

A rat model of intragastric infection with *Anisakis* spp. live larvae: histopathological study

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Abstract Anisakiasis is a fish-borne parasitic disease caused by consumption of raw or undercooked fish or cephalopods parasited by *Anisakis* spp. third stage larvae. The pathological effects of the infection are the combined result of the mechanical action of the larva during tissue invasion, the direct tissue effects of the excretory/secretory products released by the parasite, and the complex interaction between the host immune system and the *Anisakis* antigens. The aim of this study was to develop an experimental model of infection with *Anisakis* spp. live larvae in rats, useful to study the acute and chronic histopathological effects of the *Anisakis* infection. Sprague–Dawley rats were subjected to esophageal catheterization to place larvae directly into the stomach. Reinfections at different intervals after the first infection were preformed. Live larvae were found anchored to the mucosa and passing through the wall of the stomach and showed a strong resistance being able to stay alive at different sites and at the different pH. Migration of larvae from the stomach to other organs out of the

gastrointestinal tract was also observed. The histopathological study showed the acute inflammatory reaction, with predominance of polymorphonuclear eosinophils and a mild fibrotic reaction. The model of infection described is valid to study the behavior of the larvae inside the host body, the histopathological changes at the invasion site, and the effects of the repeated infections by ingestion of live larvae.

Anisakiasis is a fish-borne parasitic disease caused by consumption of raw or undercooked fish or cephalopods parasited by *Anisakis* spp. third stage larvae (Klimpel et al. 2004). Symptoms of acute gastric infection include sudden epigastric pain, nausea, and vomiting, and can occur 1–12 h after the infected meal. Anisakiasis is often associated with a strong allergic response, with clinical symptoms ranging from isolated swellings to urticaria, angioedema, and life-threatening anaphylactic shock (Audicana et al. 1996; Del Pozo et al. 1997).

Anisakiasis is classified into three categories according to the location of the lesions: gastric, intestinal, and heterologous or ectopic (extragastrointestinal location of larvae) (Ishikura et al. 1993). The pathological changes occurring during infection with *Anisakis simplex* are the combined result of the mechanical action of the larva during tissue invasion, the direct tissue effects of the excretory/secretory products released by the parasite, and the complex interaction between the host immune system and the *Anisakis* antigens (Audicana and Kennedy 2008).

A technique for induction of an oral infection with *Anisakis* spp. larvae in mice was previously described (Figueiredo et al. 2012), and studies of experimental anisakiasis have been reported based on describing the serological response of the host (Campos et al. 2004; Cho

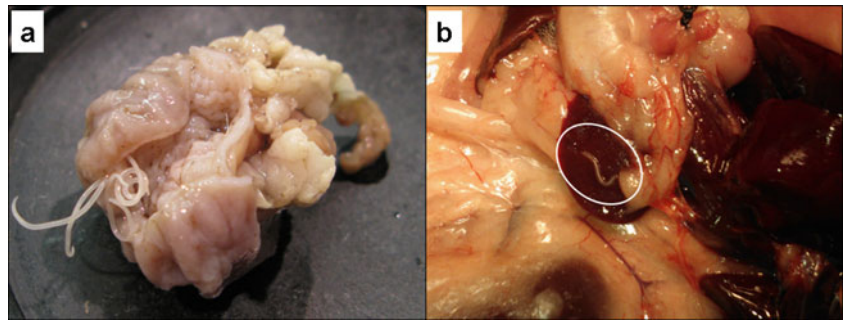
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Fig. 1 **a** Luminal side of an opened rat stomach showing several *Anisakis* spp. larvae anchored to the gastric wall; **b** *Anisakis* spp. live larva seen on the surface of the median lobe of the rat liver



and Lee 2006; Kim et al. 2005). However, neither the behavior of the larvae inside the body of the host nor the histopathological effects of infection have been described.

The aim of this study was to develop an experimental model of infection with *Anisakis* spp. live larvae in rats, useful to study the acute and chronic histopathological effects of the *Anisakis* infection.

Anisakis larvae were isolated from fish (*Merluccius merluccius*) and washed and kept in saline solution at 4 °C to use for infection.

Eight Sprague–Dawley rats (250 g weight) were subjected to esophageal catheterization to place larvae directly into the stomach. The rats fasted for 24 h before the procedure in order to facilitate the anchoring of the larvae to the gastric mucosa. The animals were under general anesthesia (5 % isoflurane for anesthesia induction and 2 % for maintenance) and at the end of the procedure, they were kept in spontaneous breathing until recovery of consciousness.

All animals were treated according to the European Union's guidelines on the protection of animals used for scientific purposes (Directive 2010/63/EU).

