely, *Contracaecum*), which, with an aquatic, mainly marine, lifecycle have marine mammals and fish-eating birds as definitive hosts and crustaceans, cephalopods and fish as intermediate/paratenic hosts. It has also been related to anisakiasis, although rarely, to raphidascarid *Hysterothylacium aduncum* (Yagi et al., 1996), which is very close to anisakids (Fig. 1). Cases of allergy to these parasites are also considered as anisakiasis (Audícana and Kennedy, 2008).

Infection by larvae of the genus *Anisakis* is specifically classified as anisakiosis, although the term anisakiasis is the term most commonly employed in medical literature, perhaps as a result of the etiological agent of 97% of cases of anisakiasis being L3 of the complex *A. simplex sensu lato*, specifically the species *A. simplex sensu stricto* and *A. pegreffii*.

The presence of *Anisakis* in fish has been known at least since the 13th century (Myers, 1976). In 1767, Linnaeus named it *Gordius marinus,* while, in 1809, Rudolphi described larvae in fish and adults in porpoise, but without relating them (Rudolphi, 1809). Finally, in 1845, Dujardin created the genus *Anisakis,* in which this parasite is included with the name *Anisakis simplex* (Rudolphi, 1809).

It was not known that it could affect humans. Hitchcock (1950) observed a larva, probably *Anisakis*, in the faeces of an Inuit in Alaska. The first cases in which symptoms were associated with the presence of *Anisakis* larvae were diagnosed by Dr. Straub in the Netherlands in 1955-59 (van Thiel et al., 1960).

The L3 of the genus *Anisakis* are very similar morphologically (Fig. 1), traditionally classified as type I or type II larvae , with the species within each type being morphologically

indistinguishable (Berland, 1961). They can however be differentiated molecularly and have been grouped into 4 clades (Mattiucci and Nascetti, 2008) (Table 1).

Transmission and epidemiology

Humans are infected on ingesting viable L3 found in host fish and squid, especially by those in the muscle tissue since those in the visceral cavity are eliminated when the fish is gutted for culinary purposes. However, some small fish are consumed whole, in which case the worm larvae in the body cavity can also cause anisakiasis. Almost all species of teleost fish studied throughout the oceans can act as hosts for *Anisakis* larvae. Consequently, the consumption of fish or some species of squid is a source of infection in humans.

Each year thousands of cases are reported globally, particularly in developed countries with a significant fishing industry, high per capita consumption of fish and where there is a culinary tradition of dishes featuring raw fish or squid. Japan is the country where most cases are diagnosed, over 500 annually (Suzuki and Murata, 2011), but estimated to be approximately 7,000 (Sugiyama et al., 2013, cited in IARS, 2017). In other countries with high fish consumption, such as Spain, the number of reported cases is much lower, at around 150/year (Herrador et al., 2019). Although increased information and raising awareness in health professionals results in more effective diagnosis (Castán et al., 2002), some authors have expressed concern regarding the continued infradiagnosis. For example, in Spain, Bao et al. (2017) used a quantitative risk assessment model to predict around 8000 cases/year of anisakiasis due to consumption of anchovies, while Herrador et al. (2019), working with hospitalization data, calculated 10,000-20,000 cases. However, only around 500 cases are reported annually in the whole of Europe. The global frequency of infection is estimated at 0.32 cases/100,000 inhabitants (Orphanet, 2020), with cases of anisakiasis diagnosed in more than 20 countries.

The lifecycle of *Anisakis* (Fig. 2) can start in the stomach chambers of odontocete cetaceans, definitive hosts, in which adult worms are free-living (Fig. 3). The female is fertilized by the male and lays eggs which are passed in the host's faeces. The eggs develop in the sea up to L3 which then hatch (Køie et al., 1995). The L3 (200-300 µm) is enclosed in a sheath, the L2 cuticle, and can survive for up to 3-4 months (Højgaard, 1998) in seawater until predated by the first suitable intermediate host, a crustacean, generally euphausiids (Smith, 1983, 1971) or large calanoid copepods (Klimpel et al. 2004), although smaller plankton crustaceans could act as paratenic hosts, prior to ingestion by the first intermediate host (Klimpel et al., 2004; Køie, 2001; Shimazu, 1974). The L3, after losing its sheath, passes from the digestive system of the crustacean into the coelomic cavity. Here it undergoes a period of growth, which can reach up to 32.7 mm in these hosts (Smith, 1983). These larvae are now infective for the definitive host. However, in order to reach an odontocete cetacean, the cycle must continue through a second suitable intermediate host, in this case squid (rarely any other cephalopod) or teleost fish which predate on euphausiids. When the infected crustacean is ingested, the L3, in order to be infective for the host, must have attained a minimum size (L3 from 8.8 mm have been recorded in host fish; Smith, 1983). The L3 then passes from the host's digestive system to the visceral cavity (Wootten and Smith, 1975). Occasionally, L3 pass from the visceral cavity to the fish musculature. This L3 will be infective for the definitive host, for which it must apparently have a suitable size (Asami and Inoshita, 1967; Iglesias et al., 1997; Kikuchi et al., 1967; vs Beverley-Burton and Pippy, 1977; Hays et al., 1998). Next, when the intermediate host is ingested by a cetacean the L3 occupy the stomach chambers, most often the first, and attach themselves to the gastric mucosa, normally in groups, where they cause an ulcer. The L3 moult to L4 which remain together in the ulcers until the final moult to adult when they generally free themselves and grow and mature in preparation for mating while roaming throughout the stomach chambers (Højgaard, 1999; Kikuchi et al., 1967; Smith, 1989, 1983; Young and Lowe, 1969). The adult female (4.5-15.0 cm) is usually larger than the male (3.5-7.2 cm), with the

cycle being completed when the male fertilizes the female (Grabda, 1976; Iglesias et al., 2001; van Banning, 1971).

In nature this lifecycle is complicated by the undefined number of paratenic hosts which can potentially intervene (Fig.2). If an L3 can to infect the next host but its paratenic host is then ingested by a host from the same level the larva will again occupy the visceral cavity, either free or encapsulated, and will not progress. This host will thus be paratenic. As the intermediate and paratenic hosts are the same they are normally known as "intermediate/paratenic hosts" as they can perform both functions according to the larval development. This large number of potential hosts for the genus *Anisakis*, especially with regard to intermediate/paratenic hosts, allows the parasite to follow alternative lifecycles, depending on both biotic and abiotic factors, for its adaptation and survival at different marine latitudes (Klimpel et al., 2004; Kuhn et al., 2016). However, many aspects of the *Anisakis* lifecycle remain unclear and must be studied to clarify them.

