

# Detection of antigens in paraffin sections of worms and snails infected by *Angiostrongylus cantonensis* by immunofluorescence reaction

## Detecção de antígenos em cortes parafinados de vermes e caramujos infectados por *Angiostrongylus cantonensis* pela reação de imunofluorescência

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### ABSTRACT

The Indirect Immunofluorescence Assay (IFA) was used to identify stage-specific antigenic structures in paraffin sections of female larvae and worms and snails tissues, infected with third stage larvae of *Angiostrongylus cantonensis*. Sera from eosinophilic meningitis cases were used to assess reactivity. Non-reactive sera from patients with other parasitic diseases and from individuals without other etiologies were used as controls for cross-reactivity. Larvae and worms showed high reactivity to IgG antibodies. IgM antibodies reacted with low intensity only to larvae. Fluorescent reactions were observed in the cuticles and internal structures on worms sections, with a marked reaction in the uterus content. In the snail tissues, the larvae were found exclusively inside the granulomas, with fluorescent markings in the cuticles of the larvae and inside the granulomatous tissues. This fluorescent pattern suggests the presence of excretory/secretory antigens distributed throughout the granulomas. Expressive cross-reactivity occurred in sera from patients with other parasitic diseases, especially strongyloidiasis. The use of IFA applied to paraffin sections to identify structures with antigenic potential and the study of new serological markers, can contribute to the improvement of the laboratory diagnosis of eosinophilic meningitis.

**Keywords.** *Angiostrongylus cantonensis*, Angiostrongyliasis, Antigens, Helminth, Histological Techniques, Immunofluorescence, Indirect.

### RESUMO

A Reação de Imunofluorescência Indireta (RIFI) foi utilizada para localizar antígenos em estruturas estágio-específicas em cortes parafinados de vermes fêmeas e em tecidos de caramujos do Gênero *Biomphalaria* infectados com larvas de terceiro estágio de *Angiostrongylus cantonensis*. Soros de casos confirmados de meningite eosinofílica foram usados para avaliação da reatividade. Soros não reagentes de casos suspeitos de pacientes com outras parasitoses e de indivíduos sem outras etiologias foram utilizados como controle de reatividade cruzada. Anticorpos da classe IgG foram reativos para antígenos presentes nos dois estágios e anticorpos IgM, somente para o estágio larvário. Nos cortes de vermes, as marcações fluorescentes foram assinaladas nas cutículas e estruturas internas, com acentuada marcação para os conteúdos uterinos. Nos tecidos dos caramujos, as larvas foram encontradas exclusivamente no interior dos granulomas, com marcações fluorescentes nas cutículas das larvas e no interior dos tecidos granulomatosos. O padrão de fluorescência no granuloma sugere a marcação de antígenos excretores/secretores. Reatividade cruzada mais expressiva ocorreu com anticorpos presentes em soros de pacientes com outras parasitoses, com destaque para estrogiloidíase. A RIFI em cortes parafinados abre novas perspectivas para identificação de antígenos e de marcadores sorológicos, que possam ser aplicados no aprimoramento do diagnóstico laboratorial da meningite eosinofílica.

**Palavras-chaves.** *Angiostrongylus cantonensis*, Angiostrongilíase, Antígenos de Helminthos, Técnicas Histológicas, Imunofluorescência Indireta.

## INTRODUCTION

*Angiostrongylus cantonensis* (Chen, 1935) is a metastrongylids nematode, a natural parasite of the pulmonary arterial system of rodents. The larval development phase occurs in tissues of different species of terrestrial molluscs (snails and slugs) and freshwater snails<sup>1</sup>. Human infection is considered occasional and occurs through the intentional or habitual ingestion of intermediate hosts parasitized with infective third-stage larvae, when consumed *in natura* or undercooked<sup>1,2</sup>. By the same mechanism, reptiles, amphibians, crustaceans, among others, considered paratenic hosts, when parasitized with the same larval stage, can also serve as sources of dissemination<sup>1,2</sup>. Vegetables contaminated by the mucus of infected molluscs, constitute a lower risk of transmission mechanism<sup>3</sup>.

In humans, ingested larvae penetrate through the intestinal wall and are disseminated through the blood or lymphatic circulation to other organs. The infective larvae have an obligatory tropism for the central nervous system (neurotropism), inducing an intense inflammatory reaction, which is the cause of eosinophilic meningitis<sup>4</sup>. The larvae remain in the organ, where they evolve to the stage of young worms and die before reaching the adult stage<sup>1,4</sup>. The mechanisms involved in the formation of the eosinophilic infiltrate in the nervous system are not fully known<sup>5</sup>. However, during experimental infection in rodents, the expression of interleukins 5 (IL-5) and 13 (IL-13) was associated with intense mobilization of eosinophils to the nervous tissue, suggesting that this is one of the effector mechanisms for the death of the parasites<sup>5</sup>.

Clinical manifestations are related to meningitis and are characterized by severe headache, fever, vomiting, diarrhea, paresthesia and nausea. Usually the infection follows as mild or moderate, but many severe and fatal cases have been reported<sup>2,6</sup>. In addition, the parasite can lodge in the ocular globe and cause ocular angiostrongyliasis, usually described in children, and may or may not be associated with meningitis<sup>2,6</sup>.

