

Cytokine signature and antibody-mediated response against fresh and attenuated *Anisakis simplex* (L3) administration into Wistar rats: implication for anti-allergic reaction

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Abstract The third larval stage (L3) of *Anisakis simplex* (Anisakidae) is one of the zoonotic parasitic nematodes in the musculature and visceral organs of marine fishes belonging to family Moronidae. The consumption of these high-commercial-value fish is widespread in many countries around the Mediterranean Sea including Egypt. The presence of these larvae in fish muscles poses a potential consumer hazard due to the parasite's ability to cause anisakidosis. Forty-two out of 60 (70 %) of the European seabass *Dicentrarchus labrax* were found to be naturally infected by L3 of *A. simplex* in the form of encapsulated juveniles in the fish musculature. Morphological examination of recovered parasites by light and scanning electron microscopy showed that, in general, all specimens examined closely resembled *A. simplex* (L3). To evaluate the allergenicity of this nematode, white blood cell count; levels of T helper 1 (Th1) [interferon (IFN)- γ and tumor necrosis factor (TNF)- α], Th2 [IL-4, IL-5, and IL-6], and Th17 [IL-17] related cytokines; total IgE and IgG antibodies; and nitric oxide (NO) were measured in the plasma of Wistar rats sensitized by oral inoculation with fresh, frozen, and heat-treated *A. simplex* L3 or rats intraperitoneally injected with L3 crude extract. Rats sensitized with fresh and

frozen L3 larvae produced significantly higher levels of IFN- γ , IL-5, IL-17, and total IgE as compared to control rats. Heat-treated larvae administration resulted in a significant rise of IFN- γ , TNF- α , IL-5, and total IgE in comparison to control rats. Intraperitoneal sensitizations enhanced release of IFN- γ , TNF- α , and total IgE. Oral sensitization led to a significant production of NO. Thereby, frozen or cooked larval L3 cannot inhibit the release of Th-related cytokines and IgE, which might impact on the overall anti-parasitic immunity.

Keywords *Anisakis simplex* (L3) · Morphology · Immune response · Wistar rats · Fresh and attenuated antigens

Introduction

Parasitic nematodes comprise one of the largest and most diverse groups of parasitic helminthes mostly of fresh, brackish, and marine water fishes. Heavily infected fish show emaciation, imbalanced swimming, and reduction of their vitality (Klimpel et al. 2011; Mehlhorn et al. 2011; Morsy et al. 2013, 2015; Abdel-Ghaffar et al. 2014, 2015). The consumption of raw or undercooked fish may lead to infection with several helminthes. The most common nematode involved in human infections is *Anisakis simplex*. Its infective larvae are found within the flesh of marine fish or cephalopods which could be ingested alive by humans causing anisakidosis when raw or uncooked infected fish is consumed (Takahashi et al. 1998; Chai et al. 2005). These larvae induce major problems including epigastric pain, nausea, vomiting, and diarrhea as well as allergic hypersensitivity reactions like urticaria and anaphylaxis (Chai et al.

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2005). The third stage (L3) larvae infecting fish are arrested in their development until ingested by some sea mammals (seals and dolphins), whereupon they develop through two more stages until the adulthood is achieved. The first case of human infection by a member of the family Anisakidae was reported at 1962 in the Netherlands by Van Thiel who described the presence of a marine nematode in the center of an eosinophilic intestinal phlegmon of a patient suffering from acute abdominal pain as a “very unusual finding.” Later, this nematode was identified as *Anisakis* spp., and the human parasitosis was named anisakiasis (Van Thiel 1962). Gutting the fish as soon as possible after capture is thought to prevent the migration of the parasite into the muscles, thereby reducing the contamination of the edible parts of the fish (Smith 1984). Although cooking at 70 °C or freezing to −20 °C for 72 h is believed to destroy the infectivity of this parasite, the allergenic capacity of its denaturated protein is not eliminated (Caballero and Moneo 2004). The secretory antigens produced by the worms can induce specific antibodies even following ingestion of fully cooked fish containing *Anisakis* antigens (Audicana et al. 1995). It has been postulated that *Anisakis* allergy may be more prevalent than any specific food allergy in the adult population and comprises as much as 10 % of idiopathic anaphylaxis (Baeza et al. 2005). Human anisakid infections frequently cause gastrointestinal symptoms, which may be associated with mild to severe immunological responses, usually allergic-type reactions (Yazdanbakhsh et al. 2002). The production of IgE tends to increase during nematode parasite infections, but the ultimate effects of IgE vary considerably, depending on the host-parasite relationships. The immune allergic reactions have been closely associated with this antibody isotype generation (Yazdanbakhsh et al. 2002). Moreover, nematode infection is characterized by the induction of immune responses which are dominated by T helper (Th) type 2 cells, involving interleukin (IL)-3, IL-4, and IL-5, which control mastocytosis, IgE class switching, and eosinophilia, respectively (Urban et al. 1992; Grecis 1996). Experimental work on nematode infections of rodent intestine has shown that a Th2 reaction is required for immune expulsion of nematodes, while parasite expulsion does proceed in those strains of mice which are genetically more likely to generate a Th1 response (Else et al. 1994). During the early phase of infection, macrophages activated by interferon (IFN)- γ and tumor necrosis factor (TNF)- α may induce nitric oxide (NO) (a free radical synthesized from L-arginine) production which is toxic for the fluke (Pavanelli and Silva 2010). This mechanism may be up-regulated by Th1-type cytokines and down-regulated by IL-10 produced by T regulatory cells. In the present study, the fish *Dicentrarchus labrax*

were examined for the prevalence of nematode infection. The morphology of the recovered worms was described by light and scanning electron microscopy. Further, the immunological responses (white blood cell (WBC) count; Th1-, Th2-, and Th17-related cytokines; total IgE and IgG antibodies; and NO) were assessed in plasma of orally and intraperitoneally administered rats with nematode parasites.

