Journal of Helminthology (2015) **89,** 620–624
doi:10.1017/S0022149X14000340
© *Cambridge University Press 2014*

Experimental anisakid infections in mice

M.A. Vericimo[1], I. Figueiredo Jr[2]*, G.A.P.B. Teixeira[1] and S.C. São Clemente[3]

[1]Departamento de Imunobiologia, Instituto de Biologia, Universidade Federal Fluminense, Outeiro São João Batista s/n, CEP 24020-150 Centro, Niterói, Rio de Janeiro, Brasil: [2]Departamento Materno-Infantil, Faculdade de Medicina, Hospital Universitário Antonio Pedro, Universidade Federal Fluminense, Av. Marques do Paraná 303, CEP 24030-210 Centro, Niterói, Rio de Janeiro, Brasil: [3]Departamento Tecnologia de Produtos de Origem Animal, Faculdade de Veterinária, Universidade Federal Fluminense, Rua Vital Brasil Filho 64, CEP 24.230-340, Niterói, Rio de Janeiro, Brasil

(Received 8 May 2013; Accepted 27 March 2014; First Published Online 29 April 2014)

Abstract

Anisakidosis is a human parasitic disease caused by infections with members of the Anisakidae family. Accidental infection after fish intake affects the gastrointestinal tract as a consequence of mechanical damage caused by migrating larvae. Infections can also trigger allergies, hives, severe asthma or anaphylaxis with angioedema. Although mouse models of intraperitoneal antigenic stimulation exist, enabling immunological studies, few models using gastric introduction of live larvae are available for the study of immunological and gastrointestinal damage in mice. This study was designed to characterize serum reactivity against *Anisakis* spp. and *Contracaecum* spp. in Balb/c mice following orogastric inoculation and to assess gastrointestinal damage. These anisakid species were classified at the Universidade Federal Fluminense (UFF) School of Veterinary Medicine and materials for live larval inoculation were developed at the UFF Immunobiology laboratory. Live larvae were inoculated following injection with a NaCl solution. Blood samples were collected and sera screened for immunoglobulin (Ig)E and IgG anti-larva responses to both nematodes, specific for somatic and excretory/secretory antigens, by enzyme-linked immunosorbent assay (ELISA). The means of the optical densities were analysed using analysis of variance (ANOVA) with Tukey's post-hoc test and the general linear model. This analysis identified the presence of anti-IgG seroreactivity to both somatic and excretory/secretory *Anisakis* antigens in inoculated animals compared with controls ($P < 0.001$), and no gastric or intestinal damage was observed. These experiments demonstrated that introduction of live *Contracaecum* spp. into the gastrointestinal tract did not elicit serum sensitization in animals.

Introduction

Anisakidosis is a parasitic disease of humans that develops following the ingestion of raw or undercooked marine fish infected with larvae of the Anisakidae family (Audicana & Kennedy, 2008). Recent studies have reported the allergenicity of *Anisakis* spp. in humans and the possible diagnosis thereof (Daschner *et al.*, 2012; Mattiucci *et al.*, 2013).

Based on data described by Huizinga (1965), Ansary *et al.* (2009) demonstrated that fish inhabiting the Mehran River in Iran were infected with third-stage (L3) *Contracaecum osculatum* and suggested that infections in

*E-mail: vericimo@vm.uff.br

https://doi.org/10.1017/S0022149X14000340 Published online by Cambridge University Press

humans might cause gastrointestinal ulcers and bleeding, triggering serious illness and death in severe infections. Additional reports have confirmed that humans can be infected with either *Contracaecum* spp. (Schaum & Müller, 1967) and/or *Pseudoterranova* sp. (Skirnisson, 2006), underscoring the need to screen fish for these pathogens before human consumption.

Despite the possibility of these parasites causing harm to humans, few reports describe serologic responses against *Contracaecum* spp. following infection. In addition, most models have examined pathology associated with *Anisakis* spp. infections following the intraperitoneal introduction of antigens. It has been suggested that the presence of only one live larva in the gastric cavity is sufficient to elicit an immune response and produce gastrointestinal pathology in humans. To date, no infection models have used live *Contracaecum* spp. larvae for antigen stimulation in mice.