Eosinophilic meningitis caused by *A. cantonensis*, also known as neuroangiostrongyliasis or meningoencephalic angiostrongyliasis, is endemic in Asian, Pacific Island and Caribbean countries<sup>1,2,6</sup> and, recently, it has taken on an emerging character in some countries of the American Continent<sup>7</sup>. In Brazil, the first cases were reported in 2007 in the municipality of Cariacica, ES, followed by reports of isolated cases and small family outbreaks in other municipalities, in different states of the federation<sup>7-9</sup>. Despite this, the parasitosis is still unknown by most health professionals, which may explain the low frequency of cases in the country<sup>7</sup>. On the other hand, reports of the parasite presence in definitive and intermediate hosts are increasing, demonstrating that it is an emerging zoonosis with a potential risk of expansion in Brazil<sup>10,11</sup>.

In laboratory diagnosis, the observation of larvae or part of them, or even the detection of DNA fragments of the parasite in the cerebrospinal fluid (CSF) proves the infection, which is not always possible, as it depends on the stage of the disease and on the parasite load acquired by the patient<sup>4,12</sup>. The investigation of antibodies in CSF and serum samples has been widely used for diagnosis, however the humoral response and the nature of the applied antigens have limited the infection confirmation in some cases<sup>4,12</sup>.

*A. cantonensis* presents a biological complexity evidenced by the diversity of adaptative forms found in its evolutionary cycle, with great adaptability to different hosts<sup>1</sup>. These biological characteristics influence the parasite-host relationship since, in physiological and pathological processes; numerous potentially antigenic molecules are expressed by the parasite, which determines a complex immune response from the host<sup>13-15</sup>.

Thus, the improvement of the immunodiagnosis of the infection involves the search for purified antigens that are able to provide higher sensitivity, specificity and predictive values for the analysis<sup>16</sup>. Low molecular weight antigens are considered as immunological markers of greater efficiency in relation to total extracts of adult worms<sup>13,16</sup>, although cross reactions have already been identified in fractions which are considered more specific, such as 31 kDa<sup>17</sup>. The use of synthetic peptides and monoclonal antibodies are promising, however they have restricted practical applications considering their low availability<sup>2</sup>.

Thus, the present study aims to locate structures with higher reactivity for human sera in paraffin sections of snail tissues, experimentally infected; and adult worms of *A. cantonensis*, using the indirect immunofluorescence assay (IFA). It is intended to relate the located structures with antigens of greater diagnostic potential for *A. cantonensis* infection, contributing to the improvement of the laboratory diagnosis of the disease.

## MATERIAL AND METHODS

### Legal ethical aspects

This study was part of a project registered in the Conselho Técnico Científico (CTC) of the Adolfo Lutz Institute (IAL) (n. CTC68I/2016); and received approval from the Comitê de Ética (CEP) of the IAL (CEPIAL n. 2.271.089). All procedures involving experimental animals were performed according to the guidelines of the Sociedade Brasileira de Ciência em Animais de Laboratório/Colégio Brasileiro de Experimentação Animal (SBCAL/COBEA), and received approval from the Comitê de Ética Animal of IAL (CEUA/IAL/Pasteur n. 042012). The rodents supply, in order to maintain the evolutionary cycle of *A. cantonensis*, was approved by the CEUA of the Institute of Tropical Medicine of São Paulo (IMT/SP n. 077/11).

### *A. cantonensis* isolate

*A. cantonensis* was isolated from *Rattus norvegicus* from the southern region of São Paulo, SP, Brazil, and has been maintained under experimental conditions in albino rats of the strain (Wistar) and in *Biomphalaria glabrata*, since 2010 at the Núcleo de Enteroparasitas (NE) of the IAL. The isolate was identified by morphological, biological and molecular criteria by the NE/ IAL and by the Laboratório de Biologia Molecular de Parasitos e Fungos, of Centro de Parasitologia e Micologia of IAL. The strain was registered in the collection as *Village*<sup>10</sup>.

### Maintenance of *A. cantonensis* in the laboratory

The adult worms of *A. cantonensis* and the infected specimens of *B. glabrata* were obtained from the experimental cycle maintained by the NE/ IAL, where groups of 15 snails of the species *B. glabrata* (strain BH) from the molluscary are routinely used. Three Wistar rats were obtained from the Institute of Biomedical Sciences (ICB/USP), with the collaboration of the Medical Investigation Laboratory (LIM 06/IMT/FMUSP).

### *Biomphalaria glabrata* infection

The first stage larvae (L1) were extracted from the feces of rodents, with patent *A. cantonensis* infection (over 45 days), using the technique of Rugai et al<sup>18</sup> technique. Each snail was individualized and exposed to 700 larvae (L1) and the inoculum was deposited directly through the shell opening. After 18 hours of contact, the specimens were collected and transferred to an aquarium until they completed 45 days of infection.

### *Infection of Wistar rats*

For rodent infection, 45 days after infection, molluscs were crushed from their shells, pooled and cut into small pieces. The tissue mass was incubated at 37 °C for two hours with 100 mL of a solution containing: 4% pepsin (Pepsin 1:10000 porcine Purex, INLAB<sup>®</sup>) and 0.7% hydrochloric acid (HCl PA, Merck<sup>®</sup>). Third stage larvae (L3) were extracted from the digested fragments using the Rugai et al<sup>18</sup> technique. Thirty L3 larvae were administered through gastric tube to each animal. Infected animals were kept under proper maintenance conditions for at least 45 days, when their feces were collected and used for new infections in molluscs, as previously mentioned.

