Original Article

Molecular Identification of the Etiological Agent of Human Gnathostomiasis in an Endemic Area of Mexico

Sylvia Paz Díaz-Camacho1, Jesús Ricardo Parra-Unda2, Julián Ríos-Sicairos2, and Francisco Delgado-Vargas2*

1Research Unit in Environment and Health, Autonomous University of Occident, Sinaloa; and 2Public Health Research Unit "Dra. Kaethe Willms", School of Chemical and Biological Sciences, Autonomous University of Sinaloa, University city, Culiacán, Sinaloa, Mexico

SUMMARY: Human gnathostomiasis, which is endemic in Mexico, is a worldwide health concern. It is mainly caused by the consumption of raw or insufficiently cooked fish containing the advanced third-stage larvae (AL3A) of Gnathostoma species. The diagnosis of gnathostomiasis is based on epidemiological surveys and immunological diagnostic tests. When a larva is recovered, the species can be identified by molecular techniques. Polymerase chain reaction (PCR) amplification of the second internal transcription spacer (ITS-2) is useful to identify nematode species, including Gnathostoma species. This study aims to develop a duplex-PCR amplification method of the ITS-2 region to differentiate between the Gnathostoma binucleatum and G. turgidum parasites that coexist in the same endemic area, as well as to identify the Gnathostoma larvae recovered from the biopsies of two gnathostomiasis patients from Sinaloa, Mexico. The duplex PCR established based on the ITS-2 sequence showed that the length of the amplicons was 321 bp for G. binucleatum and 226 bp for G. turgidum. The amplicons from the AL3A of both patients were 321 bp. Furthermore, the length and composition of these amplicons were identical to those deposited in GenBank as G. binucleatum (accession No. JF919679), corroborating our previous morphological finding that G. binucleatum is the etiological agent for human gnathostomiasis in the endemic area of Sinaloa, Mexico.

INTRODUCTION

Gnathostomiasis is a food-borne zoonotic nematodiasis in Mexico. It is mainly caused by the consumption of raw or insufficiently cooked fish that contains the advanced third-stage larvae (AL3A) of different Gnathostoma species. Gnathostomiasis induces the larva migrans syndrome that can affect different tissues (e.g., skin, eyes, viscera, and the central nervous system) and is endemic to countries such as Thailand, Japan, Mexico, and Ecuador (1,2). Globalization has also increased the number of cases among international travelers and migrants (2,3). The definitive diagnosis of human gnathostomiasis has been achieved in only a small number of cases, as it depends on the recovery and identification of the Gnathostoma larva, which is difficult due to its unpredictable migratory behavior within the infected human. Thus, the diagnosis of gnathostomiasis is based on epidemiological surveys, clinical signs and symptoms, and immunological diagnostic tests. Nevertheless, these evaluations cannot discriminate among Gnathostoma species associated with the infection and they cannot discern whether the infection is active (4,5).

Polymerase chain reaction (PCR) amplification of ribosomal DNA (rDNA) has been employed for systematics and phylogeny studies of eukaryotic organisms and diagnosis of several parasitoses such as toxocariasis, taeniasis, and schistosomiasis (6). In nematodes, the second internal transcription spacer (ITS-2) is a marker for species identification, including those of the genus Gnathostoma (7–9). The main etiological agent of human gnathostomiasis are Gnathostoma spinigerum in Asia and G. binucleatum in the Americas (7,10). The species G. binucleatum, G. turgidum, and G. lamothei have been identified in Mexico, but the latter two were found only in animals (Fig. 1) (11). Sinaloa State is located in northwest Mexico, where human gnathostomiasis is endemic (Fig. 1). In Sinaloa, the AL3A of G. binucleatum has been identified in different species of fish, birds, and reptiles, whereas adult worms and AL3A of G. turgidum have only been isolated from opossums (Didelphis virginiana) (12,13). This study aims to develop a duplex PCR amplification method of the ITS-2 region to differentiate between the G. binucleatum and G. turgidum parasites and to identify the Gnathostoma larvae obtained from the biopsies of two patients with gnathostomiasis.