Material and methods

Fish examination and parasitological study

Sixty specimens of the European seabass *D. labrax* (family: Moronidae) were collected during the period of September 2013 to May 2014 from boat landing sites and fishermen at the coasts of Hurgada City along the Red Sea in Egypt. Live fish were transported to the Laboratory of Parasitology, Zoology Department, Faculty of Science, Cairo University, where they were identified according to Randall et al. (1992). Skin surface, fins, and gills of fish were examined by the naked eye and dissecting microscope for any attached parasites, lesions, or external changes (Inoue et al. 2000). Collected fish were dissected; the internal organs were removed and examined for nematode infection. Nematode larvae were collected from the surface of visceral organs such as the stomach, intestine, and muscles; subsequently rinsed in phosphate-buffered saline (PBS); fixed in hot 70 % ethanol (60 °C); and stored in the same solution. For light microscopy, fresh and fixed worms were cleared in lactophenol. Identification was based on the comparison between the morphological characteristics of larval types including the morphology of the digestive tract, the shape and the presence of the boring tooth or the lips at the anterior end, the position of the excretory pore, and the shape of the postanal tail and its terminal mucron (Anderson 2000; Shih and Jeng 2002). Photomicrographs were taken using a Zeiss photomicroscope equipped by a Canon digital camera. For scanning electron microscopy, specimens were fixed in 3 % buffered glutaraldehyde, washed in cacodylate buffer, and dehydrated in alcohol. After passing through an ascending series of Genesolv D, they were processed in a critical point drier “Bomer-900” with Freon 13, sputter-coated with gold-palladium in a Technics Hummer V, and examined with an Etec Autoscan at a 20-kV JEOL scanning electron microscope. All measurements are in micrometers unless otherwise stated; minimum and maximum values were given, followed in parentheses by the arithmetic mean $\pm$ standard deviation (SD).

Preparation of crude nematode antigen

For crude antigen extract preparation, parasite larvae obtained from musculature and visceral organs of the infected fish were washed thoroughly in sterile 0.85 % NaCl. Twenty hundred freshly collected L3 larvae were homogenized in PBS solution; the homogenate was then centrifuged for 20 min at $12,942\times g$ in a cooling centrifuge. The supernatant was separated and its protein content was determined according to the Bradford method (Bradford 1976) to be 500 $\mu\text{g/ml}$ and then stored at $-20\text{ }^{\circ}\text{C}$ in aliquots until use.

Animals

Forty male Wistar rats, 6 to 8 weeks old and 130–150-g weight (National Research Centre, Cairo, Egypt), were kept in suitable cages with perforated covers at a temperature of $22\pm 2\text{ }^{\circ}\text{C}$, $55\pm 5\%$ humidity, and 12-h dark/light cycle and fed on standard food and water ad libitum. Rats were free of parasites as shown by repeated stool examination by direct wet saline smear and flotation method. All experimental procedures were performed following the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, National Research Council, USA. Animals were divided into five groups (eight rats each):

1. Control (non-infected and untreated) group
2. Rats were orally inoculated with freshly collected L3 larvae.
3. Rats were orally inoculated with L3 frozen at $-20\text{ }^{\circ}\text{C}$ for 1 week.
4. Rats were orally inoculated with L3 cooked in $100\text{ }^{\circ}\text{C}$ water bath for 10 min.

The larvae were cut into pieces before inoculation (three to five larvae per rat).

5. Rats were sensitized by intraperitoneal injection of crude antigen extract emulsified in an incomplete Freund's adjuvant (1:1 volume) containing 250 $\mu\text{g/ml}$. The sensitized rats were then boosted with complete Freund's adjuvant (containing 250 $\mu\text{g/ml}$) after 7 days. All groups were treated twice; the first is at day 0 followed by the second time at day 7. The boosted animals were housed for 14 days and then euthanized.

Blood collection and white blood cell count

All rats were euthanized by an over dose of sodium pentobarbital at day 21. Blood was collected in heparinized tubes.

Plasma was separated by centrifugation at $894\times g$ for 15 min and stored at $-80\text{ }^{\circ}\text{C}$ until further assays. Differential WBC count ($10^9/\text{l}$) was performed by using an automated hematology analyzer (HA-Vet, CLINDIAG SYSTEMS B.V.B.A., USA).

Cytokine assays

The levels of IFN- γ , TNF- α , IL-4, and IL-6 were measured by commercial sandwich enzyme-linked immunosorbent assay (ELISA) kits (Koma Biotech, Inc., Korea). IL-5 level was determined by sandwiched ELISA (Signosis, Santa Clara, CA, USA). IL-17 level was estimated by an indirect ELISA MAXTM Deluxe Set (BioLegend, Inc., San Diego, CA, USA). ELISA was performed following the manufacture instructions of the kits. All optical densities were measured at 450 nm. The levels of cytokines were expressed as pg/ml.

Determination of total IgE and IgG by ELISA

Total plasma levels of IgE and IgG antibodies were quantified by ELISA (Koma Biotech, Inc., Korea) according to the manufacturer's instructions. The level of antibody was expressed as ng/ml.

Quantification of nitric oxide

NO generation was detected by measuring nitrite quantity in plasma by Griess reaction (Misko et al. 1993). Heparin was removed from plasma samples by incubation with protamine sulfate for 5 min, followed by centrifugation of the mixture at $5590\times g$ for 10 min to pellet the insoluble heparin-protamine complex. Fifty microliters of modified Griess reagent (Sigma-Aldrich, St. Louis, MO, USA) was mixed with 50 μl of free heparin plasma in a microtitre plate and incubated for 15 min. The plates were read at 540 nm in a microplate autoreader. The results were expressed as mean optical densities. Nitrite concentrations (μM) were calculated from a standard curve of NaNO_2 serial dilutions.

Statistical analysis

The data were analyzed by IBM Statistical Package for the Social Sciences version 20 (copyright by IBM SPSS software, USA). One-way ANOVA was applied to analyze the effect of inoculated *Anisakis* L3 on the level of WBC count in plasma of rats, whereas ANOVA post hoc (least significant difference (LSD)) was utilized to compare between various groups. Results were expressed as mean $\pm$ standard error (SE)

Results

Morphological characteristic investigation

Forty-two out of 60 (70 %) fish specimens were found to be naturally infected by nematode juveniles which were observed as encapsulated larvae recovered from the peritoneal cavity of the infected fish. The intensity of their infection ranged from 10 to 15 worms per fish (Figs. 1 and 2). The mean intensity of the nematode larvae depended on the length of the fish. The highest parasite intensity was recorded in the hosts with a length >66 cm. The examined fish specimens showed no external visible signs of disease. Morphological and morphometric characterization showed that, in general, all specimens examined closely resembled *A. simplex* where they are identified according to the key published by Rocka (2004).

Anisakis sp. family: Anisakidae Railliet and Henry, 1912

The recorded larval stage had a slender body being attenuated at both ends and measured 0.4 to 3 cm in length and 0.5 to 1 mm in width. Lips were inconspicuous, with prominent boring teeth at the anterior extremity and four small papillae (two dorsolateral and two ventrolateral) surrounding the tri-rotate mouth opening (Figs. 3, 4, 7, 8, and 9). No head bulb and no proboscis were observed. The esophagus had a long anterior muscular part and an oblong ventriculus with an oblique esophago-intestinal junction. The excretory pore opened ventrally at the anterior end. The cuticle had annular striations covering the surface of the juveniles with parallel striations that start from the cephalic region and extend to the slit-shaped anus (Figs. 5 and 10). No intestinal cecum was observed. The tail was short and with a clear mucron seen (Figs. 6 and 11) with a length of 0.015 ± 0.001 mm (0.014–0.025 mm). Papillae were observed and distributed along the worm body (Fig. 12).