Despite the large number of hosts that may be involved in the lifecycle of these parasites not all cetaceans are suitable definitive hosts for all species: Delphinidae are ideal for species of the complex *A. simplex s.l.* and for *A. typica*, Ziphiidae for *A. ziphidarum* and *A. nascetti*, Physeteridae for *A. physeteris* and Kogiidae for *A. paggiae* and *A. brevispiculata* (Klimpel et al., 2010; Mattiucci et al., 2018; Mattiucci and Nascetti, 2008). Other cetaceans, such as misticetes (Raga et al., 1986), can serve as occasional definitive hosts, but not other marine mammals, such as the pinnipeds, in which the parasites do not attain maturity (Skrzypczak et al., 2014).

Clinics and pathogenesis

Infection in wild and farmed fish.

Fishermen have been aware of the presence of these parasites in fish for centuries, as commented. In some fish, such as herring, they were so abundant that that they were believed to be the main food source for these fish (see comment in van Beneden, 1870, p. 65).

Templeman et al. (1957) drew attention to the commercial problem represented by the presence of larvae of *Porrocaecum* (=*Pseudoterranova*) and *Anisakis* in fish, particularly cod, suggesting that a reduction in the number of seals (definitive hosts of *Pseudoterranova*) at the fishing grounds could reduce the parasite load of these fish. To date more than 200 species of fish, including many of commercial value, have been reported as hosts of *Anisakis*, with more added each year. Such valuable fish as cod, salmon, hake, saithe, redfish, blue whiting, pouting, horse mackerel, sardine, anchovy, mackerel, herring, etc. are habitual hosts, showing high prevalence and intensity, with these being affected by capture zone and the size/age of the fish (Adroher et al., 1996; Beck et al., 2008; Levsen and Lunestad, 2010; Molina-Fernández et al., 2018, 2015; Rello et al., 2009, 2008; Strømnes and Andersen, 1998; Valero et al., 2006). The presence of cetaceans in and around the fishing grounds may help to perpetuate the lifecycle of the parasite (Rello et al., 2009), while, since L3 of *Anisakis* can live within a fish for 3 years or more (Smith, 1984), parasites tend to accumulate as the fish grows (Bussmann and Ehrich, 1979; Valero et al., 2000). Nonetheless, in some cases older fish may show a reduction in parasite intensity, possibly due to a better immune response from the host (Dezfuli et al., 2016; Serrat et al., 2019) or to the death of the hosts with the highest parasite load (Levsen and Berland, 2012; Strømnes and Andersen, 1998).

The passage of *Anisakis* larvae through the stomach wall of the fish results in the formation of ulcers, although these do not seem to affect the normal performance of the organ (Jones, 1994) and nor does the presence of the parasites in the visceral cavity (Fig. 4A) appear to significantly affect the host's health, regardless of whether they are free or encapsulated on the liver (Wootten, 2012). The effects of *Anisakis* on fish have rarely been considered and are worthy of further study, particularly as *Anisakis* has recently been related to red-vent syndrome in Atlantic salmon (Beck et al., 2008). In any case, Fulton's condition factor (CF) (Fulton, 1904) for fish is a general indicator of their health (Monstad, 1990). According to this, a parasitized fish should show a lower value of CF to one free from parasites. However, this is

165 open to debate. Some authors have found CF in parasitized fish to be unaffected (Molina-
166 Fernández et al., 2018, 2015; Mouritsen et al., 2010). On the other hand, other authors have
167 suggested that CF is not only affected when the intensity of parasites is high but also by other
168 factors such as season, age/length or maturity of the fish (Richards, 1977; see Rohde, 1984,
169 and references therein). However, Serrat et al. (2019) suggest that the CF will only be affected
170 if the parasite load affects the availability of energy for the fish. This controversial topic
171 requires further study.

172 In marine fish-farming, although the feed is processed to avoid infection, it is still possible for
173 small prey animals (crustaceans, squid and small fish) infected with *Anisakis* to pass through
174 the netting of the cages and to be ingested by the fish. However, although a few cases of
175 caged fish being infected with *Anisakis* (reviewed by Lima dos Santos and Howgate, 2011; Mo
176 et al., 2014) have been reported prevalence is generally very low or nil (Brooker et al., 2016;
177 Cammilleri et al., 2018; Peñalver et al., 2010). Other practices such as the capture of wild fish
178 such as cod and tuna for subsequent growing-on in cages present a greater risk as these may
179 already be infected when captured and may cause anisakiasis when sold (Heuch et al., 2011;
180 Smrzlić et al., 2012). Consequently, the possibility of farmed fish being parasitized should
181 always be taken into consideration (Rückert et al., 2008).

182 *Infection in cetaceans.*

183 There are many species of cetaceans in which *Anisakis* has been detected in their stomach
184 chambers (Fig. 4B). As described previously, these nematodes form clusters on the gastric
185 mucosa forming an ulcer to which they remain attached until moulting to adults (Højgaard,
186 1999; Young and Lowe, 1969), some 3-5 weeks later (Iglesias et al., 2001), by penetrating the
187 mucosa with their anterior end, even reaching the submucosa (Kikuchi et al., 1967; Young and
188 Lowe, 1969). These ulcers may be associated with oedemas, haemorrhages and alterations
189 such as eosinophilic and granulomatous inflammation with giant cells, hemosiderosis, fibrosis

and areas of necrosis associated with location of parasites within the gastric mucosa (Motta et al., 2008). Fernández-Maldonado (2016) suggested that the presence of *Anisakis* in cetaceans may be related to a state of immunosupression associated with microbial systemic infections in addition to significant digestive disorders. Dermal symptoms have also been described (van Beurden et al., 2015). Recent studies seem to show that *Anisakis* populations in cetaceans could be increasing over recent decades (Pons-Bordas et al., 2020). At least, captive marine mammals can be treated, with varying degrees of success, using thiabendazole or levimasole (Smith and Wootten, 1978).

Infection in humans.

Man is an accidental host of *Anisakis* in whom the live L3, ingested on consuming raw or under-cooked fish or squid, tend to attach to the gastric mucosa, and, on occasions, the intestinal mucosa (Fig. 4C). Their attempts to penetrate the mucosa are generally unsuccessful. A single larva can cause symptoms. This L3 very rarely moults to L4 in humans (van Thiel et al., 1960). The type of anisakiasis produced depends on the location of the larva and the symptoms it is causing:

Gastric anisakiasis: the attaching of the larva to the gastroduodenal mucosa gives rise to intense epigastric pain which may be accompanied by other symptoms such as nausea, vomiting, urticaria and diarrhoea (Furuya et al., 2018) which develop between 2 to 6 hours after ingestion of the larva. The symptoms last as long as the larva is alive. This is the most common form of anisakiasis, accounting for 72% of cases (Valls Sánchez et al., 2009).
Intestinal anisakiasis: when the larva attaches to this part of the digestive system symptoms start to appear 2 to 3 days after ingestion, typically severe abdominal pain which may be accompanied by nausea, vomiting and/or diarrhoea. Occasionally, a chronic form develops, resulting in the formation of granulomas or abscesses. These may resemble episodes of

appendicitis or intestinal obstruction accompanied by an oedema with fibrinous exudate (Valls Sánchez et al., 2009).