MATERIALS AND METHODS

Clinical data and recovery of AL3A in cases of cutaneous gnathostomiasis: The two cutaneous gnathostomiasis cases from Sinaloa, Mexico included in this study were treated at the Public Health Research Unit "Dra. Kaethe Willms" (PHRU), School of Chemical and Biological Sciences, Autonomous University of Sinaloa. Gnathostomiasis was diagnosed by applying a
Molecular Identification of Gnathostoma Larvae Recovered from Human Patients

Pre-coded clinical epidemiological survey, examining the skin lesions present at the time of the medical consultation, and performing an indirect ELISA test (14) to detect IgG antibodies against a crude antigenic extract of G. binucleatum AL3 A. A 31-year-old woman presented with an edematous lesion on the right thigh, causing a hot and indurated reddish plaque associated with itching, pain, and burning sensations. The patient became ill one year prior to the consultation and showed skin inflammation and itching. Subsequently, the larva migrated to the posterior part of the thigh. A medical doctor prescribed Fig. 1. (Color online) Geographic distribution of the genus Gnathostoma in Mexico, adapted from Pérez-Álvarez, et al. (11). Map shows the states where the species have been identified (●), non-identified (□), as well as those states with cases of human gnathostomiasis (○). In Mexico, the highest number of human gnathostomiasis cases have been registered in Sinaloa and Nayarit (shadowed), the only states where G. binucleatum has been identified as the etiological agent.
Chlorpheniramine, Diclofenac, and Dexamethasone at this stage. The lesion disappeared but it reoccurred one month later, registering larval migration in the same area. Laboratory analysis revealed only a 5% of eosinophils, negative reactive protein C, negative rheumatoid factor, and a high globular sedimentation rate (24 mm/h). Moreover, the gnathostomiasis ELISA test results were positive (1:800). The patient was then treated with 200 mg Albendazole tablets (6 tabs/day for 15 days) to induce the migration of the parasite to the superficial skin layers. Seven days post-treatment, the patient exhibited a papule 10 cm in diameter. Dr. Rafael Castro-Velázquez of the Dermatologic Center of Sinaloa performed an excisional biopsy (1.8 × 0.5 × 1.0 cm) which was analyzed in the PHRU, and a live AL3A of the Gnathostoma sp. was recovered (Fig. 2). This patient has been eating raw fish 3-4 times a month since she was 10 years old.

Case report 2: A 54-year-old woman with a body weight of 63 kg presented with an erythematous and hyperthermic plaque of approximately 13 cm in diameter on her left forearm, which was associated with an itching sensation. Moreover, the edema-causing larvae migrated in a period of two or three days to different parts of the forearm. The woman informed that she was initially treated with antibiotics and Prednisone; however, her discomfort persisted. The patient went to the PHRU and was positive for the gnathostomiasis ELISA test (1:800). She was initially treated with 6 mg tablets of Ivermectin (200 μg/kg body weight/day for three days). Her discomfort disappeared for four months, but they were observed again on one of her hands, which showed inflammation and redness. The ELISA test resulted positive and treatment was changed to 200 mg Albendazole tablets (10 mg/kg body weight/day for 14 days). The discomfort disappeared again and 12 days post-treatment, the patient exhibited a pustule with a brown spot at the center of the digital pulp of the middle finger (Fig. 3). Because the lesion was very superficial, the pustule was opened with a needle and a live Gnathostoma sp. AL3A was recovered. Interestingly, the patient reported that she did not consciously consume raw fish.

The two recovered Gnathostoma AL3A were placed in isotonic saline solution (NaCl 0.85% p/v), observed with a stereoscopic microscope (4X), separated from the remaining tissue of the patients, fixed in absolute ethanol, and stored at 4°C until the extraction of genomic DNA.