### *Obtaining adult worms*

Wistar rats, after 45 days of infection, were anesthetized with 87 mg/kg of ketamine hydrochloride solution, (Dopalen, Ceva<sup>®</sup>) and 13 mg/kg of xylazine hydrochloride (Xilazin, Syntec<sup>®</sup>), intraperitoneally and euthanized in a CO<sub>2</sub> chamber. Blood was removed from the circulatory system by portal-mesenteric perfusion using 40 mL of 0.85% saline solution with 2.5% ethylenediaminetetraacetic acid (EDTA, Merck<sup>®</sup>) in continuous flow and under pressure. The heart and lungs were separated and dissected to allow the removal of worms from inside the arterial heart chambers and intrapulmonary arterial vessels. The specimens were washed in 0.85% saline solution, grouped according to sex and kept according to their use.

## **Paraffin sections for Indirect Immunofluorescence Assay (IFA)**

### *Preparation of infected Biomphalaria glabrata*

Five specimens of *B. glabrata*, with approximately 10 mm in shell diameter, from the experimental cycle and with five months of infection were used for the blocks. After breaking the shells and removing the body masses, each snail was macroscopically divided into three parts, in order to facilitate the paraffin embedding process and the better positioning of the structures in the paraffin sections.

Thus, the regions of the cephalopodium (section close to the collar of the mantle), viscera (between the collar of the mantle to the posterior third of the digestive gland), and hermaphrodite glands (final third of the digestive gland to the ovotestis) were separated.

The preparation process consisted of tissue fixation in Rossman's solution, dehydration in an alcoholic series and treatment with Methyl benzoate, following the protocol established by Deelder and Kornelis<sup>19</sup>, for paraffin sections of adult *Schistosoma mansoni* worms. At the time of embedding, the portions of *B. glabrata* were carefully positioned inside the block and received a portion of melted paraffin, according to the direction of the intended cut (transverse or longitudinal). Serial 3 µm sections were performed in a microtome (HM-315, Micron<sup>®</sup>) and five to ten sections were put on each slide.

To identify the histological structures and distribution of L3 in the tissues of infected snails, sections from each region were separated and stained with Hematoxylin-Eosin (HE), according to Michalany<sup>20</sup>. Histological descriptions were based on the observations from Pan<sup>21</sup>.

### *Preparation of adult females of A. cantonensis*

On average, six female specimens of *A. cantonensis* were used for each paraffin block. The preparation process followed the same steps used for the *Biomphalaria* specimens, according to Deelder and Kornelis<sup>19</sup>. The worms were positioned to obtain serial 5 µm cross-sections and eight sections were put on each slide.

### **Human serum samples**

A panel of 46 sera, which were part of the official serum library of the NE/IAL was used for the study. These samples were obtained from the laboratory routine. Following issuance of laboratory report, they were classified, organized, fractionated and stored at -20 °C. All procedures were in accordance with the institutional guidelines for this type of repository. The panel was composed of:

- *Group I*: 14 reagent samples for the immunodiagnosis of eosinophilic meningitis caused by *A. cantonensis*. Cases defined by clinical and laboratory criteria;
- *Group II*: 16 non-reagent samples for the immunodiagnosis of eosinophilic meningitis caused by *A. cantonensis*. Suspected cases not confirmed by laboratory analysis;
- *Group III*: 11 samples from patients with other parasites; five samples from patients with schistosomiasis and six with strongyloidiasis;
- *Group IV*: five samples from individuals classified as free from other parasites; with non-reactive results for angiostrongyliasis, schistosomiasis, toxocariasis and cysticercosis serological tests. Parasitological stool examination was negative for helminth eggs and protozoan cysts.

### **Indirect Immunofluorescence Assay (IFA)**

The reaction was performed according to the technique described by Kanamura et al<sup>22</sup> for paraffin-embedded sections of adult *Schistosoma mansoni* worms. Sera samples were diluted 1:10 and 50 µL of each dilution was added onto the slides containing the tissue sections from *B. glabrata* or female *A. cantonensis* worms. The slides were incubated for 50 minutes at 37 °C and washed with 0.01M phosphate buffered saline (PBS) pH 7.2 three times. Then, 25 µL of the diluted (1:150) fluorescein isothiocyanate-labeled anti-IgG or anti-IgM human conjugates (Biolab®) was added, following incubation for 50 minutes at 37 °C. After three PBS washes, the slides were mounted with buffered glycerin pH 9.5 and examined under a fluorescence microscope. The different fluorescent patterns were recorded using an A640 digital camera (Canon®).

