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ORIGINAL PAPER

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Morphology of *Gnathostoma* spp. isolated from natural hosts in Sinaloa, Mexico

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Abstract Gnathostomosis is an emerging public health problem in Sinaloa, Mexico, where an increasing number of human cases have been diagnosed since 1989. The present study was carried out to determine the presence of the parasite in other natural hosts from the area. Birds, fish, opossums and raccoons were captured from local dams and lagoons. The flesh from bird and fish specimens was ground and examined under a 100 W light bulb. Larvae were processed for light and electron microscopy. A total of 368 advanced stage 3 (AL3) larvae were found in 300 ichthyophagous birds, with *Egretta alba* exhibiting the highest infection rate. A total of 4,156 fish were examined, of which six species were infected with AL3 larvae: *Arius guatemalensis* (blue sea catfish), *Dormitator latifrons* (Pacific fat sleeper), *Gobiomorus* sp. (fat sleeper), *Oreochromis* sp. (Nile tilapia), *Cichlasoma beani* (Sinaloan cichlid or green guapote) and *Eleotris picta* (spotted sleeper). Twenty larvae from birds were used to infect domestic cats and dogs. Young adult worms were recovered from the stomach of a cat with a 17 day infection and from a dog with a 35 day infection. Larvae exhibited four rows of hooklets on the head bulb, whereas the young adults had nine rows of hooklets. The cuticular spines of adult worms along the body evolved from single-pointed, bi- or trifurcated spines. Nuclei were counted in intestinal cells examined in serial sections of larvae recovered from a great heron and a fish, in which a mean of 1.6 nuclei/cell was found, corresponding to data published for *Gnathostoma binucleatum*. Although the external morphology of both larvae and adults are in agreement with previous descriptions of *Gnathostoma spinigerum,* the results indicate that natural host infections in Sinaloa may be caused by either *G. spinigerum* or *G. binucleatum*.

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Introduction

Gnathostomosis is a zoonotic disease caused by several species of the nematode genus *Gnathostoma*. The adult parasites reproduce in the esophagus or stomach wall of wild or domestic mammals, such as cats, dogs, pigs, rats, tigers, leopards, wild pigs and opossums. The first intermediate hosts are copepods and the second are freshwater fish in which the advanced third-stage larvae (AL3) develop. In humans, the disease is usually acquired by ingesting raw freshwater fish infected with AL3 larvae, which migrate through the gastric epithelium causing a larva migrans syndrome with cutaneous, ocular, visceral or neurological symptoms (Beaver 1969; Miyazaki 1991). Other natural paratenic hosts include amphibians, reptiles and ichthyophagous birds.

The genus *Gnathostoma* was described by Owen in 1836 from adult worms found in the stomach of a tiger (Owen 1836). Miyazaki reviewed the literature in 1960 (Miyazaki 1960) and concluded that only 7 of the 19 described species were actually different, namely *Gnathostoma spinigerum* Owen, 1836, *Gnathostoma hispidum* Fedchenko, 1872, *Gnathostoma turgidum* Stossich, 1902, *Gnathostoma americanum* Travassos, 1925, *Gnathostoma doloresi* Tubangui, 1925, *Gnathostoma nipponicum* Yamaguti, 1941 and *Gnathostoma procyonis* Chandler, 1942. Daengsvang (1980) included two more species: *Gnathostoma didelphis* Chandler, 1932 and *Gnathostoma brasiliense* Ruiz, 1952. More recent

additions to this list have been *Gnathostoma miyazakii* (Anderson 1964), *Gnathostoma vietnamicum* (Le-Van-Hoa et al. 1965), *Gnathostoma malaysiae* (Miyazaki and Dunn 1965) and *Gnathostoma binucleatum* (Almeyda-Artigas 1991).

The morphology of adult *Gnathostoma* parasites has been used as one of the main tools for species identification within this genera. Miyazaki and co-workers (Akahane et al. 1986; Miyazaki 1991) have also proposed the morphological analysis of AL3 forms as a criterion for the identification of the species that cause human infection, namely *G. spinigerum*, *G. hispidum*, *G. doloresi* and *G. nipponicum*. The differences in the number, form and distribution of the cephalic bulb hooklets as well as the number of intestinal cell nuclei have been used to distinguish these species.

The purpose of the present study was to identify the species infecting natural animal hosts found in the state of Sinaloa, Mexico, where an increasing number of human infections have recently been described (Díaz-Camacho et al. 1998; Ogata et al. 1998).

