Gnathostoma binucleatum: Pathological and parasitological aspects in experimentally infected dogs

C. Alvarez-Guerrero a, M.A. Muñoz-Guzmán b, J.A. Buendía-Jiménez b, F. Alba-Hurtado b,*

a Laboratorio de Parasitología, Secretaría de Investigación y Posgrado, Universidad Autónoma de Nayarit, México
b Departamento de Ciencias Biológicas, Facultad de Estudios Superiores Cuautitlán, Universidad Nacional Autónoma de México, México

ARTICLE INFO

Article history:
Received 12 March 2010
Received in revised form 17 June 2010
Accepted 29 June 2010
Available online 3 July 2010

Keywords:
Gnathostoma
Experimental infection
Dog’s diseases
Gastric nodules
Pathology

Abstract
Lesions and antibody kinetics produced by inoculation of Gnathostoma binucleatum larvae into dogs are described, as well as the morphology of the recovered parasites. In four out of five infected bitches parasite phases were found in the stomach. Only one bitch eliminated eggs and adult parasite phases in feces. In this bitch, the prepatency period lasted 22 weeks and the patency period 14 weeks. Necropsy results showed a copiously vascularized 8-cm diameter fibrous nodule lodged in the greater curvature of the stomach. Two bitches that eliminated no eggs showed 1- to 2-cm diameter nodules on the gastric wall, with five juvenile phases in each. One bitch that eliminated no eggs and exhibited no gastric nodules showed juvenile parasites on the gastric wall. Results confirm dogs as definitive hosts of this parasite. New data on the pathological and parasitological aspects of canine gnathostomosis are presented.

© 2010 Elsevier Inc. All rights reserved.

1. Introduction

The nematode Gnathostoma binucleatum is the only species confirmed to produce human gnathostomosis in the American Continent. This disease constitutes a major health problem in the State of Nayarit, Mexico, where a total of 6328 cases were reported between 1995 and 2005 (SUAVE: Unique Automated System for Epidemiological Vigilance, Secretaría de Salud de Nayarit 1995–2005). The first intermediate hosts of all Gnathostoma species are considered to be copepods. Estuarine fish (Cathorops fuerthii, Pomadasys macracanthus, Mugil curema and Dormitator latifrons) and some turtles (Kinosternum integrum and Trachemys scripta) act as second intermediate and paratenic hosts, respectively (Alvarez-Guerrero and Alba-Hurtado, 2007).

The role of carnivorous (ichthyophagous) mammals as definitive hosts in the biological cycle of the different Gnathostoma species has been amply documented. Adult G. binucleatum worms have been detected in gastric nodules of naturally infected cats and ocelots (Almeyda-Artigas, 1991). Koga et al. (1999) isolated adult Gnathostoma worms from the gastric nodules of experimentally infected dogs; however, the effects of the parasite on these hosts have not been described. The present study describes pathological and parasitological aspects of experimentally infected dogs.

2. Materials and methods

2.1. Experimental animals

Five clinically healthy bitches of non-defined breed, aged 2–4 months, were used. Before the experiment, preventive anti-parasite treatment consisting of praziquantel, pyrantel pamoate, and febantel (Drontal plus, Bayer) was orally administered. Externally, animals were powdered with 2-methyletoxiphenyl carbamate (Bolfo, Bayer) and immunized against distemper and parvovirus. The bitches were kept in individual cages at the laboratory animal facilities of the Research and Postgraduate Department, Autonomous University of Nayarit, Mexico. Prior to the experiment, none of the bitches showed helminth egg deposition in feces or external parasites. The animals were cleaned daily and fed balanced commercial products and water ad libitum. The study was approved by the Internal Committee for the Care of Experimental Animals of the Postgraduate Program of Animal Production and Health (UNAM, México).

2.2. Collection of advanced third stage larvae (AdvL3) for inoculum

G. binucleatum larvae were isolated from K. integrum turtles in fishing areas near Agua Brava lagoon, in a northern region of the State of Nayarit, Mexico. The muscle tissue was dissected, ground with a kitchen grinder, compressed between two glass panes (15 cm in width by 18 cm in length) and observed against the light of a 100 W lamp. The AdvL3 were separated with entomological...
needles and the cysts were checked for integrity and counted for inoculum preparation (Alvarez-Guerrero and Alba-Hurtado, 2007).

2.3. Larval morphology

Thirty AdvL3 were morphometrically evaluated. They were fixed in 10% formaldehyde for 24 h. During morphometrical evaluation, the AdvL3 were cleared with Anmann lactophenol and the variables recommended by Miyazaki (1954) were used to identify larval species of the Gnathostoma species. Measurements are presented as mean ± standard deviation and expressed in millimeters. The mean number of nuclei per intestinal epithelium cell was determined using transversal histological sections of AdvL3.