## **RESULTS**

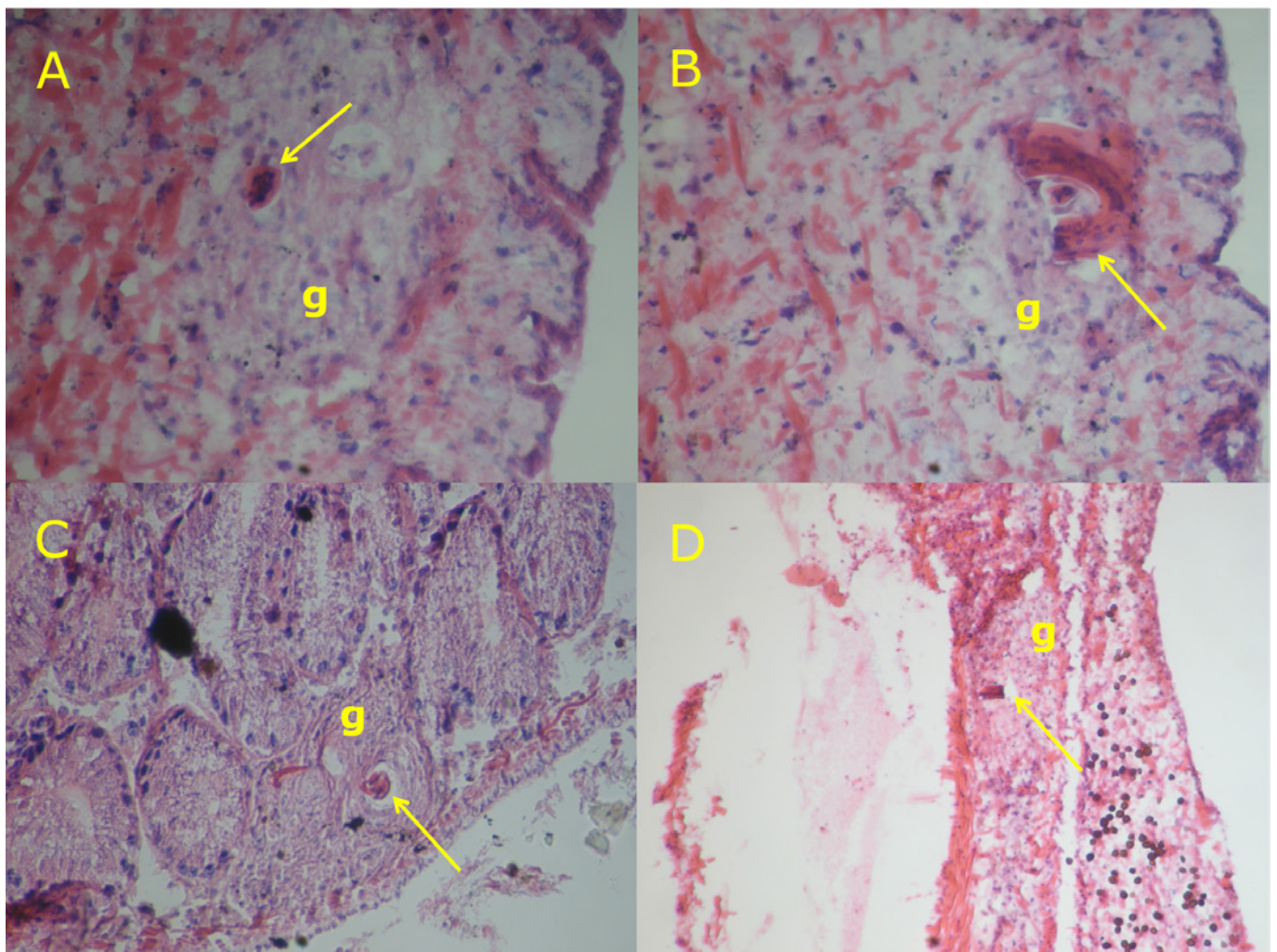
In the tissue samples of *B. glabrata* stained by HE, the parasitism was characterized by the view of nodular reactions (granulomas) containing the larval stages, with greater recurrence in the connective tissue, and distributed in different regions within the mantle and digestive glands. Granulomas were also observed in tissues adjacent to the stomach, intestine and kidney wall. In the cephalopodium, larvae were found in transversal and longitudinal sections, dispersed by the loose connective tissue. In the saccular portion of the kidney, there was presence of a larval fragment in the non-modulated fibrous



connective tissue, supported by a thin layer of muscle fibers. In addition, larvae were also identified near the epithelium of the visceral mass. The only larva found in the ovotestis was surrounded by a granuloma inside the acini. **Figure 1** shows the main histological features observed in the infection of *B. glabrata* by *A. cantonensis* in different tissues.

Despite the inoculum being considered high, of about 700 larvae per snail, and the encapsulated larvae being observed in several locations, as demonstrated in the HE-stained sections, the number of larval structures found per slice and per slide was reduced. This occurrence limited the use of all sera samples from the selected panel for the antigenic targeting study in *Biomphalaria* tissues by IFA. In this case, the technique was performed only with sera from Group I (samples from confirmed cases of the disease).