Materials and methods

Search for natural hosts

Approximately 300 birds and over 4,000 fish specimens were captured in ten fresh and brackish water bodies located in the center and south of Sinaloa. Fish captured in these dams, lakes and estuaries are sold in local and regional markets for human consumption.

Meristic data for all specimens were recorded. To identify larvae, the muscle masses were dissected, ground and compressed between two glass plates and observed by trans-illumination with a 100 W light source. Additionally, flesh was digested in artificial gastric juice (1 g pepsin in 0.7% HCl) at 37°C for 4–5 h. The sediment was washed several times in running water and examined under a stereoscopic lens (Imai et al. 1989). Recovered larvae were used for morphological analysis or for experimental infections.

In addition, 16 raccoons (*Procyon lotor*) and 12 opossums (*Didelphis virginiana*) were captured and the esophagus, stomach cavity and gastric mucosa examined.

Experimental infections

Domestic cats were maintained on a commercial cat food (Gatina, Purina, Mexico). Animals were infected by feeding them 20 viable AL3 larvae recovered from the great heron *Egretta alba*, an ichthyophagous bird captured in the Alhuate lagoon. Domestic dogs were infected with 20 larvae recovered from infected fish (Pacific fat sleeper, *Dormitator latifrons*) recovered from the Escuinapa lagoon. The viability of larvae was determined by examining them for contractile movements under a stereoscopic lens. Experimental infections were monitored by examining fecal samples every 3rd day using the methods of Faust, Ritchie or Kato (Martin and Beaver 1968; Beaver et al. 1986; Ash and Orihel 1991). Animals were killed at post-infection (p.i.) intervals of 17 and 35 days and 2, 3, 7, and 8 months.

Light and electron microscopy

Hooklets were counted on ten larval head bulbs recovered from *E. alba* by sectioning fixed larvae, compressing them between a slide and cover-slip and observing them under a light microscope.

AL3 larvae and young adult worms recovered from animal infections were rinsed in RPMI 1640 (Gibco, Gaithersburg, Pa.) and fixed in Karnovsky solution (Karnovsky 1965) for 24 h at 4°C, washed for a further 24 h in 0.15 M sodium cacodylate buffer and post-fixed in 3% osmium tetroxide for 2 h at 4°C. Samples were prepared for scanning electron microscopy by critical point drying and gold evaporation.

For transmission electron microscopy, fragments containing the larval intestines were dehydrated in ethanol and embedded in Poly-Bed (Polysciences, Warrington, Pa.). Transverse sections 1 μm thick were cut at 30 μm intervals, stained with 0.4% toluidine blue in borate buffer, and examined with a Nikon OptiPhot light microscope. The number of nuclei in intestinal cells was recorded by examining at least ten different sections and calculating a mean for each larva.

Results

Infection of natural hosts

A total of 4,156 fish were examined and classified as *Ictalurus punctatus* (catfish), *Micropterus salmoides* (bass), *Mugil* sp. (flathead mullet), *Salmo* sp. (trout), *Arius guatemaltensis* (blue sea catfish), *Dormitator latifrons* (Pacific fat sleeper), *Gobiomorus* sp. (fat sleeper), *Oreochromis* sp. (Nile tilapia), *Cichlasoma beani* (Sinaloan cichlid or green guapote) and *Eleotris picta* (spotted sleeper). A total of 1,278 AL3 larvae were found in the last six species, with *D. latifrons* and *E. picta* having the highest number (872) as well as the highest infection prevalence, of 47.5% and 80% respectively.

A total of 368 AL3 larvae were recovered from 300 ichthyophagous birds captured in the Alhuate lagoon, located near the city of Culiacán. Six species were infected with *Gnathostoma* spp. larvae: *E. alba* (great heron), *Pelecanus erythrorhynchos* (American white pelican), *Ardea herodias* (great blue heron), *Egretta thula* (snowy egret), *Phalacrocorax olivaceous* (neotropic cormorant) and *Pelecanus occidentalis* (brown pelican). The last species has not previously been recorded as a paratenic host of *Gnathostoma*. The majority of larvae (337 out of 368) were found in the great heron.

No adult worms were found in the esophagus, gastric cavity or mucosa of the 28 mammalian specimens captured.