**Purification of DNA from Gnathostoma AL3A and adult worms:** Each Gnathostoma AL3A was placed in Eppendorf tubes with alkaline lysis buffer (5 M NaCl, 1 M Tris, pH 8, 0.5 M EDTA, pH 8, and 10% SDS) and 500 μg/mL of proteinase K (Promega Co., Madison, WI, USA). Tubes were heated at 55°C for 30 min and parasites were macerated with a sterile pistil. A volume of phenol equilibrated with 0.5 M Tris-HCl (pH 8.0) was added to each tube, gently mixed for 3 min, and centrifuged (5,000 × g, 10 min). The aqueous phase was recovered into a new tube, and the extraction process was repeated with the residue. The aqueous phases were combined and two ethanol volumes at room temperature were added. The samples were then mixed and centrifuged (5,000 × g, 15 min). The pellet was recovered, washed with 70% ethanol, and centrifuged (5,000 × g, 5 min). The ethanol solution was removed and the DNA was suspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The genomic DNA was analyzed by electrophoresis on 2% agarose gels migrated for 1 h at 100 V and stained with ethidium bromide. The DNA was stored at 4°C until use (15).

**Amplification of Gnathostoma ITS-2 by PCR**

![Fig. 2](Image)

Fig. 2. (Color online) Patient with cutaneous gnathostomiasis. (A) Erythematous variant, the arrow points to the site of the biopsy. (B) Observation with magnifying lens of human tissue with the AL3A of G. binucleatum recovered by biopsy. (C) Stereomicroscopic (2X) observation of the cephalic bulb and complete body of the AL3A of G. binucleatum.

![Fig. 3](Image)

Fig. 3. (Color online) Patient with cutaneous gnathostomiasis. (A) Pseudofurunculous variety, the arrow points to the biopsy site. The stereomicroscopic image (4X) of the (B) cephalic bulb and (2X) (C) full body of the recovered G. binucleatum AL3A.
Duplex: Primers for the ITS-2 region of *G. binucleatum* and *G. turgidum* were designed based on the following sequences published: for *G. binucleatum*, GbITS2F (5′-GCT CTT CGT TTG CAG TGT GTT-3′) and GbITS2R (5′-GAT TCA TCG GGA GCC GTT CAT-3′); and for *G. turgidum*, GtITS2F (5′-CCG CCT CTC ATC CTC CAT TGT-3′) and GtITS2R (5′-TCA CAT CAC CGT CAA TCC GAA G-3′). Primers were designed using the Primer3 software v.0.4.0 (16) and Oligo Analyzer 3.1 (Integrated DNA Technologies Inc.), and synthesized by Sigma-Aldrich® Co., St. Louis, MO, USA. For the duplex PCR analysis, 200 ng of DNA samples were mixed with 2.5 μL of 10X buffer, 1.5 μL of 25 mM MgCl₂, 1.3 μL of 2.5 mM dNTPs, and 50 pM (0.5 μL) of each oligonucleotide (duplex). A total volume of 0.1 μL of Taq DNA polymerase (Promega Co, Madison, WI, USA) was added to the mixture, and nuclease-free water was added to make a final volume of 25 μL. The amplification conditions were as follows: one cycle at 94°C for 5 min, 30 cycles at 94°C for 1 min, 62°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 10 min. PCR products were separated by electrophoresis on 2% agarose gels migrated for 1 h at 100 V, stained with ethidium bromide, visualized on a UV transilluminator, and photographed using a UVP Gel Documentation System (UVP, Upland, CA, USA). The expected size of the PCR products was 321 bp (*G. binucleatum*) and 226 bp (*G. turgidum*).

