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ORIGINAL PAPER

Estuarine fish and turtles as intermediate and paratenic hosts of *Gnathostoma binucleatum* in Nayarit, Mexico

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Abstract Human gnathostomosis is a severe public health problem in the State of Nayarit, Mexico. Between 1995 and 2005, the registration of human cases numbered 6,328, which makes it one of the largest focal points of the disease in the country. The present study determined the presence of natural hosts of *Gnathostoma binucleatum* larvae at the Laguna de Agua Brava in Nayarit, Mexico. A total of 5,450 fish and 247 turtles were sampled. Muscular tissue was ground and observed against the light using a 100-W lamp to identify advanced third-stage larvae. The estuarine species *Cathorops fuerthii*, *Pomadasys macracanthus*, *Mugil curema*, and *Dormitator latifrons* were found positive for presence of larvae, and annual prevalence was 4.8, 1.83, 2.16, and 4.0%, respectively. The species *Oreochromys aureus* and *Chanos chanos* were negative. The species of estuarine turtles *Kinosternum integrum* and *Trachemys scripta* were positive with annual prevalence of 79.1 and 52.5%, respectively. The criteria of identification of the *Gnathostoma* species were: mean number of nuclei in intestinal larval cells (2.3), larval morphometry with optic microscopy, larval morphometry with scanning electron microscopy, and number and sequence of ribosomal deoxyribonucleic acid of adult parasites obtained from experimental infection in dogs. The estuarine fish *Pomadasys macracanthus* and *Mugil curema* are reported as intermediate hosts for the first time and likewise the estuarine turtle *Kinosternon integrum* as a paratenic host.

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Introduction

Gnathostoma spinigerum is the most studied species of the genus. Copepods and freshwater fish are the first and second intermediate hosts, respectively; amphibians and reptiles act as paratenic hosts and mammals as definitive hosts. Humans are accidental hosts and develop the disease after consuming raw fish infested with advanced third-stage larvae (aL3) of *Gnathostoma* (Miyazaki 1991; Daengsvang 1980, 1982). Although all species of the genus *Gnathostoma* present the same behavior in general, each species has particular intermediate and definite hosts, which results in a particular epidemiology for each species.

The first clinical cases of human gnathostomiasis in the American Continent were reported in Mexico by Peláez and Pérez-Reyes in 1970. Currently, four species of this genus have been recorded in mammals in Mexico: *G. binucleatum* (Almeida-Artigas 1991), *G. procyonis* (Almeyda-Artigas et al. 1994), *G. turgidum* (Lamothe-Argumedo et al. 1998), and *G. lamothei* (Bertoni-Ruiz et al. 2005). However, the only confirmed species that affects the human in America is *Gnathostoma binucleatum* (Almeyda-Artigas et al. 2000), although the intermediate hosts and infection source have not been entirely identified.

Human gnathostomiasis is one of the most important public health issues in the state of Nayarit, Mexico, where 6,328 cases were recorded between 1995 and 2005, this being the highest record in the country and one of the highest worldwide (SUAVE: unique system automatized for the epidemiology vigilance, Secretaría de Salud de Nayarit 1995–2005). Therefore, the intermediate hosts that represent

the infection source for humans in the region needed to be identified. In this context, common estuarine fish and turtles were collected in the largest water body, the Agua Brava lagoon, located in northern Nayarit, Mexico, Larvae were isolated from fish and turtle muscle. The average number of larval intestinal cell nuclei was determined using morphometry and scanning electron microscopy. The deoxyribonucleic acid (DNA) of the collected *Gnathostoma* species was sequenced.

Materials and methods

Collection of fish and turtles

Specimens were collected in fishing areas nearby the Agua Brava lagoon located in the northern region of the state of Nayarit, Mexico. The sampling produced 5,450 estuarine fish (3,000 *Cathorops fuerthii*, 600 *Pomadasys macracanthus*, 600 *Oreochromis aureus*, 600 *Mugil curema*, 600 *Chanos chanos*, and 50 *Dormitator latifrons*) and 247 turtles (207 *Kinosternon integrum* and 40 *Trachemys scripta*). The fish and turtle species were identified in the Ichthyology and Herpetology Departments of the Biology Institute in the National Autonomous University of Mexico based on Winckley et al. (1986); the FAO Guide (1995), Castro-Aguilar et al. (1999), Flores-Villela et al. (1995), and Alderton (1994).

Collection of larvae

Collected fish and turtles were dissected; the muscle tissue was chopped in a food processor (home appliance), compressed between two glasses (15 cm wide and 18 cm long), and observed against the light using a 100-W lamp to identify aL3 of *G. binucleatum*. The cyst wall was removed with insect pins, and larvae were fixed in 10% formaldehyde for analysis.