Experimental infections in cats and dogs

Of the five domestic cats fed AL3 larvae, one animal killed at 17 days p.i. had parasites in different stages of development in its stomach, liver and thoracic muscles. Ten adult worms were found in the gastric cavity of an experimental dog sacrificed 35 days p.i. Three worms were found free in the cavity, four were attached by the cephalic bulb to different parts of the mucosa and three were enclosed in a 1.7-cm-diameter fibrous cavity with a central orifice on the gastric mucosa.

Morphology of AL3 larvae

The larvae in bird and fish muscle were encapsulated in fibrous tissue with the body rolled into a spiral. After the

addition of artificial gastric solution, the larvae exhibited vigorous contractile movements until they migrated out of the fibrous host tissue. The morphometric analysis of larvae recovered from *E. alba* and *P. erytrorhynchos* was carried out by light, transmission and scanning electron microscopy.

Figure 1 illustrates the general morphology of AL3 larvae by SEM. Larvae measured 2–3.3 mm in length (Fig. 1a). The head bulb was clearly distinguished from the rest of the body by the bulb shape and the presence of four rows of single-pointed hooklets with an oblong base. The head bulb had the characteristic semicircular lateral labia with one pair of papillae each (46–50 × 35–40 μm) and a small amphid (1.3–2.0 μm) (Fig. 1b).The cylindrical body had over 200 rows of transverse single-pointed spines, measuring 4.4–5.5 μm in length and diminishing in size and number toward the distal end of the worm. One lateral cervical papilla (4.4 μm) was identified between the cuticular spines of rows 12–13 and 14–15, and the anal pore at the ventral end was surrounded by a cuticle and minute spines.

The results of a hooklet count on head bulbs of ten avian larvae are shown in Table 1.

The number of nuclei present in intestinal cells was counted in serial sections from one larva from *P. occidentalis* (Fig. 2) and two larvae from *E. picta*. Means of 1.7 and 1.5 nuclei/cell were estimated for the respective samples and their distribution is shown in Fig. 3.

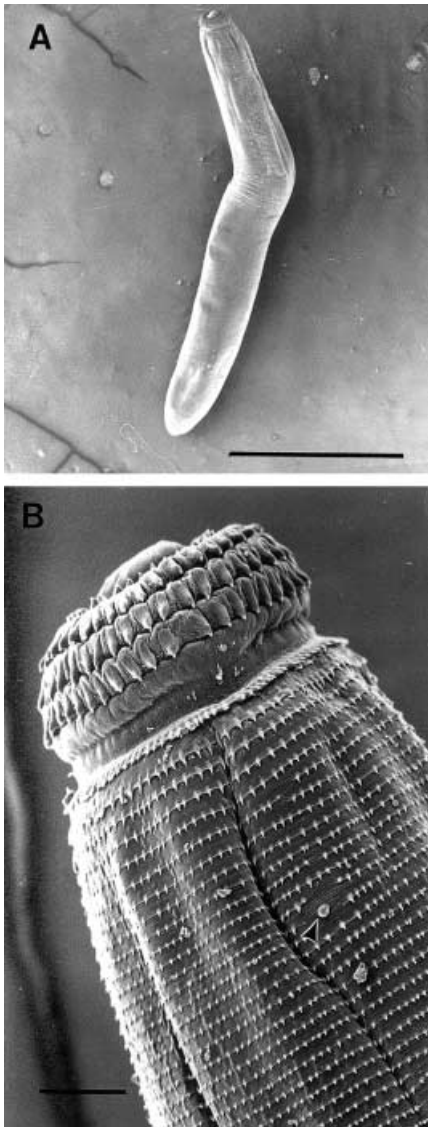

Fig. 1A, B Scanning electron micrographs of AL3 larvae recovered from the liver of a cat infected with 20 AL3 larvae from *Egretta alba*. **A** Whole larva. *Bar* = 1 mm. **B** Head bulb and upper body, with papilla (*arrowhead*) between rows 12 and 13. *Bar* = 100 μm

Morphology of young adults

In the stomach cavity of the infected cat, young adult worms were found migrating freely. Transitional stages were observed, such as the young adult shown in Fig. 4 in which a head bulb with nine rows of hooklets, as well as a molting cuticle halfway down the body, can be seen. In Fig. 5 SEM images of a young adult worm are shown, in which a head bulb with nine rows of hooklets is illustrated (Fig. 5a, b).