The PCR products were purified with the QIAquick Gel Extraction Kit (Qiagen Inc, Valencia, CA, USA), following the manufacturer’s instructions. The purity and concentration were determined using a NanoDrop Lite spectrophotometer (Thermo Scientific), whereas the integrity was determined by electrophoresis as described above. The PCR products were sent for DNA sequencing (Macrogen Inc. Rockville, MD, USA). To verify for PCR cross-reactions, amplifications were also carried out with DNA from *Escherichia coli*, *Aspergillus niger*, *Giardia intestinalis*, and *Taenia crassiceps*.

**Analysis of *Gnathostoma* ITS-2 sequences:** The nucleotide sequences of the ITS-2 region of the recovered *Gnathostoma* species were registered into the Genbank database with the accession numbers MG256498 and MG256499 for *G. binucleatum* and *G. turgidum*, respectively. The ITS-2 sequences were aligned with the sequences available in the National Center for Biotechnology Information (NCBI) and Genbank databases (accession numbers AY061740, KP941023, KP941024, KP941025, EU334739, FJ497055, KP784333, AB181157, AB181158, and JN408329) using the BLAST algorithm (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and the Clustal Omega multiple sequence alignment program version 1.2.4 (17) (<http://www.genome.jp/tools/cluster/>).

**RESULTS**

**Gnathostomiasis cases:** The two clinical cases of cutaneous gnathostomiasis were of the erythematous and pseudofurunculous variety. The medical treatments based on Ivermectin or Albendazole were not 100% effective as after drug administration, a live larva was obtained from both patients. Albendazole induced the migration of the parasite to the superficial layers of the skin and led to the formation of a pseudofurunculous lesion where the AL3A was recovered and later identified by PCR.

**Amplification and sequence analysis of the ITS-2 region of *Gnathostoma*:** The DNA concentrations of the AL3A isolated from gnathostomiasis patients were 280 ng/μL (case 1) and 350 ng/μL (case 2) and both showed a purity index (260 nm/280 nm) of 1.9. The PCR products obtained from both AL3A were 321 bp in length, whereas those of the adult *G. turgidum* DNA were 226 bp in size (Fig. 4). The PCR results of the controls were as expected; two bands (321 and 226 bp) were obtained for the mixture of the DNA of both parasites, whereas the sample without DNA and those with DNA of other organisms (i.e., *E. coli*, *A. niger*, *G. intestinalis*, and *T. crassiceps*) were unamplified.

The ITS-2 sequences (Genbank: MG256498, MG256499) of both isolated larvae from the Sinaloa patients, as well as the *G. binucleatum* ITS-2-Genbank-sequence (JF919679) from Sinaloa, were 100% identical, respectively, with the ITS-2 sequence of *G. turgidum* (FJ524380) from Sinaloa. Compared with the ITS-2 sequences of other *Gnathostoma* species, the ITS-2 sequences from larvae of gnathostomiasis patients from Sinaloa showed 69.5% identity with *G. lamothei* (EU334739), 74.1% with *G. miyazaki* (FJ497055), and values lower than 80% with other *Gnathostoma* species from Asia, such as 78.8% with *G. spinigerum* (KP784333), 46.7% with *G. nipponicum* (AB181157), 46.42% with *G. hispidum* (AB181158), and 46.38% with *G. hispidum* (AB181158).
Fig. 5. Multiple Alignment (Clustal O, 1.2.4) of nucleotide sequences of the ITS-2 region of G. binucleatum isolated from patients with gnathostomiasis in Thailand by G. spinigerum; ITS-2 from GsHThA1, GsHThA2, GsHThA3, G. binucleatum from patients with gnathostomiasis of Sinaloa, Mexico: GbHSin1 (MG256498.1), GbHSin2 (MG256499.1); Nayarit, Mexico: GbHNay1, GbHNay2, GbHNay3; G. spinigerum AY061740.1.
65% with *G. doloresi* (JN408329) (data not shown).

