Short communication

Fish-borne trematodosis: Potential risk of infection by Ascocotyle (Phagicola) longa (Heterophyidae)

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\textbf{ABSTRACT}

Owing to the veterinary and medical importance of heterophyid trematodes, a survey on Ascocotyle (Phagicola) longa in different organs of mullets Mugil liza from Rio de Janeiro was undertaken. The prevalence of metacercariae varied greatly between different organs of the mullets: spleen (100%), heart (98%), intestine wall (97%), liver (97%), muscle (87%), stomach wall (77%), brain (47%), gonads (30%) and gall bladder (30%). The high level of the intensity of the infection in relation to different fish organs was confirmed in two experimental infections performed during the spring/summer and autumn/winter seasons when 258 and 47 adult parasites were recovered from hamsters fed only with small pieces of muscle tissue. The potential risk of infection was considered to be high in view of the high prevalence and intensity of A. (P.) longa in the muscles of mullets throughout the year. Additionally new confocal imaging of metacercariae and adults experimentally obtained, enabled for the first time the description of a short genital atrium formed by the union of uterus and ejaculatory duct.

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1. Introduction

Ascocotyle (Phagicola) longa Ransom, 1920 (Heterophyidae) is a cosmopolitan species recorded from the Americas, Europe, Africa and the Middle East (Scholz, 1999; Scholz et al., 2001; Simões et al., 2010; Martorelli et al., in press), and is considered an emerging fish-borne, zoonotic disease of humans (Fried et al., 2004; Brasil, 2010). A. (P.) longa has a complex life cycle with two intermediate hosts, the snail Helix greta (d’Orbigny, 1835) and mullets Mugil spp. Adult parasites are found in the intestine of piscivorous birds and mammals (Simões et al., 2010). The extensive geographical distribution of A. (P.) longa and its intermediate hosts along with the increasing consumption of raw or undercooked fish increase the risk of human infection. However, the zoonosis is underestimated due to the absence of characteristic symptoms (Montejo et al., 2008).

In order to verify the report of Yamaguti (1975), who indicated that metacercariae of A. (P.) longa rarely occur in the body muscles of the fish and are thus less likely to infect humans, an analysis of their prevalence, abundance and intensity in different organs of Mugil liza Val., 1836 was undertaken. To evaluate the intensity of the infection in relation to different fish organs, experimental infections were performed during the spring/summer and autumn/winter seasons. Subsequently, an evaluation of the potential risk of infection was performed. New data on the metacercariae and adults experimentally obtained inferred from confocal observations, are also provided.
2. Materials and methods

2.1. Ethics statement

This research was licensed by the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA No. 15898-1) and approved by the Animal Ethics Committee of the Oswaldo Cruz Foundation (CEUA-FIOCRUZ LW-12/10), in accordance with the guidelines of the Brazilian College for Animal Experiments (COBEA).

A total of 104 mullets *M. liza* (total length 23–38 cm) (47 spring/summer, 57 the autumn/winter) were randomly obtained with fishermen at the Rodrigo de Freitas Lagoon, Rio de Janeiro, Brazil (22°57′2″S, 43°11′9″W) and examined for metacercariae. Some metacercariae were studied live and unstained or prepared as semi-permanent slides in glycerine jelly or glycerine: picric acid. For the confocal laser scanning microscopy (CLSM) analysis, specimens were treated according to the direct fluorescence technique using phalloidin conjugated with fluorescein isothiocyanate (FITC) (Sigma) (Portes Santos and Moravec, 2009).

In order to evaluate the abundance of *A. (P.) longa* nearly 2 cm³ of each organ from each fish, including the eyes, brain, gills, heart, gonads, gall bladder, liver, spleen, stomach wall, intestine wall and muscle tissue from mid-body level, were examined under a stereomicroscope to isolate and count metacercariae. The numbers of metacercariae recovered were divided into four groups as follows: 0, 1–10, 11–30 and >30. To confirm that parasite intensity varied in different organs, two experimental infections were performed, one during the autumn/winter and another in the spring/summer. The organs used in the experiments were liver, spleen, heart, gonads and muscle. For each experiment, four hamsters *Mesocricetus auratus* Waterhouse, 1814 were individually fed with almost 2 cm³ of tissue twice daily for 4 days with one of the selected organs from 8 naturally infected *M. liza* (25–33 cm long in the first infection, 31–35 cm long in the second), which has previously been analyzed for the presence of metacercariae. On the fourth day post-infection (dpi) the hamsters were killed and dissected for the collection of adults of *A. (P.) longa*. Adults were fixed in 70% alcohol, stained in alcoholic chloridic carmine, cleared in clove oil and mounted in Canada balsam. Measurements made using a Leica DM LS2 microscope are presented in micrometers as the range, with the mean in parentheses.