For the IgG marker, two fluorescent patterns were observed: the first, focal in relation to the larvae fragments inside the granulomas, with delimitation of the larvae cuticle; the second, with more intense

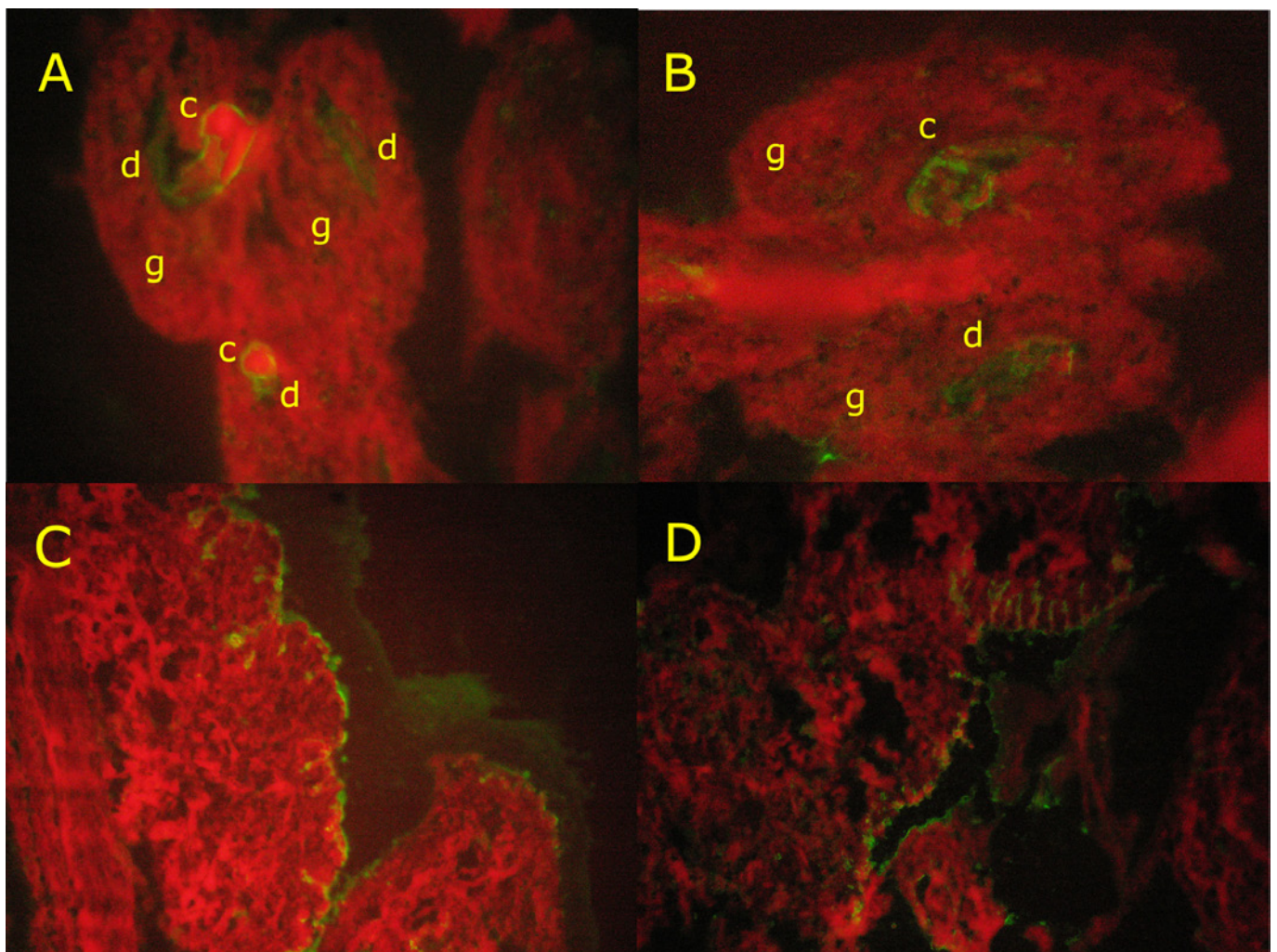


**Figure 1.** Details of granulomas (g) containing infective larvae of *A. cantonensis*. In (A) and (B) cephalopodium. (A) Arrow indicates encapsulated larva in cross section. 400X magnification. (B) Arrow indicates large larval fragment in longitudinal section. Also note a smaller fragment in cross section. 250X magnification. In (C) digestive glands. Arrow shows larva in cross section with retraction space between it and the granulomatous reaction. In (D) sacculus portion of the kidney. Arrow shows larvae in cross section and the space inside the capsule. 125X magnification