The aim of this study was to investigate serum reactivity and to assess gastrointestinal damage in mice infected with live *Anisakis* spp. or *Contracaecum* spp. larvae via orogastric inoculation.

Materials and methods

Study design

This study represents a pilot experiment designed to characterize the immune responses and pathology associated with the presence of L3 *Anisakis* spp. or *Contracaecum* spp. larvae in the gastric cavity of mice. A total of 18 Balb/c mice, aged 2 months, were inoculated via gavage with a single live *Anisakis* spp. or *Contracaecum* spp. L3 larva and five control mice received 0.9% NaCl solution via the same route. Mice were anaesthetized by subcutaneous injection of xylazine, 10 mg/kg body weight (Rompun®, Bayer, São Paulo, Brazil) and ketamine (Francotar®, Virbac SA, Brazil), 10 mg/kg body weight.

Collection and examination of larvae

Larvae of both nematodes were obtained from three fish species: pink cusk-eel (*Genypterus brasiliensis*, Regan, 1903), cutlassfish (*Trichiurus lepturus*, Linnaeus, 1758) and red porgy (*Pagrus pagrus*, Linnaeus, 1758). Fish were purchased from a local fish market in the municipality of Niterói, state of Rio de Janeiro, Brazil, and were transported to the UFF School of Veterinary Medicine for collection of nematodes from their viscera. The harvested nematodes were then transported to the UFF Laboratory of Immunobiology at the Institute of Biology. The number of larvae harvested from each fish ranged from five to eight and they were primarily located in the mesentery, stomach and intestinal serosa. Larvae were identified by optical microscopy, taking into account the established differences among the ventricles, intestinal caecum, number of rectal glands and presence of the mucron, which is absent in *Contracaecum* spp. (Saad *et al.*, 2012). Larvae were stored in 0.9% NaCl solution until the start of the experiment. Immediately before placement in the inoculation tube, larvae were transferred to a Petri dish containing 5 ml of 50 mM HCl to encourage movement.

Gastric intubation and pathology

A non-toxic, commercially available size 4 paediatric silicon tube was used to inoculate mice via gavage with *Anisakis* spp. or *Contracaecum* spp. L3 larva (Figueiredo *et al.*, 2012). Introduction of the larvae into the gastric cavity was successful. After inoculation, mice were monitored for breathing difficulties related to the procedure and for the development of any allergic symptoms (Li *et al.*, 1999) and/or gastrointestinal manifestations associated with systemic disease. After 42 days, the mice were euthanized and gastrointestinal tract specimens were collected and analysed using ImagePro 4.5® (Media Cybernetics, Silver Spring, USA). Histologically, the specimens were investigated for the presence of oedema, neutrophils, eosinophils and other inflammatory cells, and eosinophilic granuloma.

Antigens

Once the respective parasites had been identified, somatic antigens were obtained using a Potter homogenizer. The resulting homogenate was then centrifuged and supernatants collected and stored. To obtain excretory/secretory antigens, live larvae were incubated in 0.9% NaCl solution containing 50 mM HCl at 37°C for 18 h and the supernatants then collected (Caballero *et al.*, 2008), protein concentrations determined (Lowry *et al.*, 1951) and antigens stored at −70°C until use.

Enzyme-linked immunosorbent assay

The presence of serum immunoglobulin (Ig)G and IgE responses specific to *Anisakis* antigens was determined by enzyme-linked immunosorbent assay (ELISA). Briefly, 50 µl of the *Anisakis* antigen solution containing 20 µg protein/ml was added to each well of a 96-well microplate (MaxiSorp™ Surface, Nunc, Roskilde, Denmark), which was incubated overnight at 4°C. After washing with PBS-T (phosphate-buffered saline, 0.05% Tween 20), the plates were blocked with PBS-1% gelatin (PBS-G) for 2 h at room temperature. For the determination of IgG and IgE, 50 µl of duplicate serum samples were analysed from serial dilutions of 1:10 and 1:50 v/v, respectively. PBS-G was added to each well. After a 2 h incubation at 37°C, the plates were again washed with PBS-T and 50 µl of horseradish peroxidase (HRP) goat anti-mouse IgG and IgE (Sigma-Aldrich, St. Louis, Missouri, USA) was added to each well for 1 h at 37°C according to the manufacturer's instructions. Reactivity was visualized following the addition of *o*-phenylenediamine and H_2O_2 (diluted in citrate phosphate buffer, pH 5.0) and the reaction stopped after 20 min with 2 N sulphuric acid. Optical density (OD) was determined using an automated plate reader (Anthos 2010, Krefeld, Germany) at 490 nm. Results are expressed as the mean OD of duplicate wells.