Comparisons of numbers of metacercariae among the different organs were performed using a Kruskal–Wallis test. For this test, rank scores were assigned to each organ based on how many metacercariae were counted, as follows: 0 for 0 metacercariae, 1 for 1–10 metacercariae, 2 for 11–30 metacercariae and 3 for >30 metacercariae. To determine whether fish size affected the overall infection levels, a Spearman’s rank correlation coefficient was computed between fish length and the sum of the scores for all of a fish’s organs. The intensity of infection in different organs was analyzed comparatively for autumn/winter and spring/summer periods using generalized linear models (GLM). The seasons were used as response variables with a binomial distribution, probit as the link function and each organ as predictor variables. Finally, the most parsimonious model was determined using the Akaike Information Criterion (Burnham and Anderson, 2004).

3. Results

3.1. Parasite identification and characterization

*A. (P.) longa* was identified based on a morphological analysis of metacercariae from mullets and confirmed by the adults experimentally obtained from hamsters. Both were mainly characterized as follows:

Metacercaria (measurements based on 10 specimens from naturally infected *M. liza*). The body of the excysted metacercaria is pyriform, 380–590 × 100–160 (450 × 120) and covered by spines (Fig. 1A–C). The oral sucker 23–53 × 20–48 (36 × 37) is encircled by one row of 16 circumoral spines 15–18 × 5 (14 × 5) (Fig. 1A and B). The muscular posterior appendage of oral sucker is 45–85 (58) long (Fig. 1B). The ventral sucker 23–38 × 25–45 (29 × 34) is enclosed into the ventrogenital sac (Fig. 1C). The pharynx, the esophagus (Fig. 1B), and the intestinal ceca (Fig. 1D) with longitudinal and circular muscle fibers are well visible by confocal images. The testes are symmetrical. A muscular duct is well visible close to the gonotyl area (Fig. 1D). The ventrogenital sac contains a bipartite gonotyl with two pad-like lobes (Fig. 1D). The pretesticular ovary is dextral. The excretory pore is terminal (Fig. 1D).

Adult (measurements based on 10 specimens collected from the intestine of the experimentally infected hamsters, *M. auratus*). The body is pyriform, 390–660 × 170–280 (513 × 206) with tegument composed of longitudinal, circular and diagonal fibers and surface covered by spines (Fig. 2A and B). The preoral lobe is 13–25 (18) long and the oral sucker measures 28–45 × 18–43 (35–31), surrounded by one row of 16 circumoral spines 15–18 × 5 (17 × 5) (Fig. 2A and B). The posterior appendage of the oral sucker is 25–75 (50) long. The ventral sucker 28–45 × 28–48 (39 × 38) is enclosed in the strongly muscular ventrogenital sac (Fig. 2B). The muscular pharynx is 38–50 × 28–38 (46 × 32); the esophagus is mainly composed of muscular longitudinal fibers (Fig. 2C). The intestinal ceca which reach the testicular level have longitudinal and circular fibers (Fig. 2C). The left testis is 38–85 × 45–98 (61 × 69) and the right 40–75 × 58–113 (64 × 78). The seminal vesicle is sigmoid, posteroinstrial to the ventral sucker. A high-resolution confocal imaging of F-actin, using phalloidin highlighted the muscular fibers of the ejaculatory duct that unite to the terminal part of uterus to form a genital atrium. The genital pore is situated within the ventrogenital sac between the pad-like structures of the gonotyl (Fig. 2C and D). The ovary is dextral, 30–58 × 28–70 (43 × 47). The vitellaria has 2 lateral bands of 7–9 follicles that were not visible using the phalloidin stain. The uterus is sinuous with muscular walls well defined and is filled with eggs 15–23 × 10–13 (19 × 10) seen as black oval structures in the confocal images (Fig. 2D). The X-shaped excretory vesicle ends in the terminal excretory pore (Fig. 2E).
3.2. Prevalence, abundance and intensity of infection

Metacercariae of *A. (P.) longa* were found parasitizing 100% of the fishes examined. The prevalence varied greatly between organs: spleen (100%), heart (98%), intestine wall (97%), liver (97%), muscle (87%), stomach wall (77%), brain (47%), gonads (30%) and gall bladder (30%). The eyes and gills were never found to be parasitized. The differences in the numbers of metacercariae harbored by the different organs were significant (Kruskal–Wallis test: $K = 766.47$, $P < 0.0001$); high numbers of metacercariae were more frequently found in the spleen, heart, intestine wall and liver. However, considering the abundance of metacercariae in the muscle of the 104 mullets, 51 fish were ranked in the group with 1–10 metacercariae, 31 fish with 11–30 metacercariae, 9 fish in the rank score with >30 metacercariae and only in 13 fish the abundance was considered 0 (Table 1). There was no significant correlation between fish length and the overall infection intensity, measured as the sum of the scores for each organ of a fish ($r_s = 0.081$, $P = 0.413$). Thus, larger fish did not tend to harbor more metacercariae across all their organs than small fish. The GLM test used to analyze the parasite intensity of the metacercariae was not significant when compared seasonally (spring/summer × autumn/winter) for most of the organs (heart: $F = -0.375$, $P = 0.70$; spleen: $F = -0.925$, $P = 0.355$; liver: $F = 1.234$, $P = 0.217$; gall bladder: $F = 1.760$, $P = 0.07$.}