The first row of cuticular spines along the body has single points (Fig. 5c); beginning at the second and third row, the spines became bifurcated (Fig. 5d), the points becoming longer in the distal rows. In the 13th row, the

Table 1 Hooklet number in head bulb of ten different AL3 larvae obtained from *Egretta alba*

Row	Larva number										Mean
	1	2	3	4	5	6	7	8	9	10	
I	39	39	39	41	39	34	39	41	39	39	38.9
II	42	44	42	42	43	41	42	42	41	44	42.3
III	45	46	49	43	44	41	44	45	43	46	44.6
IV	48	49	46	44	50	45	49	42	50	49	47.2

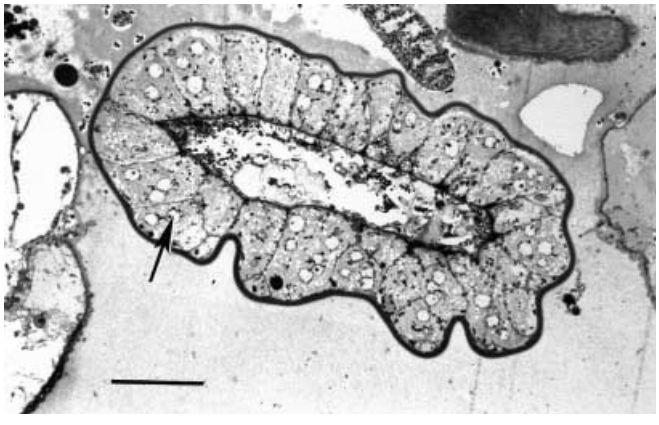

Fig. 2 Light micrograph of a 1 μm section through the intestine of an AL3 larva from *Pelecanus occidentalis* stained with toluidine blue. Intestinal cell nucleus (*arrow*). *Bar* = 30 μm

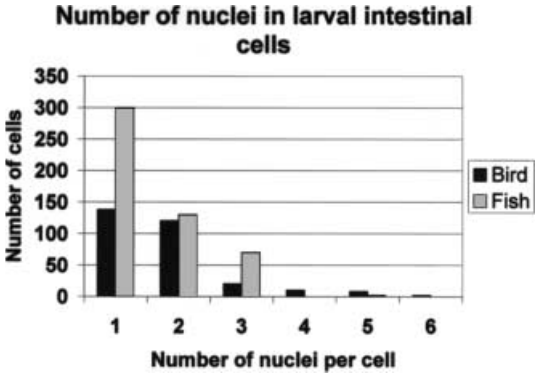

Fig. 3 Histogram showing the number of nuclei found in intestinal cells from serial sections of AL3 larvae recovered from *Pelecanus occidentalis* (*bird*) and *Eleotris picta* (*fish*)

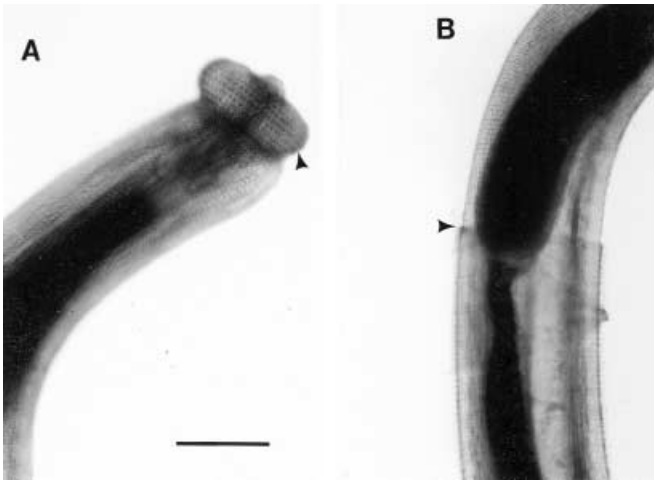

Fig. 4A, B Light micrograph of a whole young adult worm recovered from thoracic muscle of experimental cat. **A** Nine rows of hooklets on the head bulb (*arrowhead*) and **B** the rim of the cuticle (*arrowhead*) midway on the body. *Bar* = 500 μm