Data analysis

Serological data were plotted according to the best view using the general linear model. Tested parameters were expressed as mean and standard deviation. P values < 0.05 were considered statistically significant,

https://doi.org/10.1017/S0022149X14000340 Published online by Cambridge University Press

and determined using analysis of variance (ANOVA) with Tukey's post-hoc test.

Results and discussion

No complications were noted during the infection procedure, particularly respiratory distress, suggesting that the gavage procedure was successful and caused no adverse effects. Furthermore, in the days and weeks following the procedure, no behavioural or systemic changes were observed in the animals in the experimental or control groups. No animals in either group presented any signs of acute intestinal disturbances or systemic allergic conditions, and at no time was there evidence of L3 larva elimination in the faeces. No histopathological changes in the gastrointestinal tract were observed.

Significant serum IgG somatic and anti-excretory/secretory responses were elicited following gastric inoculation of *Anisakis* ($P < 0.001$) (fig. 1A). When the IgE anti-excretory/secretory *Anisakis* response was analysed by the Tukey post-hoc test, no significantly different responses to excretory/secretory antigens were observed ($P = 0.0978$); however, the IgE anti-*Anisakis* somatic antigen response was positive in consecutive samples examined between 0 and 42 days ($P < 0.001$) compared to controls. This mild reaction was not considered significant (fig. 1C). No somatic or excretory/secretory IgG or IgE antibody responses specific to *Contracaecum* spp. were observed (fig. 1B and D).

Mouse models have been developed as a means of gaining a better understanding of the pathology associated with *A. simplex* L3 infections (Cho & Lee, 2006). Previous studies have reported that the administration of *Anisakis* L3 larvae via the gastric route (Perteguer & Cuellar, 1998; Perteguer *et al.*, 2003) elicited nematode-specific antibodies; however, these studies did not quantify the number of larvae used for respective inoculations. The experiment described herein examined pathological and immune responses to inoculation with a single L3 larva, although it is possible that human infections are the result of more than one larva.

Different inoculation routes, such as intraperitoneal (Rodriguez *et al.*, 2006), intramuscular, intravenous and intranasal (Kirstein *et al.*, 2010) have been utilized to study immune responses elicited by *Anisakis* in mice. However, few analyses examining allergic responses in the gastrointestinal mucosa, or eosinophilic granuloma development, associated with *Anisakis* infections or L3 larva migration have been carried out. Jones *et al.* (1990) described several histopathological findings in an animal model in which L3 *Anisakis* larvae were surgically introduced into the abdominal cavity. Based on these observations, it is likely that T-helper (Th)1 and Th2 type responses would be elicited following orogastric inoculation, suggesting that a model of intestinal allergic

