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Pathological and parasitological traits in experimentally infected cats with *Gnathostoma binucleatum* (Spirurida: Gnathostomatidae)

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ABSTRACT

This study aims to describe some of the unknown pathological and parasitological traits of experimental feline gnathostomosis. Thirteen female cats were orally inoculated with 30 advanced third-stage *Gnathostoma binucleatum* larvae and were euthanized at various post-infection (p.i.) periods. Clinically, the cats presented with nausea, vomiting, abdominal pain and other nonspecific signs. None of the cats shed eggs in their fecal matter. One cat, euthanized at 6 months p.i., developed a fibrous vascular nodule 2-3 cm in diameter within its gastric wall. The nodule contained caverns filled with mucous and bloody fluid as well as a juvenile worm. The histological characteristics of the nodule were observed, and the morphology of the juvenile worm was revealed using scanning electron microscopy. Another cat, euthanized at 10 months p.i., was found to have a larva within its diaphragm. Infected cats developed increased antibody titers against antigens of *G. binucleatum* adults and larvae beginning in the first month p.i., and these titers were maintained until the end of the experiment, suggesting the presence of undetected migrating larvae. The low number of cats with parasites and poor development of the parasites found suggest that cats have a low susceptibility to infection by *G. binucleatum* and cast doubt on the importance of domestic cats in maintaining the biological cycle of this parasite in nature.

INTRODUCTION

Gnathostomosis is a zoonosis caused by the presence and action of *Gnathostoma* sp. (order Spirurida/family Gnathostomatidae: Mehlhorn, 2008). Have been reported 14 *Gnathostoma* species identified worldwide (Miyazaki, 1991; León-Règagnon et al., 2005; Bertoni-Ruiz et al., 2005), of which *G. binucleatum* is the only species confirmed to affect humans in Mexico and the Americas (Almeyda-Artigas et al., 2000; León-Règagnon et al., 2005). The humans become infected by consuming infected fish (second intermediate host), either raw or partially cooked ("cebiche," "callos," or "sushi"). The first clinical signs in infected patients may be fever, epigastric pain, nausea and vomit. Afterwards, when the larvae migrate symptoms may vary according to the affected area. This causes a migrant larvae

syndrome with manifestations that can be cutaneous, ocular, neurological, and visceral or a combination thereof (Daengsvang, 1980).

The first intermediate hosts of all *Gnathostoma* species are copepods, where as some freshwater fish species, including *Petenia splendida*, *Cichlasoma urophthalmus*, *Ciclasoma gadovi* and *Oreochromis* sp., and some estuarine fish, such as C*athorops fuerthi*, *Pomadasys macracanthus, Mugil curema* and *Dormitator latifrons*, act as second intermediate hosts of *G. binucleatum* (Lamothe-Argumedo et al., 1989; Almeyda-Artigas et al., 1991; Alvarez-Guerrero and Alba-Hurtado, 2007). It has been reported that some turtles (*Kinosternum integrum* and *Trachemys scripta*) and ichthyophagous birds (*Egretta thula*) can act as paratenic hosts (León-Règagnon et al., 2005; García-Márquez et al., 2001; Alvarez-Guerrero and Alba-Hurtado, 2007). Some carnivorous mammals (ichthyophagous) act as definitive hosts for the various species of *Gnathostoma*. The eggs of the parasites that are shed by these hosts through feces contaminate bodies of water where intermediate hosts reside (Miyazaki, 1954).

Recently, our group has demonstrated that dogs act as definitive hosts of *G. binucleatum*, suffering severe pathological alterations induced by the migration and establishment of this parasite within the stomach (Alvarez-Guerrero et al., 2011). Furthermore, the clinical characteristics of infection and some appropriate diagnostic techniques have been described in dogs (Alvarez-Guerrero, et al., 2012). The presence of adult *G. binucleatum* worms in gastric nodules found in cats and ocelots infested in the wild have suggested that cats can also act as definitive hosts (Almeyda-Artigas, 1991). Nevertheless, no systematic studies have confirmed this fact by examining experimental infections in cats. This study describes previously unknown pathological and parasitological characteristics of feline experimental gnathostomosis.