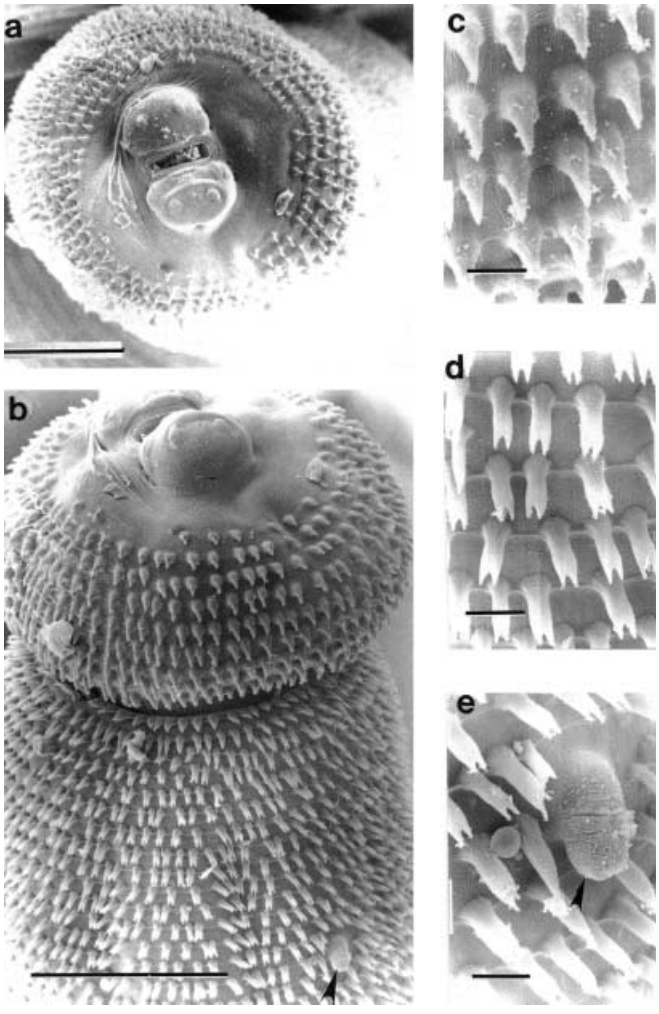

Fig. 5A–E Scanning electron micrograph of a young adult worm from the stomach of an experimental cat. **A** View showing the head bulb with an incomplete first row of hooklets. *Bar* = 100 μm. **B** Head bulb with nine rows of single-pointed hooklets and body spines. *Bar* = 100 μm. **C** Higher magnification of single-pointed body spines in first two rows. **D** Bifurcated spines, **E** trifurcated cuticular spines with cervical papilla between rows 15 and 16. *Bars* in **C–E** = 10 μm

spines are trifurcated (Fig. 5e), with a cervical papilla found between rows 15 and 16. The spines maintained a similar morphology as far down as row 45, where they again became single-pointed. The caudal end of the worm had a striated denuded cuticle with very small spines surrounding the anal pore.

Figure 6 shows light and electron micrographic images of one of the three adult worms found in the gastric cavity of the infected dog. Figure 6a, b illustrates the fibrous cavity on the gastric wall, and Fig. 6c the live worm which measured 1.3 cm in length. Figure 6d–g show SEM images of the same worm after fixation: the head bulb is covered with nine rows of hooklets, the first row apparently incomplete. It has two prominent labia with two pairs of papillae surrounding the oblong mouth cavity. The cuticular spines cover only the anterior third of the body and only the first row under the head-bulb is single-pointed. The following rows have bifurcated spines and further down, around the cervical papilla, they become trifurcated. Between rows 52 and 55, the cuticular spines become single-pointed again.

Discussion

Collection of a large number of ichthyophagous bird and fish specimens from areas close to the capital city of Culiacan, as well as from coastal water bodies along the southern coast of the state, demonstrated that gnathostoma infections are widespread. The high infection rate of several fish and bird species living around estuaries and freshwater lakes confirms the increasing spread of this zoonosis in the state of Sinaloa.

The external morphology of larvae obtained from infected birds and fish as well as young adult worms recovered from experimental infections of a cat and dog

