Research Note

Effect of Some Physical Factors on the Viability of Third-Stage Gnathostoma binucleatum Larvae

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ABSTRACT

To diminish the risk of transmission to humans of advanced third-stage larvae (A3L) of Gnathostoma binucleatum in fish foods, we evaluated the effects of some physical factors on larval viability. A3L protected within fish meatballs were subjected to freezing, refrigeration, boiling, dry heat, and immersion in lemon juice. By freezing, larvae were killed in 48 h, by refrigeration after 30 days, by boiling in 4 min, and by broiling for 60 min. By lemon juice immersion (pH 2.5), encysted larvae were killed after 5 days and nonencysted larvae in 7 h. Results show that freezing fish at −10 to −20 °C for 48 h, or cooking fish by frying, boiling, or broiling, will prevent transmission of G. binucleatum. Furthermore, results dispel the popular myth that lemon juice kills encysted larvae in fish.

Human gnathostomiasis is endemic in Asia, especially in Thailand and Japan, and it is now considered an emergent disease in Mexico (14). The only confirmed species that affects humans in North America is Gnathostoma binucleatum (9, 11). In Mexico, the State of Nayarit is presently the primary infection site in the country, where it has become a critical public health problem, with 6,328 human cases registered between 1995 and 2005 (3). There are no reports in the United States of acquired human infection; nevertheless, persons that travel to endemic places and consume traditional dishes based on raw fish could be exposed to infection. The first clinical signs in the infected are fever, epigastric pain, nausea, and vomiting. Afterward, when the larvae migrate, symptoms vary according to the area affected, causing migrant larvae syndrome, with manifestations that are cutaneous, ocular, neurological, and visceral, or a combination thereof (4). The species of the genus Gnathostoma generally use copepods as first intermediary hosts. G. binucleatum uses fresh water and estuarine fish as second hosts; turtles and some fish-eating birds participate as paratenic hosts (1, 2, 9).

Humans are accidental hosts and develop the disease after consuming traditional dishes in which fish is served raw or partially cooked, such as sushi, fresh scallops, and the Mexican ceviche (or lemon juice–cooked fish). It is difficult to identify contaminated fish, and this has hindered control of infection.

The present study evaluated the effect of some physical factors on G. binucleatum A3L viability, with the aim of proposing efficient measures to diminish the risk of transmission in humans, without affecting the organoleptic characteristics of the product.

MATERIALS AND METHODS

A3L collection. G. binucleatum larvae were isolated from estuarine fish of the species Cathorops fuerthii (known regionally as chihuil) and from turtles of the species Kinosternon integrum captured in fishing areas neighboring the Agua Brava lagoon in northern Nayarit, Mexico. Fish and turtle muscle tissue was dissected and ground in a home processor. It was pressed between two glass rectangles 15 by 18 cm and examined against the light of a 100-W bulb. A3L were separated with entomological needles.

Larval morphology. For morphometrical analysis, 30 larvae obtained from fish and 30 from turtles were fixed in 10% formaldehyde for 24 h. Larvae were cleared with Amman’s lactophenol to obtain measurements, and the variables recommended by Miyazaki (13) were used to identify the larvae of the genus Gnathostoma. Significant differences of the studied variables between turtles and fish were tested with the Student’s t test by using Statistica software (StatSoft, Inc., Tulsa, OK).

Larval sections. Larvae were subsequently included in 1 cm³ of minced meat, fixed in 10% formaldehyde for 24 h and embedded in paraffin. Transversal sections of 4-μm thickness were conventionally stained with eosin and hematoxylin. 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, DNA was extracted with the standard phenol-chloroform technique (15),

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and the internal transcribed spacer 2 (ITS2) of ribosomal DNA was amplified by PCR with the technique described by León-Régagnon et al. (11) and Martínez-Salazar and León-Régagnon (12), by using the primers NEWS2 (forward) 5′-TGTGTCGATGAA-GAACGCAG-3′ and ITS2-RIXO (reverse) 5′-TTCTATGCTTAAATTCAGGGG-3′. The obtained sequences were aligned with sequences AY734632, AY061740, and AB181159, obtained from GenBank and corresponding to *G. binucleatum*.

**Larval viability.** Three criteria were used to evaluate larval viability after physical treatments. The first was internal and external morphological integrity; the second, dark-red color of the intestine; the third, movement response on exposure to heat and light of a microscope or to 2 drops of 4% formaldehyde.

**Freezing treatment.** One hundred thirty-five larvae of *G. binucleatum* were used to evaluate the effect of freezing on larval viability. Twenty-seven 500-g fish meatballs (FMB) were used; five encysted *G. binucleatum* larvae were inserted into each FMB, and each FMB placed separately into a plastic bag (Fig. 1C). The FMB were divided into three groups of nine FMB each. The first group was frozen at −210°C, the second at −215°C, and the third at −220°C. Three FMB were thawed at room temperature after 24 h, three after 48 h, and the rest after 72 h. Immediately after thawing, larvae were recovered, and their viability was evaluated as described above.

