PATHOLOGICAL AND ULTRASTRUCTURAL STUDIES ON ANISAKIS SIMPLEX RUDOLPHI-1809 INFECTING CARANGOIDES BAJAD WITH SPECIAL REFERENCE TO INTESTINAL MATURATION IN PUPPIES

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ABSTRACT

Thirty five out-of 51 (70%) Carangoides bajad were naturally infected with Anisakis simplex during the period from September 2007 to January 2008. The fish were collected from Eastern South Coast of the Red Sea (Hurgada), Egypt. Moreover, five puppies were orally infested with fish-containing the 3rd-stage larvae as a final host model.

The body of the larvae is tapering gradually towards the anterior part, and covered by striated ornamentation longitudinal and horizontally except the anterior region which is smooth. The morphology and the ultrastructural examinations found that the anterior body end of the larvae showed a boring tooth prominent, 3 pairs of lips inconspicuous and excretory pore ventral between rudimentary subventral lips. The anal end showed mucron which is distinct and a slit–shaped anus. The pathological studies revealed encapsulated larvae with concentrically fibrous connective tissue infiltrated with granulomatous infiltrates on the surface of the liver, spleen and peritoneum of infected fish. The macrophages were aggregated together, forming the denser part of the capsule and invaded to adjacent parenchymal tissue. The hepatocytes under the affected capsule were necrotic and invaded by melanomacrophages. Meanwhile in the puppies, the larvae induced visceral migrations in the liver and kidneys with granulomatous reactions. The adult worms were seen in the intestinal lumina with catarrhal or necrotic enteritis.

Finally, it could be concluded that the cellular components of the lesions indicate a chronic pathology, which would be significantly serious in puppies (final host) and fish (intermediate host) particularly with heavy infestation.

Keywords: Anisakis simplex, SEM, Pathology, Third stage larvae, Fish, Puppies.
INTRODUCTION

Anisakiasis was first recognized in the Netherlands by Van Thiel since 1960. It is caused by the 3rd stage larvae of Anisakis species; A. simplex Sensu stricto, A. pegrefii and A simplex Rudolphi-1809; of family Ansikeidae (Nascetti et al., 1983; Mattiucci et al., 1997; 1998; 2004). Such parasite can be contracted by people eating inadequately cooked or raw marine fish or squids (Thomas and Lawrence, 1995; Eissa et al, 1998; El-Gazzar et al., 2004). The majority of cases are from Japan (about 2000 cases/year), northern Europe (Scandiavia, Holland, England) and the Pacific Coast South America were consumption of raw fish is common. The ingested larvae could penetrate the gastrointestinal mucosa inducing the disease (Nagasawa, 1990). The adults of Anisakis reside the stomach of marine mammals such as whales and dolphins; while the crustaceans are the first intermediary hosts, and the second intermediary hosts include various species of fish and some cuttle fish (Grabda, 1976; Ishikura, 1989; 1991).

Identification of the Anisakis larvae and adults are usually based on the morphology of the digestive tract, the psotion of the boring tooth in relation to the excretory pore and the morphology of the post anal tail with a typical terminal mucron (Koyama et al., 1969; Hurst, 1984; Berland, 1961 & 1989; Bruno et al, 2006). Recently, the scanning EM and genetic studies through several molecular techniques (PCR and subunite tRNA gene) have been successfully employed for the identification of the Anisakis species (Matticucci et al., 1997; Amelio et al., 2000; Abe et al., 2005; 2006).

The pathology of Anisakis is due mainly to 2 mechanisms, allergic reaction and direct tissue damage (Lopez Serrano et al., 2000; Audicana et al., 2002; Caramello et al., 2003). The former range from isolated urticaria and angioedema to life-threatening anaphylactic shock associated with gastrointestinal Anisakis (Caramello et al., 2003; El-Daly et al, 2004). Meanwhile, the tissue damage is due to invasion of the gut-wall, development of eosinophilic granuloma or perforation (Audicana et al., 2002).