Infection in animals was characterized according to the definitions proposed by Margolis et al (1982), in which prevalence represents the percentage of hosts parasitized by one or more parasite species, mean intensity is the average number of one parasite species per parasitized host, abundance is the average number of a parasite species per analyzed host, and intensity interval is the minimum and maximum number of parasites found in a sample of hosts.

Larval morphology

Thirty larvae collected from fish and 30 from turtles were measured in a calibrated compound light microscope. Larvae were fixed in 10% formaldehyde for 24 h and stored at room temperature until analyzed. At measurement time, larvae were cleared with Amann's lactophenol, and the variables recommended by Miyazaki (1954) were used to identify the species of *Gnathostoma* at larval stage. Variables were total length, maximum width, head-bulb length, head-bulb width, number of rows and hooklets in the head-bulb (1, 2, 3, 4), difference in number of hooklets between the first and fourth row in the head-bulb, position of the cervical papilla, distance from excretory pore to distal end, and number of transverse striations in larval bodies. To obtain a more accurate count of the head-bulb hooklets, they were separated with a surgical knife with a thin straight blade. The separated head-bulb was placed between a slide and a coverslip in frontal position, and micrographs were obtained with a 5-megapixel digital camera; images were amplified in the computer for hooklet count.

Scanning electron microscopy

Larvae 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, and the sample was mounted in 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.

Intestinal sections of larvae

Larvae (aL3) recovered from fish and turtle muscle were embedded in 1 cm^3 of flesh, fixed in 10% formaldehyde for 24 h, and then embedded in paraffin. Cross-sections of 4 μm thickness were obtained and stained with hematoxylin–eosin with conventional techniques. The number of intestinal epithelial cells was counted, and the average number of nuclei per cell was determined with a ×100 objective.

DNA sequencing

A segment of an adult worm was used, obtained from a dog that had been infected with larvae collected from turtle muscle. The sample was preserved in absolute alcohol, and DNA was extracted using the standard phenol–chloroform technique (Palumbi 1996), and the internal transcribed spacer 2 (ITS2) of ribosomal DNA was amplified by polymerase chain reaction with the technique described by León-Régagnon et al. (2002) and Martínez-Salazar and León-Régagnon (2005) using the primers NEWS2 (forward) 5′-TGTGTCGATGAAGAACGCAG and ITS2-RIXO (reverse) 5′-TTCTATGCTTAAATTCAGGGG. The obtained sequences were aligned with sequences AY734632, AY061740, and AB181159 obtained from GenBank and corresponded to *G. binucleatum*.

Table 1 Characterization of infection with *Gnathostoma binucleatum* larvae in fish and turtles in Nayarit, México

Species	AH	PH	FP	P	MI	A	II
Fish							
Cathorops fuerthii	3,000	154	183	4.8	1.25	0.061	1 to 3
Pomadasys macracanthus	600	11	11	1.83	1. 0	0.018	1 to 1
Mugil curema	600	13	15	2.16	1.15	0.025	1 to 2
Dormitator latifrons	50	2	2	4	1. 0	0.04	1 to 1
Oreochromis aureus	600	0	0	0	0	0	0
Chanos chanos	600	0	0	0	0	0	0
Total in fish	5,450	170	211	1.46%	0.73	0.024	1 to 3
Turtles							
Kinosternon integrum	207	163	1,070	79.1	6.28	4.91	1 to 65
Trachemys scripta	40	21	25	52.5	1.29	0.62	1 to 3
Total in turtles	247	184	1,095	65.8%	3.78	2.76	1 to 65

AH Analyzed hosts, *PH* parasitized hosts, *FP* found parasites, *P* prevalence, *MI* mean intensity, *A* abundance, *II* intensity interval

Statistical analysis

Significant differences of the studied variables between turtles and fish were tested with Student's *t* test using Statistica® (Copyright 1984–2000 by StatSoft).

Results

Infection in intermediate and paratenic hosts

Table 1 shows the infection variables in the two hosts. *Gnathostoma* larvae were detected in fish species *C. fuerthii*, *P. macracanthus*, *M. curema*, and *D. latifrons* (Fig. 1a–d) and in turtle species *K. integrum* and *T. scripta* (Fig. 2a,b). All infection variables were statistically higher ($p<0.05$) in turtles than in fish (Table 1).