WBC count

Blood analysis revealed that the differential counts of WBCs (lymphocytes and granulocytes) were significantly ($P < 0.0001$, $P < 0.05$, and $P < 0.0001$) affected by the nature of the inoculated *Anisakis* L3 larvae (fresh, cooked, and frozen), respectively, as analyzed by one-way ANOVA (Table 1). Oral (fresh, frozen, and cooked) sensitization by *Anisakis* L3 larvae and intraperitoneal injection by *Anisakis* L3 crude antigen extract resulted in a significant ($P < 0.0001$) increase in the number of granulocytes and a significant ($P < 0.0001$) decrease of the number of lymphocyte in the blood of sensitized rats as compared to control. The number of monocytes was significantly ($P < 0.01$ and $P < 0.0001$) elevated in the orally inoculated rats with fresh and cooked L3

larvae, respectively, versus control rats, whereas frozen L3 induced non-significant increase of monocyte count. Intraperitoneal sensitized rats revealed a higher significant ($P < 0.01$) elevation of monocytes than those of control rats.

Cytokine profile

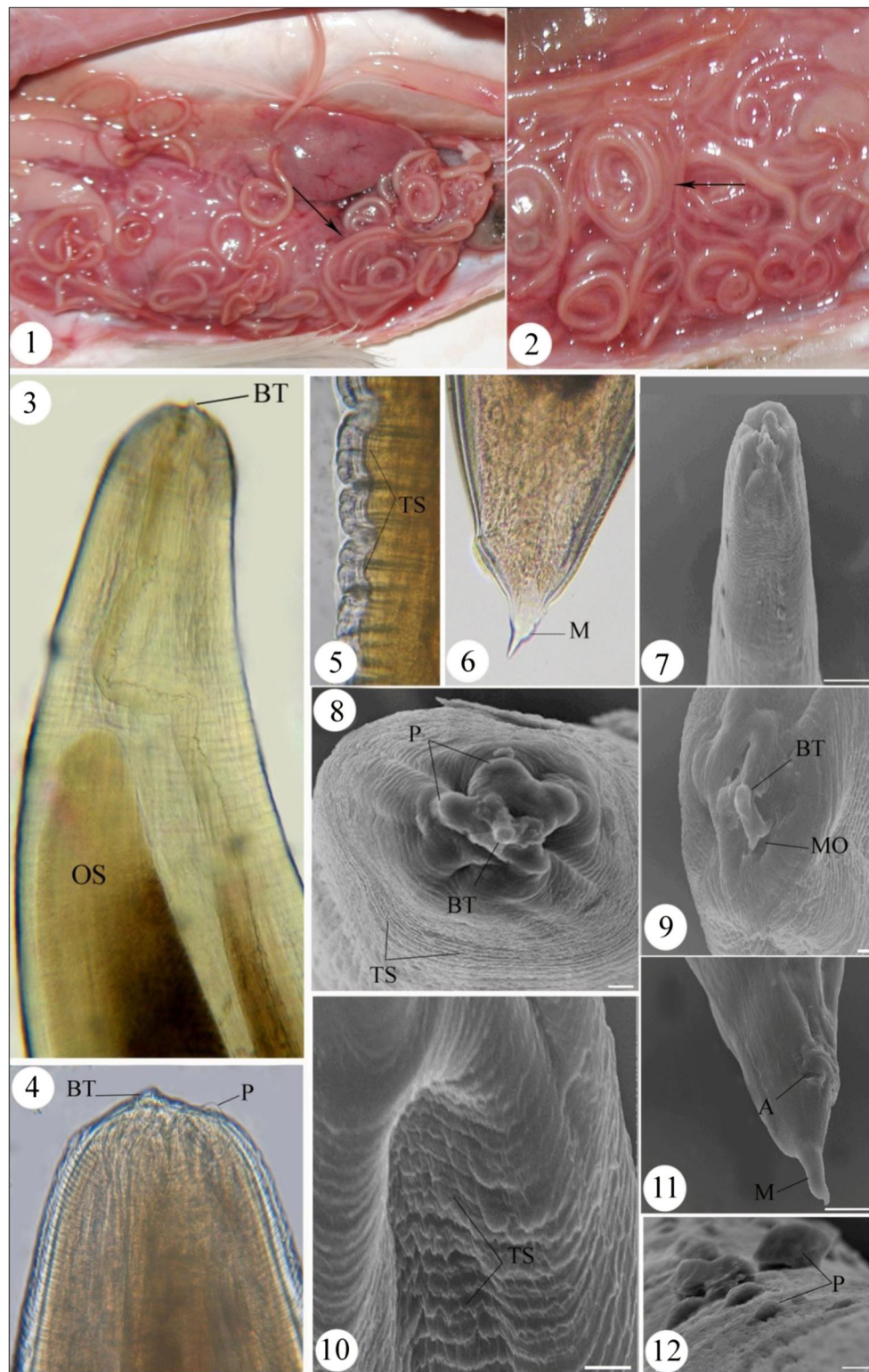
The levels of cytokines (TNF- α and IFN- γ , IL-4, IL-5, IL-6, and IL-17) were investigated in rats sensitized by *Anisakis* L3 larvae (Fig. 13). With respect to controls, this parasite induces Th1-, Th2-, and Th17-associated cytokines with different levels in sensitized rats. The plasma of rats inoculated with fresh, frozen, and cooked L3 larvae or intraperitoneally injected with larval extract exhibited significantly higher levels of IFN- γ ($P < 0.01$, $P < 0.05$, $P < 0.01$, and $P < 0.5$), respectively, as compared to control rats. Moreover, TNF- α was promoted in highly significant levels after inoculation of rats by cooked L3 larvae ($P < 0.0001$) followed by freshly ($P < 0.05$) collected L3 larvae, while frozen L3 failed to elicit the release of TNF- α . Intraperitoneal sensitization with *Anisakis* larval extract also markedly stimulated a significant ($P < 0.0001$) production of TNF- α . The Th2 cytokine (IL-5) was significantly ($P < 0.01$, $P < 0.05$, and $P < 0.01$) increased in the plasma of rats inoculated orally by fresh, frozen, and cooked L3 larvae, respectively, while intraperitoneal injection had no marked influence on the level of this cytokine. The level of IL-4 and IL-6 in all treated rats was not statistically affected in comparison to control rats. However, the level of IL-17 was detected at higher significant ($P < 0.01$) values in the plasma of rats inoculated by fresh and frozen L3 compared to untreated rats. Similar with IL-5, intraperitoneal injection with larval crude extract was also unable to trigger a significant generation of IL-17.