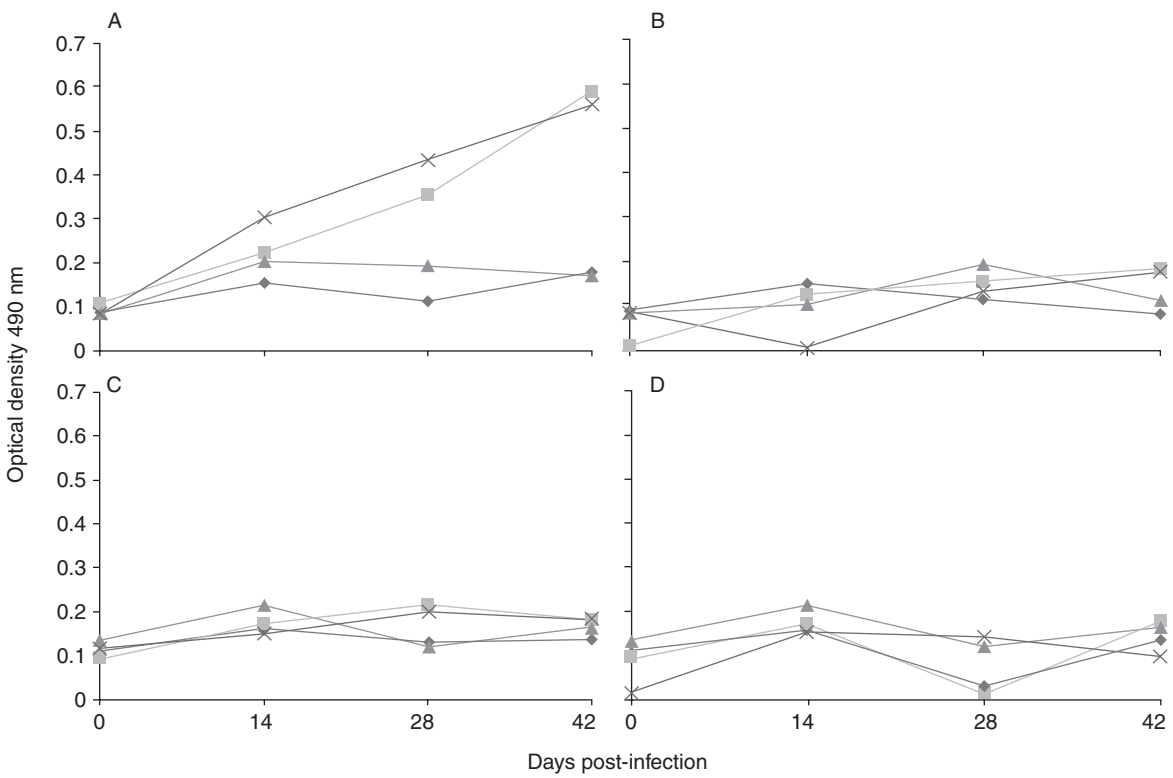


Fig. 1. Optical density values up to 42 days post-infection in mice to show (A) anti-*Anisakis* IgG (immunoglobulin G) responses specific for excretory/secretory antigens (ES) and somatic (S) antigens; (B) no anti-*Contracaecum* IgG responses; (C) no anti-*Anisakis* IgE (immunoglobulin E) responses to ES nor S antigens; and (D) no anti-*Contracaecum* IgE responses to ES nor S antigens; (–■–) IgG and IgE ES larval antigen, (–×–) IgG and IgE S larval antigen and (–▲–) (–◆–) controls.

https://doi.org/10.1017/S0022149X14000340 Published online by Cambridge University Press

responses can be developed as a means of examining disease presentation, which is poorly defined in humans.

Although this topic is still a matter of controversy, it is not known whether live worms must be present to elicit allergic symptoms (Audicana *et al.*, 2002; Alonso-Gomez *et al.*, 2004). Somatic antigens are the most abundant and cross-reactive *Anisakis* antigens, making it difficult to diagnose infections caused by related species (Weiler, 2007). These antigens are synthesized in body structures and can be obtained by homogenizing whole larvae. However, excretory/secretory antigens help parasites penetrate the gastric and intestinal mucosa (Valls *et al.*, 2003), and, as shown in this report, the presence of L3 *Anasakis* larvae in the gastrointestinal tract elicited serum IgG production with specificity for both types of antigens. It is currently accepted that *Anisakis* sensitization is only produced by living larvae and that Ani s 7 is the most important excretory/secretory *A. simplex* allergen (Anadon *et al.*, 2009).

Responses elicited against *Anisakis* spp. were significantly different from reactions elicited against *Contracaecum* spp. L3 larva of *Contracaecum* spp. are structurally similar to *Anisakis* spp. and range from 3 to 6 cm in length, with an average thickness of 0.16 cm. Both species possess a flat tooth with a ventrally located excretory pore. The excretory channel is located at the basal and lateral strands in a classic butterfly form (Laffon-Leal *et al.*, 2000). These same authors demonstrated that *Contracaecum* spp. larvae migrated into fish muscles in a manner similar to that of *Anisakis* larvae, making it difficult for health authorities to ignore this infection. *Contracaecum* spp. has only rarely been implicated as a causative agent of disease (Schaum & Müller, 1967; Shamsi & Butcher, 2011). Although *Contracaecum* spp. is described as being found infrequently in this region of the planet (Mattiucci & Nascetti, 2008), recent evidence shows the presence of larvae of *Anisakis* and *Contracaecum* in fish for human consumption (Dias *et al.*, 2011; Fontenelle *et al.*, 2013).