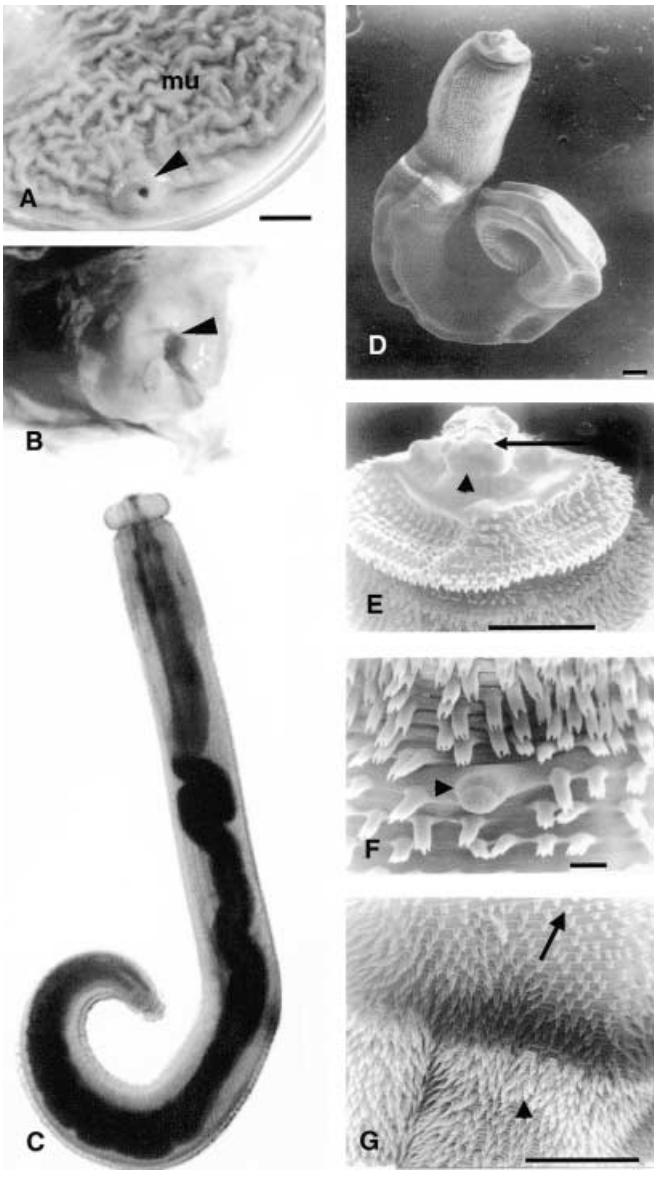

Fig. 6A–G Light and electron micrographs of a 35-day-old adult gnathostome from the gastric wall of an experimental dog infected with AL3 larvae from *Eleotris picta*. **A** Light micrograph of the stomach wall with lesion (*arrowhead*) harboring three adult gnathostomes. Mucosa is labelled *mu*. *Bar* = 1.5 cm. **B** Higher magnification of **A** illustrating the central orifice (*arrowhead*). **C** Light micrograph of live adult worm, measuring 1.3 cm. **D** SEM of whole fixed worm seen in **C**, *bar* = 100 μm. **E** SEM of the head bulb with nine rows of hooklets, two papillae and amphids. *Bar* = 100 μm. **F** SEM of trifurcated cuticular spines around cervical papilla found between rows 52 and 55. *Bar* = 10 μm. **G** SEM of cuticular body spines evolving from a trifurcate (*arrow*) to single pointed (*arrowhead*). *Bar* = 100 μm

suggests that the species involved in bird and fish infections in Sinaloa could be either *G. spinigerum* or *G. binucleatum* when compared with previous descriptions in the literature (Daengsvang 1980; Anantaphruti et al. 1982; Akahane et al. 1986; Almeyda-Artigas 1991; Miyazaki 1991; Koga et al. 1994).

In Mexico, descriptions of various adult *Gnathostoma* have been reported since 1958 (Caballero 1958). Caballero found three adult worms in an opossum (*D. marsupialis*) captured in the state of Chiapas, and classified them as *G. spinigerum*. Lamothe-Argumedo (1997) reported *G. turgidum* and *G. procyonis* in the gastric cavity of opossums captured in the states of Morelos, Oaxaca and Veracruz. In 1991, Almeyda-Artigas described a new species, *G. binucleatum*, from Mexican worms found in wild cats (*Felis pardis* and *Felis catus*) captured along the basin of the Papaloapan river that runs through the states of Oaxaca and Veracruz (Almeyda-Artigas 1991).

Larvae found in intermediate hosts have been classified as *G. spinigerum* by Lamothe in freshwater fish and ichthyophagous birds, amphibians and reptiles captured in Oaxaca, Mexico (Lamothe-Argumedo et al. 1989; Lamothe-Argumedo 1997).