Antibody detection

Anisakis L3 larvae induced a potent IgE antibody production in the plasma of all sensitized rats with significant values as compared to control rats. The concentration (ng/ml) of IgE in plasma of rats was in the order of injected > live > frozen > cooked > control (Fig. 14). Total IgG antibody level was diminished in all orally sensitized rats, while rats sensitized by crude larval extract showed a highly significant ($P < 0.0001$) IgG production as compared to the control rats.

Nitric oxide (NO)

NO, an indicator to innate immune response, was estimated by quantification of the stable degradation product nitrite in the plasma of sensitized and control rats (Fig. 15). However, significant quantities of NO were enhanced in the plasma of rats



Figs. 1–12 Photographs of *D. labrax* showing the heavy infestation (arrows) with *A. simplex* (L3) in the peritoneum as well as visceral organs of infected fish. Photomicrographs showing high magnifications of *A. simplex* (L3): 3, 4 the anterior part with a boring tooth (BT) and lateral papillae (P). (OS) esophagus. $\times 140$; $\times 400$. 5 Transverse striations (TS) of cuticle. $\times 400$. 6 The posterior part terminated at a pointed mucron

(M) $\times 400$. Scanning electron micrographs of *A. simplex* showing: 7–9 the anterior part of the worm body, with a boring tooth (BT) and four papillae (P) surrounding the mouth (MO). 10 Transverse striation (TS) of cuticle. 11 The posterior part of worm terminated at a mucron (M), A anus. 12 Body papillae (P). (Bars: Fig. 7, 100 μm ; 8–10, 12, 10 μm ; 11, 50 μm)

Table 1 White blood cell (lymphocytes, monocytes, and granulocytes) count in control as well as Wistar rats sensitized by L3 *Anisakis*

Experimental groups	WBCs	Lymphocytes	Monocytes	Granulocytes
Control	17.94±1.203	17.73±1.201	0.249±.023	0.01±0.000
Fresh	4.66±0.69**	3.59±0.7±5309**	0.473±0.113*	0.986±0.190**
Frozen	5.50±1.011**	4.51±0.4578**	0.333±0.110	1.083±0.223**
Cooked	19.13±1.44	1.76±0.14449**	0.477±0.034**	16.943±1.276**
One-way ANOVA ^a	$F_{2,6}=54.84$ $P<0.0001$	$F_{2,6}=6.60$ $P<0.05$	$F_{2,6}=0.761$ $P>0.05$	$F_{2,6}=141.52$ $P<0.0001$
Injected	17.12±1.77	4.3875±1.31**	0.789±0.139*	12.076±2.131**

Data are expressed as mean±standard error of mean (SEM)

Asterisks represent the high significance level in comparison to the L3 non-infected (control) rats (* $P<0.01$ and ** $P<0.0001$)

^a The effect of state (fresh, frozen, and cooked) of *Anisakis* larvae L3 on the levels of cytokines (pg/ml)

orally inoculated by fresh ($P<0.0001$) L3 followed by frozen ($P<0.01$) and cooked ($P<0.01$) L3 *Anisakis* compared to control rats. Non-significant increase of NO was recorded in intraperitoneally injected rats.

Discussion

Nematodes are considered as the most economically important helminth parasites infecting fishes in the world (Dick and Choudhury 1995). Adult nematodes are usually found in the intestine of fish, while their larval stages are sometimes found on the flesh and viscera, causing massive diseases for fish and their economical losses. The larval stages of these parasites are infective to humans; they have the greatest impact on the consumer acceptance of fish (Post 1987; Moravec 1994; Dick and Choudhury 1995). Anisakid larvae have been detected worldwide in a large variety of fish belonging approximately to 200 fish species (Chen et al. 2008). Humans can be infected by anisakid nematodes by consuming raw or undercooked fish. *Anisakis* infection in humans causes several symptoms such as sudden epigastric pain, nausea, vomiting, diarrhea, or allergic reactions (Audicana and Kennedy 2008). A high percentage of these worms were recorded in the present study, and a high degree of low marketability was observed where they are found in the viscera and musculature of fish. This finding agreed with Dorny et al. (2009) and Shih et al. (2010) who concluded that the high prevalence of this nematode in economically important fish species indicates that damage to the fishing industry could occur by considerably reducing the quality of fish, leading to a loss of marketing values. Recent studies have also shown high prevalence of *Anisakis* spp. in salmonid species, and the worms were mostly found in the musculature or encapsulated on the visceral organs, mesenteries, and peritoneum (Smith 1984; Sugawara et al. 2004; Noguera et al. 2009; Morsy et al. 2012).

Morphological study

All of the larval worms collected from the peritoneum of the host fish revealed that all of them are the L3 stage of *A. simplex*. According to Rocka (2004), the third-stage larvae (L3) of *A. simplex* are characterized as small worms (9–36 mm in length), with a straight anterior gut structure consisting of esophagus, ventriculus, and intestine. The cuticle is obviously striated transversely. The lips are inconspicuous but possess a prominent boring tooth on the anterior end. The living larvae are white or creamy in color and are encysted in capsules of host origin being coiled like a watch feather. A characteristic papillae structure was observed by scanning electron microscopy on the tail of juveniles which were not recorded previously. At the marine Egyptian coast, Abdou (2005) recorded *Terranova* larvae from marine fishes belonging to families Carangidae, Scombridae, Lethrinidae, Scaridae, and Serranidae. He mentioned that 19 % of the collected samples were found harboring anisakid larvae within the intestinal lumen. In these studies, the prevalence of worms had been associated with host age as reflected by body length (Platt 1975). These results agreed with Arthur et al. (1982), Bush et al. (1990), Køie (2001), Costa et al. (2003), Podolska and Horbowy (2003), and Cruz and Saraiva (2005) who mentioned that the occurrence and abundance of the anisakid larvae varied with geographic location and with fish length (age). Thus, larger fish appeared more susceptible to the infection rather than the smaller one. This may be attributed to the available niches for parasites that are more diverse in larger hosts.