2.4. Experimental design

Encysted larvae were mixed with 50 g of ground fish meat (meatballs). Five bitches were orally inoculated with approximately 50 AdvL3. Total fecal material was collected daily to search for parasite structures. A blood sample was obtained from all bitches by monthly venous puncture and the serum was isolated and stored at −20 °C for antibody determination by ELISA. Five bitches were euthanized with a sodium pentobarbital dose at (one bitch), 7 (one bitch), 9 (two bitches) and 13 (one bitch) months after inoculation. The stomach was removed and the wall was examined macroscopically for nodules and parasites. In the bitches that showed no macroscopic nodules, artificial digestion of the stomach wall was performed to search for larvae or juvenile parasites that had not produced nodules. Recovered adult and juvenile worms were processed for scanning electron microscopy and one parasite was used to sequence ribosomal DNA. One-cm³ samples were obtained from the recovered nodules, fixed in formaldehyde for 48 h, and included in paraffin. Four-mum thick sections were obtained. Sections were processed and stained with conventional hematoxylin/eosin staining procedures.

2.5. Parasitological examination

Total feces collected daily were examined macroscopically in search of eliminated parasites and a Faust coproparasitoscopic analysis was carried out in search of eggs. From the first day that eggs were observed in feces, they were counted with a modification of the McMaster technique (Alba-Hurtado, 2007) and expressed as eggs per gram of feces (egf).

2.6. Scanning electron microscopy

Adults and juvenile worms were washed in distilled water for 30 min to eliminate formaldehyde residues and dehydrated in alcohol graded from 10% to 100%. Critical point drying was performed, the sample was mounted on an aluminum sample holder with double-sided carbon adhesive and ionized with gold. Micrographs were obtained under high vacuum conditions using a JEOL SM 5410LV scanning electron microscope.

2.7. DNA sequencing

A segment of an adult worm obtained from an infected dog was used to sequence DNA. The sample was kept in absolute alcohol. DNA was extracted by the standard phenol–chloroform technique (Palumbi, 1996) and the internal spacer of the ribosomal DNA (ITS2) was amplified by PCR, as described by Martínez-Salazar and León-Régagnon (2005). NEWS2 primers (forward) 5’TTCCTAGCTGATGGAGCAAGCAG and ITS2-RIXO (reverse) 5’TCCATGCTTAAATTCAGGG were employed. The resulting sequences were aligned with sequences AB181159 obtained from the GenBank corresponding to G. binucleatum, with the computer program Clustal-W (Thompson et al., 1994) and corrected manually with the computer program Bioedit (Hall, 2001). Uncorrected distance matrices were obtained for the pairs of examined sequences with PAUP* version 4.0b10 (Swofford, 2002).

2.8. Serum antibody determination

Parasite antigens were obtained from approximately 250 G. binucleatum AdvL3 (Ag-AdvL3) and from an adult phase (Ag-AP) isolated from a gastric nodule, as described by Muñoz-Guzmán et al. (2006). The amount of total protein was determined by the Bradford method (1976).

Serum antibody levels against Ag-AdvL3 and Ag-AP were measured by ELISA. All ELISAs were performed in duplicate and optimized according to antigen concentration and serum and conjugate dilution. Antigen concentration was 10 µg/ml for both antigens; serum dilution was 1:320 and 1:80, respectively, and the conjugate (sheep anti-canine IgG and serotec AA132P) was diluted 1:5000. The plate was read at 492 nm in an ELISA Multiskan Ascent reader (Labsystems). For each serum, the unspecific reaction was discarded, using an adjacent antigen-free well. The resulting absorbance was subtracted from that obtained in the presence of antigen. O.D. results for the two wells were averaged and later transformed to percentage absorbance (%Abs), with respect to a positive control, by the following formula:

\[
\%\text{Abs} = \frac{(\text{sample serum O.D.)} - \text{negative control O.D.})}{\text{positive control O.D.}} \times 100
\]

2.9. Statistical analysis

Antibody kinetics results were analyzed by one-way ANOVA for repeated samples, using the Statistica for Windows software. Duncan’s Multiple Range Test (DMRT) was used for comparisons of the means of each week, with respect to the initial infection week.