It is not clear why the larva attaches to the gastric or intestinal mucosa. However, in Japan the intestinal form has been related to factors pertaining to the human host such as sex, habitual consumption of alcohol or cardiac risk (Yamamoto et al., 2020). Geographical factors, potentially related to culinary traditions, may also be involved. In both cases, in the zone of attachment, the organism reacts by generating an eosinophilic granuloma which normally kills the larva in 1 to 2 weeks, at which point the symptoms disappear.

Ectopic or extraintestinal anisakiasis: is rare, occurring when the larva passes through the digestive wall giving rise to 'visceral *larva migrans* syndrome'. There are no specific symptoms and these depend on the organ affected.

Allergic anisakiasis or 'allergy to *Anisakis*': occurs when the presence of larval allergens (due to live or dead parasite larvae) triggers an allergic response in the host, with symptoms ranging from urticaria (Kasuya et al., 1990) and/or angioedemas to anaphylaxis (Audícana et al., 1995; see section 3.2.1 in EFSA-BIOHAZ, 2010). These usually appear within the first hour after consuming the parasitized fish. Although it is not absolutely clear whether a dead larva (following cooking or freezing of the fish) is capable of sensitizing the subject, there is evidence to suggest that human sensitization can only be produced by contact with live larvae, although, once sensitized, the subject will also exhibit an allergic response to dead larvae (Alonso-Gómez et al., 2004; Audícana et al., 2002; Daschner et al., 2012).

Gastroallergic anisakiasis: The requirement for contact with live larvae for sensitization to occur has given rise to the name of this type of anisakiasis, in which digestive and allergic symptoms are combined (Alonso et al., 1997; Daschner et al., 2000), being defined as a severe case of IgE-mediated allergy, typically accompanied by gastric digestive symptoms (Valls

238 Sánchez et al., 2009). This acute form may evolve, in some cases, into chronic urticaria
239 (Petithory, 2007).

240 *Occupational anisakiasis*: there is evidence for occupational allergy (dermatitis, asthma,
241 conjunctivitis) caused by *A. simplex* in fishermen, fishmongers or other fish industry workers
242 (Añíbarro and Seoane, 1998; Armentia et al., 1998; Carretero Añíbarro et al., 1997;
243 Nieuwenhuizen et al., 2006; Purello-D'Ambrosio et al., 2000).

244 **Diagnosis**

245 Diagnosis of anisakiasis is not easy due to the lack of specificity of the symptoms. An
246 anamnesis is necessary (Del Rey-Moreno et al., 2008) to establish the consumption, in the 72
247 hours previous to the appearance of symptoms, of sea fish, either raw, marinated, pickled in
248 vinegar, in brine or seared, that is, inadequately cooked such that it contains live larvae of
249 *Anisakis*, for patients with acute epigastric or abdominal pain. Following confirmation there
250 are several diagnostic tests to aid the doctor's decision-making.

251 *Diagnosis of infection by* Anisakis*.*

252 From the first cases of anisakiasis to be identified up to the end of the 20th century diagnosis
253 was generally by identification of the larvae in the eosinophilic granulomas or in segments of
254 the digestive tract affected (Fig. 5), which were surgically resected. However, advances in
255 imaging techniques, especially in endoscopy, have made them more easily available and
256 financially viable for patients suspected of infection following the anamnesis. In this sense,
257 endoscopy is currently the technique of choice for diagnosing acute gastric or intestinal
258 anisakiasis (Bucci et al., 2013). In addition to being able to observe the larvae (Fig. 4C) or
259 fragments of these using gastroscopy or colonoscopy it is also possible to extract them for
260 posterior identification by their morphological features and thus avoid unnecessary surgery
261 (Fig. 6). Furthermore, it has been suggested that the computed tomography, performed before
262 endoscopy, is useful for the diagnosis (Ashida et al., 2017; Shibata et al., 2014).

On occasions, in the absence of a precise diagnosis, particularly in the case of intestinal anisakiasis, exploratory surgery is required. This enables observation and extraction of the larvae from either the abscess or the eosinophilic phlegmon. The anatomopathological study of these samples may reveal sections of the larvae: In the transverse sections we will observe a fine cuticle without wings, a well-developed polymyarian subcuticular musculature, lateral nerve cords in a "Y" shape and smaller dorsal and ventral cords. If the section is of the first third of the larva the muscular esophagus with a tri-radiate lumen can be observed, as can the excretory cell or the excretory tubule (Fig. 6). If the section is of the posterior two thirds the intestine can be observed, as can its epithelium with columnar cells with the nucleus at the base of each cell. Saggital or oblique sections are more difficult to identify. If the larva cannot be identified due to its poor state, a molecular diagnosis can be carried out following DNA extraction (D'Amelio et al., 2012; Paoletti et al., 2018).

Diagnosis of allergy to Anisakis*.*

When allergic symptoms are present a prick-test with *Anisakis* L3 antigen can be performed. Other techniques may be used to determine specific IgE in the patient's serum (Petithory, 2007) such as ImmunoCAP® with total antigen or with selected purified or recombinant antigens from the parasite, the most widely utilized being *Ani s 1*, responsible for the majority of cases of sensitization to *Anisakis*. Some authors have raised doubts concerning these techniques due to possible cross reactions leading to false positive results (Mattiucci and D'Amelio, 2014). Radioallergoabsorbence (RAST) permits differentiation between subclinical allergy and anaphylaxis (Desowitz et al., 1985), but is an expensive technique.

Currently, the most highly-rated techniques are those based on microarrays (Armentia et al., 2017) such as ImmunoCAP® ISAC (Immuno Solid-phase Allergen Chip) to detect, above all, the thermostable allergens *Ani s 1* and *Ani s 3* as these permit differentiation between patients who could experience anaphylaxis (sensitive to *Anis s 1*) and those only having a subclinical

allergy (sensitive to *Ani s 3*). Furthermore, the former is detected in 88% of patients affected by gastroallergic anisakiasis (Shimakura et al., 2004; Subiza, 2020).

Anadón et al. (2010) believed the recombinant allergens *rAni s 1* and *rAni s 7* to be the best option for serological diagnosis of anisakiasis in terms of sensitivity and specificity (Trisakis-170 ELISA kit) since *Ani s 3* (tropomyosin) is a panallergen and gives cross reactions with tropomyosins from other invertebrates such as shrimps, cockroaches or mites (Daschner et al., 2012; Guarneri et al., 2007).

Although these techniques are considered valid for the diagnosis of allergy to *Anisakis*, the fact that part of the healthy population show positive responses to the allergens of *Anisakis* (Del Rey Moreno et al., 2006) raises doubts regarding the choice of suitable techniques or allergens (Table 2). In this sense, Mazzucco et al. (2018) emphasized that the estimates of hypersensitivity varied greatly according to geographical zone, population characteristics, diagnostic criteria and laboratory assays.