The ITS-2 sequences of the larvae from patients showed 99.7% identity with a *G. binucleatum* larva isolated from one case of human gnathostomiasis in the state of Nayarit, Mexico (Genbank: AY061740). The sequences of the Sinaloa AL3A showed G201 and the one from Nayarit showed C201. In addition, alignment with the ITS-2 sequences of three cases (KP941023, KP941024, and KP941025) of human gnathostomiasis caused by *G. spinigerum* which were reported in Thailand showed an identity of 78.8% (Fig. 5).

**DISCUSSION**

Human gnathostomiasis is a foodborne disease caused by the consumption of raw or insufficiently cooked fish that contains AL3A of *Gnathostoma* species. The number of cases is high in endemic areas of the Americas (e.g., México > 10,000, Ecuador > 2,000, and Peru > 18) (2) and Asia (e.g., Thailand > 3,173, Japan > 4,000, Vietnam > 619, and China > 23) (18,19).

The first two human cases of gnathostomiasis in the Americas were reported in Mexico and one of them acquired the disease in the state of Sinaloa (20). The states of Nayarit and Sinaloa have the highest incidences of gnathostomiasis in Mexico (2). The cutaneous cases of gnathostomiasis in this study were identified using clinical and immunological data, corresponding with the ITS-2 sequences of three cases (KP941023, KP941024, and KP941025) of human gnathostomiasis.

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The ITS-2 *Gnathostoma* sequences of the AL3A isolated from patients in this study presented 100% identity with other *G. binucleatum* ITS-2 sequences, proving that AL3A is *G. binucleatum*. As mentioned above, human gnathostomiasis is associated with the consumption of raw or insufficiently cooked fish. In Sinaloa, only shrimp and fish are consumed raw in typical dishes such as “cebiche” and “callos”. Patient 2 revealed that she did not ingest any type of raw fish dish, and she only drank purified water. Thus, the infection by consumption of infected copepods could be discarded. However, seafood cocktails are common in Sinaloa and contain mixtures of shrimp and octopus, that are frequently added, but not mentioned, with small pieces of raw fish. Such pieces of raw fish are not identified because the cocktails contain tomato, onion, chili, and pepper. This suggested that patient 2 unknowingly ingested raw fish by consuming these types of dishes.

The *G. binucleatum* ITS-2 sequences showed the structure, regions, and characteristic motifs described in other trematoda and monogenea parasites (24). Their G+C content was 49.6%, which was a value similar to that of trematodes (48%), and in the range of other organisms (8). The analyzed *Gnathostoma* ITS-2 sequences show differences with other eukaryotes, e.g., in the number of helices and secondary structure (25). In Trematode and Monogenean parasites, the ITS-2 length varies from 251 bp to 431 bp (24). Moreover, the ITS-2 sequences of different species of *Gnathostoma* exhibit variations in size, G+C content, and polymorphisms (8). Nevertheless, this study only analyzed a 321 bp fragment and it will be desirable to compare the complete ITS-2 sequences with those of other species such as *Gnathostoma* sp III (359 bp), *Gnathostoma* sp II (344 bp), and *Gnathostoma* sp I (512 bp) (7). In this regard, it has been suggested that the variability of the ITS-2 *Gnathostoma* sequences is a consequence of an old diversification process or high evolution rate. Thus, the ITS-2 sequence has been proposed as a good genetic marker to differentiate between *Gnathostoma* species (7).

The ITS-2 sequences are useful genetic markers when using a duplex PCR assay for diagnosis and pathogen identification in parasitic diseases. For example, ITS-2 polymorphisms have been detected among species of *Toxocara* (i.e., *T. cantans*, *T. cati*, and *T. leonine*) (26). In this study, the sequences of the larvae isolated from gnathostomiasis patients from Sinaloa, México were identical. Moreover, they showed high identity with that of a patient from Nayarit, Mexico (10), with...
only a single nucleotide polymorphism. In addition, our previous study demonstrated that *G. binucleatum* is the etiological agent for human gnathostomiasis in an endemic area of Sinaloa, Mexico, despite the fact that the *G. turgidum* life cycle takes place in the same geographical area (12–14,23,27,28). Thus, *G. binucleatum* is the only species associated with the disease in the Americas (7).