3. Results

The morphometry of larvae recovered from turtles was as follows: total length 4.05 ± 0.52; maximum width 0.30 ± 0.06; four rings of hooks per cephalic bulb; length of the cephalic bulb 0.21 ± 0.02; width of the cephalic bulb 0.11 ± 0.02; location of cervical papillae 14.2 ± 1.4; distance from the cloacal aperture to the hind end 0.61 ± 0.028. The mean number of nuclei per intestinal epithelium cell of AdvL3 was 2.20 ± 1. All morphometric variables corresponded to G. binucleatum. The base sequence of the amplified ribosomal DNA segment showed 0.48% divergence (2–419 base pairs) with the sequence reported in the GenBank for G. binucleatum.

Four of the five bitches inoculated with G. binucleatum AdvL3 presented parasite phases in the stomach (Table 1). Only one bitch eliminated eggs (Fig. 2C) and adult phases in fecal material. Eliminations were determined using transversal histological sections of AdvL3.

### Table 1

<table>
<thead>
<tr>
<th>No.</th>
<th>Bitch</th>
<th>Nodule with adult</th>
<th>Nodule with larvae</th>
<th>Juvenile without nodule</th>
<th>Ova in adult</th>
<th>Adult in feces</th>
<th>Month at necropsia</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>*</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>*</td>
<td>–</td>
<td>9</td>
</tr>
<tr>
<td>2</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>*</td>
<td>–</td>
<td>9</td>
</tr>
<tr>
<td>3</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>13</td>
</tr>
<tr>
<td>4</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>7</td>
</tr>
<tr>
<td>5</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>5</td>
</tr>
</tbody>
</table>
ination began on week 22 p.i. (prepatency period) and was maintained until week 36 p.i. (14 week patency period). The largest number of eggs was eliminated during week 30 p.i. (3542 egf). Five adult female parasites with no eggs in uterus were detected in weeks 24, 25, 26, 28 and 36 p.i.

Necropsy of the only bitch that eliminated eggs in feces showed a copiously vascularized 8-cm fibrous nodule (Fig. 1A) lodged in the greater curvature of the stomach. Internally, the nodule presented caverns that communicated with the abdominal cavity and the gastric lumen (Fig. 1C). The caverns contained a bloody

Fig. 1. Lesions produced by experimental inoculation of bitches with *Gnathostoma binucleatum*. (A) Internal gastric surface (mucosa) of one nodule with adult worms. (B) External gastric surface (serosa) of one nodule. The arrow shows a female parasite at the entrance of a cavern, in contact with the viscera. (C) Section of a nodule with internal caverns. (D) External gastric tissue (serosa) showing small nodules with juveniles inside.

Fig. 2. Experimental inoculation of *Gnathostoma binucleatum* in dogs. (A) Histological section of a gastric nodule with eggs trapped in tissue in a zone showing strong inflammatory reaction. (B) Histological section of a gastric nodule with eggs trapped in tissue and surrounded by collagen. (C) Eggs eliminated in feces. (D) Necropsy showing gastric nodule, splenomegaly and hepatomegaly.
mucous secretion, as well as eggs at different developmental stages (immature, embryonated, and larvated). Inside the nodule, six adult males were found with the anterior half of their body attached to the abdominal cavity wall and the posterior half to the cavern light. Close to the cavern entrance, an adult female parasite was found in contact with the viscera (Fig. 1B). The peritoneal liquid was reddish in color and had probably been secreted by the nodule.

The two bitches that eliminated no eggs in feces showed 1- to 2-cm-diameter nodules attached to the gastric wall with five juvenile phases in each (Fig. 1D). One bitch eliminated no eggs in feces and showed no gastric nodules, and five juvenile parasites were recovered during artificial digestion of the gastric wall.

Necropsy of the four *G. binucleatum* infected bitches revealed muscular atrophy, hepatomegalia, splenomegaly, mesenteric lymphangitis, pancreatitis, gastric hypertrophy, chronic gastritis, and small ulcers in the gastric mucosa (Fig. 2D).

Histologically, the infected bitches presented nodules with high collagen content. Fibrotic zones, small necrotic zones, and a large number of eggs with an apparently complete covering were trapped inside the tissue (Fig. 2B). In addition, infiltrates of plasma cells, macrophages and eosinophils were seen surrounding the eggs (Fig. 2A).

Adult male parasites measured from 18 to 23 and females from 35 to 57. The anterior body half showed a cephalic bulb with 8–9 concentrically disposed hooks (Fig. 3A). The mouth presented thick lips (trilobulated), each with a pair of papillae, between which were the amphidae or sensory receptors. The largest cuticular spines were distributed over 45% of the body and the spines closest to the cephalic bulb showed 2–3 denticles. In the middle region, they displayed three denticles: the middle one was the largest (Fig. 3B). In the posterior region, the spines showed one or two denticles.