Human cases of gnathostomosis in Mexico have been increasing since the first description by Pelaez and Perez Reyes in 1970 (Pelaez and Pérez-Reyes 1970). Patients with migrating, intermittent edema of the limbs and creeping eruptions have been reported in several states, mostly from individuals with a history of eating raw fish in the form of "cebiche" (Perez et al. 1995). The morphological characteristics of human AL3 larvae recovered from patients in Sinaloa suggested that human infections were caused by *G. spinigerum* (Díaz-Camacho et al. 1998).

The results reported here indicate that the morphology of hooklets, spines, the number of spine rows and the location of cervical papillae found in adult worms recovered from experimental hosts are in agreement with those reported by Miyazaki for adult *G. spinigerum* (Miyazaki 1991), namely: head bulbs with nine rows of hooklets with single-, double- and triple-pointed spines along the upper third of the body. The average number of hooklets on the head bulb of larvae recovered from a bird also coincides with data previously described for *G. spinigerum*.

Several authors (Akahane et al 1994; Almeyda-Artigas 1991; Koga et al. 2000) have described the morphological similarities between *G. spinigerum* and *G. binucleatum* larvae and adult worms, illustrating the present difficulty in establishing them as distinct species by morphological/taxonomic criteria.

The number of nuclei in larval intestinal cells has been widely used as a parameter for distinguishing *Gnathostoma* species. Akahane et al. (1994) have reported an average of 3.4 nuclei/cell in *G. spinigerum*, whereas Almeyda-Artigas (1991) reported an average of 2 nuclei/cell in *G. binucleatum*. Our results revealed averages of 1.5 and 1.7 nuclei per intestinal cell in fish and avian larvae, falling in the range of what has been previously recorded for *G. binucleatum* and *G. doloresi*.

Also of interest was the observation that young adult worms were found after only 17 days in the stomach of a cat fed AL3 bird larvae, suggesting that the transition from larvae to adult occurs rapidly in the stomach of a definitive host, although the maturation and production of viable eggs may, of course, take longer. A recent

study of the molting patterns for *G. doloresi* in wild pigs strongly suggests that *Gnathostoma* larvae molt only once in the definitive host (Imai and Hideo 2001). In the present experiments, the adult worm found in a dog 35 days p.i. confirms that, at this stage, the worm has developed the cuticle of an adult, with rows of cuticular spines covering the upper third of the body, and isolated spines along the bottom two thirds. In addition, the parasitic cavity found in the gastric mucosa of the dog is a characteristic tissue formation induced by mating adult worms (Miyazaki 1960).

A recent study by Koga et al. (2000) describing the surface ultrastructure of AL3 *Gnathostoma* suggests that the species found in fish from Temazcal, Oaxaca and in *E. alba* from a dike in the vicinity of Culiacan, Sinaloa corresponds to *G. binucleatum*. The results obtained from nuclear counts made on sections of the intestines of larvae from *P. occidentalis*, a bird found in the proximity of Culiacan not previously recorded as a host for this infection, and from infected fish (*E. picta*) found in Escuinapa, about 300 km south of Culiacan, showed that these specimens contained between two and seven nuclei, numbers that differ from the mean of 1.5 and 1.7 nuclei per cell reported here.

The discrepancies arising from the inherent difficulties of morphological descriptions of biological material obtained from different paratenic hosts and geographic areas point to the necessity of developing molecular biological markers for the precise identification of *Gnathostoma* species. Recently, Almeyda et al. (2000) used the ITS2 spacer of ribosomal DNA to distinguish between *G. spinigerum* obtained from Thailand and several species collected in southern Mexico and in Ecuador. The authors find that under the conditions of their experiments, all of the American species corresponded to *G. binucleatum*. In light of the fact that *G. spinigerum* and *G. binucleatum* share morphological similarities and that the species responsible for human infections in Mexico have not been identified, we believe it is necessary to use molecular biological tools to analyze a wider range of specimens in all of the localities where the zoonosis has been detected, bearing in mind the rather large number of different *Gnathostoma* species which have already been reported in animal specimens in Mexico. This may be particularly important since recent evidence published by Yuko et al. (2000) suggests that *G. malaysiae* may also cause human larva migrans disease.

From the results of the present analysis, combining the surface morphology of larvae and adults, average hooklet count on larval head bulbs and the intestinal cell nuclear count, we conclude that the species involved in the fish and bird infections in the state of Sinaloa, Mexico may be either *G. spinigerum* or *G. binucleatum* and confirm a widespread distribution of this parasitic disease.

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