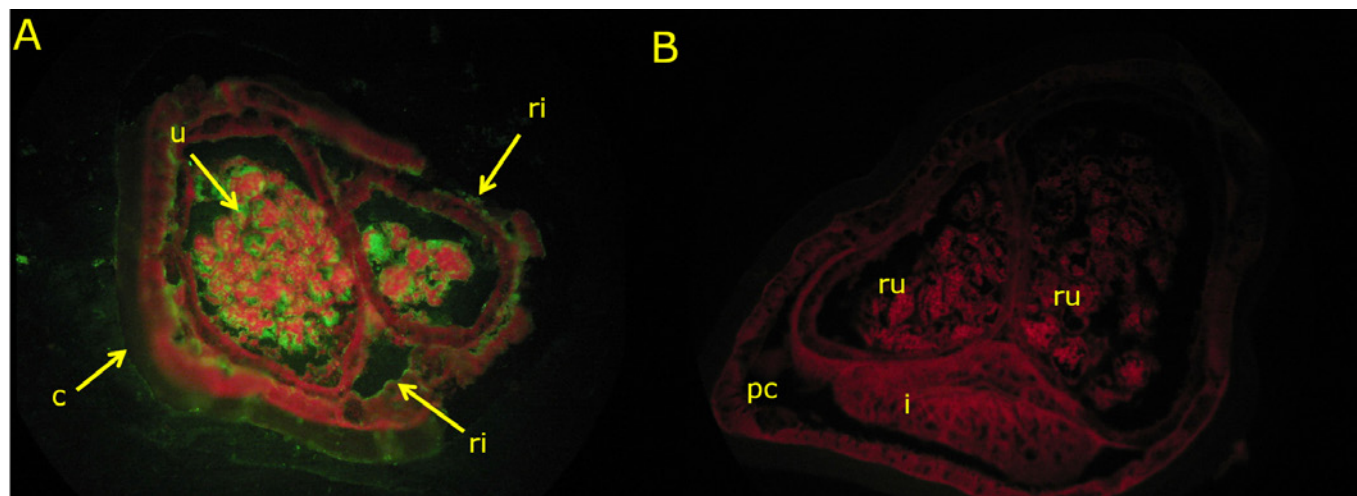
and diffuse fluorescence, observed in the space between the larvae and the granulomas. This pattern was also observed distributed among cells of some granulomas. However, other structures with no apparent relationship with parasitism, such as the epithelium of the edge of the mantle and the interior of the intestine, also showed fluorescence with this marker. For IgM antibodies, low-intensity fluorescent reactions were observed in the granuloma and in the larvae cuticles. The fluorescence patterns found for IgG antibodies related to larval stages, granulomas and other tissues are represented in **Figure 2**.

In the paraffin sections of *A. cantonensis* female worms, IFA was performed in all samples from the selected panel. Reactivity for IgM antibodies was null. However, IgG antibodies showed, in the internal structures of the worms, focal or diffuse fluorescent patterns of different intensities, which varied according to the group of samples. Homogeneous focal fluorescence was observed in the cuticle, inner lining of the pseudocoelom, and outer walls of the intestine, ovaries, and uterus. The diffuse fluorescence pattern was



**Figure 2.** Paraffin tissues sections of *B. glabrata* infected by *A. cantonensis*. In (A) and (B) granulomas (g) formed around the larvae, showing the fluorescence patterns for IgG antibodies against sera samples from Group I (confirmed cases of the disease) by IFA. Focal pattern with cuticle delineation (c) and diffuse pattern (d) in the space between the larva and the granuloma. In (C) and (D) reactivity for the IgG fraction against sera samples from Group I, with delineation of the mantle border epithelium and the intestinal epithelium, respectively

exclusively observed in the uterine contents. **Figure 3** compares a reactive sample for IgG antibodies with a non-reactive sample, regarding the characteristics of the focal and diffuse fluorescence patterns.



**Figure 3.** Paraffin sections of adult female *A. cantonensis* worms comparing the reactivity patterns for the IgG fraction in (A) a sample from Group I, and in (B) a sample from Group II. In (A) the arrows point to focal fluorescence of the cuticle (c) and internal coatings (ri); and diffuse fluorescence in the uterus (u). In (B) absence of reactivity in the pseudocoelom coating (pc), intestine (i) and internal contents of the uterine branches (ru)

The uterine contents were the regions with the highest antigenic focus for IgG, with markings of eggs and, possibly, of embryonic structures related to them. **Figure 4** shows the differences in staining patterns and fluorescence intensity in the uterine contents of *A. cantonensis* females.