Treatment

The treatment for infection by *Anisakis* consists of extracting all the larvae found in the digestive tract during the endoscopic examination (Fig. 7), either by gastroscopy or colonoscopy, depending on the zone affected (Audícana Berasategui et al., 2007). There is no completely efficacious drug available for the treatment of anisakiasis. However, since endoscopy permits the immediate removal of the larvae, leading to an improvement in the patient's condition within a few hours, no pharmacological treatment is required in these cases. The most widely-used drug is albendazole with other anthelmintics such as thiabendazole, flubendazole or ivermectine also used, although none is completely efficacious (Shimamura et al., 2016). Current studies are focusing on natural substances such as wood

creosote (Sekimoto et al., 2011) and essential oils of plants and their components (reviewed by Valero et al., 2015).

When the larva is located in zones of the intestine which are difficult to access, some authors recommend a conservative treatment with fluid therapy and antibiotics, which usually improve the patient's condition (Audícana Berasategui et al., 2007; Shrestha et al., 2014). Occasionally, however, laparotomy and resection of the affected fragment of the intestine is required.

'Allergy to *Anisakis*' should be treated immediately, with the recommendation that the patient should avoid consuming marine fish (Petithory, 2007). In allergic patients suspected of suffering anaphylactic shock, they must be provided with an injectable dose of adrenaline to cope with the contingency and, if it occurs, immediate referral to hospital (Audícana Berasategui et al., 2007).

Prevention and control

The prevention of anisakiasis is based on avoiding the ingestion of live larvae of *Anisakis* through consumption of raw or under-cooked fish or squid. These should have undergone some form of termal treatment since preparation with vinegar or lemon juice, smoking, brining, pickling, marinating, etc. does not always inactivate all the larvae (EFSA-BIOHAZ, 2010). It is generally considered that fish and squid should be cooked at >60 ºC for >1 min or frozen whole at -20 ºC for >24 h (or -35 ºC, >15 h) to ensure the death of the larvae (EU, 2011). This eliminates the possibility of infection by *Anisakis* and sensitization to its allergens. However, if the subject is already sensitized it is still possible for them to develop allergic symptoms (Alonso-Gómez et al., 2004; Audícana et al., 2002; Daschner et al., 2012).

Anisakis is difficult to control due to its complex lifecycle and the large number of potential hosts, particularly paratenic hosts. However, there is a variety of measures to reduce the

incidence of human anisakiasis. The first is health education, which should principally consist of raising awareness regarding prevention in consumers. This education should be broadened and adapted to different economic sectors of the population, such as catering or fishing. Public health agencies (FDA, 2019) and experts committees (EFSA-BIOHAZ, 2010) have recommended a series of measures to become part of a countries' legislation. In the European Union (EU, 2019, 2011, 2004) and other countries as Japan in 2012, most of these measures are now included in current legislation. These measures require:

- establishments serving food or selling prepared food, to freeze fish and squid that are to be consumed raw or under-cooked.
- high seas fish processors, to gut fish and squid to avoid migration from body cavity to muscle tissues (Borderías and Sánchez-Alonso, 2011) and to destroy these viscera rather than to dispose of them in the sea where they would disseminate parasites.
- boats, not to fish in zones where the parasites are known to be abundant, for example, those close to areas with high numbers of cetaceans, definitive hosts of *Anisakis*, where prevalence of the parasite in fish is usually higher than elsewhere (Rello et al., 2009).
- fish farms, to feed fish with products that are free of viable parasites capable of affecting human health.
- food business operators, to guarantee that fish products are inspected visually to detect visible parasites before going on sale and that fish with visible parasites are destroyed.

- food industry businesses selling fish products intended to be consumed raw, marinated, salted or other treatments that are not sufficient to inactivate the parasites, to treat them, in their entirety, at >60 °C for >1 min or, alternatively, to freeze them at -20 °C for >24 hours, or -35 °C for >15 hours, to ensure inactivity of the larvae.

Countries should incorporate these measures into their legislation. It may also be useful to establish the prevalence of anisakiasis in each country with a view to implementing and managing public health resources effectively. This would require that every country have a mandatory national register of all diagnosed cases. Finally, the recommended thermal treatment measures, applicable to many foods, prevent not only anisakiasis but many foodborne pathogens.

Future studies and research needed.

While much progress has been made, our knowledge of *Anisakis* and anisakiasis is still limited. Here, we will only address two important aspects: commercial and sanitary.

From a commercial viewpoint the presence of *Anisakis* in fish products is an aesthetic issue since worms visible to the naked eye repel the consumer. Furthermore, the well-informed consumer is also aware that this parasite can cause health problems. Consequently, the great challenge for the fishing industry is to provide the consumer with a risk-free product, i.e. free from *Anisakis*. The current parasite detection methods, based on visual inspection and candling, are far from precise, despite being endorsed by European Union legislation, as some worms may not be detected. The development of effective detection methods (Smaldone et al., 2020), accompanied by clear and precise legislation, must be a priority and both are vital for the industry (Fig. 8).

From a sanitary point of view, the cosmopolitan nature of *Anisakis* and the increased awareness and knowledge of anisakiasis and its diagnosis by health professionals are responsible for the increase in the number of cases reported in an increasing number of countries, along with cultural and commercial globalization, since gastronomic products prepared with raw or under-cooked fish or squid are consumed worldwide. So, dishes such as *sushi, sashimi, gravlax, groene haring, ceviche/cebiche, boquerones en vinagre*, etc., can be

sources of viable parasites and thus anisakiasis if not properly pre-frozen. However, it seems clear that there is an infradiagnosis in many countries (Seal et al., 2020), related both to awareness among health professionals and to diagnostic methods (Bao et al., 2017; Herrador et al., 2019). Therefore, it is necessary to search for target molecules or infection markers that can be identified with the development of new more sensitive, specific and cheaper diagnostic techniques that are more reliable and accessible to laboratories that must unify diagnostic criteria (Mazzucco et al., 2018).

A. simplex s.s. seems more pathogenic than *A. pegreffii* (Jeon and Kim, 2015; Quiazon et al., 2011; Romero et al., 2013; Suzuki et al., 2010). However, this requires confirmation, while possible differences between the two should be identified (Cavallero et al., 2020, 2018; Llorens et al., 2018; Molina-Fernández et al., 2019; Torralbo-Ramírez et al., 2019), including allergens (Arcos et al., 2014; Baird et al., 2016), as they may affect diagnostic methods. As it has also been suggested that anisakiasis constitutes a risk factor for stomach and colon cancers (Corcuera et al., 2018; García-Pérez et al., 2015), studies to determine its relationship with these and other diseases are especially relevant. Furthermore, Sánchez-Velasco et al. (2000) suggested a genetic predisposition to allergy to *Anisakis*, whose study could help prevent hypersensitivity in at-risk subjects. Likewise, the study of immunogenic molecules for vaccine development and the design allergen-specific immunotherapy are options for the future.