Morphology of larvae aL3

Recovered larvae were coiled inside a translucent fibrous cyst, through which the blood-red intestine could be seen. Mean size of the 20 measured cysts was 1.34 mm long and 1.14 mm wide. Table 2 shows the main morphometric variables of *G. binucleatum* larvae collected from fish and turtles. No statistical differences were found ($p<0.05$) between larvae obtained from fish and larvae obtained from turtles in the studied variables.

Scanning electron microscopy

This technique was used to observe the larval morphology in detail. The head-bulb presented four concentric rows of hooklets with a single tip per hook and four sac-like openings that communicate with the cervical sacs, through

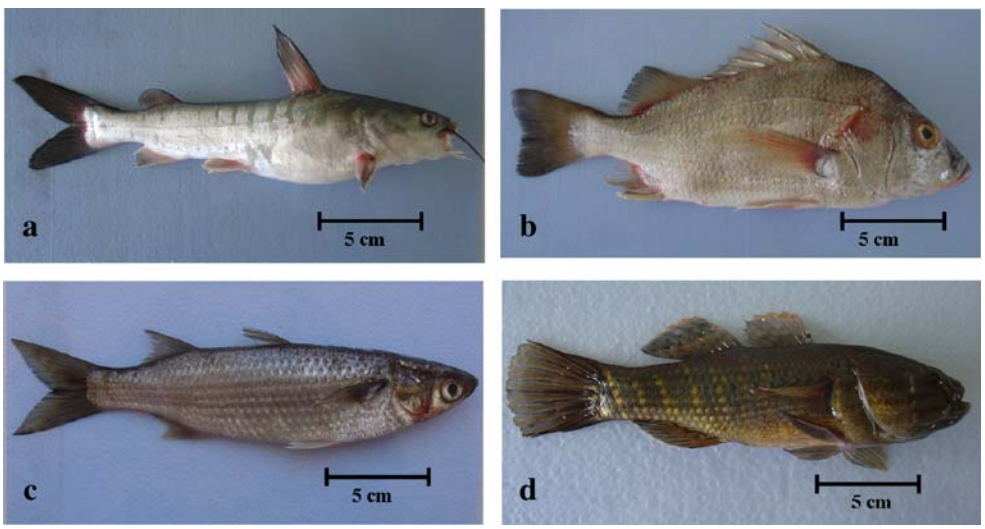

Fig. 1 Fish species infected with *Gnathostoma binucleatum* larvae in Nayarit state, México. **a** *Cathorops fuerthi*, **b** *Pomadasys macracanthus*, **c** *Mugil curema*, **d** *Dormitator latifrons*

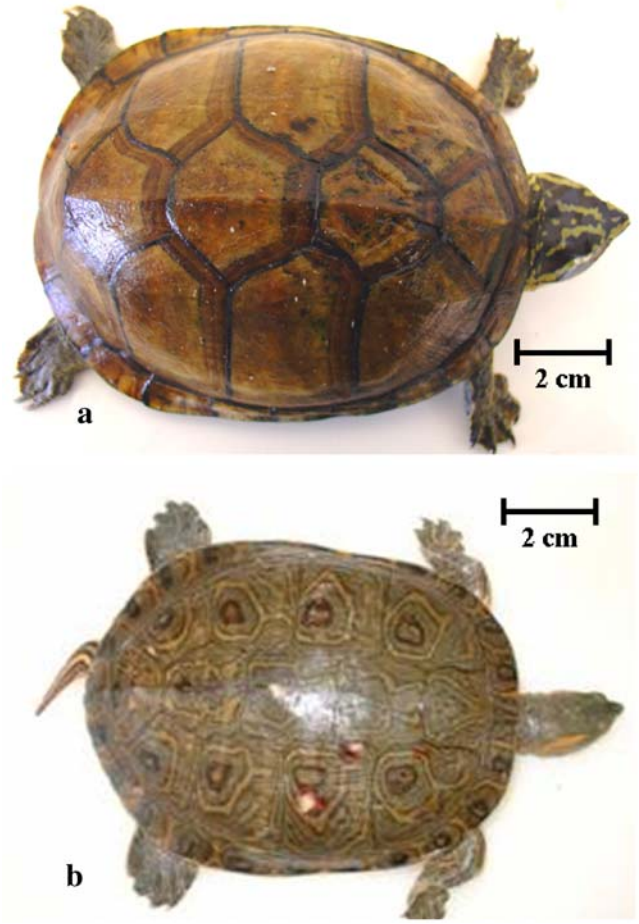

Fig. 2 Turtle species infected with *Gnathostoma binucleatum* larvae in Nayarit state, México. **a** *Kinosternon integrum*, **b** *Trachemys scripta*

which passes a fluid that causes head-bulb dilation or retraction. The mouth presented strong lips with a pair of papillae each with amphidia or sensory receptors between them (Fig. 3a). The cervical papilla was located in transversal row 14 (Fig. 3b). The cuticle presented 268 transverse striations in larvae obtained from fish and 276 in larvae obtained from turtles. Spines near the head-bulb were larger and densely abundant; in the middle third, they were smaller, and nearing the distal end, they were even smaller and less abundant. The anus was subterminal, the caudal end was point shaped, and both sides had a pair of phasmidial pores (Fig. 3c).