On the ventral side of the tail, the males presented minute unidentate spines, four pairs of large lateral papillae and three pairs in a middle position, as well as one pair of unequal spiculae. The left one measured approximately 90 μm in length and the right one, 40 μm (Fig. 3C). The cloaca was covered with minute spines. The vulva, situated in the mid-ventral region, was also surrounded with minute spines. Juveniles presented similar characteristics to adults, but smaller, measuring 1–1.5 cm, and showed none of the reproductive spiculae of adult males.

The kinetics of mean antibody production against Ag-AdvL3 and Ag-AP in infected bitches are shown in Fig. 4. The mean antibody levels against Ag-AdvL3 increased significantly *(P < 0.05)* from the second (100.56 ± 57.79% Abs) to the fifth (166.77 ± 54.89% Abs) month p.i., with respect to the moment of infection (17.72 ± 14.90% Abs). Ag-AP antibody levels showed no statistical differences throughout the months of sampling *(P < 0.05)*.

4. Discussion

Three species of the genus *Gnathostoma* have been reported in Mexico: *G. binucleatum* (Almeida-Artigas, 1991), *Gnathostoma turigidum* (Lamothe-Argumedo et al., 1998) and *Gnathostoma lamothei* (Bertoni-Ruiz et al., 2005; Hernández-Gómez et al., 2010). Their morphological and biological characteristics were used to differentiate *Gnathostoma* species. However, at present, ribosomal DNA sequencing is considered a more efficient tool to confirm species identification. The morphology of larvae used to infect bitches and the preadult development stages have been described as definitive hosts of the different *Gnathos-
had they remained longer in the host. Nevertheless, no precedent is available to explain this wide span in the prepatency period of worms of the same species. For example, in *Spiorerca lupi*, the prepatency period may range from 3 to 9 months (Van der Merwe et al., 2008).

The infected bitches developed nodules on the gastric wall. This type of lesion has been reported in most of the definitive hosts of the 14 identified *Gnathostoma* species in the world, except for *Gnathostoma nipponicum*, which lodges in the esophagus, *Gnathostoma miyazakii* and *Gnathostoma vietnamicum*, which lodge in the kidney, and *Gnathostoma didelphys* and *Gnathostoma braziliense*, in the liver (Miyazaki, 1991; Daenssangvang, 1982). The fact that eliminated eggs in fecal material also eliminated female parasites, but the necropsy revealed only the presence of males lodged in the nodule wall. There is no definitive explanation for this; however, it is probable that female parasites that had ended their egg production had exhausted their metabolic reserves and died. The completely empty uteri of eliminated female parasites and the loss of color of the pseudocelom are probably protected. However, the eggs trapped in the tissues were probably ovideposited in the light of the nodule’s caverns and thereafter dragged to the lesions by parasite movements. The presence of a large amount of inflammatory cells close to or through organs such as the liver or the kidneys, which lodge in the kidney, as well as the presence of a large amount of eggs trapped inside the tissue and a strong surf-area is probably the result of their antigenic capacity, thus contributing to the nodule’s growth. Therefore, even if these eggs probably participate in the pathogenesis and stimulation of nodule formation in the host, they play no role in the epidemiology because they are confined to the nodule.

Antibody levels against the larval phase rose significantly in infected bitches, starting at the second month p.i., and remained high until the fifth month p.i. However, antibody levels against the adult phase showed no significant increase. This difference may be because larval phase antigenic stimulation occurred earlier and was more intense. This was probably due to the migration of larvae through organs such as the liver before they finally attach to the gastric wall (Díaz-Camacho et al., 2010) and also to the high number of inoculated larvae (approximately 50). Adult phases of the parasite were recovered from only one infected bitch. Thus, the response against the adult phase probably takes longer.

The difficulty in obtaining enough viable *G. binucleatum* larvae is the main obstacle in performing studies with a larger number of experimental animals. Results obtained in the present study contribute relevant data on unknown pathological and parasitological aspects of canine gnathostomosis.

**Acknowledgments**

The authors would like to thank to Dra. Virginia León-Régagnon for DNA sequencing of *G. binucleatum*. This study was supported in part by a grant from the Programa de Apoyo a Proyectos de Investigación e Innovación Tecnológica (PAPIIT-UNAM).

**References**


E. C. Alvarez-Guerrero et al. / Experimental Parasitology 127 (2011) 84–89

**Fig. 4.** *Gnathostoma binucleatum* anti-antigens of advanced third larva (Ag-AdvL3) and anti-antigens of adult phase (Ag-AP) antibody kinetics in five experimentally infected bitches. *Significant difference (P < 0.05)*, with respect to the time of inoculation.


SUAVE. Sistema único automatizado para la vigilancia epidemiológica. Secretaría de Salud de Nayarit.