**Refrigeration treatment.** Forty-five *G. binucleatum* larvae were used to evaluate the effect of refrigeration on larval viability. Nine 500-g FMB were used; five encysted larvae of *G. binucleatum* were inserted into each FMB, and each FMB placed separately into a plastic bag (Fig. 1C). All FMB were refrigerated at 4°C. Larval viability was evaluated in three FMB after 10 days, in three FMB after 20 days, and in three FMB after 30 days of refrigeration.

**Boiling treatment.** Forty-five *G. binucleatum* larvae were used to evaluate the effect of boiling at 100°C on larval viability. Each larva was placed inside an FMB with an approximately 2-cm diameter, and these were each in turn enclosed in a small gauze bag to avoid the loss of one larva contained within (boiling). Three groups of larvae (15) were removed after 3 min, three groups after 4 min, and three groups after 5 min.

**FIGURE 1.** Gnathostoma *binucleatum* A3L preparation to evaluate the effect of different physical treatments on viability. (A) Larvae deposited within a circle of methylene blue in fish muscle (*Mugil curema*), (B) larvae recovered from grilled fish muscle, (C) fish meatballs (FMB) into which five larvae were placed (freezing and refrigeration), and (D) FMB enclosed in a small gauze bag to avoid the loss of one larva contained within (boiling).
TABLE 1. Morphometry of 30 Gnathostoma binucleatum larvae obtained from estuarine fish and 30 larvae obtained from turtles in the state of Nayarit, Mexico

<table>
<thead>
<tr>
<th>Animal</th>
<th>Parameter</th>
<th>TL (mm)</th>
<th>MW (mm)</th>
<th>RB</th>
<th>HBL (mm)</th>
<th>HBW (mm)</th>
<th>CP (mm)</th>
<th>ADE (mm)</th>
</tr>
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<tbody>
<tr>
<td>Fish</td>
<td>MD</td>
<td>3.98</td>
<td>0.3</td>
<td>4</td>
<td>0.14</td>
<td>0.23</td>
<td>14.6</td>
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<tr>
<td></td>
<td>SD</td>
<td>0.58</td>
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<td>0.01</td>
</tr>
<tr>
<td>Turtle</td>
<td>MD</td>
<td>4.05</td>
<td>0.3</td>
<td>4</td>
<td>0.21</td>
<td>0.11</td>
<td>14.2</td>
<td>0.06</td>
</tr>
<tr>
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<td>0</td>
<td>0.02</td>
<td>0.02</td>
<td>1.4</td>
<td>0.02</td>
</tr>
</tbody>
</table>

a TL, total length; MW, maximum width; RB, number of rings per head-bulb; HBL, head-bulb length; HBW, head-bulb width; CP, location of cervical papilla; ADE, distance from anus and distal end.

Dry heat treatment. Forty-five G. binucleatum larvae were used to evaluate the effect of dry heat (a process known regionally as *tatemar*) fish on larval viability. Five larvae were placed within a circle marked with methylene blue (to prevent their loss) in the muscle mass of each of nine fish of the species *Magil curena*, weighing approximately 400 g, as shown in Figure 1A. Fish were then cooked approximately 60 cm from the heat generated by burning wood (120 to 150°C). Three fish were removed from the heat after 40 min, another three after 60 min, and the last three after 80 min of cooking. Larvae were extracted from each fish, and viability was evaluated (Fig. 1B).

Lemon juice immersion. Ninety G. binucleatum larvae were used to evaluate the effect of lemon juice immersion (pH 2.5) on larval viability. Forty-five larvae were extracted from their cyst covers with an entomological needle, and 45 larvae kept their cyst covers. All larvae were immersed in lemon juice and kept at room temperature. Fifteen nonencysted larvae were removed from the juice after 1, 3, and 7 h, and their viability was evaluated. Fifteen encysted larvae were removed from the juice after 24, 72, and 120 h, and their viability was evaluated.

TABLE 2. Effect of different physical treatments on the viability of Gnathostoma

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of larvae treated</th>
<th>Living</th>
<th>Dead</th>
<th>No. of larvae treated</th>
<th>Living</th>
<th>Dead</th>
<th>No. of larvae treated</th>
<th>Living</th>
<th>Dead</th>
<th>Total (360)</th>
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<tbody>
<tr>
<td>Freezing</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>10°C</td>
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<td>6</td>
<td>9</td>
<td>15</td>
<td>0</td>
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<td>15°C</td>
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<td>13</td>
<td>15</td>
<td>0</td>
<td>15</td>
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<td>0</td>
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<td>15</td>
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<td>15</td>
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<tr>
<td>10°C</td>
<td>15</td>
<td>2</td>
<td>13</td>
<td>15</td>
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<td>15</td>
<td>15</td>
<td>0</td>
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<td>45</td>
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<tr>
<td>Lemon juice (pH 2.5)</td>
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<td>15</td>
<td>0</td>
<td>15</td>
<td>5</td>
<td>10</td>
<td>15</td>
<td>0</td>
<td>15</td>
<td>45</td>
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<td>Encysted</td>
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</tbody>
</table>

RESULTS

Table 1 shows the main morphometric parameters of larvae obtained from fish and turtles. The average number of nuclei in intestinal epithelium cells of fish larvae was 2.24, and the average in turtle larvae was 2.2. No differences in morphometric variables and average number of larval intestinal epithelium cell nuclei were found in both groups of larvae; their morphology corresponded to *G. binucleatum*.