Finally, there are many potentially useful topics for research and development in the field of anisakiasis and new omic tools will undoubtedly facilite the task (D'Amelio et al., 2020). Here, we have only mentioned those that we consider most urgent regarding the prevention, diagnosis and control of anisakiasis.

404 *Acknowledgements*

405 Funding: This research did not receive any specific grant from funding agencies in the public,

406 commercial, or not-for-profit sectors. The authors are grateful to Drs Irene Adroher-Benítez,

407 Lola Molina-Fernández and Carolina Fernández-Maldonado for their help with the photos and

408 figures. Translation to English was by Robert Abrahams, BSc.

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902

Table 1.- Molecular classification of known *Anisakis* species in clades (after Mattiucci et al., 2018).

Clades	*Anisakis* species	L3 type[a]	Anisakiasis	Observations
Clade 1	*A. simplex sensu stricto*	Type I	Human[b]	
	A. pegreffii	Type I	Human[b]	
	A. berlandi	Type I	?[c]	Low frequency in commercial fish
Clade 2	*A. ziphidarum*	Type I	?	Low frequency in commercial fish
	A. nascetti	Type I	?	Low frequency in commercial fish
Clade 3	*A. physeteris*	Type II	Lab animals[b,d]	Human anisakiasis by *Anisakis* larva type II or *A. physeteris* (morphological diagnosis) reported[e].
	A. brevispiculata	Type II	?	
	A. paggiae	Type II	Lab animals[b,d]	
Clade 4	*A. typica*	Type I	?	Low frequency in fish muscle tissue

([a]) Sensu Berland (1961), larvae of the same type (I or II) are morphologically indistinguishable. ([b]) Confirmed by molecular diagnosis. ([c]) ?, Human and experimental infections have not been proved. ([d]) Experimental infection by Romero et al. (2014). ([e]) See references in Molina-Fernández et al. (2018a).

865

Table 2.- Characteristics of the described allergens of third larval stage (L3) of *Anisakis simplex s.l.*

Allergen[a]	Origin	Protein features	Mw (kDa)[a]	References
Ani s 1	ES	Kunitz-type serine-protease inhibitor; domain lustrine-type cystein-rich. Major allergen. Thermostable.	21/24	Moneo et al., 2000 Shimakura et al., 2004
Ani s 2	Somatic	Paramyosin. Cross-reactivity with other helminth and arthropod paramyosins.	97/100	Pérez-Pérez et al., 2000
Ani s 3	Somatic	Tropomyosin. Cross-reactivity with arthropod tropomyosins.	41	Asturias et al., 2000a, 2000b
Ani s 4	ES	Cysteine protease inhibitor. Thermostable. Resistant to pepsin digestion.	9/12.7	Moneo et al., 2005 Rodríguez-Mahillo et al., 2007
Ani s 5	ES	SXP/RAL-2 family protein. Thermostable.	15	Kobayashi et al., 2007 Caballero et al., 2008
Ani s 6	ES	Serine protease inhibitor. Resistant to pepsin digestion.	7	Kobayashi et al., 2007
Ani s 7	ES	Glycoprotein with tandem repeat sequences. Major allergen. Specific for *Anisakis*-infected patiens.	139	Rodríguez et al., 2008 Anadón et al., 2009
Ani s 8	ES	SXP/RAL-2 family protein. Thermostable. Crossreactivity with Ani s 5.	15	Kobayashi et al., 2007b
Ani s 9	ES	SXP/RAL-2 family protein. Thermostable. Share identity partial with Ani s 5 and Ani s 8.	14	Rodríguez-Pérez et al., 2008
Ani s 10	Somatic	Unknown function. With repeat sequences. Thermostable.	21	Caballero et al., 2011
Ani s 11	?	Unknown function. With repeat sequences. Ani s 11-like 78% similarity (17 kDa)	27	Kobayashi et al., 2011 Carballeda-Sangiao et al., 2016
Ani s 12	?	Unknown function. With repeat sequences.	31	Kobayashi et al., 2011
Ani s 13	?	Haemoglobin. No cross-reactivity to *Ascaris suum* haemoglobin.	37	González-Fernández et al., 2015
Ani s 14	?	Unknown function. Share identity partial with Ani s 7 and Ani s 12.	24/27	Kobayashi et al., 2015

[a] Allergens nomenclature according to WHO/IUIS Allergen Nomenclature Sub-Committee (2020). ES, excretory-secretory. ?, no reported.

903 Legends to Figures:

904 Figure 1.- Differential morphological features among third stage larvae (L3) of nematodes that
905 frequently occur in fish and that can potentially cause anisakidosis. Left: A freshly collected L3
906 from the body cavity of a blue whiting, host fish. Right: Drawings of the cephalic end,
907 ventricular area and caudal end of the larvae showing its external morphology and digestive
908 system. Note the differences between species, especially in the number of cecums in the
909 ventricular area. Also, note the position of the excretory pore to differentiate *Contracaecum*
910 and *Hysterothylacium*. Finally, note the differences between *Anisakis* type I (elongated
911 ventriculus with oblique join to the intestine, tail with mucron) and type II larvae (shorter,
912 thicker ventriculus with straight join to the intestine, conical tail). Abbreviations: a, anus; bt,
913 boring tooth; e, esophagus; ep, excretory pore; i, intestine; ic, intestinal caecum; m, mouth; nr,
914 nerve ring; p, papilla; r, rectum; rg; rectal gland; s, spine or mucron; v, ventriculus; va,
915 ventricular appendage. Drawing by Irene Adroher-Benítez.

916

917 Figure 2.- Lifecycle of *Anisakis* spp. L1, L2 and L3 are the first, second and third larval stages of
918 these parasitic nematodes into the eggs. L3 are also found in the body cavity of all
919 intermediate and paratenic hosts throughout the lifecycle of the worms. The fourth larval
920 stage (L4) and adults develop in the digestive tract of the final hosts. Note that L3 is the
921 infective stage for all hosts. Drawing by Lola Molina-Fernández.