Immunological study

Several studies estimated that the third-stage larvae of *A. simplex* do not develop into further stages in humans but can penetrate the mucosa of the gastrointestinal tract, producing acute or chronic abdominal syndromes (Kinoshita et al. 2014). Multiple cases of human disease have been described

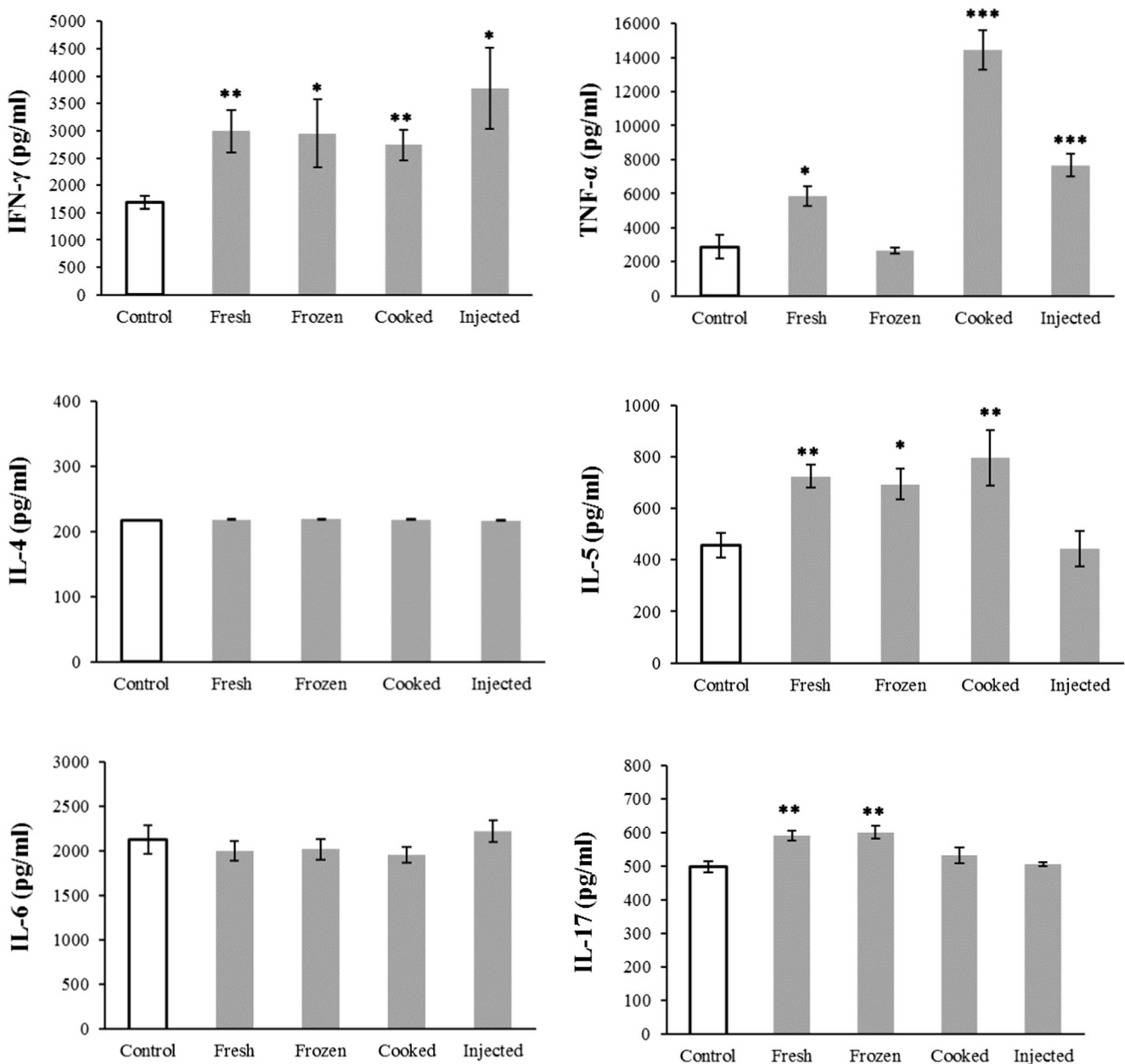


Fig. 13 Levels of Th1 (IFN- γ and TNF- α), Th2 (IL-4, IL-5, and IL-6), and Th17 (IL-17) cytokines in Wistar rats orally inoculated by *Anisakis* L3 larvae (fresh, frozen, and cooked) as well as intraperitoneally injected

rats by larval crude extract of L3. Asterisks represent the significance level in comparison to the non-infected (control) rats (* P <0.05, ** P <0.01, *** P <0.001)

worldwide as a result of the ingestion of raw fish, sushi, and sashimi (Williams and Jones 1994; Bouree et al. 1995). In Egypt, where fish tends to be cooked before consumption, the general belief is that cooking at 60 °C for 10 min or freezing for 24 h can kill the parasite and prevent human anisakidosis. The public has been warned of the risk of eating raw fish, all fish to be eaten raw is previously frozen to kill the parasites (Petithory and Marti 1988). It is considered that these measures can be successful in preventing parasitic zoonosis. The immunological hallmark of helminth infection is characterized by an elevation of IgE and eosinophils. Whether IgE and eosinophil rise is a

protective immune measure or plays a damaging role (Yazdanbakhsh 1996), this question is ill-defined. To the best of our knowledge, the present study is considered as the first large-scale epidemiologic analysis of occupational *Anisakis*-induced allergy using a Wistar rat experimental model. We investigated the immune reactions and allergic responses to *Anisakis* L3 larvae. A characteristic of nematode infections is the induction of immune responses which are dominated by Th cells which included Th1 cells which are the key regulators of the cell-mediated immune response through IFN- γ and TNF- α release, whereas Th2-type cells generate the cytokines IL-13, IL-

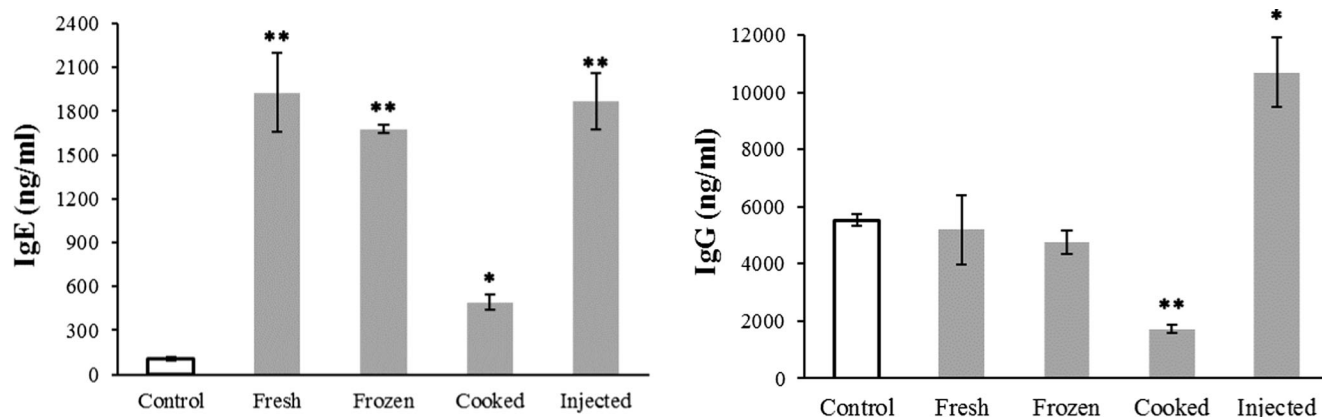


Fig. 14 Levels of total IgE and IgG assayed in plasma of Wistar rats orally inoculated (fresh, frozen, and cooked) by L3 *Anisakis* and the intraperitoneally injected rats by larval crude extract. Asterisks represent

the significance level in comparison to the L3 non-infected (control) rats (* $P < 0.01$, ** $P < 0.001$)