The absence of serological conversion in mice inoculated with *Contracaecum* spp. is difficult to explain, since this nematode shares similar basic structures and similar somatic and excretory/secretory antigens with *Anisakis* spp. Perhaps genetic analyses will identify differences between these species that may explain the different disease manifestations observed, and may explain further why there was an absence of sensitization following inoculation with a live *Contracaecum* spp. larva.

In conclusion, the data presented in this report demonstrate that introduction of a live *Contracaecum* spp. larva into the gastrointestinal tract failed to elicit sensitization in mice.

Financial support

This research was partially supported by Proppi – Universidade Federal Fluminense (Fopesq-2013).

Conflict of interest

None.

Ethical standards

This study was approved by the Animal Research Ethics Committee of the Universidade Federal Fluminense (UFF) Centre for Laboratory Animals (038/2009).

References

Alonso-Gomez, A., Moreno-Ancillo, A., Lopez-Serrano, M.C., Suarez-De-Parga, J.M., Daschner, A., Caballero, M.T., Barranco, P. & Cabanas, R. (2004) *Anisakis simplex* only provokes allergic symptoms when the worm parasitises the gastrointestinal tract. *Parasitology Research* **93**, 378–384.

Anadon, A.M., Romaris, F., Escalante, M., Rodriguez, E., Garate, T., Cuellar, C. & Ubeira, F.M. (2009) The *Anisakis simplex* Ani s 7 major allergen as an indicator of true *Anisakis* infections. *Clinical and Experimental Immunology* **156**, 471–478.

Ansary, T.H., Moghaddar, N. & Esmaeili, H.R. (2009) *Iranocichla hormuzensis* (Coad 1982), a new paratenic host of *Contracaecum* sp. and *Phocanema* sp. (Nematoda: Anisakidae). *Comparative Clinical Pathology* **19**, 335–337.

Audicana, M.T. & Kennedy, M.W. (2008) *Anisakis simplex*: from obscure infectious worm to inducer of immune hypersensitivity. *Clinical Microbiology Reviews* **21**, 360–379.

Audicana, M.T., Ansotegui, I.J., De Corres, L.F. & Kennedy, M.W. (2002) *Anisakis simplex*: dangerous – dead and alive? *Trends in Parasitology* **18**, 20–25.

Caballero, M.L., Moneo, I., Gomez-Aguado, F., Corcuera, M.T., Casado, I. & Rodriguez-Perez, R. (2008) Isolation of Ani s 5, an excretory–secretory and highly heat-resistant allergen useful for the diagnosis of *Anisakis* larvae sensitization. *Parasitology Research* **103**, 1231–1233.

Cho, S.W. & Lee, H.N. (2006) Immune reactions and allergy in experimental anisakiasis. *Korean Journal of Parasitology* **44**, 271–283.

Daschner, A., Cuellar, C. & Rodero, M. (2012) The *Anisakis* allergy debate: does an evolutionary approach help? *Trends in Parasitology* **28**, 9–15.

Dias, F.J., Sao Clemente, S.C., Pinto, R.M. & Knoff, M. (2011) *Anisakidae* nematodes and *Trypanorhyncha* cestodes of hygienic importance infecting the king mackerel *Scomberomorus cavalla* (Osteichthyes: Scombridae) in Brazil. *Veterinary Parasitology* **175**, 351–355.

Figueiredo, I. Jr, Cardoso, L., Teixeira, G., Lopes, L., Sao Clemente, S.C. & Vericimo, M.A. (2012) A technique for the intra-gastric administration of live larvae of *Anisakis simplex* in mice. *Experimental Parasitology* **130**, 285–287.

Fontenelle, G., Knoff, M., Felizardo, N.N., Lopes, L.M. & Clemente, S.C. (2013) Nematodes of zoonotic importance in *Cynoscion guatucupa* (Pisces) in the state of Rio de Janeiro. *Revista Brasileira de Parasitologia Veterinária* **22**, 281–284.