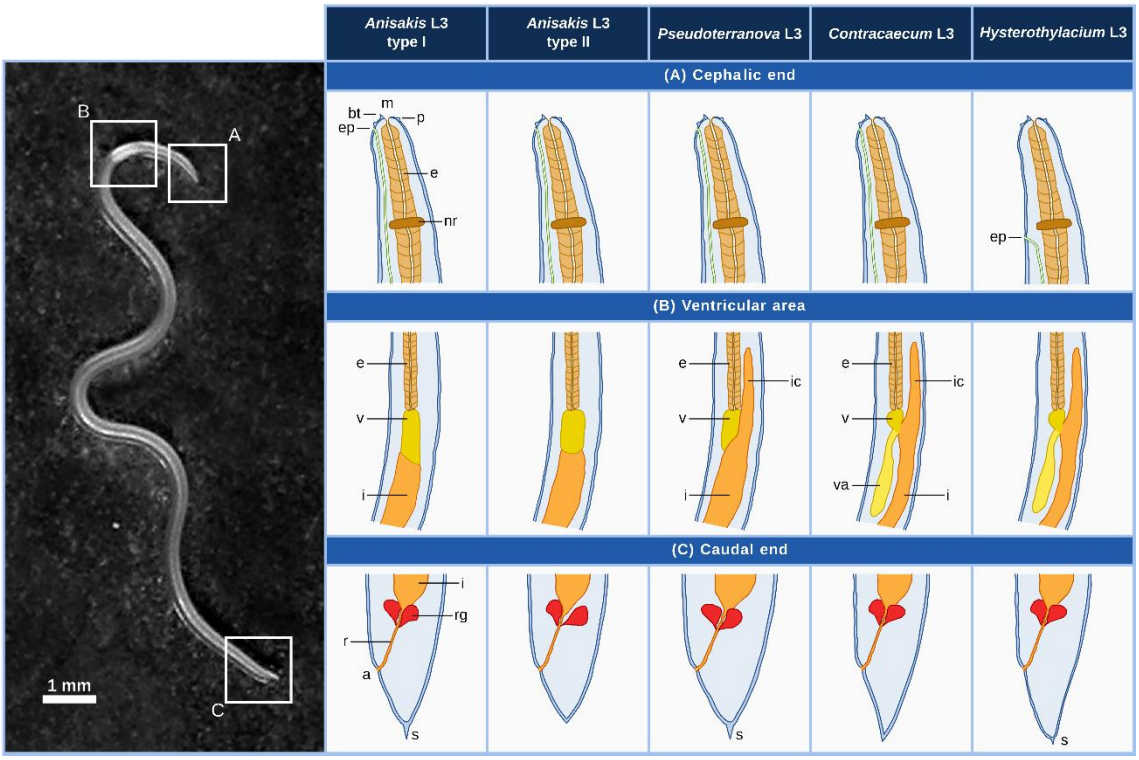
922

923 Figure 3.- *Anisakis simplex s.l.*, female adult obtained from *in vitro* culture described by Iglesias
924 et al. (2001). Scale in cm.

925

926 Figure 4.- Infection by *Anisakis simplex s.l.*: Worms (L3) in the body cavity of blue whiting,
927 *Micromesistius poutassou* (A), adults in the first gastric chamber of the bottlenose dolphin,

928 *Tursiops truncatus* (B), and L3 in the human stomach (C). Credits: Image B courtesy of
929 Fernández-Maldonado (2016), and C from Shimamura et al. (2016).
930
931 Figure 5.- *Anisakis simplex s.l.* L3 section in human. A) L3 penetrating the digestive wall
932 surrounded by acute inflammatory cells (bar, 1 mm). B) Cross section of L3 surrounded by a
933 thick cuff of acute inflammatory cells with numerous eosinophils "showing transmural
934 infiltration of acute inflammatory cells with diffuse subserosal (ss) involvement" (bar, 500 μm).
935 From Takei and Powell (2007), courtesy from Elsevier ©.
936
937 Figure 6.- Cross section through the L3 of *Anisakis simplex s.l.* (M: polymyarian muscle layer;
938 LEC, lateral nervous cord; EG, excretory cell; DT, esophageal lumen). From Takei and Powell
939 (2007), courtesy from Elsevier ©.
940
941 Figure 7.- Extraction of a larva of *Anisakis simplex s.l.* from human digestive tract (after
942 Shimamura et al., 2016).
943
944 Figure 8. Observation of pressed and frozen fillets of blue whiting in UV-light allows the
945 detection of *Anisakis* larvae as bright fluorescent spots. The straight lines mark the boundary
946 between the epiaxial (dorsal) and hypoaxial (belly) muscles of each side of fish. Photo by A.
947 Levsen (EFSA-BIOHAZ, 2010).



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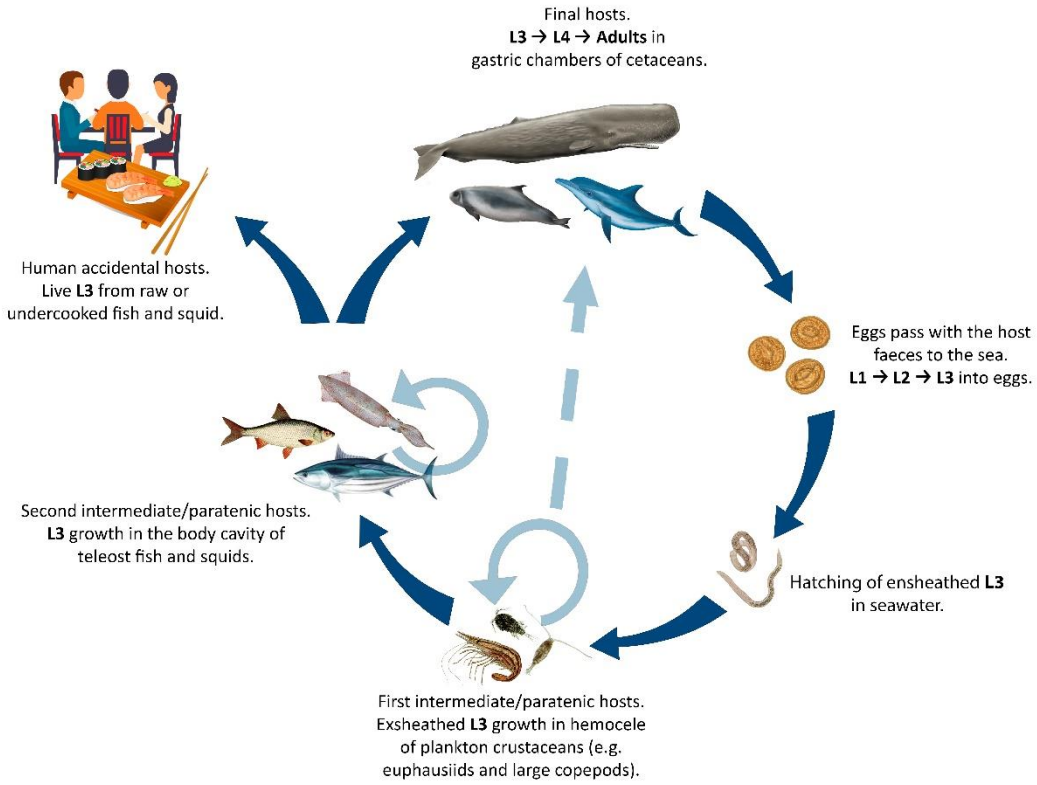