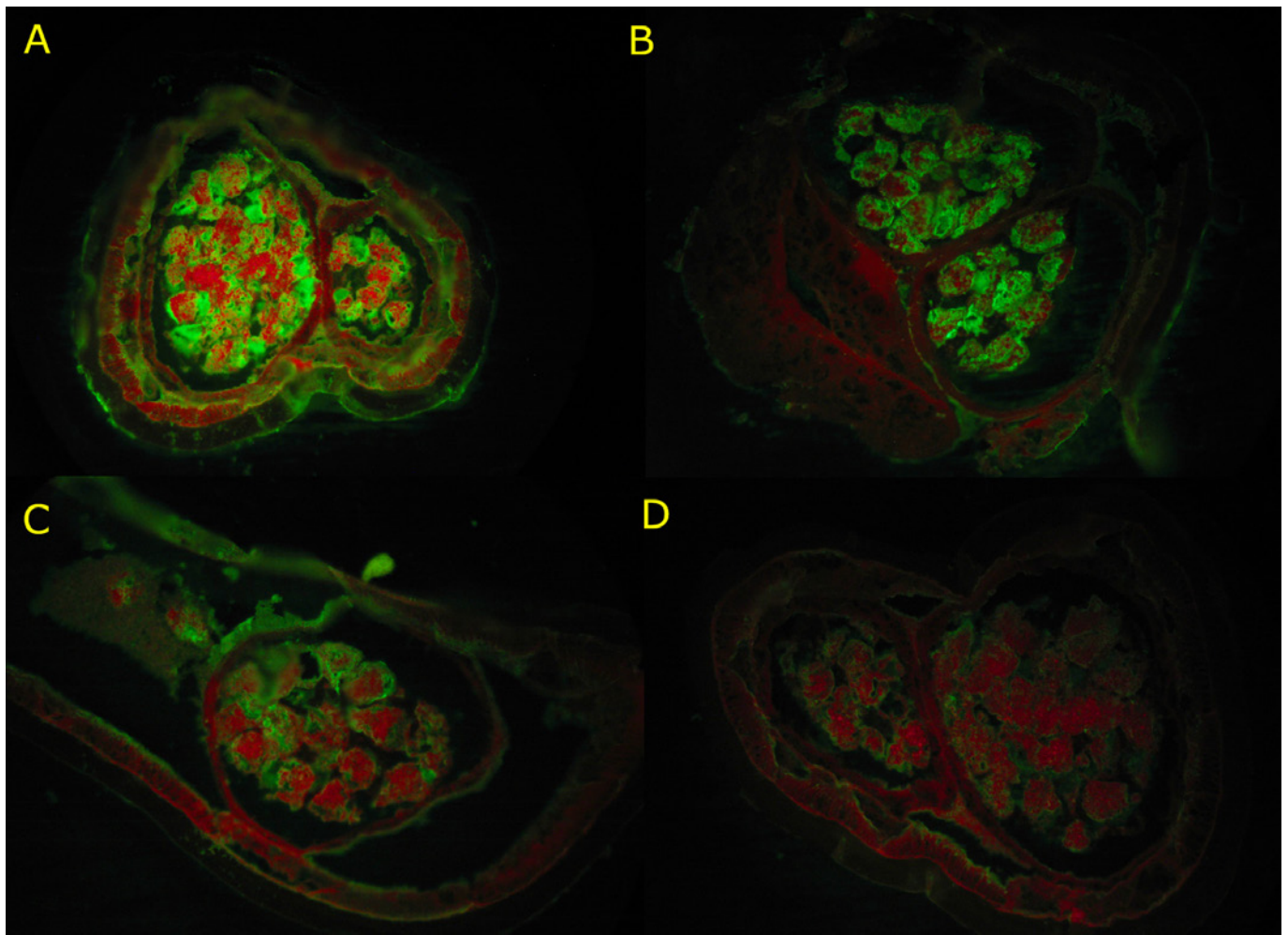
There were variations in the reactivity for IgG antibodies among the 46 samples evaluated, and according to the category of the group of sera used in the assays. In Group I, 100% of the samples (14/14) were reactive for uterine structures; in 78.5% (11/14) the intensity of the reactions ranged from high to moderate, and the other samples (3/14) exhibited low intensity. In this group, reactivity for the cuticle and inner coatings occurred less frequently than for the uterus. In Group II, there was no reactivity for the uterine contents with the exception of a single sample that showed a low-intensity reaction. The reactivity for uterine structures in Group III was 80% (4/5) for schistosomiasis and 83% (5/6) for strongyloidiasis, with a predominance of medium and low intensity reactions. Reactivity for the cuticle and internal linings occurred in this group, mainly in samples from patients with strongyloidiasis. In Group IV there was no reactivity for the uterine contents or for other structures, with the exception of one sample that presented a low-intensity reaction both for the uterus and cuticle.

## DISCUSSION

For more than six decades, IFA has been used for the immunodiagnosis of parasitic diseases caused by protozoa and helminths. In general, the technique has good sensitivity and specificity rates, which may vary according to the nature of the antigens used and the immunoglobulin class involved in the host's humoral response. Whole, fragmented or cut parasites, from different evolutionary stages, have been used as sources of antigens for the reaction<sup>23</sup>.

In our field, the immunofluorescence test is used in sero-epidemiological surveys and in the





**Figure 4.** Paraffin sections of adult female *A. cantonensis* worms showing marking and fluorescence intensity patterns in uterine contents by IFA-IgG. In (A) high fluorescence intensity; (B) mean fluorescence intensity; (C) low fluorescence intensity; (D) very low fluorescence intensity

complementary diagnosis of the acute and chronic phases of schistosomiasis. The reaction uses paraffin sections of adult *S. mansoni* worms, which expose polysaccharide and proteoglycan antigens, related to the digestive tract, with high reactivity for IgM antibodies. This technique was originally described by Nash<sup>24</sup> who introduced Rossman's solution as a fixative, a reagent that promotes the denaturation of protein epitopes. Later, the polysaccharide nature of the antigens was confirmed by other authors using immunofluorescence, and these components were demonstrated in other evolutionary forms of *S. mansoni*<sup>25,26</sup>.

In the present study, it was possible to identify, using the same protocol, stage-specific antigenic structures in tissue sections of experimentally infected *B. glabrata* and female worms, against sera from confirmed cases of eosinophilic meningitis caused by *A. cantonensis*. In addition, a panel composed of samples which were not reactive for the disease; samples from patients infested with other parasites, and samples from individuals free from other parasites served as controls to evaluate cross-reactivity in sections of adult females. The results pointed to high antigenicity of the two evolutionary stages (larvae and worms) in paraffin sections. Likewise, Bender et al<sup>27</sup>, using IFA in frozen sections, also demonstrated high antigenicity of different evolutionary stages of *A. costaricensis*, another metastrongylid species of medical interest. However, it is noteworthy that the use of

paraffin sections of infected *B. glabrata* tissues and female worms, for this purpose, seems to be unprecedented, since there was no reference in the literature about this approach for *A. cantonensis*.