MATERIALS AND METHODS

Experimental animals

A total of 13 clinically healthy female cats aged 2 to 4 months of undefined breed were used. Before the start of the experiment, a preventive deworming was conducted using praziquantel, pyrantel pamoate and febantel (Drontal plus®, Bayer), which were

administered orally and 2-methylethylphenyl carbamate (Bolfo®, Bayer), which was administered externally by spraying. Additionally, cats were examined to ensure that before the start of the experiment, there were no external parasites or helminth eggs present in their feces (Faust method, Alba-Hurtado, 2007). Cats were kept in individual cages and were fed with a commercial balanced feed and water *ad libitum*. This study was approved by the Internal Subcommittee for the Care of Experimental Animal (SICUAE) of the Postgraduate Program of Animal Production and Health (National Autonomous University of Mexico, Mexico).

Collection of advanced third stage larvae (AdvL3) for inoculums

AdvL3 of *G. binucleatum* were isolated from approximately 50 turtles of *Kinosternon integrum* captured in fishing areas in the northern part of the State of Nayarit (Mexico) in the manner described by Alvarez-Guerrero and Alba-Hurtado (2007). The identification of larvae was carried out using the morphometric variables recommended by Miyazaki (1954). A representative sample of the larvae used in this study was identified by amplifying and sequencing the second internal transcribed spacer (ITS-2) of the ribosomal DNA using the PCR technique described by Martínez-Salazar and León-Règagnon (2005).

Experimental design

The 13 cats received an oral dose of 30 AdvL3 of *G. binucleatum* mixed with 50 grams of ground fish meat formed into a meatball. All fecal matter was collected daily and processed using Faust's technique to search for parasite structures (Alba-Hurtado, 2007), and a weekly exhaustive clinical examination was conducted on each cat. Blood was obtained monthly from each cat, and isolated serum was stored at -20 ºC until measured for antibody titers using ELISA. Two cats per time point were euthanized with sodium pentobarbital at 2, 3, 4, 6, 8 and 9 months p.i. The final cat was euthanized at 10 months p.i. The stomachs of all cats were removed at necropsy, and the gastric wall and organs of the abdominal and thoracic cavities examined for the presence of nodules and/or parasites. A tissue sample (1 cm^3) was obtained from a recovered nodule, fixed in formaldehyde for 48 h, and embedded in paraffin. Four-µm-thick sections of the nodule were obtained. Sections were processed and stained with conventional hematoxylin/eosin staining procedures. The collected

juvenile parasite was fixed in 10% formaldehyde and processed for scanning electron microscopy.

Serum antibody determination

Parasite antigens were obtained from approximately 250 *G. binucleatum* AdvL3 (Ag-AdvL3) and from an adult worm (Ag-AW) isolated from the gastric nodule of an experimentally infected dog, as described by Alvarez-Guerrero et al. (2011). The amount of total protein was determined by the Bradford method (1976).

Serum antibody levels against Ag-AdvL3 and Ag-AW were measured by ELISA as described by Muñoz-Guzmán et al. (2010). All ELISAs were performed in duplicate and optimized according to antigen concentration and serum and conjugate dilution. The concentration of both antigens was 10 µg/ml, serum was diluted 1:320 and 1:80, respectively, and the conjugate (goat anti-feline IgG, serotec AA126P) was diluted 1:5000. Plates were read at 492 nm in an ELISA Multiskan Ascent reader (Labsystems). For each serum sample, the nonspecific absorbance of an adjacent antigen-free well was subtracted from the absorbance obtained in the presence of antigen. O.D. results for duplicate wells were averaged and a percentage absorbance (%Abs), in relation to a positive control, was calculated using the following formula:

$$\%Abs = \frac{(\text{sample serum O.D.})\,(100\%)}{\text{Positive control O.D.}}$$

Scanning electron microscopy

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

Statistical analysis

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

RESULTS

All morphometric variables (total length, number of head-bulb hooklets, position of the cervical papillae, number of transverse striae, number of intestinal cells, and average number of nuclei per intestinal cell) of the AdvL3 that were recovered from turtles corresponded to the variables reported for *G. binucleatum* (Alvarez-Guerrero and Alba-Hurtado, 2007). The base sequence of ITS-2 rDNA of the larvae showed a 0.48% divergence (2 to 419 base pairs) from the sequence reported in GenBank (AY734632, AY061740 and AB181159) for *G. binucleatum*.