The aim of this investigation was to identify the morphological and ultrastructural features of Anisakis larvae and adults in marine fish Carangoides bajad and puppies besides its pathology.
MATERIALS AND METHODS

Adult 51 *Carangoides bajad* (fish host) specimens were collected from September 2007 to January 2008, of the Eastern South Coast of Hurgada, Egypt. Each fish was dissected and the liver, stomach, intestine, gallbladder, gonads were examined to the recovered larvae. The latter were washed in physiological saline, fixed in 5% formalin for morphological examinations. While, for scanning electron microscopy, the 3rd stage larvae were fixed in 4% glutaraldehyde for 12 hr. at 4°C and post fixed with OSO4 for 1 hr. They were dehydrated, critically dried sputtered coated and then photographically examined by 100 J. SEM Unit, Faculty of Science, Ein Shams University, Egypt. Specimens from the liver and intestine of fish were taken and fixed in 10% buffered neutral formalin solution for histopathological examination.

Five puppies (anthelmintically treated before the experiment) were orally infested with fish-containing the 3rd-stage larvae of *Anisakis* and then daily examined their feces for nematode-eggs. One-month later, the puppies were necropised and specimens from the liver, kidneys and intestine (ileocecal segment) were collected and fixed in 10% buffered neutral formalin solution. Five-micron thick paraffin sections were prepared from fish and puppies specimens stained by hematoxylin and eosin and PAS-reaction for parasitic elements and then examined microscopically (Bancroft *et al.*, 1996).

RESULTS

Thirty-six out-of 51 adult *Carangoides bajad* (70%) showed *A. simplex* in the tissues of fish.

I-SEM description of third stage larvae of *Anisakis simplex*:

The nematodes measures were 18.8 mm body length, 0.36 mm width, 1.8 mm esophagus length, 0.015 mm length of larval boring tooth and 0.019 mm mucron length. The cuticle was multilayered of an obvious cellular external cuticle and ornamented with striations (Fig 1, G-H), which has ridges. A boring tooth was observed projecting anteroventral ventral to the mouth, three biloped lips were present (Fig 1, C-D), one dorsal and two ventral lips, excretory pore in the form of transverse slit situated between the ventral lips. The posterior end of the larvae was distinguished
as the transverse striations became wider and a cone-shaped posterior end showed a slit–shaped anus and a characteristic mucron, (Fig. 1, A-E).

II- Morphological Examination of Adult Worm:

The body of adult nematode is fusiform and tapering at the posterior end. The cuticle is smooth. The esophagus is cylindrical with anterior muscular and posterior glandular parts and expanded at the anterior third. The excretory pore lies near the posterior third where presence of large excretory gland cell (Fig. 2A-D). The tail is conical and short. The male is long and thin, while the female is long and thicker and with the same measures of SEM.

III- Pathological Findings:

A- Anisakis Larvae Lesions in Fish:

Macroscopically, the serosal surface of the intestine was irregularly thickened or rarely nodular. The outer surface of the liver, kidneys, pancreas and peritoneum wall was focally thickened with whitish fibrous connective tissue. The nodules or the outer surface showed coiled larvae. Grayish white foci were seen throughout the hepatic and renal tissues. Microscopically, these larvae were encapsulated with concentrically fibrous connective tissue infiltrated with granulomatous infiltrates (macrophages, lymphocytes) besides numerous eosinophils (Fig 3). The macrophages were aggregated together, forming the denser part of the capsule and invaded to adjacent parenchymal tissue. Larger numbers of lymphocytes appeared to be associated with the capsule of lightly infected fish than of heavily infected ones. The hepatocytes under the affected capsule were degenerated or necrotic and invaded by lymphocytes and macrophages (Fig 4). Some larvae were visualized among the hepatic cells with no evidence of inflammatory or necrotic responses. The parasitic larvae were positive for PAS reaction. In some cases, the fibrous capsule around the larvae was impregnated with PAS-positive material (Fig 5). Almost all of the hepatic cells were depleted from glycogen and rarely infiltrated by fat to give the picture of hepatic steatosis besides periductal lymphocytes aggregation (Fig 6). In some cases, the liver showed diffuse hydropic degeneration (Fig 7), congestion and activation of melanomacrophages centers (Fig 8). The peritoneum revealed larvae on the hepatic surface (Fig 9) and serosal coat of the intestine with or without capsule. The intestine
containing the parasites (Fig 10) showed catarrhal enteritis with severe mucinous degeneration and leukocytes infiltration (Figs 11 and 12). The muscular coat was focally hyalinized.