For tissue sections, it was decided to use snails with infections that lasted longer than usual (five months), as a guarantee that the evolutionary forms were in the stage of infective L3 larvae. Under the previously described conditions, encapsulated larvae were observed inside granulomas and distributed in different tissues of the snails, as revealed by HE staining. According to Harris and Cheng<sup>28</sup>, encapsulation results from the host tissue reaction, which is not capable of making the larvae unviable. The authors observed that encapsulated larvae survived for more than 12 months in snail tissues after infection.

The two fluorescence patterns found in paraffin sections of infected snails are associated with L3 larvae within the granulomas. The reactivity of IgG antibodies present in the entire Group I sera, against these structures, showed that the infective larvae retained their antigenicity when transmitted, through the food chain, to other hosts.

Focal fluorescence, with cuticular delineation, suggests that the host's humoral response is directed against the surface constituents of the larvae. The use of Rossman's solution as a fixative in histological preparations prevents the binding of antibodies to protein epitopes, which is why labeling did not occur within the larvae. The diffuse fluorescence found between larvae and granulomas points to dispersed antigenic constituents, which suggests the presence of excretion/secretion antigens produced by the parasites, throughout their lives. The excretory/secretory activity is common to parasites as a form of adaptation to the evolutionary cycle and, therefore, related to the mechanisms of host's immune response invasion, migration, evasion, among other functions<sup>29</sup>. The ability of L3 larvae and other evolutionary stages of *A. cantonensis* to produce excretion/secretion antigens has been proven *in vitro*<sup>30</sup>, and their antigenicity was evaluated by monoclonal antibodies<sup>31,32</sup>.

In paraffin sections of female worms, IgG antibodies were reactive for cuticle and internal structures, with accentuated marking of uterine structures. Considering the variations in both the reactivity and fluorescence intensity found among the different groups of serum samples, the focal fluorescence pattern, with cuticular delineation and internal delineation of the viscera, was interpreted as nonspecific. Although all samples from Group I were reactive for uterine contents, cross-reactivity to IgG antibodies was observed in most samples from Group III, mainly in patients with strongyloidiasis. The diffuse fluorescence pattern observed in the organ suggests that more than one component is responsible for the reactivity of IgG antibodies. Bender et al<sup>27</sup> related the high antigenicity of eggs and uterine content of adult females of *A. costaricensis* to the maturation of the reproductive tube.

The results of the present study only allow us to infer that the markings observed in tissue sections and female worms of *A. cantonensis* indicate the existence of components of high antigenicity; but do not provide a basis for a deeper discussion about the biochemical nature of these antigens. However, some considerations must be pointed out based on new knowledge generated in the last two decades. A wide variety of molecules with different functions have already been identified in all forms of the life cycle of *A. cantonensis*, derived from excretion/secretion products, crude extracts of worms and purified fractions<sup>14,16,31-35</sup>. Glycoconjugates play an important role in the host's innate and adaptive immune response<sup>36</sup>. It is assumed that these molecules participate in the parasite/host interaction and the glycan portion is responsible for inducing the immune response in the host<sup>37</sup>. Glycoproteins have already been identified in different antigenic preparations of *A. cantonensis*, including the purified fraction of 31 kDa used in immunodiagnosis<sup>37,38</sup>. Cross-reactions with other parasitic diseases have already been reported, with emphasis on those that can

determine clinical conditions of eosinophilic meningitis, such as toxocariasis, gnathostomiasis, strongyloidiasis, among others<sup>13,15</sup>. These data demonstrate the need to improve the immunodiagnosis of eosinophilic meningitis and introduce the differential diagnosis as a medical practice in the routine investigation of cases<sup>13</sup>.

## CONCLUSION

In this study, IFA applied to paraffin sections was able to focus antigens on structures present in infected *B. glabrata* tissues and in female *A. cantonensis* worms. Based on the different patterns of fluorescence, the observed results pointed to the possibility of the presence of excretory and secretory antigens inside the granulomas that involve L3 larvae in the tissues of *B. glabrata*, and for antigens present in the uterine structures of the worms, which reacted with antibodies IgG present in sera from confirmed cases of the disease. Cross-reactivity, especially with antibodies present in sera from patients with other parasites, was observed in uterine structures of female worms, which confirms the results of other studies. Therefore, IFA in paraffin sections opens a new perspective for the identification of structures with antigenic potential and other serological markers that can be applied to improve the laboratory diagnosis of eosinophilic meningitis.

The difficulty in obtaining purified antigens has limited the laboratory response in relation to case confirmation, which makes Laboratory Surveillance less efficient. The implementation of a Health Surveillance Program associated with Laboratory Surveillance is essential for the identification and monitoring of new cases, a measure that contributes to the knowledge of the disease in our environment.

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## CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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## AUTHORS CONTRIBUTIONS

All authors contributed to the design, planning, analysis, interpretation of data. All authors read and approved the final manuscript.

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## PRESENTATION NOTE

The partial results of this article integrate the doctoral thesis by Leyva Cecília Vieira de Melo, entitled “Immunodiagnosis of meningoencephalic angiostrongyliasis against different antigenic preparations of *Angiostrongylus cantonensis*”, defended in 2020 in the Programa de Pós-Graduação em Ciências of the Coordenadoria de Controle de Doenças da Secretaria de Estado da Saúde de São Paulo.



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