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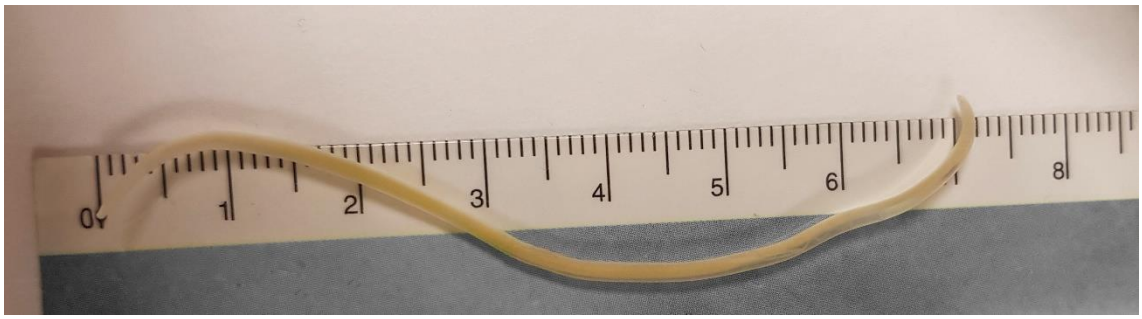

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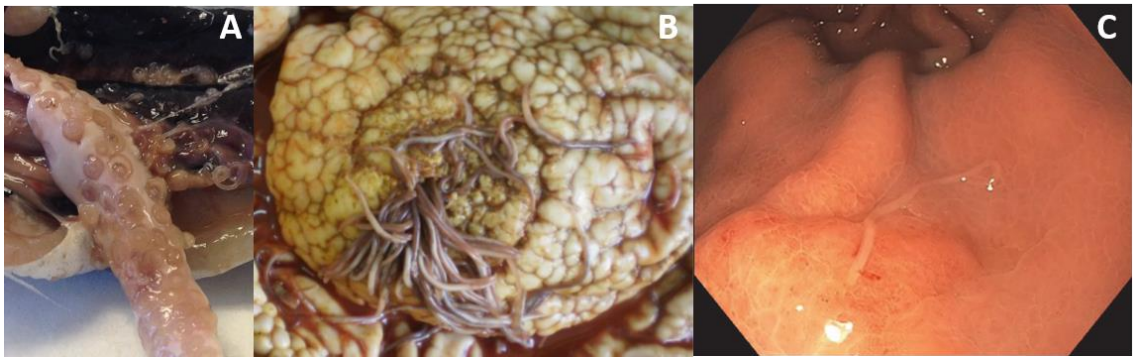

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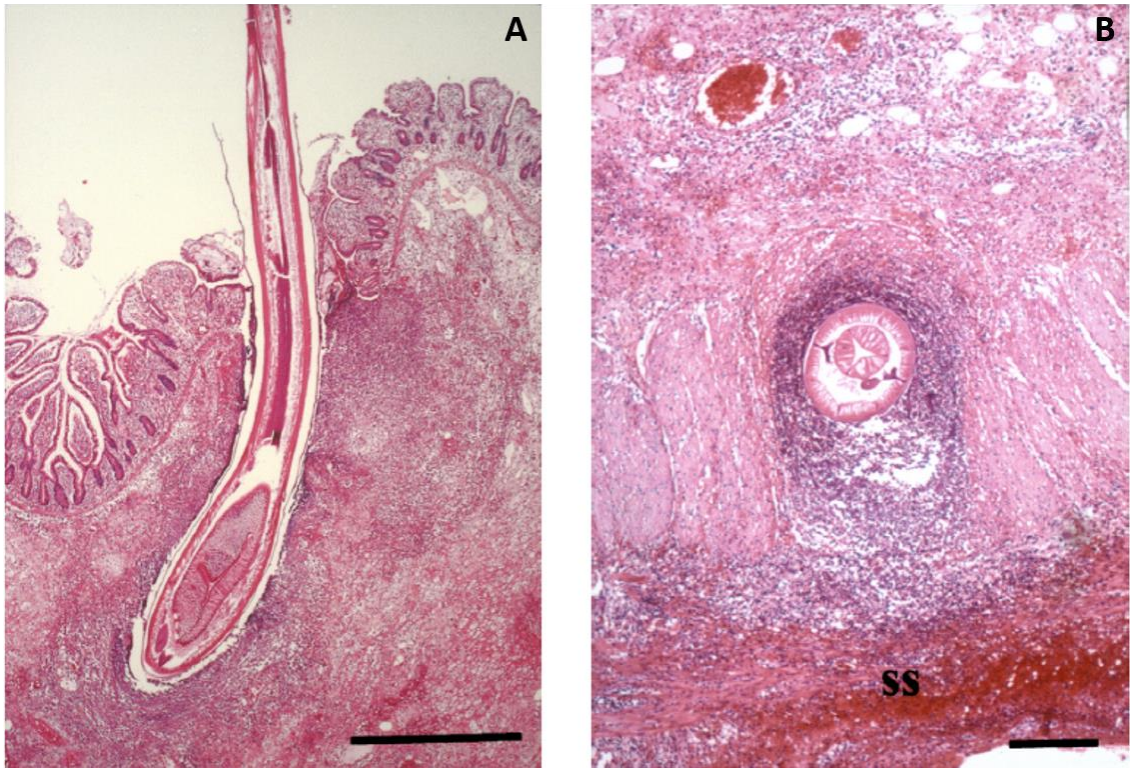

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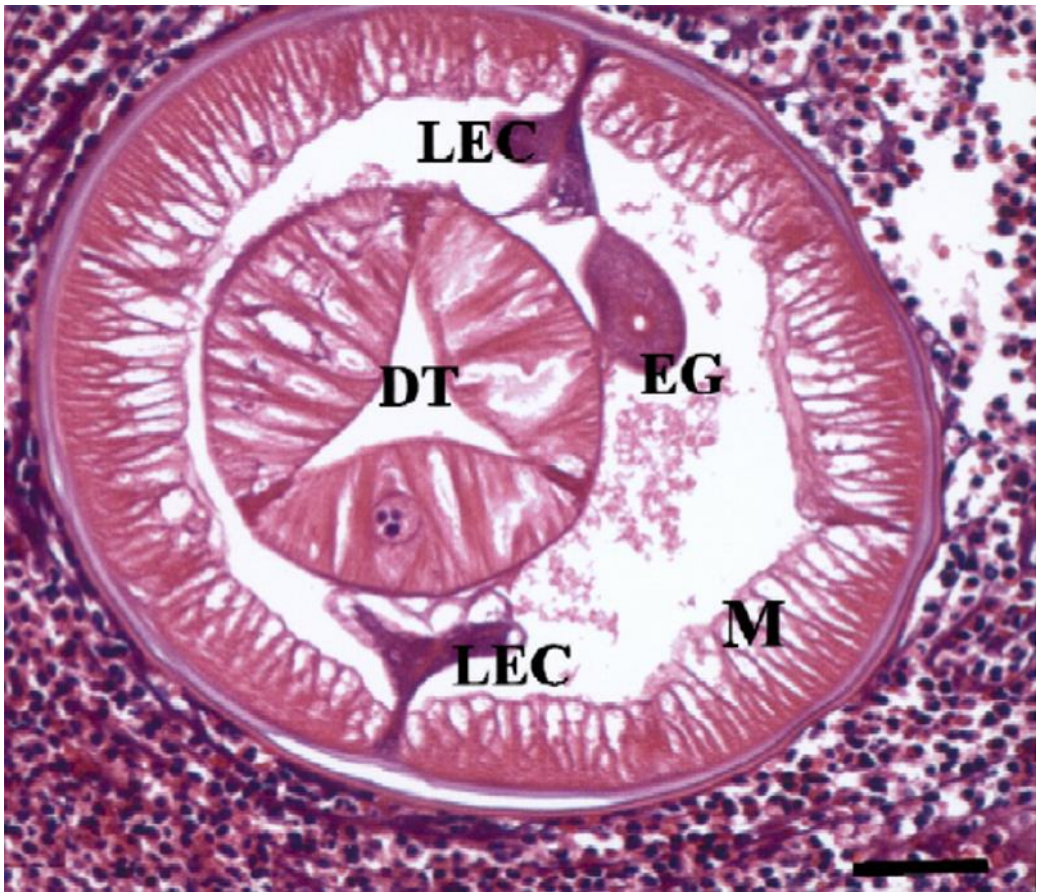