4, IL-5, and IL-6 (Urban et al. 1992; Grecis 1996; Yazdanbakhsh 1996). In the present study, *Anisakis*-stimulated cytokines (INF- γ , TNF- α , IL-5, and IL-17) were produced in higher significant quantities in most of the experimental rats with some exceptions which may be related to the route of administration in accordance with Audicana et al. (2002). Production of IgE showed higher levels after the second infection in rats denoting the relation between allergy and repeated infection with *Anisakis* larvae (Cho et al. 2006). We identified a significant increase of total IgE levels in all sensitized rats. In contrast to this profound elevation of IgE, levels of total IgG were markedly dropped in all orally sensitized rats. The discrepancy between IgE and IgG responses could be due to that each is provoked by a different Th subset; it was documented that production of IgE depends mainly on Th2 cytokines (IL-4 and IL-13), whereas IgG depends on IFN- γ which is a Th1 product (Kirstein et al. 2010). The present data demonstrated a dominance of INF- γ in parallel with IgE production in sensitization

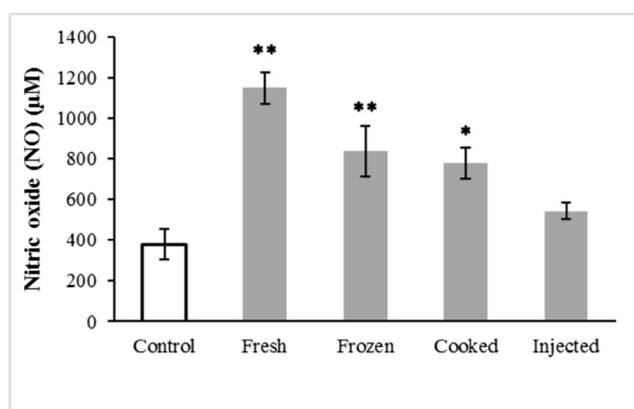


Fig. 15 Levels of Th1 (INF- γ and TNF- α), Th2 (IL-4, IL-5, and IL-6), and Th17 (IL-17) cytokines in Wistar rats orally inoculated by *Anisakis* L3 larvae (fresh, frozen, and cooked) as well as intraperitoneally injected rats by larval crude extract of L3. Asterisks represent the significance level in comparison to the non-infected (control) rats (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$)

to *A. simplex* L3 larvae that has been previously reported, whereas IgE formation was not blocked by INF- γ production, and consequently, allergic symptoms could not be avoided against *A. simplex* (Baeza et al. 2005). IL-4 and IL-5 are associated with their role in antibody production, as they promote and control IgE and IgG antibody production and class switching (Shimoda et al. 1996). Acute helminth infection leading to an allergic disease is a result of up-regulation of IL-4, IL-5, IL-13, total IgE, and eosinophilia being part of host immune response (Flohr et al. 2009). Indeed, the present IgG response showed a difference between oral and intraperitoneal administration that may be attributed to the natural way of exposure to L3 larvae and presence of adjuvants with injection. In the current study, it was observed that different cytokine profiles might be associated with various states (fresh, frozen, and cooked) of *Anisakis* larvae. *A. simplex*-sensitized rats by fresh or frozen L3 larvae demonstrated Th1, Th2, and Th17 immune responses, whereas cooked larvae provoked a mixture of pro-inflammatory Th1 cytokines and Th2. Th1 mechanisms are marked mainly by the significant elevation in INF- γ release that can be implicated in resistance and is perhaps especially important in killing of the incoming larval stages migrating through tissues (Baeza et al. 2005). Exactly how Th2 responses are naturally initiated in nematode infections remains a matter of debate. It has been proposed, for example, that its effect and quantity depend on the level of antigen exposure, the site at which it occurs, the route of administration, or the intrinsic property of parasite antigens (Urban et al. 1992). Triggering of the Th2 response programed by intestinal nematodes was orchestrated by cytokines including interleukins (IL-4, IL-33, IL-5, IL-9, IL-13, and IL-21) that leads to a stereotyped cascade of effector mechanisms, potentially including immunoglobulin E isotype-switched B cell responses, mast cells, eosinophils, basophils, and increased permeability, smooth muscle contractility, and mucus production in the gut (Min 2,008). The increased count of granulocytes in the present study may be contributed to IgE-mediated allergic inflammation (Flohr et al. 2009) and

IgG1-mediated systemic anaphylaxis (Mukai et al. 2005; Tsujimura et al. 2008). Of these, basophils constitutively express FcεRI, high-affinity IgE receptor, and upon cross-linking of FcεRI-bound IgE with specific antigen, they release effector mediators such as histamine, leucotrienes, Th2 cytokines (IL-4, IL-5 and IL-13), and thymic stromal lymphopoietin in response to protease allergens of the invading parasites, causing immediate-type hypersensitivity (Min 2008). TNF-α is produced by many cell types, including macrophages, and is released when macrophages are exposed to lipopolysaccharide or upon infection with a pathogen. TNF-α with IFN-γ, induced the production of microbicidal reactive nitrogen intermediates by macrophages (Flesch and Kaufmann 1990). Here, sensitization by heat-treated L3 larvae showed marked elevation of TNF-α than those of fresh and frozen L3 larvae. Therefore, we could suggest that cooking of parasitized fish could not inhibit the vigorous release of Th1 cytokine responses of IFN-γ and TNF-α, denoting the importance of Th1 response in our model of sensitized Wistar rats. NO is recognized as a potent microbicidal agent, and its role in host defense against intra- and extracellular parasites has been demonstrated against larvae of nematodes such as *A. simplex* (Audicana and Kennedy 2008). In the present work, highly significant increase in the level of NO was observed in all of the orally sensitized rats with respect to control. Inducible NO synthesis (iNOS) requires stimulation by bacterial lipopolysaccharide and/or cytokines which upregulate the transcription of iNOS enzymes, leading to the conversion of L-arginine to L-citrulline. Also, the significant increase in the levels of TNF-α may be attributed to the NO increase (Wink et al. 2011). The obtained data from the nematode *A. simplex* infection have shown that cooking and freezing of worms before oral administration to rats might not protect against stimulation of Th1, Th2, and Th17 immune responses inducing allergic and hypersensitivity reactions with various degrees. So, it is important to note that although cooking or freezing kills the parasite, these procedures do not necessarily destroy the allergenic capacity of *A. simplex* and that infected organisms will vary in both susceptibility to atopic responses and immune recognition of *A. simplex* antigens (Kennedy et al. 1988). Thus, it is important to create a wider awareness of this potential risk to human health. *A. simplex* can not only cause symptoms directly associated with the gastrointestinal infection but also cause IgE-mediated hypersensitivity reactions of greater severity. It is becoming increasingly likely that the impact of *A. simplex* on human health has been underestimated and it is perhaps time to consider more sweeping measures than the ones currently enforced to protect the public.