Huizinga, M.W. (1965) Studies on the life cycle of nematode parasite *Contracaecum* in fish eating birds. *Northeast fish and wildlife conference*, Harrisburg, Pennsylvania, 17 January.

https://doi.org/10.1017/S0022149X14000340 Published online by Cambridge University Press

Jones, R.E., Deardorff, T.L. & Kayes, S.G. (1990) *Anisakis simplex*: histopathological changes in experimentally infected CBA/J mice. *Experimental Parasitology* **70**, 305–313.

Kirstein, F., Horsnell, W.G., Nieuwenhuizen, N., Ryffel, B., Lopata, A.L. & Brombacher, F. (2010) *Anisakis pegreffii*-induced airway hyperresponsiveness is mediated by gamma interferon in the absence of interleukin-4 receptor alpha responsiveness. *Infection and Immunity* **78**, 4077–4086.

Laffon-Leal, S.M., Vidal-Martinez, V.M. & Arjona-Torres, G. (2000) 'Cebiche' – a potential source of human anisakiasis in Mexico? *Journal of Helminthology* **74**, 151–154.

Li, X.M., Schofield, B.H., Huang, C.K., Kleiner, G.I. & Sampson, H.A. (1999) A murine model of IgE-mediated cow's milk hypersensitivity. *Journal of Allergy and Clinical Immunology* **103**, 206–214.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randll, R.J. (1951) Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193**, 265–275.

Mattiucci, S. & Nascetti, G. (2008) Advances and trends in the molecular systematics of anisakid nematodes, with implications for their evolutionary ecology and host–parasite co-evolutionary processes. *Advances in Parasitology* **66**, 47–148.

Mattiucci, S., Fazii, P., De Rosa, A., Meucci, C., Megna, A.S., Glielmo, A., De Angelis, M., Costa, A., Meucci, C., Calvaruso, V., Sorrentini, I., Palma, G., Bruschi, F. & Nascetti, G. (2013) Anisakiasis and gastroallergic reactions associated with *Anisakis pegreffii* infection, Italy. *Emerging Infectious Diseases* **19**, 496–499.

Perteguer, M.J. & Cuellar, C. (1998) Isotype-specific immune responses in murine experimental anisakiasis. *Zentralblatt für Veterinärmedizin. Reihe B* **45**, 603–610.

Perteguer, M.J., Cuellar, C., Guillen, J.L., Aguila, C., Fenoy, S., Chivato, T. & Laguna, R. (2003) Cross-reactivity between *Anisakis simplex* sensitization and visceral larva migrans by *Toxocara canis*. *Acta Tropica* **89**, 85–89.

Rodriguez, E., Romaris, F., Lorenzo, S., Moreno, J., Bonay, P., Ubeira, F.M. & Garate, T. (2006) A recombinant enolase from *Anisakis simplex* is differentially recognized in natural human and mouse experimental infections. *Medical Microbiology and Immunology* **195**, 1–10.

Saad, C.D.R., Vieira, F.M. & Luque, J.L. (2012) Larvae of Anisakidae Skrjabin & Karokhin, 1945 (Nematoda, Ascaridoidea) in *Lophius gastrophysus* Miranda-Ribeiro, 1915 (Actinopterygii, Lophiidae) from the coastal zone of the state of Rio de Janeiro, Brazil. *Neotropical Helminthology* **6**, 159–177.

Schaum, E. & Müller, W. (1967) Heterocheilidiasis (case report). *Deutsche medizinische Wochenschrift* **92**, 2230–2233.

Shamsi, S. & Butcher, A.R. (2011) First report of human anisakidosis in Australia. *The Medical Journal of Australia* **194**, 199–200.

Skirnisson, K. (2006) [*Pseudoterranova decipiens* (Nematoda, Anisakidae) larvae reported from humans in Iceland after consumption of insufficiently cooked fish]. *Læknafélag Íslands Laeknabladid* **92**, 21–25 (in Icelandic).

Valls, A. & Pascual, C.Y. (2003) [Anisakis and anisakiosis]. *Allergologia et immunopathologia (Madrid)* **31**, 348–355 (in Spanish).

Weiler, C.R. (2007) *Anisakis simplex* and cross-reacting antigens. *International Journal of Dermatology* **46**, 224–225.

https://doi.org/10.1017/S0022149X14000340 Published online by Cambridge University Press