Intestinal sections of larvae

In the present study, 101 larval intestinal epithelium cells were firstly obtained from fish larvae and analyzed; 25 cells presented a single nucleus, 35 presented two nuclei, 26 presented three nuclei, 11 presented four nuclei, and 4 presented five. The total number of nuclei was 227, and the average number of nuclei per cell was 2.24. Likewise, 84 larval intestinal epithelium cells obtained from turtle larvae were analyzed; 26 presented a single nucleus, 27 presented two nuclei, 23 presented three nuclei, 7 presented four nuclei, and 1 presented five. The total number of nuclei was 182, and the average number was 2.20.

DNA sequencing

The base sequence of the amplified segment presented a divergence lower than 0.48% (2 of 419 bp) with *G. binucleatum* sequences reported in GenBank. Therefore, we concluded that the studied species is in fact *G. binucleatum*.

Discussion

Most intermediate hosts of *Gnathostoma* spp. reported in the literature are freshwater fish (Daengsvang 1980). The state of Nayarit (Mexico) is of special epidemiological interest, as more than 6,000 human gnathostomiasis cases have been reported in 10 years (SUAVE: unique system automatized for the epidemiology vigilance, Secretaría de Salud de Nayarit 1995–2005). This high prevalence can only be explained by the huge quantity of intermediate hosts finding in the region. Most are marine and estuarine fish, and only few are freshwater fish. Estuarine fish are the

Table 2 Morphometry of 30 *Gnathostoma binucleatum* larvae obtained from estuarine fish and 30 larvae obtained from turtles in the state of Nayarit, México

	TL	MW	RB	HBL	HBW	HPR				IV−I	CP	ADE
						1	2	3	4			
Fishes												
MD	3.988	0.308	4.0	0.147	0.235	38.4	41.6	43.8	46.2	7.3	14.6	0.065
SD	0.586	0.054	0.0	0.020	0.042	3.1	3.1	3.1	3.2	3.5	1.4	0.018
Turtles												
MD	4.057	0.308	4	0.218	0.118	38.2	41.2	43.7	45.9	7.47	14.2	0.061
SD	0.522	0.069	0	0.023	0.025	2.80	2.7	3.1	3	1.3	1.4	0.028

TL Total length, *MW* maximum width, *RB* rings per head-bulb, *HBL* head-bulb length, *HBW* head-bulb width, *HPR* hooklets per ring, 1, 2, 3, 4. *IV−I* difference between averages of fourth and first rows, *CP* location of cervical papilla, *ADE* distance from anus to distal end