Parasites at various stages of development were found in 2 of the 13 cats inoculated with AdvL3 of *G. binucleatum*. One of the cats, euthanized at 6 months p.i., had a nodule within its gastric wall, and another, euthanized at 10 months p.i., had a larva in its diaphragm. During the necropsy of the cat euthanized at 6 months p.i., a fibrous vascular nodule of 2-3 cm in diameter was found in the greater curvature of its stomach (Figure 1a). This nodule had caverns that were not continuous with the abdominal cavity or the gastric lumen (Figure 1b). The caverns contained mucous and bloody fluid as well as a juvenile worm (Figure 1c). Histologically, the nodule contained large amount of collagen, areas of fibrosis, areas of degenerative necrosis, and small amounts of lymphoplasmocytic and macrophage infiltrate. The cat that was euthanized at 10 months p.i. did not have macroscopic lesions associated with the *G. binucleatum* larva found in the diaphragm. None of the cats shed eggs in their fecal matter.

The juvenile worm found measured 11.9 mm long and 0.9 mm wide. Its anterior portion had a cephalic bulb with 8 full rows of concentric hooks and one incomplete row (Figure 2a). Its mouth had a pair of strong lips (trilobulated) with a pair of papillae each. The largest cuticular spines were distributed over 45% of the body, and the spines closest to the cephalic bulb showed 2 to 3 denticles. In the middle region, three denticles were displayed, with the central denticle being the largest (Figure 2b). In the posterior region, the spines had one or two denticles. The cloaca was covered with minute spines and surrounded by 3 pairs of

cloacal papillae (Figure 2c). Neither spicules nor a clearly differentiated vulva were observed. The recovered larva measured 4.035 mm long and 0.385 mm wide, its anterior portion dad a cephalic with 4 rows of concentric hooks. The larva has no evidence of ecdisis in the cuticle. The morphometric values (total length, number of head-bulb hooklets, position of the cervical papilla and number of transverse striae) of these larvae corresponded to those reported by Alvarez-Guerrero and Alba-Hurtado (2007) for *G. binucleatum*. Clinically, the cats presented with nausea, sporadic vomiting, abdominal pain and prostration during the first two months p.i. Afterwards, they presented nonspecific signs such as apathy and a reduction in feed consumption. The cat in which the nodule was detected did not show any additional clinical signs.

The kinetics of average IgG antibody production against Ag-Adv-L3 and Ag-AW in infested cats are shown in Figure 3. The average levels of anti Ag-AdvL3 antibodies increased ($p<0.05$) since the first month p.i. (96.7±5.4 Abs) when compared to month zero (22.3±6.8 Abs), no statistical differences ($p>0.05$) were found between the first and subsequent months p.i. The anti Ag-AW antibody levels gradually increased throughout the experiment, becoming significant ($p<0.05$) when compared to month zero (9±1.7 %Abs) from the fourth month p.i. (103.70±18.3 %Abs) onward. No statistical differences ($p>0.05$) were found in antibody levels between the fourth and subsequent months p.i.

DISCUSSION

In order to guarantee the reliability and reproducibility of an experimental infection model, it is important to fully identify the parasite to be inoculated. Originally, morphological characteristics were used for identifying the various *Gnathostoma* species. Currently ribosomal DNA sequencing is considered the most specific tool to confirm the identity of these species (Miyasaki, 1954; Almeyda-Artigas, 1991; Almeyda-Artigas et al., 2000). The morphological characterization of the larva and the recovered juvenile worm and the sequencing of the ribosomal DNA amplicon of the larvae in this study confirmed that the species used for the experimental infection of cats was *G. binucleatum*.

Some mammalian species that feed on fish have been described as definitive hosts of the various *Gnathostoma* species (Daengsvang, 1982; León-Règagnon et al., 2005). In the specific case of *G. binucleatum*, it has been reported that dogs can act as definitive hosts

(Koga et al., 1999; Alvarez-Guerrero et al., 2011). Also, the presence of nodules and adult worms in felines has been reported (Almeyda-Artigas, 1991), however information on the pathological and parasitological characteristics in this host is limited. In this study, 1 of 13 infected cats developed a nodule containing a juvenile worm, (this worm had morphological characteristics similar to those reported for adults but lacked mature sexual structures and did not produce eggs), whereas in another cat, a larva was found within the diaphragm. This suggests that cats have a low susceptibility to infection by *G. binucleatum*.