Human gnathostomiasis is still a public health problem in Mexico. *Gnathostoma binucleatum* was revealed to be the species associated with human gnathostomiasis in the endemic area of Sinaloa, Mexico, where the life cycle of *G. turgidum* also takes place. The PCR method designed to amplify the ITS-2 region discriminated between *G. binucleatum* and *G. turgidum* and it allowed, for the first time, the identification of *G. binucleatum* as the causal agent of human gnathostomiasis in northwest Mexico through a molecular technique. Furthermore, considering that three species of *Gnathostoma* (*G. binucleatum*, *G. lamothei*, and *G. turgidum*) are found in Mexico, future studies must develop a multiplex PCR including these species.

**Acknowledgments**  Authors acknowledge the financial support provided by Autonomous University of Sinaloa (PROFAPI, “Programa de Fomento y Apoyo a Proyectos de Investigación”) and National Council for Science and Technology of Mexico (CONACyT). We thank Dr. Edgar Adán Valenzuela-García (Center of Studies of Foreign Languages of the Autonomous Occidental University) for the language assistance in the manuscript preparation.

**Conflict of interest**  None to declare.

**REFERENCES**

1. Gaviria Giraldo CM, Velásquez C, Ruiz AC. Gnathostomosis an increasingly common disease in Colombia. Ces Medicina. 2017;3:199-206. Spanish.
2. Nawa Y, Maleewong W, Intapan PM, et al. *Gnathostoma*. In: Xiao L, Ryan U, Feng Y, editors. Biology of Foodborne Parasites. 1st ed. Boca Raton, Florida, FL: CRC Press; 2015. p. 520.
3. Hamilton WL, Agranoff D. Imported gnathostomiasis manifesting as cutaneous larva migrans and Löffler’s syndrome. BMJ Case Rep. 2018;pii:bcr-2017-223132.
4. Anantaphruti MT, Nuamtanong S, Dekumyoy P. Diagnostic values of IgG4 in human gnathostomiasis. Trop Med Int Health. 2005;10:1013-21.
5. Saksirissampant W, Choomchuay N, Kraivichian K, et al. Larva migration and eosinophilia in mice experimentally infected with *Gnathostoma spinigerum*. Iran J Parasitol. 2012;7:73-81.
6. Wong SS, Fung KS, Chau S, et al. Molecular diagnosis in clinical parasitology: when and why? Exp Biol Med (Maywood). 2014;239:1443-60.
7. Almeyda-Artigas RJ, Bargues MD, Mas-Coma S. ITS-2 rDNA sequencing of *Gnathostoma* species (Nematoda) and elucidation of the species causing human gnathostomiasis in the Americas. J Parasitol. 2000;86:537-44.
8. Ando K, Tsunemori M, Akahane H, et al. Comparative study on DNA sequences of ribosomal DNA and cytochrome c oxidase subunit 1 of mitochondrial DNA among five species of gnathostomes. J Helminthol. 2006;80:7-13.
9. Martínez-Salazar EA, León-Régagnon V. Confirmation of *Gnathostoma binucleatum* Almeyda-Artigas, 1991, advanced third-stage larvae in Tres Palos Lagoon, Mexico, by morphological and molecular data. J Parasitol. 2005;91:962-5.
10. León-Régagnon V, Osorio-Sarabia D, García-Prieto L, et al. Study of the ethiological agent of gnathostomosis in Nayarit, Mexico. Parasitol Int. 2002;51:201-4.