The base sequence of the ribosomal DNA–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*.

The effects of the physical treatments to which larvae were exposed are shown in Table 2. All larvae were killed by freezing after 48 h, by refrigeration after 30 days, by boiling in 4 min, and by dry heat after 60 min. Encysted larvae immersed in lemon juice (pH 2.5) were killed after 5 days of exposure, and nonencysted larvae after 7 h.

Figure 2 shows the effects of the physical treatments presently tested on A3L morphology of *G. binucleatum*. Boiling induced color changes, detachment of the cuticle, and loss of internal morphology in A3L (Fig. 2A). Dry heat induced color changes and detachment of the cuticle in A3L (Fig. 2B). Freezing rendered larvae dehydrated, crystallized, brittle, and broken (Fig. 2C), and nonencysted larvae treated with lemon juice showed color changes (Fig. 2D).

DISCUSSION

Human gnathostomiasis is acquired by consuming traditional dishes in which fish is served raw or partially cooked, such as sushi, fresh scallops, and Mexican ceviche. Some techniques can reveal the presence of *Gnathostoma* spp. in fish (meat compression, digestion, or histopathology). However, sanitary inspection is ineffective, since it cannot detect larvae without damaging fish to the point of making it useless for human consumption. Finding a larvae-inactivation...
method to that can be applied to all fish before being consumed would reduce the risk of acquiring the disease.

Treatment with certain physical factors such as gamma radiation and thermal treatment (freezing and boiling) has been found to inactivate some helminthes (7, 10, 16). Freezing at $-210$ and $-215^\circ C$ for at least 48 h killed all A3L of *G. binucleatum* that had been inserted into FMB. This information is useful to prevent infections at the household level. A standard home refrigerator may be used to freeze fish for 48 h before using it to prepare raw fish dishes, and this will protect family health. Freezing at $-220^\circ C$ killed larvae after 24 h. This technique may be used by fish enterprises that store large volumes (stocking centers, fish shops, restaurants, supermarkets, fish packers, etc.) to protect the fish consumer’s health. Freezing has also been shown to be effective in killing larvae of *Trichinella spiralis* in pork and *Taenia saginata* in beef (8, 10).

Refrigeration at 5°C, used to keep perishable food fresh for short periods, did not kill *G. binucleatum* larvae after 10 to 20 days (mortality rate of 26%). Although all larvae were killed after 30 days of refrigeration, this was probably due to fish tissue self-lysis, during which the processes of decay and microorganism proliferation increase. This option is definitely not recommended for human consumption. Other parasitoses react similarly to refrigeration; for example, *Taenia solium* cysticerci can survive 25 days in pork meat refrigerated at 4°C (5).

Boiling FMB containing *G. binucleatum* larvae for 4 to 5 min was enough to kill all larvae. This treatment during 10 min was also enough to kill *T. solium* cysticerci in pork meat (17). In both cases, meat had to be cut into small enough pieces (2 to 3 cm) to ensure that the required temperature would be reached by all tissue long enough to kill all microorganisms. Boiling has also been shown to prevent transmission of other fish-dwelling helminthes such as *Anisakis* spp. and *Contracaecum* spp. (6).

Dry heat (dry heat with burning wood) can be considered effective for larvae destruction because it takes approximately 90 min to cook fish by this method, and on examination, only two viable larvae were found after 40 min of dry heat. The much longer period necessary to complete the cooking process guarantees that all larvae will be killed and the risk of infection will be eliminated.

The traditional Mexican dish ceviche is prepared with minced fish, which is immersed in lemon juice. Encysted *G. binucleatum* larvae died after 5 days of being subjected to this treatment. Nonencysted larvae took 7 h of exposure to lemon juice to die, which, on the one hand, illustrates the resistance of the cyst to low pH levels (2.5), but on the other, is a much longer period than that time used for the preparation of this dish. Ceviche is normally prepared 4 to
5 h before being consumed, and it is eaten on the same day it is prepared. It not considered apt for human consumption after storing for 48 h, so this could be a possible source of infection with *G. binucleatum* larvae.

Results obtained in this study show that freezing fish for 48 h, or cooking it by frying or boiling guarantees that *G. binucleatum* larvae will not be transmitted. However, cooking times longer than 5 min alter the palatability of fish, which motivates traditional consumers to eat fish raw or partially cooked in the form of ceviche or sushi, consequently exposing these people to *G. binucleatum* larvae. As shown in this work, the popular myth of the Nayarit population that lemon juice kills larvae is false. However, freezing fish, which will later be eaten raw or partially cooked, would be an effective method for killing *G. binucleatum* larvae.

**ACKNOWLEDGMENT**

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**REFERENCES**