The size of the gastric nodules containing adult worms reported in experimentally infested dogs reaches approximately 8 cm and these nodules are continuous with the abdominal cavity and the gastric lumen (Alvarez-Guerrero et al., 2011). The nodule found in a cat at 6 months p.i. was smaller (2-3 cm), was not continuous with the gastric lumen and had only a juvenile worm within it. Differences in the size nodules may be the result of the number of worms found within each nodule. The absence of continuity between the cat nodule and the gastric lumen may also be due to the absence of adult stages. Furthermore, histological differences were observed between the nodules found in dogs and the one found in the cat in this study. Although both have large amounts of collagen and fibrotic areas in their walls, only the dogs nodules had a large number of eggs trapped within the tissue surrounded by macrophages and eosinophils (Alvarez-Guerrero et al., 2011). The absence of eggs in the feline nodule may explain the reduced development of the nodule and the absence of an eosinophilic infiltrate.

The prepatent period of *G. binucleatum* in dogs is 22 weeks, whereas other spiruloid nematodes such as *Spirocerca lupi* the prepatent period can take up to 9 months (Alvarez-Guerrero et al., 2011; Van der Merwe et al., 2008). It is possible that if the cat had been euthanized several months later, the juvenile worm that was found could have matured into its adult stage. Nevertheless, since it was a single worm it would have been unable to reproduce and therefore could not repeat the life-cycle.

The erratic migration of larvae from various *Gnathostoma* species to organs such as the skin, liver, lungs, kidneys, brain and eyes has been reported in humans (Miyasaki, 1991; Bhattacharjee et al., 2007). Therefore, an erratic migration of *G. binucleatum* in cats cannot be ruled out. This hypothesis is supported by the fact that even in cats with no detectable parasite stages in the stomach, increases in serum antibody levels could be observed against

both larvae and adults beginning at the first month p.i. and continuing throughout the experiment. Although larvae could not be observed macroscopically in the stomach or abdominal cavity, the examination was not exhaustive, and the artificial digestion and examination of other organs in addition to the stomach were not carried out.

The production of specific antibodies results from stimulation by an antigen. In this study, antibody titers against larvae increased after infection, which, in itself, implies that at least some larvae migrated within the cat and came into contact with the immune system. The maintenance of antibody levels against larva during the course of the experiment suggests the presence of these larvae or at least their antigens in some feline tissues even if they were not physically detectable, like to reported in other helmints such as *Toxocara* or *Trichinella* (Yepez-Mulia et al., 1999; Alba-Hurtado et al., 2009). In this context, the constant and gradual increase in the levels of antibodies against adult worm antigens suggests a change in the antigens that can be associated to the degree of maturation of the larvae.

Adult stages of *G. binucleatum*, *G. americanum* or *G. spinigerum* have been found in some naturally infected felines such as ocelot (*Leopardus pardalis*), tiger cat (*Leopardus tigrinus*) and domestic cat (*Felis catus*) respectively (Travassos, 1925; Almeyda-Artigas, 1991), however studies evaluating the experimental infection by *G. binucleatum* in felines are scarce or insufficient. In this study, the infected cats presented nonspecific signs and developed increased antibody titers against antigens of *G. binucleatum* suggesting the presence of migrating larvae. Only 1 of 13 (7%) cats had a few-developed gastric nodule containing a juvenile worm and another cat had a larva within its diaphragm. None of the cats developed adult worms or shed eggs in their feces. Almeyda-Artigas (1991), found *G. binucleatum* adult worms in 1 of 4 (25%) ocelot and 2 of 9 (22%) feral cats naturally infected, and only immature worms (juvenile?) in 2 of 4 (50%) domestic cats with experimental infection. Own results show the ability of the parasite to produce gastric nodules and clinical signs in domestic cats, however, the absence of adult worms suggests that domestic cats are nonpermissive host for the development of biological cycle, or possibly they need more time than others permissive hosts (such as the ocelot?).

The difficulty of obtaining a sufficient number of viable *G. binucleatum* larvae and restrictions to experiment with wild species in danger of extinction, limits the development

of more specific studies using a greater number of animals. The results of this study do not conclusively establish the role of cats as definitive hosts of *G. binucleatum* and cast doubt on their significance in the maintenance of this parasite's biological cycle in nature.