**B- Adult Nematode Lesions in Puppies:**

Macroscopically, multifocal grayish foci were detected allover the hepatic and renal surfaces. Both organs were slightly enlarged, congested and oozed blood from their cut surfaces. The intestine revealed hemorrhagic necrotic mucosa. In some cases the intestine showed mucoid content. Microscopically, the larvae could induce visceral migration particularly in the liver and kidneys. In the liver, such migrations were represented by migratory tracks filled with eosinophils and erythrocytes besides few lymphocytes and macrophages (Fig 13). The contagious portal areas showed congested blood vessels, edema and eosinophils infiltration (Fig 14). Cloudy swelling, vacuolar and hydropic degenerations were noticed. The migratory tacks in the kidneys were represented by granulomatous nodules consisting of central caseation. The latter was surrounded by macrophages, lymphocytes and eosinophils and followed by fibrous connective tissue (Fig 15). In some nodules revealed central calcification and giant cells of foreign body and Langhans types (Fig 16). The renal parenchyma was focally replaced by eosinophils, lymphocytes and macrophages besides severe vacuolar and hydropic degenerations in the renal tubular epithelia (Fig 17). The intestine showed the adult nematodes (used for parasitological examination). The intestinal mucosa revealed atrophied villi with round tips and subepithelial acellular zone (Fig 18). Hyperplasia, mucinous degeneration and desquamation of the lining epithelium besides pale eosinophilic mucus in the lumen were seen (Fig 19). The remaining mucosa, submucosa and lamina propria were heavily infiltrated with leukocytes of lymphocytes, macrophages and eosinophils. Severe congestion, edema and hemorrhages were detected (Fig 20). Sometimes, the mucosa was necrotic with round cells and eosinophils infiltration (Fig 21). Such necrosis was extended to include the muscular coat. These areas (caseous necrosis) were surrounded by palisading fibroblasts and round cells (Fig 22). Such muscle fibers were hyalinized.

**DISCUSSION**
The present investigation was conducted to study the prevalence, morphological and ultrastructural features of the third stage larvae and adult worm of *Anisakis simplex* in *Carangoides bajad* marine fish (IMH) and puppies (definitive host). Thirty-six out-of 51 fish (70%) were infected with the nematodes which reside the peritoneal cavity, liver, intestine and spleen. Such prevalence was relatively high to that obtained by many authors (Berland 1961, Shiraki 1974, Chao, 1985, Hochbergh, 1990 and Sun *et al* 1991) who reported up to 40% prevalence. On the other hand, the very lowest rate 2.2% was reported among the orange-spotted trevally, *Carangoides bajad* (Abdou and Dronen 2007). The differences in the intensities of the natural infection of the examined hosts may be due to differences in the ecological, seasonal variations and nutritional status of the host that may lead to variations in the immunity against parasites (Mehlhorn, 2001).

Concerning morphology and scanning electron microscope of *Anisakis simplex* larvae were characterized by the body of the larvae is tapering gradually towards the anterior part; the anterior body end showed a boring tooth prominent, 3 pairs of lips inconspicuous and excretory pore ventral between rudimentary sub-ventral lips. The anal end has mucron which was distinct and a slit–shaped anus. These results are in agreement with those described by Abdou and Dronen (2007). The nematodes were 18.8 mm body length, 0.36 mm width, 1.8 mm esophagus length, 0.015 mm length of larval boring tooth and 0.019 mm mucron length. The aforementioned findings were similar to those obtained by Berland (1961) and Shiraki (1974). Meanwhile, the major morphological criteria of the adult *A. simplex* worms in our work were similar to those obtained by Smith and Wootten (1978), Abollo *et al* (20021), and Takei and Powell (2007).

*Anisakis simplex* is a nematode parasite belonging to the Ascaroidea group which includes several species of medical importance. Nematodes as *Anisakis* parasitize a variety of marine fish as intermediate hosts. Anisakiasis is difficult to diagnose which may explain the relatively recent recognition of its importance as a human disease (Smith and Wootten 1978 and Bruno *et al* 2006) and its frequent misdiagnosis as other conditions. The liver, gallbladder, kidneys and gonads of fish
may be grossly discolored if infected with certain protozoa, microsporidi or myxosporea.

The pathological findings revealed presence of encapsulated *Anisakis* larvae with fibrous connective tissue infiltrated with macrophages, lymphocytes and eosinophils. Capsule formation around these larvae in the *Carangoides bajad* appears similar to that described in other species (Dezfui et al 2007). A thin hyalinized layer of degenerative tissue was located adjacent to the larvae. The presence of the macrophages may be due to its participation in the capsule formation (Caramello et al 2003). Numerous eosinophils were observed around the parasitic larvae. Our findings disagree with Sakanari (1997) who mentioned that the eosinophilia is no usual inflammatory reaction of fish hosts against parasites. Such response is common in human anisakiasis (Coutare et al 2003).