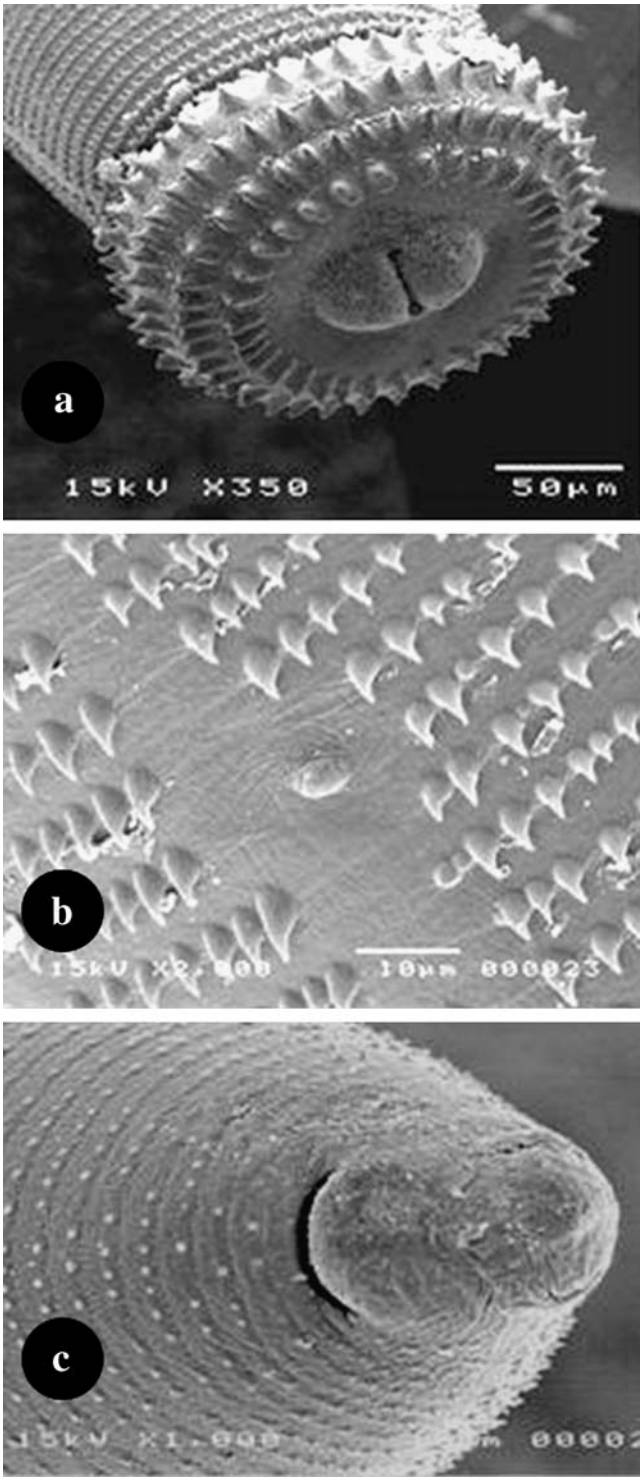

Fig. 3 Scanning electron micrographs of *Gnathostoma binucleatum* larvae obtained from turtles. **a** Lateral view of the head bulb. **b** Cervical papilla and transverse striations. **c** The terminal end where the anus and phasmidials pores are clearly visible

most consumed in the state and, thus, the first suspects of being the intermediate hosts.

Four fish species presented *G. binucleatum* larvae in the muscle. Among these species, *M. curema* and *P. macracanthus* are reported for the first time as intermediate hosts of *G. binucleatum*. The two species *C. fuerthii* and *D. latifrons* have been previously reported as intermediate hosts of the parasite (Alvarez-Guerrero and Lamothe-Argumedo 2000). These species are distributed in fish markets and restaurants in the state and are used to prepare a regional dish based on raw fish known as "cebiche"; therefore, this is a probable source of infection for humans and may account for the high prevalence of gnathostomiasis in the state.

The turtles *K. integrum* and *T. scripta* presented a prevalence of muscle-encysted larvae higher than that recorded for fish, and this is the first report of these species as hosts of *G. binucleatum*. From a cultural perspective, people attribute curative properties to the turtle, and given its alimentary properties as well, this finding is important, as these paratenic hosts may be directly involved with the disease in coastal populations and increase the incidence in the state of Nayarit.

In the present study, larvae from estuarine fish and turtles were compared, and no differences in morphology or in average number of intestinal epithelium cell nuclei were found between both groups of larvae, which suggests that they are the same species of *Gnathostoma*. The morphometric values (total length, number of head-bulb hooklets, position of the cervical papilla, number of transverse striae, number of intestinal cells, and average number of nuclei per intestinal cell) of these larvae corresponded to those reported for *G. binucleatum* (Almeida-Artigas 1991; Koga et al. 2000). However, several authors have considered that morphological features are not enough to differentiate *Gnathostoma* species; hence, in the present study, ribosomal DNA of an adult parasite obtained from a dog, experimentally infected with larvae collected from turtles, was sequenced. The base sequence of the amplified segment in the adult worm corresponded to *G. binucleatum* (León-Régagnon et al. 2002; Bertoni-Ruiz et al. 2005), which matched the species identity of the larvae found in turtles. The morphological similarities between larvae found in fish and larvae found in turtles, with the facts that both were simultaneously collected in the habitat and that this *Gnathostoma* species that infects had already been reported in the same region by León-Régagnon et al. in 2002, suggest that the species in both hosts is *G. binucleatum*.

The only species undisputably associated with human gnathostomiasis in America is *G. binucleatum* (Almeyda-Artigas et al. 2000), and, especially in Mexico (León-Règagnon et al. 2002), humans become infected by consuming the intermediary host, either raw or partially cooked ("cebiche," "callos," or "sushi"). The four fish species and two turtle species reported in this study as *G. binucleatum* hosts are consumed by humans, which can be

the infection source for human gnathostomiasis in the state of Nayarit, Mexico.

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