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FIGURE CAPTIONS

Figure 1. Experimental infection with *Gnathostoma binucleatum* in a female cat. A), Necropsy showing gastric nodule (white arrow). B), Internal gastric surface (mucosa) of the nodule (discontinuous circle). C), Section of the nodule with internal caverns and a juvenile worm (black arrow).

Figure 2. Scanning electron micrograph of a juvenile specimen of *Gnathostoma binucleatum*. A), Lateral view of the cephalic bulb that showed 9 concentrically disposed hooks and spines closest to the cephalic bulb showed 2–3 denticles (white arrow). B), Spines of the middle region of the body displaying three denticles (the middle is the largest). C), posterior region with spines showed one denticle and three pairs of ventral papillae (white arrow) around the cloaca.

Figure 3. Antibody kinetics of anti-advanced third larvae antigens (Ag-AdvL3) and anti-adult worm (Ag-AW) antigens of *Gnathostoma binucleatum* in 13 experimentally infected female cats. *Significant difference ($P<0.05$) with respect to the time of inoculation.

FIGURE 1

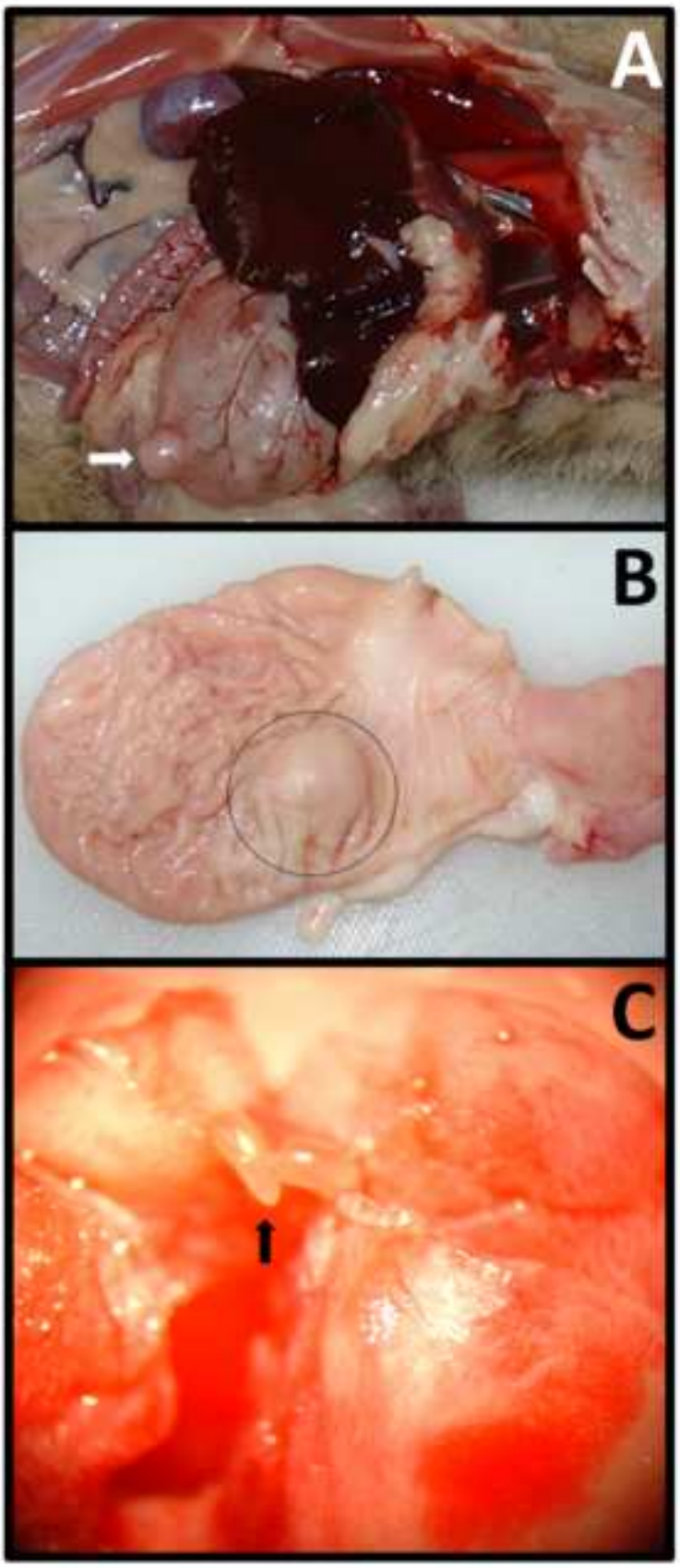

FIGURE 2

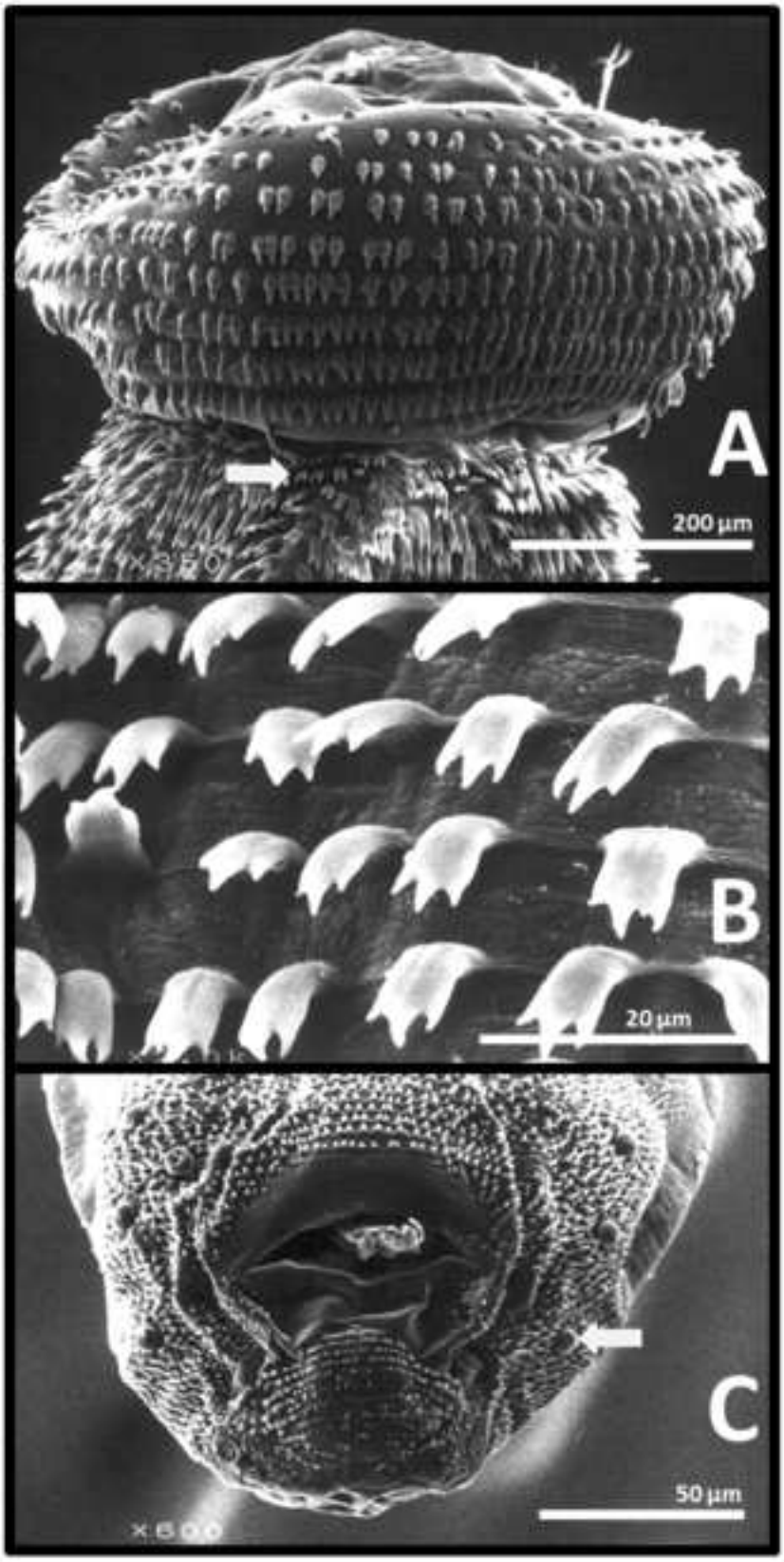

FIGURE 3