Anisakis live larvae were directly placed into the stomach of the rat by a probe Nelaton Catheter CH10, 40 cm (Maersk Medical A/S, Copenhagen, Denmark). This probe was selected based on its blunt end and an appropriated diameter to be introduced through the rat esophagus. This catheter has two lateral orifices in the distal area. The most distal orifice

was expanded longitudinally in order to transport between 10 and 12 live larvae, whereas the other hole was closed with a thin strip of laboratory film (Parafilm M, Neenah, WI, USA). The probe was fitted to the rat esophagus length and was then adapted to an animal feeding needle 16 G×3 in., 7.62 cm (Popper & Sons, New Hyde Park, NY, USA) and a syringe filled with 1.5 mL of distilled water whose slow injection facilitated the expulsion of larvae into the stomach.

Reinfections at different intervals 24, 48, 72, and 96 h after the first infection were performed following the protocol described.

The animals were killed at 24, 48, and 72 h, and 5 and 10 days after infection or reinfections by introducing them in a CO₂ chamber for morphological and histopathological examination. During the necropsy, the pH of the content of different sections of the gastrointestinal tract was measured by a universal pH indicator strip (Nahita, Navarra, Spain). The complete gastrointestinal tract, liver, pancreas, spleen, lungs and kidneys were fixed with 4 % formaldehyde for 24 h and embedded in paraffin using an automated vacuum tissue processor Leica TP 1050, following the histological conventional protocol. Thin sections (4 μm) were stained with hematoxylin–eosin and Masson trichrome dyes for general histological study.

Twenty-four hours after intragastric infection, live larvae were found anchored to the mucosa (mainly at the fundus) and passing through the wall of the stomach (Fig. 1a). Other

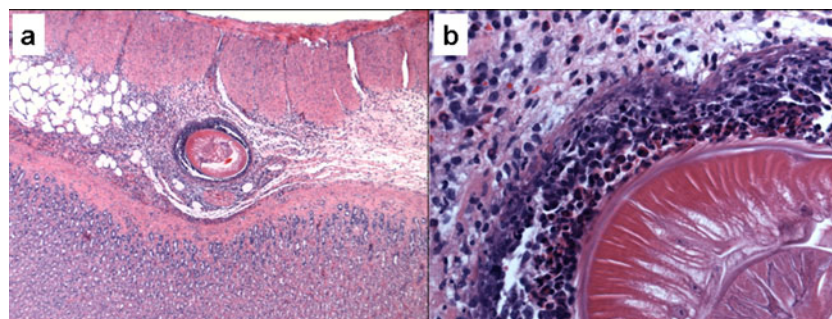


Fig. 2 Microscope image of rat stomach stained with hematoxylin–eosin. **a** Cross-section of an *Anisakis* spp. larva located in the submucosa and surrounded by an acute inflammatory reaction,

(original magnification ×50); **b** Detail of the previous image. There is a predominance of polymorphonuclear eosinophils and a mild fibrotic reaction, (original magnification ×400)

live larvae were seen into the colon and cecum. *Anisakis* spp. larvae showed a strong resistance being able to stay alive at different sites of the rat until 48 h after the intragastric infection. Live larvae were found in the lumen of different sections of the gastrointestinal tract and at the different pH conditions measured, which ranged between pH4 in the fasted rat stomach and pH6.5 in the cecum. Migration of larvae from the stomach to other organs out of the gastrointestinal tract was also observed (Fig. 1b). Generally, one third of the injected larvae were not found and these larvae might have been destroyed and/or evacuated. Five days after first infection, hyperemia, and edema were observed in different bowel sections (jejunum, colon, and cecum). All these effects observed after infection of rats with *Anisakis* spp. larvae resembled the features described in human anisakiasis (Arizono et al. 2012).

Anisakis spp. larvae use a mechanical disruption of tissue combined with the release of potent proteolytic enzymes to burrow into the gastrointestinal mucosa. These proteases are produced by the excretory cell of the larva to be secreted through the excretory pore (Audicana and Kennedy 2008). Figure 2a, shows an *Anisakis* spp. larva invading the gastric submucosal layer while preserving its structure. Figure 2b shows in detail the eosinophilic infiltration in the tissue surrounding the larva. Eosinophils adhere to the nematode's epicuticle in the presence of antibodies releasing cytotoxic factors which, although apparently not capable of damaging the nematode's cuticle, are probably responsible for a great deal of tissue damage observed surrounding the parasite in both acute and chronic infections (Deardorff et al. 1991).

Human anisakiasis is linked to the habit of eating raw or undercooked fish, this leads to frequent reinfections after the primary one, mainly if the symptoms associated to the first episode were mild. This fact explains that 15 % of Spanish patients who attended the consultation for suspected allergy without fish-related allergy have IgE antibodies against the parasite allergens, which implies that these individuals have been in contact with the parasite more than once (Caballero et al. 2012).

To conclude, the model of infection described is valid to study the behavior of the larvae inside the host body, the histopathological changes at the invasion site, and the effects of the repeated infections by ingestion of live larvae.

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Ethical standard The experiments comply with the current laws of the country in which they have been performed.

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