![Fig. 1. Metacercaria of *Ascocotyle* (*Phagicola*) longa, CLSM micrographs. A: Whole mount, ventral view; B: internal layer of body showing the circumoral spines (s), posterior appendage of oral sucker (a), pharynx (p) and ventral sucker (vs); C: aperture of ventrogenital sac (hollow) above the ventral sucker; D: gonotyl with pad-like lobes (g), ceca with longitudinal and circular fibers (c), testis (t) and muscular duct (*).](image-url)
stomach wall: $F = 1.937, P = 0.052$; muscle: $F = -0.916; P = 0.359$; gonads $F = -0.347, P = 0.728$; intestine wall: $F = -0.08, P = 0.936$), except for those recovered from the brain ($F = -3.455, P < 0.001$).

### Table 1

Number of fish parasitized by metacercaria of *A. (P.) longa* distributed in rank scores of parasite abundance in various fish organs.

<table>
<thead>
<tr>
<th>Abundance</th>
<th>0</th>
<th>1–10</th>
<th>11–30</th>
<th>&gt;30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gills</td>
<td>104</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Eyes</td>
<td>104</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Gall bladder</td>
<td>72</td>
<td>24</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Gonads</td>
<td>63</td>
<td>36</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Brain</td>
<td>55</td>
<td>36</td>
<td>11</td>
<td>2</td>
</tr>
<tr>
<td>Stomach wall</td>
<td>23</td>
<td>44</td>
<td>25</td>
<td>12</td>
</tr>
<tr>
<td>Muscle</td>
<td>13</td>
<td>51</td>
<td>31</td>
<td>9</td>
</tr>
<tr>
<td>Liver</td>
<td>3</td>
<td>15</td>
<td>40</td>
<td>46</td>
</tr>
<tr>
<td>Intestine</td>
<td>3</td>
<td>16</td>
<td>22</td>
<td>63</td>
</tr>
<tr>
<td>Heart</td>
<td>2</td>
<td>16</td>
<td>28</td>
<td>58</td>
</tr>
<tr>
<td>Spleen</td>
<td>0</td>
<td>5</td>
<td>33</td>
<td>66</td>
</tr>
</tbody>
</table>

3.3. Experimental infections

In the experimental infection performed in spring/summer, a total of 2664 adult parasites were recovered from the intestine of the four hamsters, with 1446 parasites recovered from the hamster fed with liver and spleen, 852 from that fed with hearts, 258 from the one fed with muscle and 108 from that fed with gonads. The experimental infection performed in autumn/winter, a total of 833 adult parasites were recovered from the intestine of the four hamsters, comprising 650 from the one fed with liver and spleen, 119 from that fed with hearts, 47 from the hamster fed with muscle and 17 from that fed with gonads.

4. Discussion

The morphology of *A. (P.) longa* has been already studied by light and scanning electron microscopy but the high-resolution confocal imaging enabled us to identify for the...
first time the union of the uterus and ejaculatory duct to form a short genital atrium that opens via a genital pore situated within the ventro genital sac.

The present results showed that, apart from the liver, spleen, heart and intestine, the muscles of mullets are also heavily parasitized. In addition, the GLM test indicated no significant seasonal variation in parasite intensity in the different organs of *M. liza*, when comparing the seasons, except in the brain, which presented a higher intensity during autumn/winter. Despite the fact that these seasonal differences were not significant, the experimental infection performed in spring/summer resulted in a higher intensity of parasites than that completed during the autumn/winter.

Martorelli et al. (in press) reported the occurrence of metacercariae of *A. (P.) longa* parasitizing *M. liza* with a general account of prevalence, intensity and abundance, not considering the specificity in each organ. We have shown specifically that a high prevalence (87.5%) of metacercariae does occur in the muscle of mullets, contrary to the information of Yamaguti (1975) which reported that *A. (P.) longa* rarely parasitizes in this site. These data were demonstrated by the experimental infections, where large numbers of adult parasites (47 and 258) were recovered from hamsters fed only with small pieces (2 cm³ per day) of muscle tissue. If we take into account that *A. (P.) longa* may remain viable for at least 3 days at low temperatures, which, as previously reported (Antunes and Almeida-Dias, 1994), is equivalent to the ‘sell by date’ for fish intended for consumption raw, our results show that the muscles of mullets do in fact represent a source of a zoonotic infection. In view of the high prevalence and intensity of *A. (P.) longa* in muscle of mullets, this work has shown that the risk of human infection is high throughout the year.

Although this risk assessment of potential human infection alone cannot provide all information for a governmental risk management decision, the identification of the emerging risk of *A. (P.) longa* as human health hazard regarding the ingestion of raw mullets is a preventive instrument at the disposal of authorities.

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**References**


**Conflict of interest statement**

The authors declare there is no conflict of interest related to this research.