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References

- Abdel-Ghaffar F, Bashtar AR, Abdel-Gaber R, Morsy K, Mehlhorn H, Al Quraishy S, Mohammed S (2014) *Cucullanus egyptae* sp. nov. (Nematoda, Cucullanidae) infecting the European eel *Anguilla anguilla* in Egypt. Morphological and molecular phylogenetic studies. Parasitol Res 113(9):3457–3465
- Abdel-Ghaffar F, Abdel-Gaber R, Bashtar AR, Morsy K, Mehlhorn H, Al Quraishy S, Saleh R (2015) *Hysterothylacium aduncum* (Nematoda, Anisakidae) with a new host record from the common sole *Solea solea* (Soleidae) and its role as a biological indicator of pollution. Parasitol Res 14:513–522
- Abdou NE (2005) Studies on the anisakid nematode juveniles infecting some Red Sea fishes in Egypt. J Zool Invert Parasitol 47:147–160
- Anderson RC (2000) Nematode parasites of vertebrates: their development and transmission. CAB, Wallingford
- Arthur JR, Margolis L, Whitaker DJ, McDonald TF (1982) A quantitative study of economically important parasites of walleye pollock (*Theragra chalcogramma*) from British Columbian waters and effects of postmortem handling on their abundance in the musculature. J Fish Res Board Can 39:710–726
- Audicana MT, Kennedy MW (2008) *Anisakis simplex*: from obscure infectious worm to inducer of immune hypersensitivity. Clin Microbiol Rev 21(2):360–379
- Audicana M, Fernández de Corres L, Muñoz D, Fernández E, Navarro JA, Del Pozo MD (1995) Recurrent anaphylaxis due to *Anisakis simplex* parasitizing sea-fish. J Allergy Clin Immunol 96:558–560
- Audicana MT, Ansotegui IJ, de Corres LF, Kennedy MW (2002) *Anisakis simplex*: dangerous—dead and alive? Trends Parasitol 18:20–25
- Baeza ML, Conejero L, Higaki Y, Martín E, Pérez C, Infante S, Rubio M, Zubeldia JM (2005) *Anisakis simplex* allergy: a murine model of anaphylaxis induced by parasitic proteins displays a mixed Th1/Th2 pattern. Clin Exp Immunol 142(3):433–440
- Bouree P, Paugam A, Petithory JC (1995) Anisakidosis: report of 25 cases and review of the literature. Comp Immunol Microbiol Infect Dis 18:75–84
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
- Bush AO, Aho JM, Kennedy C (1990) Ecological versus phylogenetic determinants of helminth parasite community richness. Evol Ecol 4: 1–20
- Caballero ML, Moneo I (2004) Several allergens from *Anisakis simplex* are highly resistant to heat and pepsin treatments. Parasitol Res 93: 248–251
- Chai J, Murrell KD, Lymbery AJ (2005) Fish-borne parasitic zoonoses: status and issues. Int J Parasitol 35:1233–1254
- Chen Q, Yu HQ, Lun ZR, Chen XG, Song HQ, Lin RQ, Zhu XQ (2008) Specific PCR assays for the identification of common anisakid nematodes with zoonotic potential. Parasitol Res 104:79–84
- Cho TH, Park HY, Cho S, Sohn J, Yoon YW, Cho JE, Cho SW (2006) The time-course of biological and immunochemical allergy states induced by *Anisakis simplex* larvae in rats. Clin Exp Immunol 143: 203–208
- Costa G, Pontes T, Mattiucci S, Amelio SD (2003) The occurrence and infection dynamics of *Anisakis* larvae in the black-scabbard fish, *Aphanopus carbo*, chub mackerel, *Scomber japonicas*, and Oceanic horse mackerel, *Trachurus picturatus* from Adeira, Portugal. J Helminthol 77:163–166
- Cruz CA, Saraiva VA (2005) Larval anisakids from horse mackerel in Portugal. Helminthologia 42(1):3–7
- Dick TA, Choudhury A (1995) Phylum Nematoda. In: Woo PTK (ed) Fish diseases and disorders, volume I protozoan and metazoan infection. Cambridge University Press, Cambridge, pp 415–446