The pathology in the puppies infested with 3rd-stage larvae of *Anisakis simplex* showed visceral migrations with eosinophilic granulomatous migratory tracks in the liver and kidneys with maturation of adult nematodes in the intestine. The latter revealed catarrhal or necrotic enteritis with round cell and eosinophil infiltrations. The aforementioned lesions were similar to intestinal anisakiasis in human (Takei and Powel 2007). The cellular components of the lesions indicate a chronic pathology, which would be significantly serious in puppies (final-host model) and fish (intermediate host).

In summary we described that the cases of puppies with intestinal anisakiasis caused by the consumption of raw fish. The pathologists in this country should consider this disease as one of the differential diagnosis when confronted with intestinal reaction in patient with signs of acute abdominal pain after ingestion of raw or undercooked fish.

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Fig. 1: A–H: *Anisakis simplex* third larva SEM micrograph showing: A- Posterior with a slit shape anus (A) and characteristic mucron (M); B- Top view of the anal slit-shaped opening; C- Interior top view showing Lips (L) and tooth (T); D-ventral position of the anal opening; E-Interior position; F-H, body cuticle striations., A-E X500; F X750; G X1500; H X100.
Fig. 2: A-B The anterior end of adult *Anisakis simplex*: A- buccal capsule (bc) and esophagus (e), B- ventral sucker (vs). C-D The posterior end of adult *Anisakis simplex*: C- male with tapred end, D- female with anus.
Fig. 3: A- larva focally replaced the hepatic parenchyma and surrounded with granulomatous infiltrates (macrophages, lymphocytes) besides numerous eosinophils. B-Fibrous connective tissue capsule around the larva and infiltrated with macrophages. C-The capsule was hyalinized and positive for PAS reaction. D-hepatic steatosis with glycogen depletion, (arrows on the lesions); H & E X 50.

Fig. 4: A-Dense fibrous connective tissue capsule surrounding the larva which was positive for PAS reaction. B-Numerous melanomacrophages were scattered the hepatic parenchyma. C and D-Anisakis larvae attached to the liver capsule and in the peritoneal cavity, PAS reaction. X 50
**Fig. 5:** A-Numerous sections of the larvae in the intestinal lumen with severe necrotic mucosa. B-The necrotic mucosa with increased goblet cells and leukocyte infiltration. C-Migratory track in the liver tissue with round cells and eosinophils aggregation. D-Migratory track in the kidney with granulomatous reaction consisting of central caseation, surrounded by fibrous connective tissue capsule infiltrated with macrophages and lymphocytes, H & E X 50.

**Fig. 6:** A- A high power of the affected kidney to show giant cells and dystrophic calcification B-Atrophied intestinal villi with round tips and submucosal leukocytic infiltrations. C-Excessive mucus in the intestinal lumen with mucinous degeneration. D-Necrosis and round cells infiltration in the intestinal wall. X 50
تم دراسة مدى انتشار الطور الثالث للانساسكس سميلكس في 51 سمكة من نوع كارنجويدز بيضاء وكان معدل الإصابة 70%. والتي تم جمعها على طول شاطئ البحر الأحمر خلال الفترة من سبتمبر 2007 إلى يناير 2008. وأوضحت الدراسة باستخدام الميكروسكوب الإلكتروني الماسح أن جسم اليرقة مدبوس تدريجيا باتجاه القمة ومزين بخطوط طويلة وعرضية ويحمل ثلاث شفاه في المقدمة. كما أوضح الدراسة أنه يميز أيضا وجود فتحة إخراج بطنية بين الشفاه البطنية وفتحة الشرج على شكل شق ويوجد أيضا زائدة دبوسية واضحة (المكرون). وقد تم من خلال الدراسة أن هذه اليرقات متصلة داخل الأنسجة العضدية التي في الكبد والطحال والمنعخة بكثير من الخلايا الالتهابية. كما أوضح الدراسة أنها مع الخلايا الكبدية المجاورة. أما العائل الأساسي (الكرو) فقد أوضحت الدراسة هجرة هذه اليرقات إلى الأعضاء الداخلية لكل من الكبد والكلي ثم عودتها واستقرارها في الأمعاء لتكون الطور اليافع (الدودة البالغة) مع وجود التهابات بها.