11. Pérez-Alvarez Y, García-Prieto L, Osorio-Sarabia D, et al. Present distribution of the genus *Gnathostoma* (Nematoda: Gnathostomatidae) in Mexico. Zootaxa. 2008;1930:39-55.
12. Díaz-Camacho SP, Delgado-Vargas F, Willms K, et al. Intrahepatic growth and maturation of *Gnathostoma turgidum* in the natural definitive opossum host, *Didelphis virginiana*. Parasitol Int. 2010;59:338-43.
13. Nawa Y, de la Cruz-Otero MdC, Zazueta-Ramos ML, et al. Is *Gnathostoma turgidum* an annual parasite of opossums? drastic seasonal changes of infection in *Didelphis virginiana* in Mexico. J Parasitol. 2009;95:908-12.
14. Díaz-Camacho SP, Zazueta-Ramos M, Ponce-Torrecillas E, et al. Clinical manifestations and immunodiagnosis of gnathostomiasis in Culiacan, Mexico. Am J Trop Med Hyg. 1998;59:908-15.
15. Sambrook J, Fritsch EF, Maniatis T. editors. Molecular cloning: A laboratory Manual. 2nd ed. New York: Cold Spring Harbor Press; 1989.
16. Undergasser A, Cutcutache I, Koressaar T, et al. Primer3-ner new capabilities and interfaces. Nucleic Acids Res. 2012;40:e115.
17. Larkin MA, Blackshields G, Brown NP, et al. Clustal W and Clustal X version 2.0. Bioinformatics. 2007;23:2947-8.
18. Li DM, Chen XR, Zhou JS, et al. Short report: case of gnathostomiasis in Beijing, China. Am J Trop Med Hyg. 2009;80:185-7.
19. Waikagul J, Díaz-Camacho SP. Gnathostomiasis. In: Murrell KD, Fried B, editors. Food-Borne Parasitic Zoonoses. 1st ed. Vol. 11. New York: Springer; 2007. p. 235-61.
20. Peláez D, Pérez-Reyes R. Gnathostomiasis in America. Rev Latinoam Microbiol. 1970;12:83-91.
21. Bertoni-Ruiz F, Lamothe-Aranguedo MR, García-Prieto L, et al. Systematics of the genus *Gnathostoma* (Nematoda: Gnathostomatidae) in the Americas. Rev Mex Biodiver. 2011;82:453-64.
22. Díaz-Camacho SP, de la Cruz-Otero MdC, Torres-Montoya EH, et al. Infection status of the estuarine turtles *Kinosternon expansum* and *Trachemys scripta* with *Gnathostoma sp*. isolated from natural hosts in Sinaloa, Mexico. Parasitol Res. 2002;88:639-45.
23. Díaz-Camacho SP, Willms K, Ramos MZ, et al. Morphology of *Gnathostoma* spp. isolated from natural hosts in Sinaloa, Mexico. Rev Mex Biodiver. 2010;81:569-71.
24. Morgan JA, Blair D. Trematode and monogenean rRNA ITS2 secondary structures support a four-domain model. J Mol Evol. 1998;47:406-19.
25. Coleman AW. Pan-eukaryote ITS2 homologies revealed by RNA secondary structure. Nucleic Acids Res. 2007;35:3322-9.
26. Jacobs DE, Zhu X, Gasser RB, et al. PCR-based methods for identification of potentially zoonotic ascarioid parasites of the dog, fox and cat. Acta Trop. 1997;68:191-200.
27. Díaz-Camacho SP, de la Cruz-Otero MdC, Zazueta-Ramos ML, et al. Identiﬁcation of estuarine fish *Dormitator latifrons* as a paratenic host for *Gnathostoma spinigerum*. J Parasitol. 2008;94:1421-5.
28. Díaz-Camacho SP, Willms K, Rendón-Maldonado JG, et al. Discovery of an endemic area of *Gnathostoma turgidum* infection among opossums, *Didelphis virginiana*, in Mexico. J Parasitol. 2009;95:617-22.