- Dorny P, Praet N, Deckers N, Gabriel S (2009) Emerging food-borne parasites. *Vet Parasitol* 163:196–206
- Else KJ, Finkelman FD, Maliszewski CR, Grecnis RK (1994) Cytokine mediated regulation of chronic intestinal helminth infection. *J Exp Med* 179:347–351
- Flesch IE, Kaufmann SH (1990) Activation of tuberculostatic macrophage functions by γ -interferon, interleukin-4, and tumor necrosis factor. *Infect Immun* 58:2675–2677
- Flohr C, Quinnell RJ, Britton J (2009) Do helminth parasites protect against atopy 408 and allergic disease? *Clin Exp Allergy* 39:20–32
- Grecnis RK (1996) T cell and cytokine basis of host variability in response to intestinal nematode infections. *Parasitology* 112:S31–S37
- Inoue K, Oshima SI, Hirata T, Kimura I (2000) Possibility of anisakid larvae infection in farmed salmon. *Fish Sci* 66:1049–1052
- Kennedy MW, Tierney J, Ye P, McMongle FA, McIntosh A, McLaughlin D, Smith JW (1988) The secreted and stomach antigens of the third stage larvae of *Anisakis simplex*, and antigenic relationship with *Ascaris suum*, *Ascaris lumbricoides*, and *Toxocara canis*. *Mol Biochem Parasit* 31:35–46
- Kinoshita Y, Fujimoto K, Lee M, Shinohara R, Kobayashi Y, Kawana S, Saeki H (2014) Two cases of allergies due to *Anisakis simplex*, positive to specific IgE for ani S 12 allergen. *Arerugi* 63(10):1348–1352
- Kirstein F, Horsnell WG, Kuperman DA, Huang X, Erle DJ, Lopata AL (2010) Expression of IL-4 receptor alpha on smooth muscle cells is not necessary for development of experimental allergic asthma. *J Allergy Clin Immunol* 126:347–354
- Klimpel S, Abdel-Ghaffar F, Al-Rasheid KA, Aksu G, Fischer K, Strassen B, Mehlhorn H (2011) The effects of different plant extracts on nematodes. *Parasitol Res* 108(4):1047–1054
- Køie M (2001) Experimental infections of copepods and sticklebacks *Gasterosteus aculeatus* with small ensheathed and large third stage larvae of *Anisakis simplex* (Nematoda: Anisakidae). *Parasitol Res* 87:32–36
- Mehlhorn H, Al-Quraishy S, Al-Rasheid KA, Jatzlau A, Abdel-Ghaffar F (2011) Addition of a combination of onion (*Allium cepa*) and coconut (*Cocos nucifera*) to food of sheep stops gastrointestinal helminthic infections. *Parasitol Res* 108(4):1041–1046
- Min B (2008) Basophils: what they “can do” versus what they “actually do”. *Nat Immunol* 9(12):1333–1339
- Misko TP, Schilling RJ, Salvemini D, Moore WM, Currie MG (1993) A fluorometric assay for the measurement of nitrite in biological samples. *Anal Biochem* 214(1):11–16
- Moravec F (1994) Parasitic nematodes of freshwater fishes of Europe. Academia and Kluwer Academic Publishers, Prague, p 473
- Morsy K, Bashtar AR, Abdel-Ghaffar F, Mehlhorn H, Quraishy SA, Mahdi ME, Al-Ghamdi A, Mostafa N (2012) First record of anisakid juveniles (Nematoda) in the European seabass *Dicentrarchus labrax* (Family: Moronidae), and their role as bioindicators of heavy metal pollution. *Parasitol Res* 110(3):1131–1138
- Morsy K, Bashtar AR, Abdel-Ghaffar F, Mostafa N (2013) New host and locality records of two nematode parasites *Dujardinascaris mujibii* (Heterocheilidae) and *Hysterothylacium aduncum* (Anisakidae) from the common seabream *Pagrus pagrus*: a light and scanning electron microscopic study. *Parasitol Res* 112(2):807–815
- Morsy K, Bashtar AR, Mostafa N, El Deeb S, Thabet S (2015) New host records of three juvenile nematodes in Egypt: *Anisakis* sp. (Type II), *Hysterothylacium patagonense* (Anisakidae), and *Echinocephalus overstreeti* (Gnathostomatidae) from the greater lizard fish *Saurida undosquamis* of the Red Sea. *Parasitol Res* 114(2):513–522
- Mukai K, Matsuoka K, Taya C, Suzuki H, Yokozeki H, Nishioka K, Hirokawa K, Etori M, Yamashita M, Kubota T, Minegishi Y, Yonekawa H, Karasuyama H (2005) Basophils play a critical role in the development of IgE-mediated chronic allergic inflammation independently of T cells and mast cells. *Immunology* 23(2):191–202
- Noguera P, Collins C, Bruno D, Pert C, Turnbull A, McIntosh A, Lester K, Bricknell I, Wallace S, Cook P (2009) Red vent syndrome in wild Atlantic salmon *Salmo salar* in Scotland is associated with *Anisakis simplex* sensu stricto (Nematoda: Anisakidae). *Dis Aquat Org* 87:199–215
- Pavanelli WR, Silva JJN (2010) The role of nitric oxide in immune response against *Trypanosoma cruzi* infection. *Open Nitric Oxide J* 2:1–6
- Petithory JC, Marti B (1988) L'anisakiase en France. *Lett l'Infectiologue* 2:96
- Platt NE (1975) Infestation of cod (*Codus morhua* L.) with larvae of cod worm (*Terranova decipiens* Krabbe) and herring worm, *Anisakis* sp. (Nematoda: Ascaridata) in the north Atlantic. *J Appl Ecol* 12:437–450
- Podolska M, Horbowy J (2003) Infection of the Baltic herring (*Clupea harengus membras*) with *Anisakis simplex* larvae, 1992–1999: a statistical analysis using generalized linear models. *J Mar Sci* 60:85–93
- Post G (1987) Animal parasites of fishes: textbook of fish health. T.F.H. Publications, Inc. USA, pp. 159–214
- Randall DA, Shao Q, Moeng CH (1992) A second order bulk boundary-layer model. *J Atmos Sci* 49:1903–1923
- Rocka A (2004) Nematodes of the Antarctic fishes. *Pol Polar Res* 25(2):135–152
- Shih HH, Jeng MS (2002) *H. aduncum* (Nematoda: Anisakidae) infecting a herbivorous fish, *Siganus fuscescens*, off the Taiwanese Coast of the Northwest Pacific. *Zool Stud* 41(2):208–215
- Shih HH, Ku CC, Wang CS (2010) *Anisakis simplex* (Nematoda: Anisakidae) third-stage larval infections of marine cage cultured cobia, *Rachycentron canadum* L., in Taiwan. *Vet Parasitol* 171:277–285
- Shimoda K, van Deursen J, Sangster MY (1996) Lack of IL-4 induced Th2 responses and IgE class switching in mice with disrupted STAT 6 gene. *Nature* 380:630–633
- Smith JW (1984) The abundance of *Anisakis simplex* L3 in the body-cavity and flesh of marine teleosts. *Int J Parasitol* 14:491–495
- Sugawara Y, Urawa S, Kaeriyama M (2004) Infection of *Anisakis simplex* (Nematoda: Anisakidae) larvae in chum salmon (*Oncorhynchus keta*) in the North Pacific Ocean, Bering Sea, and a river of Hokkaido. North Pacific Anadromous Fish Commission Doc 791. Hokkaido Tokai University, Sapporo
- Takahashi S, Ishikura H, Kikuchi K (1998) Anisakidosis: global point of view. In: Ishikura H, Aikawa M, Itakura H, Kikuchi K (eds) Host response to international parasitic zoonoses. Springer, Tokyo, pp 109–120
- Tsujimura Y, Obata K, Mukai K, Shindou H, Yoshida M, Nishikado H, Kawano Y, Minegishi Y, Shimizu T, Karasuyama H (2008) Basophils play a pivotal role in immunoglobulin-G-mediated but not immunoglobulin-E-mediated systemic anaphylaxis. *Immunology* 28(4):581–589
- Urban JF Jr, Madden KB, Svetic A (1992) The importance of TH2 cytokines in protective immunity to nematodes. *Immunol Rev* 127:205–220
- Van Thiel PH (1962) Anisakiasis. *Parasitology* 52:16–17
- Williams HH, Jones A (1994) Parasitic worms of fish. Taylor & Francis, London
- Wink DA, Hines HB, Cheng RY, Switzer CH, Flores-Santana W, Vitek MP, Ridnour LA, Colton CA (2011) Nitric oxide and redox mechanisms in the immune response. *J Leukoc Biol* 89(6):873–891
- Yazdanbakhsh M (1996) IgE, eosinophils and mast cells in helminth infections. *Ned Tijdschr Klin Chem* 21:213–216
- Yazdanbakhsh M, Kremsner PG, van Ree R (2002) Allergy, parasites, and the hygiene hypothesis. *Science* 296:490