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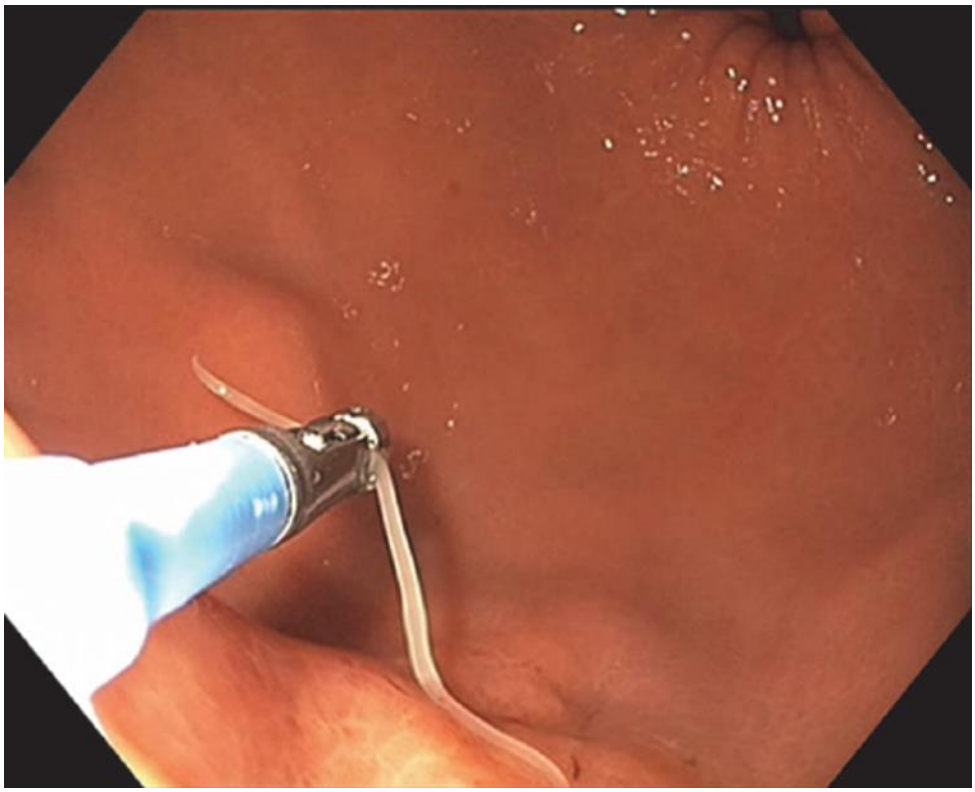

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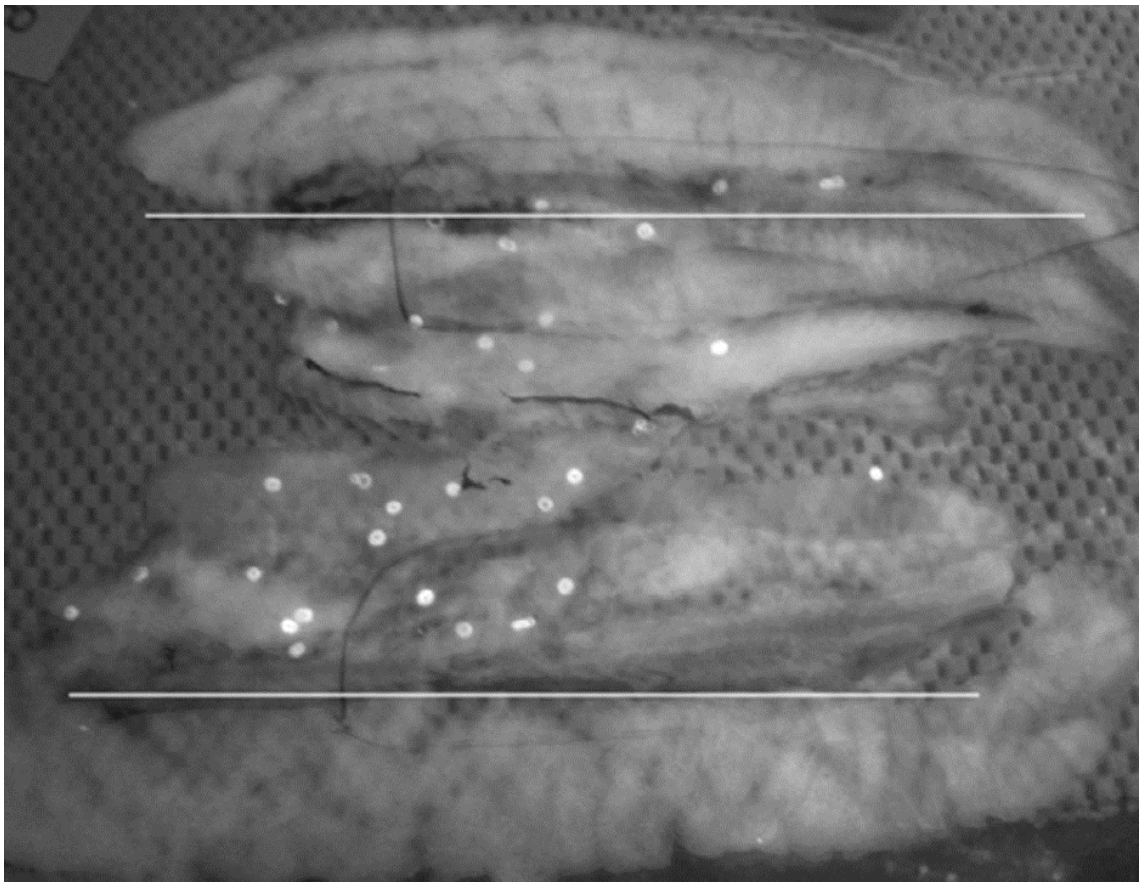

Figure 8.